Title	Studies on quinoa (Chenopodium quinoa) for novel food and beverage applications
Authors	Mäkinen, Outi
Publication date	2014
Original Citation	Mäkinen, O. 2014. Studies on quinoa (Chenopodium quinoa) for novel food and beverage applications. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
Rights	© 2014, Outi M#kinen. - http://creativecommons.org/licenses/by-nc-nd/3.0/
Download date	2024-05-12 12:07:29
Item downloaded from	https://hdl.handle.net/10468/2018

UCC

University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Ollscoil na hÉireann
THE NATIONAL UNIVERSITY OF IRELAND

Coláiste na hOllscoile, Corcaigh
UNIVERSITY COLLEGE, CORK

SCHOOL OF FOOD AND NUTRITIONAL SCIENCES

STUDIES ON QUINOA (*CHENOPODIUM QUINOA*) FOR NOVEL FOOD AND BEVERAGE APPLICATIONS

Thesis presented by

Outi Mäkinen

M.Sc. Cereal Technology (University of Helsinki)
B.Sc. Food Technology (University of Helsinki)

For the degree of
Doctor of Philosophy
(PhD, Food Science and Technology)
Under the supervision of
Prof. DSc. Dr. Elke K. Arendt

May 2014

Table of Contents

Abstract

Quinoa (*Chenopodium quinoa*) is a seed crop native to the Andes, that can be used in a variety of food product in a similar manner to cereals. Unlike most plants, quinoa contains protein with a balanced amino acid profile. This makes it an interesting raw material for e.g. dairy product substitutes, a growing market in Europe and U.S. Quinoa can however have unpleasant off-flavours when processed into formulated products. One means of improving the palatability is seed germination. Also, the increased activities of hydrolytic enzymes can have a beneficial influence in food processing. In this thesis, the germination pattern of quinoa was studied, and the influence of quinoa malt was evaluated in a model product. Additionally, to explore its potential for dairy-type products, quinoa protein was isolated from an embryo-enriched milling fraction of non-germinated quinoa and tested for functional and gelation properties. Quinoa seeds imbibed water very rapidly, and most seeds showed radicle protrusion after 8-9 h. The α-amylase activity was very low, and started to increase only after 24 hours of germination in the starchy perisperm. Proteolytic activity was very high in dry ungerminated seeds, and increased slightly over 24 h. A significant fraction of this activity was located in the micropylar endosperm. The incorporation of germinated quinoa in gluten-free bread had no significant effect on the baking properties due to low α-amylase activity.

Upon acidification with glucono-δ-lactone, quinoa milk formed a structured gel. The gelation behaviour was further studied using a quinoa protein isolate (QPI) extracted from an embryo-enriched milling fraction. QPI required a heat-denaturation step to form gel structures. The heating pH influenced the properties drastically: heating at pH 10.5 led to a dramatic increase in solubility, emulsifying properties, and a formation of a fine-structured gel with a high storage modulus (G') when acidified. Heating at pH 8.5 varied very little from the unheated protein in terms of functional properties, and only formed a randomly aggregated coagulum with a low G'. Further study of changes over the course of heating showed that the mechanism of heat-denaturation and aggregation indeed varied largely depending on pH. The large difference in gelation behaviour may be related to the nature of aggregates formed during heating. To conclude, germination for increased enzyme activities may not be feasible, but the structure-forming properties of quinoa protein could possibly be exploited in dairy-type products.

Acknowledgements

Declaration

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any degree. Where other sources of information have been used, they have been acknowledged.

Signature:

Date: 09/05/2014

"Men love to wonder, and that is the seed of science." – Ralph Waldo Emerson

"Education is a girl's best friend." - Mum

Chapter 1

Introduction

Introduction

Plants are a significant food source for humans, but most plant proteins are used as animal feed to produce milk, eggs and meat. The conversion from plant to animal protein is very inefficient, and as much as 85% is lost, which is problematic as the growing world population is increasingly pushing the "planetary boundaries" (Aiking, 2011; Day, 2013). The environmental impacts related to both meat and dairy production include loss of biodiversity, the disruption of nitrogen and carbon cycles, changes in land use and diminishing freshwater reserves (Aiking, 2011; Goodland and Anhang, 2009). Increasing the utilisation of plant proteins in the human diet would reduce these adverse influences (Day, 2013). Possible approaches include plant protein based meat and dairy analogues and an increased use of protein-rich legumes and grains (Goodland and Anhang, 2009).

Plant proteins have generally a poorer nutritional value than animal-derived proteins. They have a lower digestibility, and are deficient in one or more amino acids, e.g. lysine in cereals and sulphur amino acids in legumes (Friedman, 1996). Quinoa seed protein is high in lysine and sulphur amino acids and thus has a high biological value (Abugoch, 2009; Ranhotra et al., 1992). Although not a protein crop as such, quinoa can have a higher protein content (12-23%) than other grains, but the range is wide (Abugoch, 2009). Quinoa seeds also have other desirable nutritional properties, e.g. higher levels of minerals and vitamins than conventional cereals (Repo-Carrasco et al., 2003). Quinoa is native to the Andes but can be adapted to very different environmental conditions due to its wide genetic variability (Jacobsen et al., 2003). It can thus be cultivated in various parts of the world, including Europe,

North America, North Africa and China (Hirich et al., 2014; Jacobsen et al., 2003; Zurita-Silva et al., 2014).

Quinoa can be processed into a variety of products, e.g. gluten-free baked goods, weaning formulae and pasta (Repo-Carrasco et al., 2003). Some studies have however reported grassy and bitter off-flavours that limit the level of quinoa in a product (Lorenz and Coulter, 1991; Schonlechner et al., 2006). Germination can be used to improve the palatability of grains (Kaukovirta-Norja et al., 2004). In addition, the enzyme activities that arise during germination of seeds can have desired functionalities cereal and pseudocereal-based foods. Studies on the germination of quinoa in terms of food applications are however scarce (Atwell et al., 1988; Park, 2005; Zarnkow et al., 2007).

Increasing the know-how in the processing of novel plant protein based foods is fundamental for the development of desirable products, as there is much room for growth in the sales (Goodland and Anhang, 2009). These products include plant-based dairy substitutes, such as "plant milk" and its derivatives e.g. fermented yoghurt type products, that are a growing market as a part of the "free from" trend (Letherhead, 2011). Because of the high nutritional quality of quinoa protein, the formulation of these products could potentially be feasible.

References

Abugoch James, L., E., 2009. Quinoa (*Chenopodium quinoa Willd.*): Composition, chemistry, nutritional, and functional properties. Adv. Food Nutr. Res. 58, 1–31.

Aiking, H., 2011. Future protein supply. Trends Food Sci. Technol. 22, 112–120.

Atwell, W.A., Hyldon, R.G., Godfrey, P.D., 1988. Germinated quinoa flour to reduce the viscosity of starchy foods. Cereal Chem. 65, 508–509.

Day, L., 2013. Proteins from land plants − Potential resources for human nutrition and food security. Trends Food Sci. Technol. 32, 25–42.

Friedman, M., 1996. Nutritional value of proteins from different food Sources. A review. J. Agric. Food Chem. 44, 6–29.

Goodland, R., Anhang, J., 2009. Livestock and climate change. What if the key actors in climate change were pigs, chickens and cows? Worldwatch Institute, Washington DC. 10–19.

Hirich, A., Choukr-Allah, R., Jacobsen, S.-E., 2014. Quinoa in Morocco - Effect of sowing dates on development and yield. J. Agron. Crop Sci. 200, 371–377.

Jacobsen, S.E., Mujica, A., Ortiz, R., 2003. The global potential for quinoa and other Andean crops. Food Rev. Int. 19, 139–148.

Kaukovirta-Norja, A., Wilhelmson, A., Poutanen, K., 2004. Germination: a means to improve the functionality of oat. Agric. Food Sci. 13, 100-112.

Lorenz, K., Coulter, L., 1991. Quinoa flour in baked products. Plant Foods Hum. Nutr. 41, 213–223.

Park, S.H., 2005. Dough and breadmaking properties of wheat flour substituted by 10% with germinated quinoa flour. Food Sci. Technol. Int. 11, 471–476.

Ranhotra, G.S., Gelroth, J.A., Glaser, B.K., Lorenz, K.J., Johnson, D.L., 1992. Composition and protein nutritional quality of quinoa. Cereal Chem. 70, 303−305.

Repo-Carrasco, R., Espinoza, C., Jacobsen, S.-E., 2003. Nutritional value and use of the Andean crops Quinoa (*Chenopodium quinoa*) and Kañiwa (*Chenopodium pallidicaule*). Food Rev. Int. 19, 179–189.

Leatherhead Food Research, 2011. Food Allergies and Intolerances: Consumer Perceptions and Market Opportunities for "Free From" Foods, Leatherhead Food International, Surrey, UK.

Schonlechner, R., Linsberger, G., Kaczyk, L., Berghofer, E., 2006. Herstellung von glutenfreien Keksen aus den Pseudo-cerealien Amaranth, Quinoa und Buchweizen mit Gartenbohnen. Ernähr/Nutr 30, 101–107

Zarnkow, M., Geyer, T., Lindemann, B., Burberg, F., Back, W., Arendt, E.K., Kreisz, S., 2007. The use of response surface methodology to optimise malting conditions of quinoa (*Chenopodium quinoa*) as a raw material for gluten-free foods and beverages. Monatsschrift für Brauwiss. 60, 118–126.

Zurita-Silva, A., Fuentes, F., Zamora, P., Jacobsen, S.-E., Schwember, A.R., 2014. Breeding quinoa (*Chenopodium quinoa Willd.):* potential and perspectives. Mol. Breed. 34, 13–30.

Chapter 2

Quinoa: composition, applications and potential future uses

1. Introduction

Seeds are a major food staple for the mankind, mainly those of cereals and legumes (Bewley and Black, 1994a). The cultivation of the first domesticated grains during the Neolithic Revolution, wheat and barley in the Fertile Crescent and rice in China for example, was the basis of early agriculture that led to the rise of civilisation. The intensified food production lead to a radical transformation from nomadic hunter-gatherers to agricultural societies, and the subsequent development of literature, science and technology (Li et al., 2007; Prakash, 2001). In addition to being of high importance for our past and present day nutrition, seeds are a vital part of plant reproduction as the propagation and dispersion units of *Spermatophytes*, seed producing plants. As plants are attached to their place of growth, seeds provide them a way to travel through space and time. Potentially a lot of time: the oldest viable seed known to mankind has been dated back 32 000 years (Yashina et al., 2012). The seeds of angiosperms are essentially an embryo and food reserves trapped in one or more protective layers. They vary in structure and patterns of reserve deposition and mobilisation. For example, cereal grains consist largely of a central endosperm with starch and protein storage. The embryo is a thin and flattened tissue nearly absent of storage reserves. In legumes on the other hand, the cotyledons act as storage tissues, making up nearly all of the seed mass (Bewley and Black, 1994a).

Out of about 250 000 identified plants found in the world 30 000 are edible, but only 30 species "feed the world". Wheat, maize and rice alone provide 50% of the world's calories (FAO, 1998). There is however an increasing interest in so-called minor crops, as they may promote sustainability and agrodiversity in farming systems (Williams and Haq, 2002). Also consumer interest has increased as these crops are often perceived as healthy by the Western consumer, and minor seed crops

can be devoid of peptides that can not be tolerated by individuals suffering from the coeliac disease (Bergamo et al., 2011).

One such crop, quinoa, was the most important seed crop in in South America in the pre-Columbian times. It was of such importance to Inca people that it was considered sacred and called the "mother grain" in the Quechua language. After the Spanish conquest (1532 A.D.) the use of quinoa nearly vanished and remained the food of Quechua and Aymara people in rural mountain areas (Abugoch, 2009). Quinoa seeds have desirable nutritional properties, with considerably higher levels of minerals and some vitamins than conventional cereals, as well as high-lysine protein with good digestibility (Ranhotra et al., 1992; Repo-Carrasco et al., 2003). Quinoa also has a wide genetic variability and can thus be adapted to very different environmental conditions, including European countries (Jacobsen et al., 2003). Due to these factors, FAO has declared it as "one of humanity's most promising crops", and it has been considered as a potential crop for NASA's Controlled Ecological Life Support System (CELSS) (FAO, 2011; Schlick and Bubenheim, 1996).

Quinoa has been used for a variety of products, including gluten-free baked goods, pasta, infant food, extrudates and other processed foods (Repo-Carrasco et al., 2003). The limiting factor in its incorporation in products is the grassy and bitter aftertaste it causes when used at higher levels, as has been reported by some authors (Lorenz and Coulter, 1991; Schonlechner et al., 2006). A way to improve the palatability and also nutritional properties of grains is germination (Kaukovirta-Norja et al., 2004). In addition, the enzyme activities formed during germination can have the potential to modify the properties of cereal and pseudocereal-based foods. The germination of quinoa has however mainly been studied from the agronomical perspective (Hariadi et al., 2011; Rosa et al., 2009), and work related to food uses is

limited to a few publications (Atwell et al., 1988; Park, 2005; Valencia et al., 1999; Zarnkow et al., 2007).

Another potential area of application for quinoa which has received little research interest is plant-based dairy substitute products. This product segment is growing as a part of the "free from" trend, but yet scientific literature on systems other than soy is hard to find. These products include plant milks, water extracts of seeds that resemble cow's milk in appearance, and their derivatives, such as fermented yoghurt-type products and cream substitutes. Because of the high nutritional quality of quinoa protein, the formulation of these products could potentially be feasible. This review aims to give an overview about quinoa, its composition and current and potential uses in the food and beverage industry, for example plant-based dairy substitute products.

2. Quinoa

Quinoa (*Chenopodium quinoa*) is a dicotyledonous plant native to the Andean highland region in South America. It grows 1-3 m tall, and produces starchy seeds that have a composition similar to cereal grains (Table 2.1) (Galwey, 1995; Jacobsen et al., 2003). Quinoa is a domesticated species, and has been cultivated in the Andes for more than 5000 years. Its edible relatives include *C. pallidicaule*, "kañiwa" and *C. berlandieri* subsp. *nuttalliae*, "huahzontli", that are used as grains and vegetables, and *C. album*, "lambs quarter", a weed that has been used for food in prehistoric Europe (Bazile et al., 2013; Heiser and Nelson, 1974).

Quinoa has a wide genetic variability, with cultivars adapted to very different environmental conditions ranging from a cold highland climate to subtropical conditions (Jacobsen, 2003). Some cultivars also show good tolerance for adverse

conditions including drought, frost, soil salinity and hale (Bonifacio, 2003; Jacobsen, 2003). Because of its adaptability, quinoa has a potential for cultivation outside the Andean region. In the past decades, field trials have been conducted in various European countries, Kenya, United States and Asia, and it is grown commercially in at least France and Finland at the time of writing (Abbottagra, 2013; Jacobsen, 2003; Vähämäki and Kasvinen, 2013).

Table 2.1. Chemical composition of quinoa and some cereals (oat and wheat), and oil seeds (soy and linseed); g per 100 g dry weight (dw), n.r. = not reported.

Component	Quinoa [a]	Oat [b]	Wheat [a]	Soy [c]	Linseed [d]
Carbohydrate	72.6	69.8	78.6	n.r.	6.2
Protein	14.4	11.6	10.5	42.3	26.4
Fat	6.0	5.2	2.6	19.8	24.6
Fibre	4.0	10.4	2.5	11.5	33.0
Ash	2.9	2.9	1.8	5.2	3.2

Adapted from: [a] Repo-Carrasco et al., 2003; [b] Valentine et al., 2011; [c] Grieshop and Fahey, 2001; [d] Giacomino et al., 2013;

Quinoa seeds are disc-shaped and usually 1.4-1.6 mm in diameter (Abugoch James, L., 2009). The hulls can have a variety of colours ranging from white to orange, and pink to black (Figure 2.1), but the seed can exists in three colours; white, brown ("red") and black (Jacobsen, S-E., personal communication Sep. 12th, 2014). The pigments responsible for the orange-red and red-purple are betalains, found in only species in the *Caryophyllales* order in which they replace the more common anthocyanins (Brockington et al., 2011; Wohlpart and Mabry, 1968).

Quinoa seeds are perispermic: they consist of a central perisperm that is surrounded by a peripheral embryo (Figure 2.2). The endosperm is a 1-2 cell layer thick cap covering the micropyle. The storage reserves are strictly compartmentalised (Prego et al., 1998). The central perisperm acts as a starch storage, while lipid and protein bodies are found in the embryonic tissues and endosperm. Seeds with a similar structure and storage reserve compartmentalisation include grain amaranth (*Amaranthus cruentus*) and sugar beet (*Beta vulgaris*).

Figure 2.1. Colour variation of quinoa seeds (Hermann, 2014).

P - perisperm

R – radicle

H – hypocotyl

C – cotyledons

EN – endosperm cap

SC – seed coat
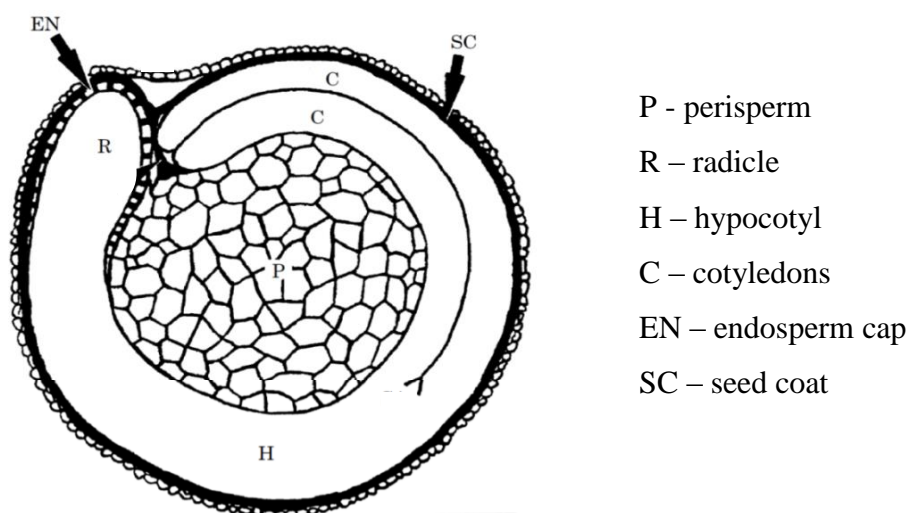
Figure 2.2. Quinoa seed structure showing (Adapted from Prego et al. 1998).

2.1. Chemical and nutritional properties of quinoa constituents

2.1.1. Carbohydrates

Starch is the main storage reserve in most seeds that are cultivated as food, including quinoa (Bewley and Black, 1994a; Prego et al., 1998). It is found exclusively in the central perisperm at a content of 58-64% of the seed weight (Prego et al., 1998; Repo-Carrasco et al., 2003). Starch is a polymer consisting of α-1,4-linked glucose units that exists in two forms: the linear amylose and branched amylopectin. In amylopectin, blocks of α-1,4-linked glucose units are attached by α-1,6-linkages, forming a ramified, very large macromolecule (Zobel and Stephen, 2010). In plants, starch is laid down as complicated granular structures (Buléon et al., 1998). Starch granules consist of amylose and amylopectin in crystalline and amorphous states, and vary largely in size and shape among plant species.

Quinoa starch granules are very small, ranging from 0.6 to 2.0 μm in diameter, and they are found as individual or spherical/oblong compound granules in perisperm cells (Figure 2.3) (Lorenz, 1990). The amylose content ranges from 3 to 20%, which is lower than in most other species (Lindeboom et al., 2005). Quinoa amylopectin has low average degree of polymerisation, but is highly branched (Tang, 2002). The physicochemical properties of quinoa starches vary widely (Lindeboom et al., 2005). Thermal properties, such as starch paste peak viscosity and gelatinisation onset and peak temperatures, as well as the tendency for retrogradation, are correlated with the amylose content (Lindeboom et al., 2005). According to data based on eight quinoa varieties, the onset and peak gelatinisation temperatures vary between 44.6 to 53.7°C and 50.5-61.7°C, respectively, because of different amylose contents (Lindeboom et al., 2005). These temperatures are lower than in many common food crops (Singh et al., 2003; Wolter et al., 2013). Quinoa

starch has been reported to have excellent freeze-thaw stability (Ahamed, 1996), but a later study found that it varies between cultivars and is generally linked to low amylose content as a result of decreased tendency to retrogradation (Ahamed, 1996; Lindeboom et al., 2005).

The digestibility of starch is characterised by the degree of digestion and absorption, that depends on e.g. starch characteristics and processing techniques used (Hager et al., 2013; Singh et al., 2010). Glycemic index (GI) relates the glycemic response of a food to a reference food such as glucose or white bread (Singh et al., 2010). Ruales and Nair (1994) studied the digestibility of quinoa starch. The obtained a value of 72% for raw, untreated quinoa starch. Pre-cooking increased the digestibility slightly to 77% (Ruales and Nair, 1994). The *in vitro* GI of quinoa bread is 95, which is nearly as high as that of wheat bread (Wolter et al. 2013). Other gluten free breads had much lower values, ranging from 69 to 80. Quinoa starch has very small granules, which was suggested to cause the high glycaemic index (Wolter et al. 2013).
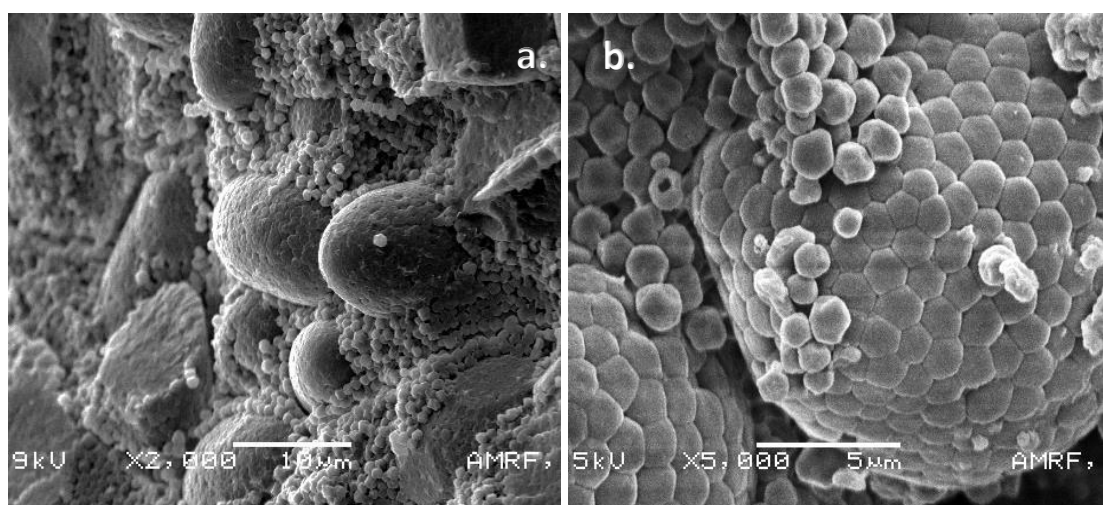
Figure 2.3. Individual and compound starch granules in quinoa perisperm at magnification 2000x (a) and 5000 (b).

Dry seeds contain small quantities of sugars as energy reserves during the early stages of germination (Bewley and Black, 1994b). Quinoa contains 1.7% glucose, 2.9% sucrose and 1.4% maltose, which is higher than the contents in common grains (Repo-Carrasco et al., 2003). The high content of sugars may be related to the frost resistance mechanism of quinoa. Dissolved sugars act as "anti-freeze" and lower the freezing point of water to prevent ice formation in the tissues (Jacobsen et al., 2007).

Dietary fibre is collective term for polysaccharides that are resistant to digestion and absorption in the human small intestine, including non-starch plant polysaccharides, resistant starch and oligosaccharides, and lignin. They thus enter the large intestine undigested, where they can be fully or partially degraded by gut microbes (Buttriss and Stokes, 2008; Lattimer and Haub, 2010). High fibre intake has been linked to reduced risks in e.g. coronary heart disease, certain cancers, obesity and diabetes by various mechanisms (Buttriss and Stokes, 2008). Fibres are commonly categorised as soluble or insoluble based on their water-solubility, the former being fermented in the colon. However, research has shown that neither of these groups completely fit in these categories and solubility does not predict the physiological effect, e.g., some insoluble fibres are also fermented (Buttriss and Stikes, 2008). Still, the physiological properties vary between different fibre forms, e.g., only highly viscous, soluble fibres lower cholesterol (Buttriss and Stokes, 2008).

Reported values for the fibre content of quinoa range from 4% to 14.4% (Alvarez-Jubete et al., 2009; Hager et al., 2012b; Repo-Carrasco et al., 2003). Of the total fibre, 25% is soluble (Hager et al., 2012b). More detailed compositional characterisation of the fibre fraction is still missing, but (Cordeiro et al., 2012) isolated a linear (1→5)-linked arabinan, and a rhamnogalacturonan I type branched

polysaccharide from quinoa seeds. These polysaccharides also showed gastroprotective activity in rats with ethanol-induced gastric lesions (Cordeiro et al., 2012).

2.1.2.Protein

Seed storage proteins act as a nitrogen, carbon and sulphur source for the developing plantlet (Shewry, 1999). In seed protein research, the early Osborne classification based on solubility is still used: albumins are soluble in water, globulins in saline, prolamins in 60-70% alcohol and glutelins insoluble in neutral aqueous solvents and alcohol, but can be extracted in alkali (Shewry, 1999). Storage proteins are found in all Osborne fractions. Globulins and to some extent albumins make up the main storage proteins in most dicotyledonous seeds, while cereal proteins are mostly prolamins (Shewry, 1999). Quinoa has a slightly higher protein content than common food grains, with literature values ranging from 12% to 24% (Abugoch, 2009; Repo-Carrasco et al., 2003). The major storage proteins are a legumin type (11S) globulin named chenopodin (37% of total protein) obtained from the salt-soluble protein fraction by isoelectric precipitation, and a 2S type protein (35% of total protein), that is the residual protein in the salt-soluble fraction after the removal of chenopodin, collected by ammonium sulphate precipitation (Brinegar and Goundan, 1993; Brinegar et al., 1996).

The 11S globulins are hexameric, and consists of six subunits with molecular weight of 50-57 kDa (Casey, 1999). Each subunit consisting of an acidic (~30-39 kDa) and a basic (~20-27 kDa) polypeptide, that are linked by a disulphide bond. The subunits are synthesised as a single entities that then assemble into trimers in the endoplasmatic reticulum. The mature trimers are then stacked face-to-face to form a

hexamer, leaving the side with a higher number of hydrophobic residues buried inside the molecule (Figure 2.4 a) (Adachi et al., 2003, 2001). The tertiary structure of the protein is very sensitive to even small shifts in pH and ionic strength, that alter the association/dissociation behaviour of the subunits (Marcone, 1999). For example, the 11S globulin from sunflower seeds (*Helianthus annuus*) exists as a hexamer at pH 6-9, but dissociates into trimers at higher pH and monomers at very low pH (Figure 2.4 b).
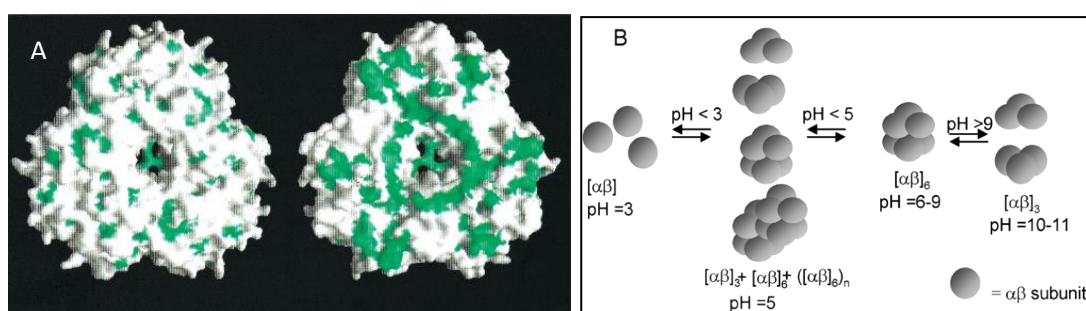
Figure 2.4. (a) Diagrams of the hydrophobic surface of the two faces of the soy proglycinin trimer. Hydrophobic amino acid residues are shown in green (Adachi et al., 2001). (b) The oligomeric state of the 11S globulin from sunflower seeds at varying pH (Molina et al., 2004).

Generally, 2S storage proteins are found in the water-soluble protein fraction and are thus called 2S albumins. These proteins are heterodimers of a 30-40 residue and a 60-90 residue subunit connected by two disulphide bonds. They are highly polymorphic, and the structures and properties can show large variation between cultivars and species. The cysteine and methionine contents are usually high, but can also vary widely between species (Shewry and Pandya, 1999). In contrast, the 2S protein reported in quinoa is salt-soluble. It consists of polypeptides of 8-9 kDa, and possibly smaller subunits but those could not be detected in the study. Quinoa 2S protein is high in cysteine (15.6 mol%) but low in methionine (0.6 mol%) (Brinegar et al., 1996).

The nutritional value of protein depends on their amino acid composition, the ratios of essential amino acids and physiological utilisation (Friedman, 1996). The most abundant amino acids in quinoa protein are glutamic acid, aspartic acid, arginine and leucine in descending order (Abugoch, 2009). Essential amino acids cannot be synthesises *de novo*, and hence they have to be supplied in foods. Table 2.2 shows the essential amino acid profiles of quinoa, some cereals and oil seeds, and milk. FAO amino acid scoring pattern used for the evaluation of protein quality are shown for comparison. This scoring pattern expresses the amino acid composition of an "ideal" protein, against which other proteins are evaluated. Quinoa protein is especially high in lysine (6.0 g/100 g protein), the limiting amino acid in most cereal grains (Friedman, 1996). The lysine content is nearly as high as that of soy bean (6.3 g/100 g protein), and the quantity of sulphur amino acids (methionine and cysteine) is nearly twice as high. Quinoa protein meets the FAO values for both infants and adults, expect for histidine (Table 2.2).

The physiological utilisation of proteins, namely their digestibility, is generally lower for plant proteins (Friedman, 1996). Several methods of evaluating the protein quality exist. These include the protein efficiency ratio (PER) that is based on the weight gain of an experimental animal, and amino acid chemical score

Table 2.2. Essential amino acids in quinoa and other foods (g/100 g protein)

Amino acid	Quinoa[a]	Oat[b]	Linseed[c]	Wheat [a]	Soy [d]	Milk protein [e]	FAO* infant	FAO* adult
Histidine	3.2	2.2	2.4	2.0	2.6	3.2	2.0	1.5
Isoleucine	4.9	3.9	3.9	4.3	4.9	5.0	3.2	3.0
Leucine	6.6	7.4	5.7	6.7	8.2	9.4	6.6	5.9
Lysine	**6.0**	**4.2**	**3.8**	**2.8**	**6.3**	**7.6**	**5.7**	**4.5**
Threonine	3.7	3.3	4.1	2.9	3.7	4.0	3.1	2.3
Tryptophan	0.9	n.r.	1.5	1.2	1.3	n.r.	0.85	0.6
Valine	4.5	5.3	4.8	4.6	5.1	6.2	4.3	3.9
SAA [§]	4.8	4.1	2.6	3.5	2.6	3.5	2.7	2.2
AAA [‡]	6.2	8.4	7.1	8.6	9.1	10.2	5.2	3.8

Adapted from: [a] Repo-Carrasco et al., 2003; [b] Pomeranz et al., 1971; [c] Giacomino et al., 2013; [d] Hughes et al., 2011;

[e] Rutherfurd and Moughan, 1998;

*FAO amino acid scoring pattern requirements (g/100 g protein). Infant 0.5 years; adult >18 years. (FAO, 2011)

[§] SAA: Sulphur amino acids (methionine + cysteine)

[‡] AAA: Aromatic amino acids (phenylalanine + tyrosine)

based on comparison to a reference protein (Friedman, 1996). The method currently preferred by WHO/FAO is the protein digestibility-corrected amino score (PDCAAS), which compares the concentration of the first limiting amino acid to a reference pattern (child 2-5 years), that is corrected for the digestibility (Schaafsma, 2000). Table 2.3 lists the PDCAAS and PER values of some plant proteins and cow's milk. The PDCAAS of quinoa has been reported by Ruales et al. (2002) with a value on 67.7%. This is higher than the values for cereals, but notably lower than milk and soy proteins. The PER value is 2.7, which is higher than that of soy protein, and slightly higher than for casein used as reference in the study (Ranhotra et al., 1992). Ruales et al. (1994) reported significantly lower values for *in vitro* digestibility for raw quinoa when compared to casein. Also, the removal of saponins, bitter compounds covering unprocessed quinoa seeds, improved the protein digestibility.

2.1.3. Lipids

Seeds contain varying amounts of lipids used as food reserves during germination. These are laid down in subcellular organelles called lipid or oil bodies, that range from 0.2 to 0.6 µm in diameter (Bewley and Black, 1994a). Quinoa has a lipid content of 5-6%, which is fairly high compared to most cereals but too low for it to have value as an oil seed (Repo-Carrasco et al., 2003; Wood et al., 1993). The lipids in the quinoa seeds including hulls consist of 56% of neutral and 25% of polar lipid, and 19% of free fatty acids (Przybylski et al., 1994). In bran (embryo) and flour (perisperm), most of the neutral lipids are triglycerides (82-87%), the second major group being diglycerides (10-13%). In the polar lipid fraction, the most abundant phospholipid was

Table 2.3. Protein efficiency ratio (PER) and protein digestibility corrected amino acid score (PDCAAS) values of some plant protein compared to cow's milk in descending order. Values in italics indicates heat-treatment.

	PDCAAS (%) [*]	PER
Cow's milk [a]	120	3.1
Soy	91 [a]; 93 [b]	0.46 [c]; *2.28* [c]
Quinoa	***67.7*** [d, §]	**2.7** [e]
Amaranth [f]	63; 83 [g, ‡]	1.9 ; *2.6*
Hemp [h]	63-66	-
Oat	45-51 [j]; 60 [b]	2.3 [i]
Rice	54 [b]	2.0 [k]
Wheat [a]	42; 37 [b]	1.5
Linseed [l]	*64*	-
Almond [m]	30	-

Adapted from: [a] Schaafsma, 2000; [b] Michaelsen et al., 2009; [c] Friedman et al., 1991; [d] Ruales et al., 2002; [e] Ranhotra et al., 1993; [f] Garcia et al., 1987; [g] Escudero et al., 2004; [h] House et al., 2010; [i] Hischke et al., 1968; [j] Pedo et al., 1999; [k] Juliano et al., 1971; [l] Giacomino 2013; [m] Ahrens et al., 2005

[*] Nontruncated values

[§] Value from weaning food formula

[‡] Value from protein concentrate

lysophosphatidyl ethanolamide in the hulls, but phosphatidyl choline in bran and flour fractions (Przybylski et al., 1994). The fatty acids in the total lipids consist ~85% of unsaturated and 11% saturated fatty acids (Wood et al., 1993). The most abundant fatty acid is linoleic acid at 52.3%, followed by oleic acid at 31.6% (Table 2.4). Quinoa fat is slightly higher in α-linoleic acid, an essential omega 3 fatty acid, than oat and soy oils. The content is however significantly lower than in flax seeds. Overall, the fatty acid profile of quinoa is similar to that of soy bean oil (Wood et al., 1993).

2.1.4. Minor constituents

Although carbohydrate, protein and lipids are quantitatively the most significant components of seeds, the minor constituents are important from the

nutritional perspective (Delcour and Hoseney, 2010). These include minerals, vitamins and sone bioactive compounds, such as phytosterols. Phytic acid, a compound that inhibits mineral absorption, is discussed in more detail with other anti-nutrients in a separate paragraph.

Table 2.4. Fatty acid composition of quinoa, some cereals and oil seeds (%)

Fatty acid	Quinoa [a]	Oat [b]	Wheat [c]	Soy [c]	Linseed [d]
16:0 palmitic	8.5	17.4	21	10.3	4.8
18:0 stearic	0.7	1.1	2	3.8	2.4
18:1 oleic	31.6	38.5	15	22.8	19.2
18:2 linoleic	52.3	39.1	58	51.0	15.0
18:3 α-linoleic	8.1	2.0	4	6.8	58.2

Adapted from: [a] Wood et al., 1993; [b] Saastamoinen et al., 1989; [c] Delcour and Hoseney, 2010; [d] Cunnane et al., 2007

The content of some minerals, vitamins and phytosterols are shown in Table 2.5. Quinoa contains nearly twice as much magnesium and 2-3 times as much iron as oat and wheat (Repo-Carrasco et al., 2003). Also zinc and calcium contents are higher, except for oat that has a a similar calcium content. Phosphorus content, likely to be found in the form of phytic acid, is equal in all compared species (Konishi et al., 2004).

Konishi et al. (2004) studied the distribution of minerals in quinoa using energy dispersive X-ray microanalysis (EDX). Phosphorus (P), potassium (K) and magnesium (Mg) are located in the embryonic tissues in the form of globoids, visible as colorful reagions at the narrow ends of seed cross-sections (Figure 2.5). Prego et al. (1998) also observed P, K and Mg containing globoids in the protein bodies located in endosperm and embryo tissues. Calcium and also potassium were found

in the seed coat, possibly associated with cell wall polysaccharides (Konishi et al., 2004).

Table 2.5. Contents of some minerals, vitamins and phytosterols in quinoa, some cereals and oil seeds (mg/100g dw). n.r. = not reported.

Component	Quinoa	Oat	Wheat	Soy	Linseed
Calcium [a]	94	94	48	81	163 [b]
Magnesium [a]	270	138	152	133	316 [b]
Iron [a]	16.8	6.2	4.6	4.9	6.6 [b]
Zinc [a]	4.8	3.0	3.3	2.4	9.2 [b]
Phosphorus [a]	387	385	387	n.r.	731 [b]
α-tocopherol [c]	2.1	n.r.	0.6 [§]	0.9 [d]	0.1
β + γ-tocopherols [c]	3.1	n.r.	0.5 [§]	n.r.	8.2
Folate [e, ‡]	180	30	34	40.4 [f]	n.r.
Phytosterols [c,*]	82.5	39.1 [f]	68.8 [§]	n.r.	98.2

Adapted from: [a] Repo-Carrasco et al., 2003; [b] Gambuś et al., 2009; [c] Ryan et al., 2007; [d] Plaza et al., 2003; [e] Hager et al., 2012b; [f]Arcot et al., 2002; [e] Normén et al., 2002
[*] b-sitosterol + campesterol + stigmasterol
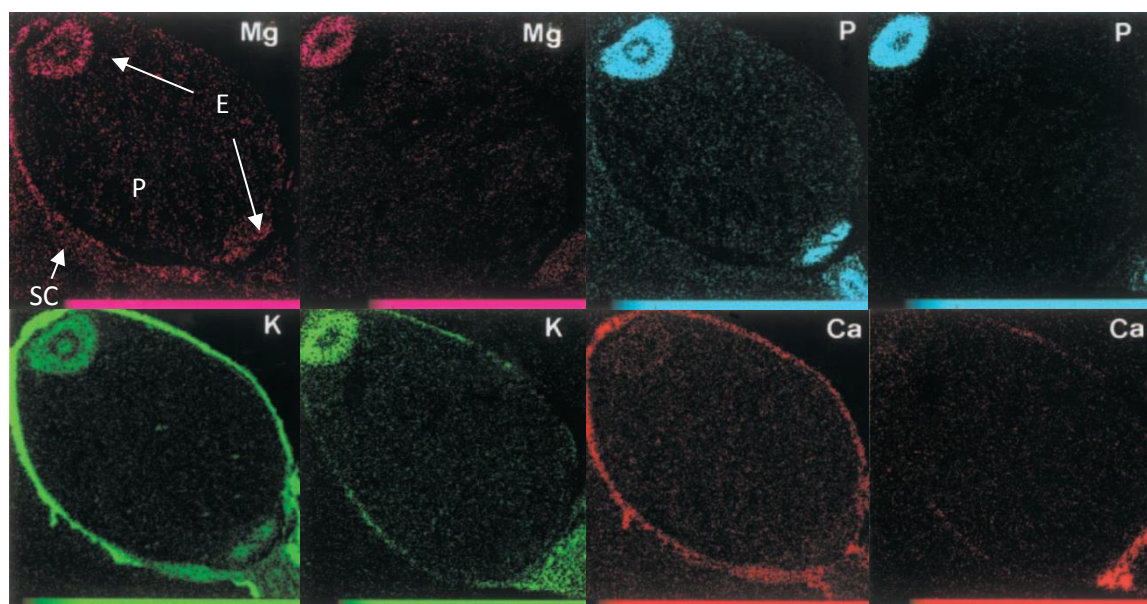[§] Value for spelt
[‡] µg/100 g

Figure 2.5. Element mapping (EDX) of cross-sections of whole (left of each pair of images) and de-hulled (right of each pair of images) quinoa seeds. P = perisperm, E = embryo, and SC = seed coat. Adapted from Konishi et al. (2004).

Vitamin E refers to a group of tocopherols and tocotrienols that are only found in plant foods (Kamal-Eldin and Appelqvist, 1996). These compounds are strong antioxidant, and protect for example cell membranes from oxidation damage. Tocotrienols also have neuroprotective effects (Sen et al., 2006). Tocopherols exist as different isomers (α, β and γ) with varying antioxidant activity, with α-tocopherol being the strongest antioxidant *in vivo* (Kamal-Eldin and Appelqvist, 1996). Compared to wheat and, quinoa has a very high content of both α- and $\beta+\gamma$-tocopherols (Table 2.5). The $\beta+\gamma$-tocopherol level of linseed is however 2-3 times higher.

Folate is a term for tetrahydrofolate and its derivatives, that belong in the group of B vitamins. Folate deficiency may increase the risk of megaloblasic anemia, cardiovascular disease, and neural tube defects on fetuses. Because of the latter, folate supplementation is recommended for pregnant women (Bekaert et al., 2008). Plant foods are the primary source of folates, however the levels in most staple cereals are very low and cannot satisfy the recommended 170-400 µg/d (Bekaert et al., 2008; de Bree et al., 1997). Compared to oat and wheat (30-34 µg/100 g), quinoa is extremely high in folate with 180 µg per 100 g (Table 2.5).

Phytosterols (β-sitosterol, campesterol, and stigmasterol) are compounds found in plants similar to cholesterol in structure and function (Kritchevsky and Chen, 2005). They have a well-documented beneficial impact on serum cholesterol levels on individuals suffering from hypercholesterolemia, and are used to enrich a number of foods (Kritchevsky and Chen, 2005). Quinoa contains 82.5 mg phytosterols in 100 g, which is again higher than oat, wheat and barley (Table 2.5). This is however much lower than the dose required for a hypocholesterolemic effect (2 g/d), which can only be achieved by supplementation (Katan et al., 2003).

2.1.5. Anti-nutritional factors

Anti-nutritional factors in foods are compounds that reduce the nutrient utilisation (Soetan, 2008). Anti-nutrients, including tannins, saponins, protease inhibitors and phytates, are found in plant foods (Singh et al., 2003). Tannins are water-soluble polyphenols that are synthesised by plants as a part of their defence mechanisms against insects and herbivores. They influence protein bioavailability by binding proteins or decreasing the activity of proteases (Singh et al., 2003). Quinoa contains ~0.5% tannins, most of which is located in the outer layers of the seed (Chauhan et al., 1992). Ruales and Nair, (1993) could not detect tannins in whole or polished quinoa, and concluded that their levels are likely to be related to differences in varieties and growing conditions.

Saponins are amphiphilic triterpene or steroidal glucosides, named after their ability to form stable foams in water due to their surface active properties (Osbourn, 1996; Singh et al., 2003). They are found in many plant species, and act as antimicrobial agents (Osbourn, 1996). Saponins do not only have a bitter taste that renders plants and seeds unpalatable, but they interfere with protein digestion and can cause cell damage at high concentrations (Singh et al., 2003). Saponins can be found in seed and the vegetative tissues of plants (Ahamed et al., 1998). Two major saponins have been identified in quinoa seeds (Figure 2.6). According to Ridout et al. (1991), the three major aglycones in quinoa saponin mixture are phytolaccagenic acid, hederagenin and oleanolic acid. The latter has been found to correlate with the saponin content of quinoa, and can be used as an index of saponin content (Ahamed et al., 1998).
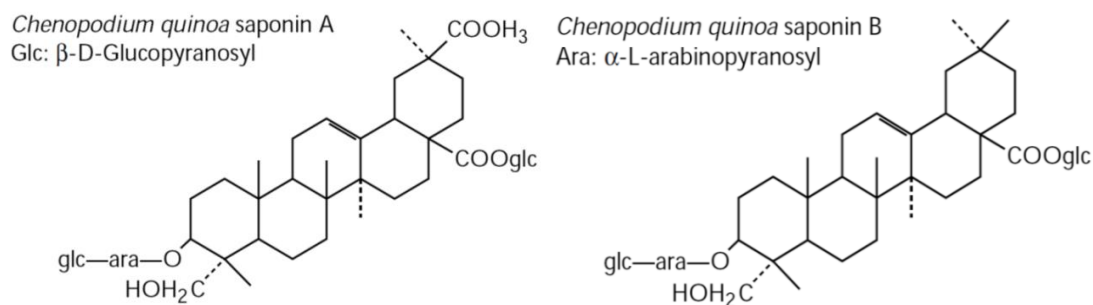
Figure 2.6. Proposed structures of the major quinoa saponins Adapted from Ruales and Nair (1993).

Quinoa contains 0.9-1.2% saponins in total, that is found mainly in the seed coat, and can be removed nearly completely by washing and polishing (Ridout et al., 1991; Ruales and Nair, 1993). These treatments also removes the bitter taste (Ruales and Nair, 1993). There is considerable variation in the seed saponin content (Mastebroek et al., 2000). Cultivars with a low saponin content (sweet quinoa) are of interest, as they do not require desaponification before use (Ruiz et al., 2013). The most widespread of such new varieties is called Kancolla (Dini et al., 2002). However the sweet varieties are late maturing which is an undesirable trait, and perform poorly at higher latitudes, and are more susceptible to pests (Ruiz et al., 2013; Ward, 2001).

Phytic acid (myo-inositol hexaphosphate; IP_6) is an inhibitor of mineral absorption present in many plant foods (Sandberg et al., 1999). They are found in seeds, where they probably act as storage compounds for phosphorus and inositol (Reddy et al., 1982). Phytic acid and its derivatives bind minerals, especially calcium, zinc magnesium and iron, making them unavailable for absorption (De Boland et al., 1975; Maga, 1982; Sandberg et al., 1999). Phytic acid can be hydrolysed during processing as a result of phytase activity into lower inositol

phosphates with less than six phosphate groups, of which IP_5 inhibits iron absorption (Sandberg et al., 1999). Also, IP_4 and IP_3 present in processed foods may contribute to the negative effect of higher inositol phosphates on mineral absorption (Sandberg et al., 1999). The phytic acid content is ~1% in whole unprocessed quinoa, which is similar to cereals and legumes (Ruales and Nair, 1993; Valencia et al., 1999). However, iron solubility in quinoa is double compared to cereals despite a comparable phytic acid content (Valencia et al., 1999). Washing and polishing of quinoa reduces the content to 0.78%. This indicates that phytic acid is also found in abundance in tissues other than the outermost layers (Hídvégi and Lásztity, 2003.; Ruales and Nair, 1993). The content of IP_6+IP_5 is reduced by 39% by germination, 68% by cooking and soaking, and 98% by fermenting the germinated quinoa (Valencia et al., 1999). The resulting increase in iron solubility was 2-4-fold after soaking and germination, and 5-8-fold after germination and fermentation (Valencia et al., 1999).

Protease inhibitors regulate protein metabolism in plant tissues, and act as defence against insects and phytophatogenic microorganisms that secrete proteolytic enzymes (Habib and Fazili, 2007; Liener, 1970; Murdock and Shade, 2002). They can be found in leaves, tubers and cotyledons or whole seeds, depending on the plant species (Liener, 1970). Protease inhibitors reduce the protein digestibility by inhibiting the action of proteolytic enzymes in the gastrointestinal tract. Early animal studies showed that soybeans did not support the growth of rats unless thoroughly cooked to inactivate the inhibitors (Liener, 1970). Most plant protease inhibitors can be destroyed by heat (Liener, 1970), as can be seen when comparing the PER values or raw and heated (italic) soy and amaranth proteins in Table 2.3. Ruales and Nair

(1993) could not detect trypsin inhibitors in quinoa, but Chauhan et al. (1992) reported a low level of inhibitors located in the seed coat.

2.2. Applications of quinoa and its constituents

2.2.1. Post-harvest processing

The outer layer of quinoa seeds contains saponins that taste bitter and may interfere with protein digestion (Repo-Carrasco et al., 2003; Singh et al., 2003). These have to be removed before the seed can be consumed (Repo-Carrasco et al., 2003). The traditional method utilised by farmers is washing the grains while rubbing them with hands or a stone. This method can however be costly when applied on an industrial scale because of a necessary drying step and wastewater disposal (Repo-Carrasco et al., 2003). Also the seeds may start to germinate during the washing step. Dry methods remove the saponins by polishing the grains, which is cheaper and more efficient, but may not remove the saponins completely. A combination method with a polishing step followed by quick washing is the most recommended method (Repo-Carrasco et al., 2003). After saponin removal, quinoa can be used as whole seeds in a similar manner to rice, or milled into a flour and processed into a variety of foods, such as bread, pasta, infant formula, extrudates and meat substitutes (Abugoch, 2009). The separation of the anatomical parts of the seed by milling is widely utilised in grain processing (Delcour and Hoseney, 2010). Milling and can also be used to obtain fraction enriched in certain tissues, e.g. for the production of fractions high in antioxidants or fibre (Glitsø and Bach Knudsen, 1999; Gray et al., 2000). As the storage reserves in quinoa seeds show marked compartmentation with protein and fat reserves located in the embryo and starch in the perisperm, dry fractionation could have a lot of potential (Prego et al., 1998). Some studies exist on

the composition and properties of seed fractions obtained by milling and sieving, but to the author's knowledge these are not commercially available (Ando et al., 2002; Becker and Hanners, 1990; Elgeti et al., 2014).

2.2.2. Baked goods

Bread is a staple food in many parts of the world. Wheat is by far the most common ingredient for bread because of the unique dough forming properties of its proteins. Because of these proteins, wheat dough has the ability to retain gas and form a light, porous structure when baked (Delcour and Hoseney, 2010). Other grains such as rye, oats and sorghum are used in traditional local bread-like products (Duodu and Taylor, 2012). Celiac disease (CD) is an immune-mediated enteropathy with a worldwide prevalence of 1%. The condition is triggered by wheat gluten and related proteins in rye and barley. The only existing treatment to CD is the strict life-long exclusion of cereals containing coeliac toxic proteins (Catassi and Fasano, 2008).

The gluten-free market segment has expanded enormously in the recent years, and the growth has been predicted to continue (Robinson, 2014). In addition to the increasing number of people with a diagnosed coeliac disease, gluten-free diet has also gained popularity as a lifestyle choice. Gluten-free bread is usually based on e.g. rice flour, pseudocereals (quinoa, buckwheat, amaranth) or purified starches (Hager et al., 2012a). As the key to breadmaking lies in the structure forming properties of wheat proteins, the formulation of high-quality gluten-free bread is challenging. Often hydrocolloids (e.g. hydroxypropylmethylcellulose (HPMC), xanthan gum, guar gum) are used to mimic the viscoelasticity provided by wheat proteins. Also proteins from various sources, e.g. egg white, milk or soy beans are useful

ingredients because of their foaming, emulsifying and gelation properties (Hager et al., 2012a). When used as the only flour in a gluten-free bread recipe with no added hydrocolloid, quinoa flour produces loaves with low specific volume, hard crumb and low liking scores due to a pea-like odour (Hager et al., 2012a). Quinoa flour as a part of a complex formulation with rice flour and xanthan gum however yields loaves with a higher specific volume, softer crumb and equal acceptability when compared to a standard gluten-free recipe based in rice flour and potato starch (Laura Alvarez-Jubete et al., 2009). Elgeti et al. (2014) discovered that a perisperm-enriched milling fraction of quinoa gives a high volume bread with a fine crumb structure when used in gluten free baking. Perisperm flour is an interesting ingredient for gluten-free bread, as the absence of large quantities of embryonic tissue also prevents the formation of grassy off-flavours. It is however not necessarily any healthier than using purified starches, as the protein, minerals and vitamins that make quinoa nutritionally interesting are largely located in the embryo.

The inclusion of quinoa for nutritional enhancement has been studied also in wheat-based products. Chauhan et al. (1992) substituted wheat flour with quinoa in the production of bread. The inclusion of quinoa in the product increased the dislike by a sensory panel, but addition level ≤10% was still acceptable. Lorenz and Coulter (1991) concluded that 5-10% quinoa produced bread and cake of good quality, but higher levels decreased the loaf volume and deteriorated the crumb texture. Also, a 30% addition led to a formation of a bitter aftertaste. Park (2005) compared the influence of the addition (10%) of non-germinated and germinated quinoa flour in wheat bread. Germination for 24 h increased the hardness of the dough, but led to no other significant differences. When the seeds were allowed to germinate for 48 or 72 h, they deteriorated the bread quality. According to a report, the production of short

dough biscuits from quinoa is technologically possible, but the product is of low quality when 100% quinoa flour was used because of poor texture, and a strong bitter aftertaste (Schoenlechner et al., 2006). As is the case in gluten-free products, the off-flavour is a major obstacle when whole grain quinoa is included in bakery products.

2.2.3. Pasta and extrudates

Pasta in its most common form is an extruded product that consists of durum wheat semolina, water, salt and sometimes egg (Delcour and Hoseney, 2010). Like in bread, wheat proteins play a major role in the quality of pasta, and gluten-free alternatives tend to show high stickiness and cooking loss, and low elasticity (Hager et al., 2012b). Schoenlechner et al. (2010) studied the production of pasta using amaranth, quinoa and buckwheat. Pasta produced from 100% quinoa showed decent agglutination and performed better than amaranth overall, but had a high cooking loss. The combination of pseudocereals (60% buckwheat, 20% quinoa and 20% amaranth) was suitable for the production of good quality gluten-free pasta. Chillo et al. (2008) investigated the addition of quinoa, chick pea and broad bean (30%) in an amaranth-based pasta formulation, of which quinoa and broad bean improved the quality. Low to medium (1.6-10%) inclusion of quinoa flour in corn-based gluten-free pasta resulted in desirable technological properties (Caperuto et al., 2001).

Extrusion cooking is a hydrothermal process used for the production of ready-to-eat cereals, snacks, and textured meat-like products based on plant proteins. Extrusion also influences the digestibility of starch and protein, as well as other properties of macromolecules. It can for example be used for the modification of fibre solubility and the functional properties of protein ingredients (Camire, 1991;

Gualberto et al., 1997; Singh et al., 2007). Ramos Diaz et al. (2013) studied the inclusion of pseudocereals in gluten-free extruded snacks based on corn flour. Quinoa (20%) increased the expansion of the extrudates when compared to a corn flour control. However, quinoa extrudates showed considerable hexanal production after 9 weeks of storage at a low relative humidity, resulting from lipid oxidation (Ramos Diaz et al., 2013). The extrusion cooking of snack products using 100% quinoa flour requires a high shear environment because the low amylose content makes starch granules hard to disrupt (Dogan and Karwe, 2003). The best product, with high expansion, low density and high degree of starch gelatinisation, was achieved when low moisture and low temperature were used (Dogan and Karwe, 2003).

2.2.4. Other food applications

Due to its high mineral and vitamin content, as well as the good quality of the protein, quinoa is a good raw material for infant foods (Repo-Carrasco et al., 2003). Arginine and histidine are especially important for infants and children, and are found at high concentrations in quinoa proteins (Berghofer and Schoenlechner, 2008). Despite the increasing prevalence of obesity in Latin America, a significant number of children suffer from malnutrition (21% according to a 2002 report) in Bolivia, Peru, Equador and Columbia due to social inequality (Larrea and Freire, 2002). Hence, it is important to have a locally produced raw material for these products. An infant food for supplementary feeding was developed by Ruales et al. (2002) by drying a pre-cooked slurry prepared from quinoa flour. The product could be reconstituted with water and used as porridge or beverage. The product had a protein content of 16%, and was a good source of vitamin E, thiamine, iron, zinc and

magnesium. The supplementation of the diets of children recovering from malnutrition with this product led to an increase in insulin-like growth factor 1 levels after 15 days (Ruales et al., 2002).

Peñaloza et al. (1992) investigated the solid-state fermentation of quinoa tempeh, a product traditionally made of legumes and fermented with a fungus (*Rhizopus oligosporus)* that can be used like meat in vegetarian cooking. They used both a sweet and a bitter variety, of which the latter led to a decreased fungal biomass formation possibly because of differences in hyphal penetration. Schumacher et al. (2010) added toasted quinoa in dark chocolate to develop a product for health-conscious consumers with an increased protein and vitamin E content. The inclusion of up to 20% quinoa did not significantly decrease the acceptability of the product.

2.2.5. Non-food applications

Some components of grains can be used for non-food applications, such as the manufacture of biodegradable plastic-like materials and fibres (Lawton, 2000). The formation of biodegradable films and coatings involves cross-linking of polymer chains. A number of plant-derived macromolecules are suitable for this purpose, e.g. wheat gluten, maize zein, chitin, starch and cellulose (Tharanathan, 2003). Both quinoa protein and starch have been used to develop biodegradable films. Quinoa protein extracts form mechanically resistant films when blended with chitosan in the absence of a plasticiser (Abugoch et al., 2011). When compared to pure chitosan films, 1:1 chitosan/quinoa protein blend films showed lower tensile strength but higher elongation at break, and better water-vapor permeability properties. Quinoa starch forms colourless films with good mechanical and barrier properties when

produced with 21% glycerol as a plasticiser (Araujo-Farro et al., 2010). Although biodegradable plastics decrease the need for oil, and also reduce problems with waste disposal, they should be ideally manufactured out of by-products not usable for human consumption (Gillgren and Stading, 2007; Tharanathan, 2003). Especially using the edible parts of a crop like quinoa that is both nutritious and at the moment, pricey, does not appear feasible.

Due to its very small granule size, quinoa starch has attracted interest in some other applications. Microencapsulation is a way to achieve controlled release of compounds such as flavouring. Tari et al. (2003) studied the formation of spherical aggregates for entrapping vanillin, using starches with small granules. The ability of the starch to entrap vanillin appeared to depend on the amylose content, with an extremely low amylose amaranth starch showing the best performance (Tari et al., 2003). The quinoa starch used had a rather high amylose content for quinoa (22.5%), and it is thus possible that lower amylose cultivars would be more suitable for such an application. Pickering emulsions are stabilised by intact particles that accumulate on the interface instead of surfactants (Rayner et al., 2012). These emulsions are very stable even at large droplet size of the disperse phase. Quinoa starch granules were succinylated to increase the hydroprobicity, and successfully used for the stabilisation of an oil-in-water emulsion. The authors suggested its suitability for food, cosmetic and pharmaceutical applications (Rayner et al., 2012).

3. Germination in food processing

3.1. Germination and dormancy

Germination is the process during which a new plant is formed from a dry seed. If the dry seed is non-dormant and the conditions are favourable it enters the

germination process upon imbibition (Weitbrecht et al., 2011). In *sensu stricto*, germination is completed once the seed coat is protruded by the radicle, and the following growth is referred to as seedling establishment or post-germinative growth (Bewley and Black, 1994a). However, the whole process including the early seedling growth, is often called the germination by e.g. cereal scientists and hortonomists to whom the difference between these phases is irrelevant. From a physiological point of view however, germination and post-germinative growth are clearly separate events. In this review, the grain processing including both germination, subsequent growth will be called germination process, but otherwise accurate terminology will be used. In malt production, the metabolic events are ended by heat-treatment (kilning) (Kaukovirta-Norja et al., 2004).

The process starts by water uptake (imbibition), that leads to the rise of metabolism and the subsequent growth (Bewley and Black, 1994b). Imbibition is a three-stage process (Figure 2.7). Once the seed is exposed to water, the water content increases rapidly because of the low water potential of the dry seed (Weitbrecht et al., 2011). Water potential is the tendency of water to travel into a tissue, and it depends on the pressure (e.g. hydrostatic), osmotic, and matrix (e.g. potential for capillary action) potentials of the material (Bewley and Black, 1994b). Respiration and energy metabolism are reactivated, protein synthesis starts, and damage to membranes and DNA caused by dehydration, storage and rehydration are repaired during this phase (Weitbrecht et al., 2011). Once the water uptake and swelling starts to stagnate, the germination process enters phase II. During this phase, respiration and metabolism are further increased, and the reserve mobilisation in embryonic tissues and the micropylar endosperm (if present) begin. The embryonic axis starts to elongate, and the weakening of tissues surrounding the protruding radicle may occur

in some seeds, eventually leading to the rupture of these tissues (Weitbrecht et al., 2011).

The water uptake starts to increase again during the post-germinative phase (III), as the cells in the embryo extend. The mobilisation of storage reserves also occurs after radicle protrusion, leading to the accumulation of osmotically active low-molecular weight compounds resulting from storage reserve hydrolysis, that increase the water uptake (Bewley and Black, 1994b).

For germination to commence, water and oxygen must be available and the physical conditions including temperature, light quality (spectral distribution) and quantity (intensity, duration) must be suitable for that particular species (Bewley and Black, 1994). Even if these conditions are right, seeds may fail to germinate because they are dormant. Dormancy is a mechanism that regulates the timing of germination: it will only occur
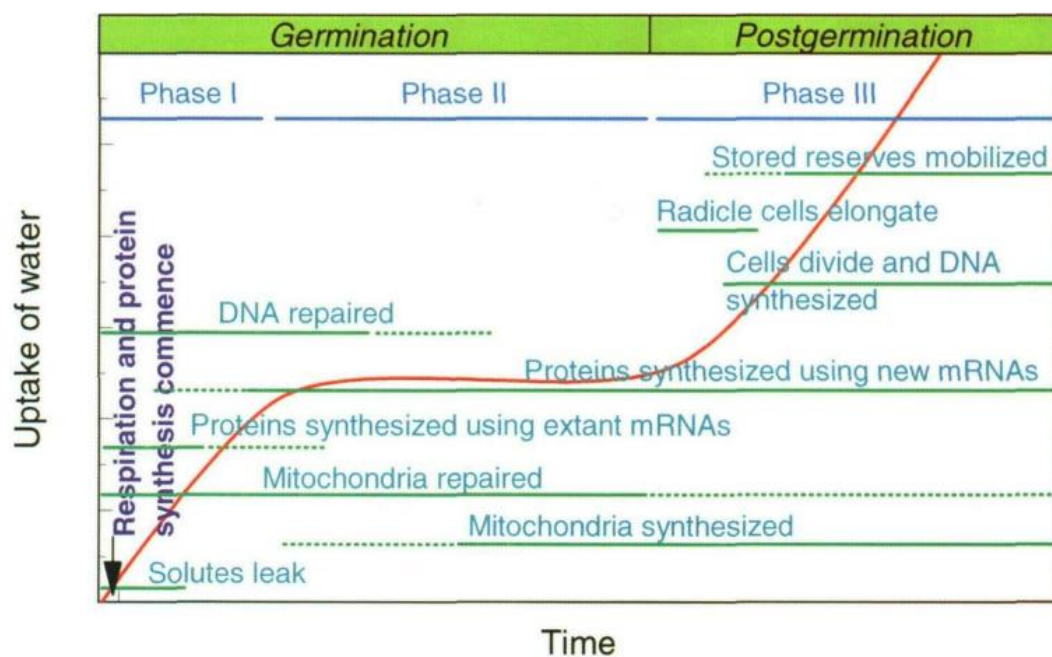

Figure 2.7. The time course of germination and post-germinative growth. Adapted from Bewley (1997).

when conditions are suitable for the survival of the plant (Finch-Savage and Leubner-Metzger, 2006). In other words, dormancy is the inability of the seed to complete germination under conditions otherwise favourable for germination (Bewley, 1997). Different types of dormancy mechanism exist, including coat-enhanced dormancy in which germination is prevented by physical constraint of the structures surrounding the embryo, and embryo dormancy in which the embryo itself cannot grow (Bewley, 1997). Seeds are usually dormant to varying degrees when dispersed by the mother plant (primary dormancy), or they can enter secondary dormancy when exposed to unfavourable conditions, such as high or low temperatures (Bewley and Black, 1994). The regulation of dormancy and its release are complex events not fully understood, but abscisic acid (ABA) has an essential role in the onset and maintenance of the dormant state in many species, while gibberellic acid (GA) promotes germination (Hilhorst and Karssen, 1992).

The topics of interest for food scientists include the increase in of hydrolytic enzyme activities and the subsequent hydrolysis of storage macromolecules in the seed tissues, the change in mechanical properties seed tissues, as well as the nutritional aspects including modified nutrient bioavailabilities and secondary metabolites with possible bioactivities (Kaukovirta-Norja et al., 2004). These issues will be discussed in the following sections.

3.2. Impact of germination process on nutritional properties of seeds

During germination and early seedling growth, a range on hydrolytic enzymes will be activated and synthesises *de novo* to mobilise the high-molecular-weight storage reserves deposited in the storage tissues of the seed, or to loosen cellular structures. These enzyme activities include amylolytic enzymes, endo-β-

glucanases and other cell-wall degrading enzymes, lipases, proteases and phytase. The storage compounds are converted into soluble forms that are transported to metabolising and growing organs (Bewley and Black, 1994c). The influence of these changes on the nutritional profile of the seed depends on the extent of modification, i.e., how far the germination process is allowed to continue. In germinating finger millet (*Eleusine coracan*), starch content starts to decline after 48 h, as it is converted into sugars (Mbithi-Mwikya et al., 2000).

Protein digestibility is influenced by, e.g., limited hydrolysis and the inactivation of protease inhibitors. Reports on the influence of germination vary between species. Chang and Harrold (1988) studied the fate of lectin and trypsin inhibitors in navy and pinto beans. Lectin activity was reduced in only navy beans, and *in vitro* protein digestibility remained nearly unchanged in both beans. Nnanna and Phillips (1990) reported an increase in *in vivo* protein digestibility tested on rats, but no impact on *in vitro* digestibility. Germinating fluted pumpkin seeds (*Telfairia occidentalis Hook*) for 7 d increased the in vitro protein digestibility from 58% to 77% (Giami et al., 1999). The inclusion of malted sorghum in non-malted sorghum flour led to a significant dose-dependent increase in protein digestibility (Elkhalil et al., 2001).

The breakdown of cell walls occurring during germination leads to changes in the solubility of fiber components. In legumes, germination for 96 h generally increased the soluble fiber content. The extent of the soluble fiber increase and the required processing conditions varied between species (Cabrejas et al., 2008). However, as the health effect of soluble fibers depends on the molecular weight, excessive hydrolysis is detrimental for the nutritional benefits (Theuwissen and Mensink, 2008). This may be controlled with process parameters: Wilhelmson et al.

(2001) developed a procedure for malting oats without a considerable loss of high-molecular-weight beta-glucan. A short germination period (72 h) at 15°C retained 55-60% of the beta-glucan. Legumes contain high quantities of raffinose series oligosaccharides, that are resistant to digestive enzymes and are metabolised by the colon microbiota, causing gastrointestinal symptoms. Even a short germination process is efficient in the removal of these compounds, after 24, the content has decreased to 50% of the initial, and <20% after 60 h (Figure 2.8 a).
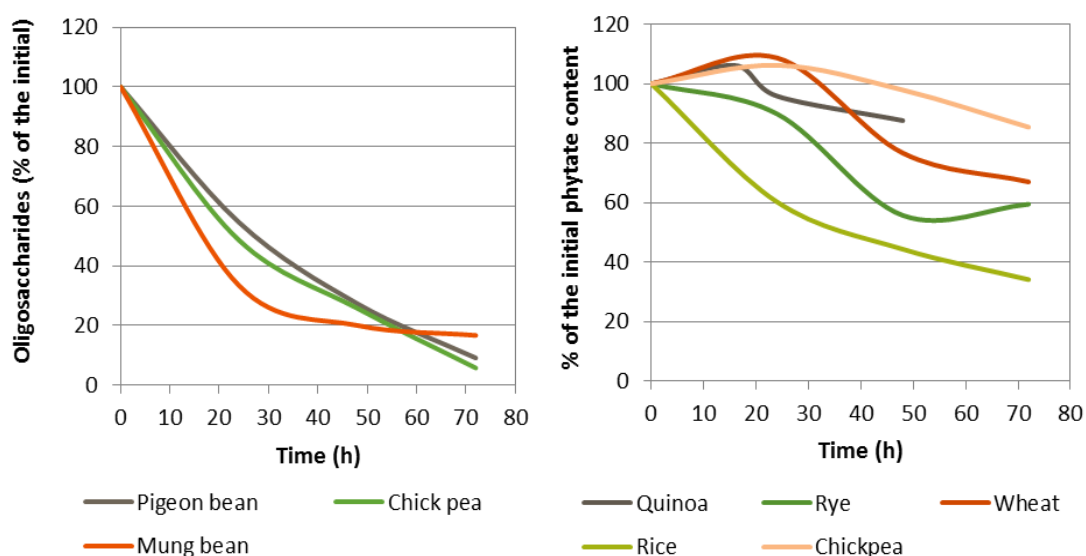
Figure 2.8. Germination as a means of oligosaccharide reduction in pulses (a) and phytate reduction in selected grains and pulses (b). Adapted from Egli et al. (2002) and Rao and Belavady (1978).

Phytate is a severe antinutrient present in seeds, as discussed under section *2.1.5*. Attempts to remove it by the means of germination have been explored with a variety of grains and legumes (Egli et al., 2002; Larsson et al., 1996; Valencia et al., 1999). The results depend greatly of the species in question: for example chick pea shows very little changes, while the phytate content on rice decreases to nearly 30% of its original value during 72 h of germination (Figure 2.8 b). Subsequent processing

such as soaking, cooking or fermentation may increase the phytate removal. Zinc absorption from a breakfast porridge was doubled, and iron absorption increased by 47%, when oats were malted and soaked before use (Larsson et al., 1996). Also the highest increase in zinc and iron solubility was achieved by germination and fermentation (Valencia et al., 1999).

Bioactive compounds in seeds include phenolic compounds and other antioxidants, plant sterols and vitamins, that have been the subject of an increased interest in cereal processing (Kaukovirta-Norja et al., 2004). The content of phenolic compounds, avenanthramides and phyrosterols has been shown to increase during germination of oat (Oksman-Caldentey et al., 2001). Also the contents of folate and lignin increased by 3- and 2-fold during 6 d germination of rye (Liukkonen et al., 2003). Active oxygen species accumulate in seed tissues resulting from the reactivation of metabolism during early germination. The increase in antioxidant compounds and detoxifying enzymes are the seed's mechanism for protecting the tissues from radical damage (Bailly, 2004). Antioxidant activities appear to increases during germination in a variety of species, including amaranth, quinoa, buckwheat, wheat, barley and oat (Alvarez-Jubete et al., 2010; Pike et al., 2007). The activities against lipid oxidation found in methanol extracts of malted oat were comparable to that of a synthetic antioxidant butylated hydroxytoluene (BHT), suggesting potential uses in food preservation (Pike et al., 2007).

A combination of germination and fermentation on nutritional modification of grains and legumes has been explored by several authors. It increases the hydrolysis of protein and starch drastically, and has also been shown to be more efficient in the enhancement of mineral solubility than fermentation alone (Sripriya et al., 1997; Valencia et al., 1999). Katina et al. (2007) compared the influence of

sourdough fermentation of germinated and non-germinated rye on the formation of bioactive compounds, and found that the combination treatment led to higher levels of folates, phenolic compounds and alkylresorcinols than fermentation only.

3.3. Significance of germination on food processing

In addition to being used for nutritional enrichment or modification of products, malted grains and legumes may have technological functionalities in food systems due to their increased enzyme activities. Hydrolytic enzyme activities and the degradation of the endosperm reserves are of course a prerequisite for malting and brewing (Briggs, 1998). Barley is the main malting cereal worldwide, but the technology can be applied to other grains and legumes. Less common malting grains include tropical cereals sorghum, maize, millet and tice, and cereals from the temperate zone, wheat, rye and oat (Briggs, 1998). The latter resemble barley in their malting properties, the malts have high amylolytic activities, especially α-amylase. Such malts hydrolyse starch efficiently into sugars, which can be measured as so-called diastatic power (Table 2.6). Tropical grains and pseudocereals often have low amylolytic activities with some exceptions.

Generally, these alternative grains produce malts with poor performance. Low activities of essential enzymes leads to problems with fermentability and high wort viscosities (Hager et al., 2014; Phiarais et al., 2006; Zarnkow et al., 2007). Also other factors such as high starch gelatinisation temperatures or complex cell-wall poly saccharides not readily hydrolysed, may contribute to the poor performance in brewing (Hager et al., 2014). Brewing with alternative grains often requires the use of exogenous enzymes (Hager et al., 2014), but also additional

processing steps such as alkaline steeping, and careful process optimisation may yield acceptable malt quality (Meo et al., 2011).

In addition to brewing, germinated grains can be used as a source of enzymes to alter the other grain-based foods. Barley and wheat malt can be used to optimise the α-amylase levels in wheat flour as an alternative to fungal α-amylase, or as a source of colour and flavour when inactivated (Briggs, 1998; Delcour and Hoseney, 2010). The high amylolytic activity has also been exploited in, e.g., the manufacture of breakfast cereals and Mämmi, a traditional Finnish rye-based Easter dish, and for the production of dextrins (Ba et al., 2013; Briggs, 1998; Loponen et al., 2009). Germination is also an efficient way of decreasing the viscosity of starchy weaning

Table 2.6. Enzyme activities of malts produced from a variety of grains.

	α-amylase [*]	β-amylase [§]	Diastatic power [‡]	Protease [†]	Lipase [‡]
Barley [a]	134	12.0	264 [b]	9.4	3.0
Wheat [a]	119	23.5	355 [b]	8.9	6.9
Oat [a]	48	2.7	n.r.	11.9	29.5
Rye [c]	224	8.3	n.r.	27.3	n.r.
Rice [d]	37	1.9	225	n.r.	n.r.
Buckwheat [e]	19.9	24.7	72 [f]	5.5	n.r.
Sorghum [g]	40	2.0	89	n.r.	n.r.
Proso millet [h]	124	1.8	n.r.	n.r.	n.r.
Teff [i]	68	7.5	n.r.	n.r.	n.r.

Adapted from: [a] Mäkinen and Arendt 2012; [b] Briggs, 1998; [c] Hübner et al. 2010; [d] Ceppi and Brenna, 2010; [e] Phiarais et al. 2005; [f] Phiarais et al. 2010; [g] Agu and Palmer, 1997; [h] Zarnkow et al. 2010; [i] Gebremariam et al. 2012.

[*] Analysed with Megazyme Ceralpha kit. One unit of activity corresponds to the liberation of 1 μmol p-nitrophenol/min.

[§] Analysed with Megazyme Betamyl-3 kit. One unit of activity corresponds to the liberation of 1 μmol p-nitrophenol/min. Unit converted from BU to B3U when necessary using a factor of 58.6.

[‡] Windisch–Kolbach units (°WK), converted from L when necessary

[†] Analysed against haemoglobin. One unit of activity corresponds to the liberation of 1 mg l-leucine/h.

[‡] Analysed against triolein using the dough method. One unit of activity corresponds to the liberation of 1 μmol oleic acid/h under assay conditions.

foods traditionally used in rural communities in developing countries, as it does not require sophisticated equipment (Atwell et al., 1988; Griffith et al., 1998).

The influence of other germinated grains and legumes on wheat dough and bread has been studied to some extent. The addition of up to 5% oat malt led to drastic weakening of the gluten network and subsequent increase in extensibility, but nevertheless yielded bread with specific volume higher than all wheat control, and even crumb grain possibly because of its high lipase activity (Mäkinen and Arendt, 2012). Germinated brown rice also weakens the dough, which led to decreased loaf specific volumes at malt levels higher than 10% but higher nutritional value (Watanabe et al., 2004). Several papers discuss the incorporation of germinated legumes in bread for nutritional enhancement. For example, Hallén et al. (2004) compared the replacement of wheat flour by non-germinated and germinated cowpea flour. Germinated cowpea flour increased the specific volume slightly at levels 5-10%, whereas non-germinated flour had no such effect. However, at levels >15%, germinated flour decreased the volume, while non-germinated flour did not. Morad et al. (1980) supplemented wheat bread with starches isolated from non-germinated and germinated yellow peas, lentils and faba beans. All starches deteriorated the loaf volumes and crumb grain, but the effects were worse for germinated starches.

Germinating and kilning influence the sensory properties of grains, giving them a typical flavour and odour generally perceived as pleasant. During germination, reducing sugars and amino acids are released, that subsequently react during heating, giving rise to Maillard reaction products (Kaukovirta-Norja et al., 2004). Heiniö et al. (2001) profiled the sensory characteristics of native, germinated and kilned oats. Germination and kilning decrease the undesirable cereal-like flavour, and musty and earthy odour notes. The main flavour notes were roasted,

nutty and intense, and dominating textural attributes were crisp and brittle. Germinating brown rice increased the hedonic response to texture, taste and appearance when cooked (Parnsakhorn and Langkapin, 2013). The inclusion of malted raw materials in products has also been shown to alter the sensory properties favourably. Extrudates produced from malted millet and soybean scored higher in flavour and texture compared to their unmalted counterparts, and the malted flavour masked the unpleasant beany flavour originating from soybean (Obatolu, 2002). Malting of raw materials also changes the sensory properties of rye extrudates drastically (Heiniö et al., 2003).

4. Plant based dairy type products

Plant milk substitutes are water extracts of legumes, oil seeds, cereals or pseudocereals that resemble cow's milk in appearance. These extracts can be processed further into related products, such as cream, yoghurts and ice cream substitutes. Milk, by definition, is a mammary secretion, and according to Codex Alimentarius, the term "milk" (and other dairy terms) can be used for products if "the exact nature of which is clear from traditional usage", unless the product is intended to substitute dairy products (Codex Alimentarius, 1999). Still, the terms like "almond milk" and "soy yoghurt" are used in everyday language, and the issue has been a long and still ongoing battle between the National Milk Producers Federation (NMPF), FDA and plant milk producers in the US (Soyfood Association od America, 1997; NMPF, 2010). At least at the time of writing, several commercial products carry the word milk in the US, and although their European counterparts are call their liquid products drinks or beverages, terms like plant-based yoghurt

alternatives and oat ice cream are used (Silk.com; Almondbreeze.com; Alpro.com, Oatly.com, Carlshamnmejeri.se, 2014).

There is a wide variety of traditional plant based beverages around the world, for example Horchata, "tigernut milk" in Spain; Sikhye, a beverage made of cooked rice, malt extract and sugar in Korea; Boza, a fermented drink made of wheat, rye, millet and maize consumed in Bulgaria, Albania, Turkey and Romania; Bushera, a fermented sorghum or millet malt based beverage from Uganda, and traditional soy milk originating from China (Cortés et al., 2005; Prado et al., 2008; Kim et al., 2012; Chen, 1989). The most widely consumed plant milk substitute is soy milk, a product that started its journey from Asia to the supermarket shelves in Europe and the US less than hundred years ago. The first commercially successful product was launched in Hong Kong in 1940 and the market grew rapidly during the 1970s and early 1980s in Asia after the development of technologies for large scale production of mild flavoured soy milk (Chen, 1989). The demand for soy milk in the Western world was initiated by consumers intolerant to cow's milk, but the market expanded in the 1990's and 2000's as a part of a health trend, and grew from USD 300 m to USD 4 bn between 1992-2008 in the U.S. (Organic Monitor, 2005; Patisaul and Jefferson, 2010). After soy received an FDA approved health claim for lowering the risk for coronary heart disease in 1999, more than 2700 new soy products were introduced to the market (Patisaul and Jefferson, 2010).

Soy products are still dominating the market in the Western world, but the emerging of alternative products from other plant sources such as coconut, oat and almond have decreased its share because of increased options and worries about GMO and allergenicity of soy (Mintel, 2011). Overall, the dairy alternative market is still growing: Packaged Facts (2012) estimated the U.S. market for plant based milk

substitutes to have a total value on USD 1.33 bn in 2011, which is expected to increase to USD 1.7 bn by 2016. Also the market for lactose- and dairy free products in general, estimated to be worth USD 3.6 bn in 2010, is growing in the U.S. and Western Europe. The figure includes lactose-free dairy products, but much of the growth has been attributed to soy milk like products (Leatherhead Food Research, 2011).

According to an estimate, 15% of European consumers avoid dairy products for a variety of reasons, including medical reasons such as lactose intolerance (LI), cow's milk allergy (CMA), cholesterol issues and phenylketonuria, as well as lifestyle choices like a vegetarian/vegan diet or concerns about growth hormone or antibiotic residues in cow's milk (Jago, 2011) (Leatherhead Food Research, 2011). LI is generally an inherited condition (primary hypolactasia) that disables lactose digestion due to lactase deficiency, causing abdominal pain, bloating and flatulence upon the consumption of lactose containing foods (Swagerty et al., 2002). The prevalence of LI varies between ethnic groups, being below 20% only among white Europeans and their descendants. The significantly higher prevalence in other ethnic groups (50-80% among Hispanic and Black and nearly 100% among Asian and Native American populations) has led to a theory that lactase deficiency is a normal condition for adult humans and the frequency of the lactase persistency gene has increased in cultures where milk has offered a selective advantage (Sahi, 1994). LI can also be caused by injuries to the intestinal mucosa (secondary hypolactasia), resulting from diseases such as untreated celiac disease, cystic fibrosis and gastroenteritis (Bode and Gudmand-Høyer, 1988; Swagerty et al., 2002). Sufferers of the inflammatory bowel disease have a higher dairy sensitivity prevalence compared to the average population (10-20%), and are often advised to avoid dairy products

(Mishkin, 1997). The main treatment for LI is the avoidance of lactose containing foods and replacing milk and dairy products with lactose-free dairy or dairy-free alternatives.

CMA is a disorder in which the immune system reacts to one or more milk proteins causing an inflammatory response. Cow's milk is the most common allergen in infants, but 80-90% of sufferers acquire a tolerance by the age of 5 years. The true prevalence of CMA is 2-6% in infants and 0.1-0.5% in adults, but the number of self-diagnosed cases is 10-fold higher possibly due to confusion with LI or misdiagnosis without clinical evaluation (Crittenden and Bennett, 2005). The only treatment for CMA is the complete avoidance of cow's milk antigens. Infants with CMA may be fed with hypoallergenic formulas based on extensively hydrolysed whey or casein (Kneepkens and Meijer, 2009).

4.1. Processing

Plant milk substitutes are colloidal suspensions or emulsions consisting of dissolved and disintegrated plant material. They are prepared traditionally by grinding the raw material into a slurry and straining it to remove coarse particles. Although countless variations of the process exist, the general outline of a modern industrial scale process is essentially the same; the plant material is soaked and wet milled to extract the milk constituents, or alternatively the raw material is dry milled and the flour is extracted in water (Figure 2.10). The grinding waste is separated by filtering or decanting. Depending on the product, standardisation and/or addition of other ingredients such as sugar, oil, flavourings and stabilisers may take place, followed by homogenisation and pasteurisation/UHT treatment to improve

suspension and microbial stabilities. These extracts can also be spray dried to produce powders (Diarra et al., 2005).

4.1.1. Raw material pre-treatments

Plant milk substitutes can be produced by extracting the soluble material directly either ground plant material with water or wet grinding soaked grains or legumes into a slurry (Diarra et al., 2005). Alternatively, the product can be reconstituted using protein isolates or concentrates and other ingredients, e.g. oils, sugars, salts and stabilisers (Debruyne, 2006). This approach also allows the formulation of a range of related products such as pharmaceutical beverages, nutritional supplements, infant formulas, meal replacers, cream alternatives and fruit smoothies (Paulsen et al., 2006). Possible raw material pre-treatments include dehulling, soaking and blanching (Debruyne, 2006). Blanching is required to inactivate trypsin inhibitors and lipoxygenase that would produce off-flavours in soy milk (Giri and Mangaraj, 2012). Roasting of the raw material enhances the aroma and flavour of the final product, but heating decreases the protein solubility and extraction yield (Hinds et al., 1997a; Rustom et al., 1991).

4.1.2. Extraction

The extraction step has a profound effect on the composition of the resulting product. To increase the yield of the process, the efficiency of this step may be improved by increasing the pH with bicarbonate or NaOH, elevated temperatures or the use of enzymes. Most cereal and legume proteins have an isoelectric point under 5, translating to the lowest solubility (Wolf, 1970). Alkaline pH during extraction increases the protein extractability, but a neutralisation step may be required in the

process (Rustom et al., 1991; Aidoo et al., 2012). A higher extraction temperature increases the extractability of fat, but the denaturation of proteins decreases their solubility and yield (Rustom et al., 1991). Hot water extraction of cowpea milk decreases the yield and protein content compared to cold water extraction, but improves the protein digestibility slightly due to trypsin inhibitor inactivation, and leads to a reduced extraction of phytic acid (Akinyele, 1991).

Partial hydrolysis of proteins and polysaccharides using enzymes is another way to increase the extraction yields (Table 2.7). Papain and enzymes extracted from *Pestulotiopsis westerdijkii* increased the protein yield of peanut and soy milks (Rustom et al., 1993; Abdo and King 1967).
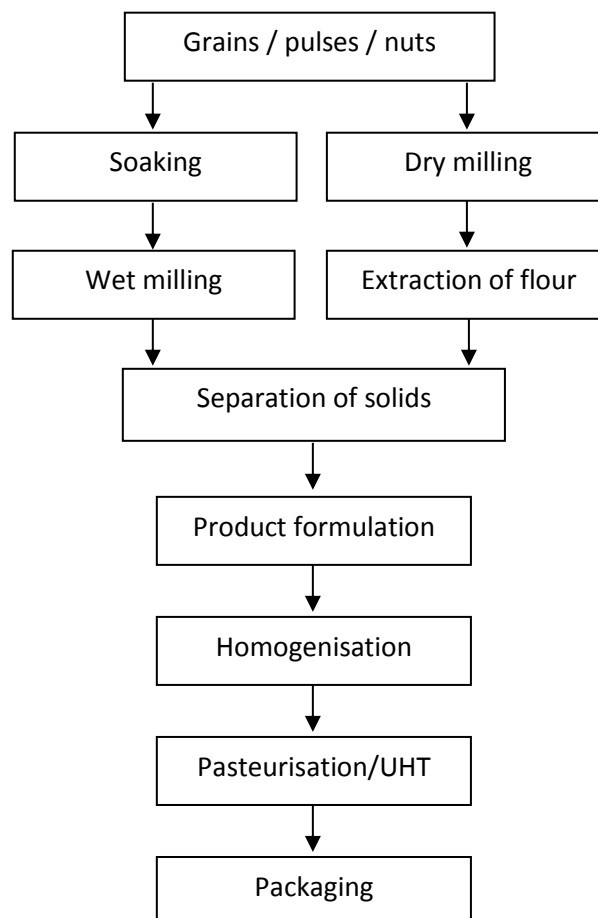

Figure 2.9. The general manufacturing process of plant milk substitutes.

In addition to proteolytic enzymes, a mixture of amyloglucosidase and a cellulase cocktail has been shown to increase the carbohydrate recovery of peanut milk (Rustom et al., 1993). Eriksen (1983) used a variety of enzymes in soy milk extraction, and found that the highest protein and total solids yield was attained using a neutral or alkaline proteinases at their optimum pH, while pectinase and β-glucanase had little effect. Enzymes with low pH optima may not be the most efficient extraction aids even if the enzyme action *per se* increases the yield, as the pH decrease influences the protein solubility, and thus neutral and alkali proteases may be the best options. In addition to increasing the extraction yield, proteolytic enzymes improve the suspension stability (Rustom et al., 1991). Also, a cellulase treatment after homogenisation has been reported to decrease the particle size and yield a more stable suspension (Rosenthal et al., 2003).

Table 2.7. Effect of enzymes in extraction yields of plant milk substitute substitutes.

Raw material	Enzyme	Dosage/pH/T	Increase in yield
Peanut	Papain	1:50 protein/ 6.95; 8.0/20 °C; 60 °C	"Significant increase in protein extraction"
Peanut	Cellulase cocktail (Viscozyme) and amyloglucosidase	- / 4.5 / 50°C	13.4% (carbohydrate)
Soy	Enzyme isolate from *Pestulotiopsis westerdijkii*	- / 4.6 / 37 °C	22% (protein)
Soy	Neutral protease and β-glucanase cocktail (Neutrase)	0.5%/7.0 / 50 °C	31% (protein) 20% (total solids)
Soy	Pectinase	2% / 5.5 / 50 °C	11% (protein) 7% (total solids)
Soy	Pectinase, cellulase, hemicellulase and protease cocktail (SP-249)	2% / 4.5 / 50 °C	26% (protein) 16% (total solids)

Adapted from: Rustom et al. (1991); Rustom et al. (1993); Abdo and King, (1967) and Eriksen, (1983).

4.1.3. Separation and starch liquefaction

After the extraction step coarse particles are removed from the slurry by filtration, decanting or centrifugation (Diarra et al., 2005; Lindahl et al., 2001). When using raw materials high in fat, such as peanuts, the excess fat can be removed using a separator as in dairy processing (Diarra et al., 2005). Soy beans and nuts contain little starch, but when using cereals or pseudocereals the starch forms a thick slurry when heated above the gelatinisation temperature (55-65 °C). To prevent this in the further processing steps, starch can be gelatinised and liquefied with α-amylase or a malt enzyme extract (Mitchell and Mitchell, 1990; Tano-Debrah et al., 2005). The patented process of Lindahl et al. (2001) employs α- and β-amylases to hydrolyse the starch until a desired level of sweetness and viscosity is reached. The liquefaction step may take place before or after the removal of coarse particles. However, according to Mitchell and Mitchell (2010) and Giri and Mangaraj (2012), heating the slurry above 50 °C before filtration compromises the mouthfeel of rice and soy milks.

4.1.4. Product formulation

Other ingredients can be added to the product base after the removal of coarse plant material. These include vitamins and minerals used for fortification as well as sweeteners, flavourings, salt, oils and stabilisers. As suspension stability is an issue in plant milk substitute substitutes, hydrocolloids are often used to increase the viscosity of the continuous phase, and emulsifiers have also been proven to be beneficial in some beverages. Rustom et al. (1995) yielded the most stable peanut milk by using a stabiliser mix for dairy products containing mono and diglycerides, glyceryl monostearate, guar gum and carrageenan, while Hinds et al. (2007b)

achieved good results with 0.02-0.04% carrageenan and 0.2-0.4% mono- and diglycerides. Lee and Rhee (2003) used pine nuts to improve the stability of a rice based beverage, as they contain proteins with good emulsifying properties. Sodium stearoyl-2 lactylate (SSL), a lipid surfactant, has been found to bind specifically to partially hydrolysed oat proteins and thus enhance the stability of oat protein suspensions (Chronakis et al., 2004).

The addition of nutrients in food substitutes may be necessary to ensure the nutritional quality of the product. The nutrients used must be bioavailable and sufficiently stable, and not cause excessive changes in product quality. The stability of vitamins is influenced by several factors during food processing, and may be reduced as a result of e.g. heating oxygen exposure (Richardson, 1990). The challenge in mineral enrichment is the reactivity of metal ions with other food components, and the use of sequestrants such as citric acid may thus be necessary (Richardson, 1990; Zhang et al., 2007a). Some mineral sources used in plant milk substitutes include ferric ammonium citrate and ferric pyrophosphate as iron sources and tricalcium phosphate and calcium carbonate as calcium sources (Zhang et al., 2007a; Zhao et al., 2005).

4.1.5. Homogenisation and suspension stability

Plant milk substitutes contain insoluble particles, such as protein, starch, fibre and other cellular material. These particles, being denser than water can sediment, making the product unstable (Durand et al., 2003). The suspension stability can be increased by decreasing the particle size, improving their solubility or by using hydrocolloids and emulsifiers (Durand et al., 2003; Rustom et al., 1995). Many plant

milk substitutes coagulate when heating. When proteins unfold as a result of heating, the nonpolar amino acid residues are exposed to water increasing the surface hydrophobicity. This enhances protein-protein interactions that can result in aggregation and sedimentation or gelling (Phillips et al., 1994). The heat stability of proteins depends on the pH, ionic strength and the presence of other compounds such as minerals and carbohydrates (McSweeney et al., 2004).

Homogenisation improves the stability of plant milk substitutes by disrupting aggregates and lipid droplets and thus decreasing the particle size distribution (Malaki Nik et al., 2008). When enough lipids are present, an emulsion is formed resulting in a creamier more homogenous product (Chen, 1989). Homogenisation in the conventional dairy processing pressure range (ca. 20 MPa) increases the suspension stability sufficiently of at least soy, peanut and rice milk substitutes (Hinds et al., 1997b; Lee and Rhee, 2003; Rustom et al., 1995). Ultra high pressure homogenisation (UHPH) of soy milk at 200 MPa reduces the particle sizes intensely, from 0.55 μm to 0.13 μm (volume weighted mean diameter), and improves the stability compared to conventionally processed products. The treatment also reduces microbial counts and can be used for preservation (Cruz et al., 2007). A higher homogenisation temperature (72-82 °C) has been reported to increase the stability of peanut milk (Hinds et al., 1997a; Rustom et al., 1995).

In soy milk, heat denaturation of proteins is required for suspension stability. Malaki Nik et al. (2008) studied the effect of heat denaturation alone and in combination with homogenisation (69 MPa) by characterising fractions obtained by stepwise centrifugation. The protein and solids content decreased after the first centrifugation (8000 g) in the untreated samples, while significant decrease in both treated samples occurred after the third centrifugation (40 000 g), indicating an

increase in the resistance to sedimentation upon heating and homogenisation. Also the ratios of β-conglycinin (7S) and glycinin (11S) in the fractions were influenced by the treatments. This indicates, that although heating decreased the solubility of β-conglycinin, large glycinin aggregates were disrupted, resulting in suspensions with smaller particles and a narrower size distribution.

4.1.6. Microbial shelf life extension

Commercial plant milk substitutes are pasteurised or UHT treated to extend the shelf life. However heat may cause changes in protein properties that can influence the stability, as well as changes in flavour, aroma and colour (Kwok and Niranjan, 1995; Rustom et al., 1996). Pasteurisation is carried out at temperatures below 100 °C, and destroys enough micro-organisms to enable a shelf-life of ca. 1 week at refrigerated temperatures. In the UHT treatment, the product is heated to 135-150 °C for a few seconds, yielding a commercially sterile product (Kwok and Niranjan, 1995). Rustom et al. (1996) treated a peanut beverage for 4 and 20 s at 137 °C. The longer treatment time decreased the suspension stability slightly, but led to higher taste and acceptability scores. Both treatments were effective in increasing the microbial shelf-life; no vegetative bacteria, spores or moulds were detected in the products.

The manufacturing process of Horchata (tiger nut milk) takes another approach: the product is not heated to prevent the starch from gelatinising and the occurrence of other sensory changes resulting from heating. Prepared this way, the product has an extremely short shelf-life. In commercial products, pulsed electric fields has been suggested to extend the microbial shelf life (Cortés et al., 2005).

Also, other non-thermal processes such as ultraviolet sterilisation, high pressure throttling, high pressure processing and ultra high pressure homogenisation (UHPH) have been explored as methods of soy milk preservation (Bandla et al., 2011; Cruz et al., 2007; Smith et al., 2009; Sharma et al., 2009). Sikhye, a Korean rice beverage, is commonly sold frozen to avoid UHT related changes in flavour. However *Bacillus cereus* spores are a risk, and their number has successfully been reduced by tyndallisation with CO_2 injection, a procedure consisting of heating to 80 °C to activate spore germination and subsequent cooling, followed by CO_2 injection and heating to 95 °C (Kim et al., 2012).

4.1.7. Fermented products

Fermentation with lactic acid bacteria improves the sensory and nutritional properties, and microbial shelf life of foods (Leroy and De Vuyst, 2004). Plant milk substitutes can be fermented to produce dairy free yoghurt type products while rendering the raw material into a more palatable form. For example, the levels of hexanal responsible for the undesired beany flavour in peanut milk is efficiently reduced with fermentation (Lee and Beuchat, 1991). Also, the levels of aflatoxin B1 commonly found in peanuts, is reduced by fermentation with *Flavobacterium aurantiacum* (Hao and Brackett, 1988). Fermentation of soy milk reduced the amount of flatulence inducing oligosaccharides depending on the α-galactosidase activity of the strain, and increased the angiotensin-converting enzyme (ACE) inhibitory activity (Donkor et al., 2007). The storage proteins of various cereals contain known ACE inhibitory peptides that can be released using fermentation and exogenous proteases as has been demonstrated with rye malt (Hu et al., 2011;

Loponen, 2004). These cereals may have potential as raw materials for dairy type functional products.

In order to produce fermented products, the starter cultures must be able to grow and dominate the microflora in the plant medium and produce a desired texture. Lactic acid bacteria have been used for cereal fermentations for centuries and many cereals and pseudocereals are known to support their growth, but low levels of fermentable sugars present in some grains may pose a problem (Zannini et al. 2012). To overcome this, sugars and food grade yeast extract can be added to the media (Diarra et al., 2005). Also, germinating the raw material to increase the amount of fermentable sugars and amino acids before processing improves the growth performance of probiotic strains (Charalampopoulos et al., 2002). Mårtensson et al. (2000) studied the growth and product characteristics of an oat milk medium fermented with a range of starter cultures. They found, that strains of *Leuconostoc mesenteriodes*, *Leuc. dextranicum*, *Pediococcus damnosus* and *Lactobacillus kefiri* produced the highest levels of lactic acid, resulting in a pleasant flavour. In addition, an EPS producing strain of *L. delbrueckii ssp. bulgaricus* yielded a viscosity comparable to dairy yoghurts after 72 h fermentation at 25 °C when glucose was used as a carbon source. Jiménez-Martínez et al. (2003) obtained a product with a viscosity similar to dairy yoghurt but slightly lower hedonic rating by fermenting milk extracted from Lupinus campestris seeds with *Streptococcus thermophilus* and *L. delbrueckii ssp bulgaricus*.

Probiotic dairy products have been available for years, but also non-dairy raw materials can be used as vehicles for probiotic strains for the dairy intolerant or vegetarian/vegan consumers (Prado et al., 2008). Donkor et al. (2007) reached desired therapeutic levels of cells (10^8 cfu/ml) after fermenting soy milk with a range

of probiotic strains for 48 h. Mårtensson et al. (2002) reported inhibition of some probiotic strains in an oat product when used in combination with a yoghurt starter culture, as the pH of the medium decreases excessively due to a lower buffering capacity in comparison to cow's milk. However a strain of *L. reuteri* was able to survive at a therapeutic levelfo at least for 30 days (Mårtensson et al., 2002).

Some authors have used additives such as CMC, coagulants (calcium citrate), milk powder and gelatin to enhance the texture and reduce syneresis in the final product (Cheng et al., 2006; Yadav et al., 2010). However, the use of animal ingredients in this product category in the Western market excludes the vegetarian/vegan consumer segment. Yazici et al. (1997) aimed to increase the calcium content of peanut yoghurt to the level of fat free dairy yoghurt, but the calcium salts decreased the gel strength and promoted syneresis. In addition to plant milk substitutes, also suspensions of solid grain material can been used as media for fermentation, yielding a gruel like product (Salovaara, 2004). This enables a more economic utilisation of the raw material, as well as better preservation of the nutritional properties such as high fibre content.

4.1.8. Other products

Other products mimicking, e.g., cream, cream cheese, ice cream and dairy desserts exist in the market. These products are not widely studied, but some reports dealing with soy-based products are available. Zulkurnain et al. (2008) developed a soy cream cheese from blended tofu, carrageenan, salt, pectin and maltodextrin were used to modify the textural properties to resemble those of dairy cream cheese The resulting product was, however, lacking the elasticity of its dairy counterpart, despite

the modifiers. A later study from the same group showed that this product supports the growth of probiotic *Lactobacilli*, and can be used as a carrier for probiotics (Liong et al., 2009). Granato et al. (2010) optimised the composition for a high protein dessert based on soy protein and guava juice. The resulting product was of acceptable sensory quality even at a protein concentration of 3%. Rheological or textural properties were not reported. Production of an ice cream substitute is possible using soy bean powder and oil to replace dairy ingredients (Her et al., 2005). The overrun and meltdown were significantly lower in the soy product compared to dairy ice cream and also low sensory scores were attained, especially for mouth feel and melting feel (Her et al., 2005).

4.2. Nutritional properties

Plant milk substitutes are often perceived as healthy, possibly due to negative perceptions about the nutritional properties of cow's milk and the health claims associated with soy (Bus and Worsley, 2003; Patisaul and Jefferson, 2010). In reality, the nutritional properties vary greatly, as they depend strongly on the raw material, processing, fortification and the presence of other ingredients such as sweeteners and oil. The nutritional values of plant milk substitutes purchased from a local store in Ireland are presented in Table 2.8. When comparing the products, it is evident that only soy milk has values comparable to cow's milk, with protein contents ranging from 2.9-3.7%. All other products are very low in protein, with only quinoa, hemp and Oatly oat milk containing ≥1% protein. This may pose a risk if plant milk substitutes are used to replace cow's milk without knowledge about the differences, especially when given to young children: several cases of kwashiorkor, a protein-energy malnutrition typical for areas of famine, have been reported in

Western countries as a result of using rice milk (0.1-0.2% protein) as a weaning food (Carvalho et al., 2001; Katz et al., 2005). Also, milks produced from legumes other than soy, such as peanut and cowpea, can have a protein content as high as 4% (Rustom et al., 1991; Tano-Debrah et al., 2005). Although plant milk substitutes are low in saturated fats and most products have caloric counts comparable to skim milk, some products contain as much energy as full fat milk, originating mostly from sugars and other carbohydrates.

Plant proteins are generally of a lower nutritional quality compared to animal derived proteins due to limiting amino acids (lysine in cereals, methionine in legumes) and poor digestibility (Friedman, 1996). PDCAAS and PER values of some raw materials used in commercially available plant milk substitutes are listed and compared to the values of cow's milk were presented previously in Table 2.3 [*2.1.2*]. Both values are the highest for cow's milk followed by heat treated soy. PDCAAS values for all other raw materials fall below 67.7%, quinoa and hemp scoring highest, with the exception of amaranth protein concentrate with a value of 83%. PER of cow's milk is 3.1, while the closest plant protein sources are quinoa, amaranth and soy (all heat treated) with values 2.7, 2.6 and 2.28, respectively. The extremely low PER value for raw soy (0.46) reflects the presence of protease inhibitors that are inactivated upon heating (Friedman, 1996).

In addition to containing high value protein, milk and other dairy products provide 30–40% of dietary calcium, iodine, vitamin B_{12} and riboflavin, and population groups with low milk intakes often have a poor status for these nutrients (Millward and

Table 2.8. Some plant milk substitutes on the market. Nutritional values per g/100 ml.

Beverage (manufacturer)	Energy (kcal)	Protein	Carbohydrate (sugars)	Fat (saturated)	Fibre	Fortification
Cow's milk (full) [1]	64	3.3	4.6 (4.6)	3.9 (2.5)	-	-
Cow's milk (skim) [1]	33	3.5	4.8 (4.8)	0.3 (0.1)	-	-
Soy (Alpro, UK)	38	2.9	2.8 (2.7)	1.7 (0.3)	0.5	Ca, B_2, B_{12}, D, E
Soy (Tesco, UK)	32	3.4	0.2 (0.1)	1.9 (0.3)	0.6	Ca, E, D, B_{12}
Oat (Alpro, UK)	66	0.4	12.7 (5.7)	1.5 (0.57)	0.0	-
Oat (Oatly, SE)	35	1	6.5 (4.0)	0.7 (0.1)	0.8	Ca, D_2, B_2, B12
Kamut, (La Finestra Sul Cielo,IT)	46	0.7	7.5 (4.6)	1.4 (0.2)	0.5	-
Amaranth (Ecomil, SP)	52	0.6	8 (5.0)	1.9 (0.5)	0.3	-
Sesame (Ecomil, SP)	51	0.6	6.7 (3.4)	2.4 (0.5)	0.2	-
Quino (Ecomil, SP)	46	1.5	3.7 (2.5)	2.8 (0.7)	0.6	-
Hemp (Braham and Murray, UK)	36	1.3	2.2 (2.1)	2.4 (0.3)	0.2	Ca [*], D_2
Rice (Hain Europe, BE)	47	0.1	9.4 (4.0)	1.0 (0.1)	0.1	-
Rice (Alpro, UK)	60	0.2	12.2 (5.0)	1.2 (0.2)	0.0	Ca, B_1, B_6, B_{12}
Almond (Alpro, UK)	24	0.5	3.0 (3.0)	1.1 (0.1)	1.6	Ca, B_2, B_{12}, D_2

[1] Food Standards Agency (2002) McCance and Widdowson's The Composition of Foods, 6th Ed. Cambridge: Royal Society of Chemistry.

Garnett, 2010; Black et al., 2002). To combat these shortcomings, some plant milk substitutes are fortified with calcium and vitamins, mainly B_{12}, B_2, D and E (Table 2.8). However, consumer awareness is important as many of these products are not fortified.

Calcium absorption depends on the salt used for fortification as well as the food matrix (Rafferty et al., 2007). A comparison between cow's milk and soy milk fortified with tricalcium phosphate revealed a 75% absorption in soy milk compared to cow's milk, while no differences have been observed when calcium carbonate was used (Heaney et al., 2000; Zhao et al., 2005). Ionic calcium precipitates soy proteins especially when subjected to thermal treatments, which may influence the calcium content of the beverage consumed (Pathomrungsiyounggul et al., 2010). Indeed, 82% to 89% of the calcium in soy and rice milks, respectively, are separable by centrifugation at 3740 g, whereas the value for cow's milk is 11%, which may indicate a decrease in the calcium content of a beverage not properly shaken before use (Heaney et al., 2005). Despite these shortcomings, fortified plant milk substitutes may be a valuable source of calcium for individuals with medical conditions that prevent the consumption of dairy products, and offering soy milk as an option in elementary schools has been reported to increase the selection of a calcium rich beverage slightly (Reilly et al., 2006).

Some plant derived components have favourable health effects, that may be present in the beverages produced from that raw material. For example, replacing low fat cow's milk with oat or soy milks has been reported to decrease the plasma cholesterol and low density lipoprotein (LDL) concentrations of healthy individuals after a 4 week consumption period (Önning et al., 1998). Soy has been perceived as a

health food due to its isoflavone content with reported impacts on the prevention of e.g. cardiovascular diseases, prostate cancer and osteoporosis (Patisaul and Jefferson, 2010). The health benefits of isoflavones have however become increasingly controversial and concerns have been raised especially about maternal soy intake and the use of soy in infant formulas. Isoflavones have a complex interaction in the endocrine network, and the effect of long term effect of a soy based diet in early childhood is not known. The serum isoflavone concentration of infants on soy formula can be as high as 10-fold compared to the concentrations in Japanese adults (Patisaul and Jefferson, 2010; Andres et al., 2011).

Processing influences the nutritional properties of foods. For example, the beneficial effects of oat β-glucan on serum LDL cholesterol and postprandial glucose levels are attributed mainly to the viscosity it forms in aqueous solutions, which is sensitive to processing (Wood, 2010). Both homogenisation and thermal treatments have been reported to alter the molecular properties of oat β-glucan (Kivelä et al., 2011; Kivelä et al., 2010). No significant loss of isoflavones occurs during soy milk processing, but coagulating the soy proteins in tofu processing decreases the total isoflavones by 44% (Wang and Murphy, 1996). Another study reports a recovery of 54% isoflavones during soymilk processing and 36% for tofu production (Jackson et al., 2002).

Water-soluble vitamins can be lost if the raw material is soaked and/or blanched before the manufacturing process (Kwok and Niranjan, 1995). Also high amounts of minerals (Ca, Fe, P, Zn) (45-74%) are lost during the decanting step in oat milk production (Ca, Fe, P, Zn) and 47% of native vitamin B_6 (Zhang et al., 2007b). The destruction of heat sensitive vitamins depends on the time temperature exposure (Kwok and Niranjan, 1995). UHT treatment caused a 60% loss of D_3 after

5 s holding time, while increasing the holding time to 20 s led to a 30% decrease in B_{12} concentration. The loss of thiamine (B_1) can be minimised by favouring high temperature short time heat exposure in the manufacturing process in soy milk production (Kwok and Niranjan, 1995). Significant losses of A, D_3 and B_{12} occurred during the storage of oat milk, while the levels of folic acid and vitamins C, B_6 and B_{12} are reduced in soy milk (Zhang et al., 2007b; Kwok and Niranjan, 1995).

4.3. Impact on climate and land use

At the moment climate change is considered one of the most important and serious phenomena caused by human action. Greenhouse gases (GHG) varying in their global warming potential are a very probable cause of global warming (IPCC, 2008). GHG emissions originating from food production are remarkable: In the European Union about 29% of total contributions to global warming are estimated to come from the food chain (Huppes et al., 2008). According to FAO, livestock is responsible of 18% of the global GHG emissions, of which dairy production and processing is estimated to contribute 4% (Steinfeld et al., 2006) (Gerber, 2010). Main contributors to global warming from livestock sector are methane from enteric fermentation, nitrous oxide from manure and fertilizer, carbon dioxide from land use changes and agricultural energy use (Steinfeld et al., 2006). GHGs differ in their radiative properties and lifetimes in the atmosphere. The warming potentials are commonly expressed as CO_2 equivalents (CO_2-eq), the amount of CO_2 emission that would have the same warming effect (IPCC, 2008).

In addition to GHG emissions, another major environmental impact of food production is land use and changes in soil such as eutrophication and acidification. Fertile land is a scarce resource, and foods requiring large production areas are less

sustainable even if the direct emissions are low (Sonesson et al., 2010). On a per kg basis, the production of plant foods generally emits less GHG and requires less land than does the production of meat and dairy products (Sonesson et al., 2010; Nijdam et al., 2012).

The global warming potential of cow's milk varies in the range of 0.84–1.3 CO_2-eq/ kg product (De Vries and De Boer, 2010). Studies dealing with the GHG emissions of plant milk substitutes are scarce, but the few reports published suggest lower values compared to cow's milk. According to Smedman et al. (2010) the GHG emissions produced during a life cycle of oat and soy drinks are 0.21 and 0.31 kg CO_2-eq/kg product. The global warming potential for commercial Oatly oat milk is 0.32 g CO_2-eq/l product (Dahllöv and Gustafsson, 2008). Mikkola and Risku-Norja (2008) compared the pre-farm gate GHG emissions from optional milk production systems in Finland. The estimated emissions expressed as kg CO_2-eq per capita per year were 4-8 times higher for cow's milk compared to oat and soy milks.

The nutritional profiles of dairy and plant based products are different, which makes the direct comparison of the GHG emissions challenging. One approach is to relate the environmental impact to the protein content. Nijdam et al. (2012) evaluated the GHG emissions and land use of protein from different sources. The productions of one kg protein from milk emits 28-43 CO_2-eq and requires 26-54 m^2 land, whereas the figures are 4-10 CO_2-eq and 10-43 m^2 for pulse protein and 6-17 CO_2-eq and 4-25 m^2 for vegetable based meat analogue protein. González et al. (2011) estimated so-called protein delivery efficiency GHG values (g protein/kg CO_2-eq) for a range of foodstuffs. The values were 31 g for milk and 505 g, 359 g and 56 g for unprocessed soybean, oat and rice protein, respectively. Smedman et al. (2010) developed a so-called nutrient density to climate impact index (NDCI), aiming to

reflect the proportion of daily nutrient requirements and the contribution of each nutrient to the Swedish diet in relation to the GHG emissions. As a result the index for cow's milk was superior to oat and soy drinks. The equation used in this study has however been criticised as biased and the finding questioned by other scientists in the field (Scarborough and Rayner, 2010).

Judging from the very limited literature, plant milk substitutes have a lower impact on the climate and require less land to produce, but the issue is more complex as cow's milk contains several key nutrients (calcium, high quality protein). It is important for the plant milk industry to either formulate the products to match cow's milk in composition, or ensure that consumers are well aware of the limitations.

References

Abbottagra, 2013. Quinoa - AbbottAgra. URL: http://www.abbottagra.com/2.html (accessed 3.3.14).

Abdo, K. M., and King, K. W. 1967. Enzymic modification of the extractability of protein from soybeans, Glycine max. J. Agric. Food Chem. 15, 83-87.

Abugoch, L., E., 2009. Quinoa (*Chenopodium quinoa Willd*.): Composition, Chemistry, Nutritional, and Functional Properties. Adv. Food Nutr. Res. 58, 1–31.

Abugoch, L.E., Tapia, C., Villamán, M.C., Yazdani-Pedram, M., Díaz-Dosque, M., 2011. Characterization of quinoa protein–chitosan blend edible films. Food Hydrocoll. 25, 879–886.

Adachi, M., Kanamori, J., Masuda, T., Yagasaki, K., Kitamura, K., Mikami, B., Utsumi, S., 2003. Crystal structure of soybean 11S globulin: glycinin A3B4 homohexamer. Proc. Natl. Acad. Sci. U. S. A. 100, 7395–400.

Adachi, M., Takenaka, Y., Gidamis, A.B., Mikami, B., Utsumi, S., 2001. Crystal structure of soybean proglycinin A1aB1b homotrimer. J. Mol. Biol. 305, 291–305.

Agu, R.C., Palmer, G.H., 1997. The effect of temperature on the modification of sorghum and barley during malting. Process Biochem. 32, 501–507.

Ahamed, N., 1996. Studies on Chenopodium quinoa and Amaranthus paniculatas starch as biodegradable fillers in LDPE films. Carbohydr. Polym. 31, 157–160.

Ahamed, N.T., Singhal, R.S., Kulkarni, P.R., Pal, M., 1998. Nutrients and antinutrients in quinoa seed. Food Nutr. Bull. 19, 61–70.

Ahrens, S., Venkatachalam, M., Mistry, A. M., Lapsley, K., and Sathe, S. K. 2005. Almond (*Prunus dulcis L*.) protein quality. Plant Food Hum. Nutr. 60, 123-128.

Aidoo, H., Sakyi-Dawson, E., Abbey, L., Tano-Debrah, K., and Saalia, F. K. 2012. Optimisation of chocolate formulation using dehydrated peanut–cowpea milk to replace dairy milk. J. Sci. Food Agric. 92, 224-231.

Akinyele, I. 1991. Effect of process method on the energy and protein content, antinutritional factors and in-vitro protein digestibility of cowpea milk (*Vigna unguiculata*). Food Chem. 42, 129-134.

Almondbreeze.com, 2014. Website of the Almond Breeze brand of Blue Diamond® Growers. http://www.almondbreeze.com/ (accessed 25.9.2014).

Alpro.com, 2014. Website of the Alpro™ brand by The WhiteWave Foods Company. http://www.alpro.com (accessed 25.9.2014).

Alvarez-Jubete, L., Arendt, E.K., Gallagher, E., 2009. Nutritive value and chemical composition of pseudocereals as gluten-free ingredients. Int. J. Food Sci. Nutr. 60 Suppl 4, 240–57.

Alvarez-Jubete, L., Auty, M., Arendt, E.K., Gallagher, E., 2009. Baking properties and microstructure of pseudocereal flours in gluten-free bread formulations. Eur. Food Res. Technol. 230, 437–445.

Alvarez-Jubete, L., Wijngaard, H., Arendt, E.K., Gallagher, E., 2010. Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. Food Chem. 119, 770–778.

Ando, H., Chen, Y.-C., Tang, H., Shimizu, M., Watanabe, K., Mitsunaga, T., 2002. Food components in fractions of quinoa seed. Food Sci. Technol. Res. 8, 80–84.

Andres, S., Abraham, K., Appel, K. E., and Lampen, A. 2011. Risks and benefits of dietary isoflavones for cancer. Crit. Rev. Toxicol. 41, 1-30.

Araujo-Farro, P.C., Podadera, G., Sobral, P.J.A., Menegalli, F.C., 2010. Development of films based on quinoa (*Chenopodium quinoa*, *Willdenow*) starch. Carbohydr. Polym. 81, 839–848.

Arcot, J., Wong, S., Shrestha, A.K., 2002. Comparison of folate losses in soybean during the preparation oftempeh and soymilk. J. Sci. Food Agric. 82, 1365–1368.

Atwell, W.A., Hyldon, R.G., Godfrey, P.D., 1988. Germinated quinoa flour to reduce the viscosity of starchy foods. Cereal Chem. 65, 508–509.

Ba, K., Aguedo, M., Tine, E., Paquot, M., Destain, J., Thonart, P., 2013. Hydrolysis of starches and flours by sorghum malt amylases for dextrins production. Eur. Food Res. Technol. 236, 905–918.

Bailly, C., 2004. Active oxygen species and antioxidants in seed biology. Seed Sci. Res. 14, 93–107.

Bandla, S., Choudhary, R., Watson, D. G., and Haddock, J. 2011. UV-C treatment of soymilk in coiled tube UV reactors for inactivation of *Escherichia coli* W1485 and *Bacillus cereus* endospores. LWT - Food Sci. Technol. 46, 71-76.

Bazile, D., Fuentes, F., Mujica, A., 2013. Historical perspectives and domestication, in: Bhargava, A., Srivastava, S. (Eds.), Quinoa: Botany, Production and Uses. CABI, Wallingford.

Becker, R., Hanners, G.D., 1990. Compositional and nutritional evaluation of quinoa whole grain flour and mill fractions. LWT - Food Sci. Technol. 23, 441–444.

Bekaert, S., Storozhenko, S., Mehrshahi, P., Bennett, M.J., Lambert, W., Gregory, J.F., Schubert, K., Hugenholtz, J., Van Der Straeten, D., Hanson, A.D., 2008. Folate biofortification in food plants. Trends Plant Sci. 13, 28–35.

Berghofer, E., Schoenlechner, R., 2008. South American traditional pseudocereals. In: Thomas Gillgren (Ed.), Traditional grains for low environmental impact and good health, Gothenburg, Sweden, pp. 28–31.

Bergamo, P., Maurano, F., Mazzarella, G., Iaquinto, G., Vocca, I., Rivelli, A.R., De Falco, E., Gianfrani, C., Rossi, M., 2011. Immunological evaluation of the alcohol-soluble protein fraction from gluten-free grains in relation to celiac disease. Mol. Nutr. Food Res. 55, 1266–70.

Bewley, J.D, Black, M., 1994. Dormancy and the Control of Germination, in: Seeds - Physiology of Development and Germination. Springer US, Boston, MA, pp. 199–271.

Bewley, J.D., 1997. Seed Germination and Dormancy. Plant Cell 9, 1055–1066.

Bewley, J.D., Black, M., 1994a. Germination, Structure and Composition, in: Seeds - Physiology of Development and Germination. Plenum Press, New York, pp. 1–33.

Bewley, J.D., Black, M., 1994b. Cellular events during germination and growth, in: Seeds - Physiology of Development and Germination. Plenum Press, New York, pp. 147–197.

Bewley, J.D., Black, M., 1994c. Mobilization of stored seed reserves, in: Seeds - Physiology of Development and Germination. Plenum Press, New York, pp. 293–343.

Black, R. E., Williams, S. M., Jones, I. E., and Goulding, A. 2002. Children who avoid drinking cow milk have low dietary calcium intakes and poor bone health. Am. J. Clin. Nutr. 76, 675-680.

Bode, S., and Gudmand-Høyer, E. 1988. Incidence and clinical significance of lactose malabsorption in adult coeliac disease. Scand. J. Gastroenterol. 23, 484-488.

Bonifacio, A., 2003. Chenopodium Sp.: Genetic Resources, Ethnobotany, and Geographic Distribution. Food Rev. Int. 19, 1–7.

Briggs, D.E., 1998. Malts and Malting. Blackie Academic and Professional, London, UK.

Brinegar, C., Goundan, S., 1993. Isolation and characterization of chenopodin, the 11S seed storage protein of quinoa (*Chenopodium quinoa*). J. Agric. Food Chem. 41.

Brinegar, C., Sine, B., Nwokocha, L., 1996. High-Cysteine 2S Seed Storage Proteins from Quinoa (*Chenopodium quinoa*). J. Agric. Food Chem. 44, 1621–1623.

Brockington, S.F., Walker, R.H., Glover, B.J., Soltis, P.S., Soltis, D.E., 2011. Complex pigment evolution in the Caryophyllales. New Phytol. 190, 854–864.

Buléon, A., Colonna, P., Planchot, V., Ball, S., 1998. Starch granules: structure and biosynthesis. Int. J. Biol. Macromol. 23, 85–112.

Bus, A., Worsley, A. 2003. Consumers' health perceptions of three types of milk: a survey in Australia. Appetite. 40, 93-100.

Buttriss, J.L., Stokes, C.S., 2008. Dietary fibre and health: an overview. Nutr. Bull. 33, 186–200.

Cabrejas, M., Diaz, M., Aguilera, Y., Benitez, V., Molla, E., Esteban, R., 2008. Influence of germination on the soluble carbohydrates and dietary fibre fractions in non-conventional legumes. Food Chem. 107, 1045–1052.

Camire, M.E., 1991. Protein functionality modification by extrusion cooking. J. Am. Oil Chem. Soc. 68, 200–205.

Caperuto, L.C., Amaya-Farfan, J., Camargo, C.R., 2001. Performance of quinoa (*Chenopodium quinoa Willd*.) flour in the manufacture of gluten-free spaghetti. J. Sci. Food Agric. 81, 95–101.

Carlshamnmejeri.se, 2014. Website of the Carlshamn brand of O. Kavli AB. http://www.carlshamnmejeri.se (accessed 25.9.2014).

Carvalho, N. F., Kenney, R. D., Carrington, P. H., and Hall, D. E. 2001. Severe nutritional deficiencies in toddlers resulting from health food milk alternatives. Pediatrics. 107, 46.

Casey, R., 1999. Distribution and some properties of seed globulins, in: Shewry, P.. (Ed.), Seed Proteins. Springer Netherlands, pp. 159–169.

Catassi, C., Fasano, A., 2008. Celiac disease. Curr. Opin. Gastroenterol. 24, 687–691.

Ceppi, E.L.M., Brenna, O. V, 2010. Experimental studies to obtain rice malt. J. Agric. Food Chem. 58, 7701–7.

Chang, K.C., Harrold, R.L., 1988. Changes in selected biochemical components, *in vitro* protein digestibility and amino acids in two bean cultivars during germination. J. Food Sci. 53, 783–787.

Charalampopoulos, D. Pandiella, S.S., and Webb, C. 2002. Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. J Appl. Microbiol. 92, 851-859.

Chauhan, G.S., Eskin, N.A.M., Tkachuk, R., 1992. Nutrients and antinutrients in quinoa seed. Cereal Chem. 69, 85–88.

Chen, S., 1989. Preparation of fluid soymilk. In: Applewhite, T. N., (Ed.), Proceedings of the World Congress on Vegetable protein Utilization in

Human Foods and Animal Feedstuffs. American Oil Chemists Society, Champaign, Illinois, pp. 341-351.

Cheng, Y., Thompson, L., and Brittin, H., 2006. Sogurt, a yogurt-like soybean product: development and properties. J. Food Sci. 55, 1178-1179.

Chillo, S., Laverse, J., Falcone, P.M., Del Nobile, M.A., 2008. Quality of spaghetti in base amaranthus wholemeal flour added with quinoa, broad bean and chick pea. J. Food Eng. 84, 101–107.

Chronakis, I. S., Fredholm, A., Triantafyllou, A. Ö., and Öste, R., 2004. Complex formation in aqueous medium of partially hydrolysed oat cereal proteins with sodium stearoyl-2 lactylate (SSL) lipid surfactant and implications for bile acids activity. Colloids Surf., B. 35, 175-184.

Codex Alimentarius. 1999. CODEX STAN 206-1999.

Cordeiro, L.M.C., Reinhardt, V. de F., Baggio, C.H., Werner, M.F. de P., Burci, L.M., Sassaki, G.L., Iacomini, M., 2012. Arabinan and arabinan-rich pectic polysaccharides from quinoa (Chenopodium quinoa) seeds: Structure and gastroprotective activity. Food Chem. 130, 937–944.

Cortés, C., Esteve, M., Frıgola, A., and Torregrosa, F., 2005. Quality characteristics of horchata (a Spanish vegetable beverage) treated with pulsed electric fields during shelf-life. Food Chem. 91, 319-325.

Crittenden, R. G., and Bennett, L. E., 2005. Cow's milk allergy: a complex disorder. J. Am. Coll. Nutr. 24, 582-591.

Cruz, N., Capellas, M., Hernandez, M., Trujillo, A., Guamis, B., and Ferragut, V., 2007. Ultra high pressure homogenization of soymilk: Microbiological, physicochemical and microstructural characteristics. Food Res. Int. 40, 725-732.

Cunnane, S.C., Ganguli, S., Menard, C., Liede, A.C., Hamadeh, M.J., Chen, Z.-Y., Wolever, T.M.S., Jenkins, D.J.A., 2007. High α-linolenic acid flaxseed (*Linum usitatissimum*):some nutritional properties in humans. Br. J. Nutr. 69, 443.

Dahllöv, O., and Gustafsson, M., 2008. Livscykelanalys av Oatly havredryck. M. Sc. thesis. Lund University, Lund, Sweden.

De Boland, A.R., Garner, G.B., O'Dell, B.L., 1975. Identification and properties of phytate in cereal grains and oilseed products. J. Agric. Food Chem. 23, 1186–1189.

De Bree, A., van Dusseldorp, M., Brouwer, I.A., van het Hof, K.H., Steegers-Theunissen, R.P.M., 1997. Folate intake in Europe: recommended, actual and desired intake. Eur. J. Clin. Nutr. 51, 643–660.

De Vries, M., De Boer, I., 2010. Comparing environmental impacts for livestock products: A review of life cycle assessments. Livest.Sci. 128, 1-11.

Debruyne, I., 2006 Soy base extract: soymilk and dairy alternatives. In: Soy applications in foods. Riaz, M. N., (Ed.), Taylor & Francis, Boca Raton, Florida, U.S.A, pp. 111-134.

Delcour, J., Hoseney, R.C., 2010. Principles of Cereal Science and Technology, 3rd ed, Principles of Cereal Science and Technology. AACC International. Inc, St. Paul.

Diarra, K., Nong, Z. G., and Jie, C., 2005. Peanut milk and peanut milk based products production: A review. Crit. Rev. Food. Sci. Nutr. 45, 405-423.

Dini, I., Tenore, G.C., Dini, A., 2002. Oleanane saponins in "Kancolla", a sweet variety of *Chenopodium quinoa*. J. Nat. Prod. 65, 1023–1026.

Dogan, H., Karwe, M. V., 2003. Physicochemical properties of quinoa extrudates. Food Sci. Technol. Int. 9, 101–114.

Donkor, O., Henriksson, A., Vasiljevic, T., and Shah, N. P., 2007. α-Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk. Food Chem. 104, 10-20.

Duodu, K.G., Taylor, J.R., 2012. The quality of breads made with non-wheat flours, in: Cauvain, S.P. (Ed.), Breadmaking. Woodhead Publishing, Cambridge, UK, pp. 754–782.

Durand, A., Franks, G., and Hosken, R., 2003. Particle sizes and stability of UHT bovine, cereal and grain milks. Food Hydrocoll. 17, 671-678.

Egli, I., Davidsson, L., Juillerat, M.A., Barclay, D., Hurrell, R.F., 2002. The influence of soaking and germination on the phytase activity and phytic acid content of grains and seeds potentially useful for complementary feeding. J. Food Sci. 67, 3484–3488.

Elgeti, D., Nordlohne, S.D., Föste, M., Besl, M., Linden, M.H., Heinz, V., Jekle, M., Becker, T., 2014. Volume and texture improvement of gluten-free bread using quinoa white flour. J. Cereal Sci. 59, 41–47.

Elkhalil, E.A., El Tinay, A., Mohamed, B., Elsheikh, E.A., 2001. Effect of malt pretreatment on phytic acid and in vitro protein digestibility of sorghum flour. Food Chem. 72, 29–32.

Eriksen, S., 1983. Application of enzymes in soy milk production to improve yield. J. Food. Sci. 48, 445-447.

Escudero, N., De Arellano, M., Luco, J., Giménez, M., and Mucciarelli, S., 2004. Comparison of the chemical composition and nutritional value of Amaranthus cruentus flour and its protein concentrate. Plant Food Hum. Nutr. 59, 15-21.

FAO, 1998. The State of the World's Plant Genetic Resources for Food and Agriculture. FAO, Rome, Italy.

FAO, 2011. Dietary protein quality evaluation in human nutrition - Report of an FAO expert consultation. FAO Food and nutrition paper 92. Auckland, New Zealand.

Finch-Savage, W.E., Leubner-Metzger, G., 2006. Seed dormancy and the control of germination. New Phytol. 171, 501–23.

Friedman, M., 1996. Nutritional value of proteins from different food sources. A review. J. Agric. Food. Chem. 44, 6-29.

Friedman, M., Brandon, D. L., Bates, A. H., Hymowitz, T., 1991. Comparison of a commercial soybean cultivar and an isoline lacking the Kunitz trypsin inhibitor: composition, nutritional value, and effects of heating. J. Agric. Food Chem. 39, 327-335.

Galwey, N., 1995. Quinoa and relatives. In: Evolution of crop plants. Longman scientific and technical, Harlow, pp. 41-46.

Gambuś, H., Gambuś, F., Wrona, P., Pastuszka, D., Ziobro, R., Nowotna, A., Kopeć, A., Sikora, M., 2009. Enrichment of gluten-free rolls with amaranth and flaxseed increases the concentration of calcium and phosphorus in the bones of rats. Polish J. Food Nutr. Sci. 59.

Garcia, L. A., Alfaro, M. A., and Bressani, R., 1987. Digestibility and protein quality of raw and heat-processed defatted and nondefatted flours prepared with three amaranth species. J. Agric. Food. Chem. 35, 604-607.

Gebremariam, M.M., Zarnkow, M., Becker, T., 2012. Effect of drying temperature and time on alpha-amylase, beta-amylase, limit dextrinase activities and dimethyl sulphide level of teff (*Eragrostis tef*) malt. Food Bioprocess Technol. 6, 3462–3472.

Gerber, P., Vellinga, T., Opio, C., Henderson, B., Steinfeld, H., 2010. Greenhouse gas emissions from the dairy Sector - a life cycle assessment. Food and Agriculture Organization of the United Nations, Rome, Italy.

Giacomino, S., Peñas, E., Ferreyra, V., Pellegrino, N., Fournier, M., Apro, N., Carrión, M.O., Frias, J., 2013. Extruded flaxseed meal enhances the nutritional quality of cereal-based products. Plant Foods Hum. Nutr. 68, 131–6.

Giami, S.Y., Chibor, B.S., Edebiri, K.E., Achinewhu, S.C., 1999. Changes in nitrogenous and other chemical constituents, protein fractions and in vitro protein digestibility of germinating fluted pumpkin (*Telfairia occidentalis Hook*) seed. Plant Foods Hum. Nutr. 53, 333–342.

Gillgren, T., Stading, M., 2007. Non-food use of cereal by-products. Proceedings of Traditional Grains for Low Environmental Impact and Good Health. Pretoria, South Africa.

Giri, S., Mangaraj, S., 2012. Processing Influences on Composition and Quality Attributes of Soymilk and its Powder. Food Eng. Rev. 4, 149-164.

Glitsø, L.V., Bach Knudsen, K.E., 1999. Milling of whole grain rye to obtain fractions with different dietary fibre characteristics. J. Cereal Sci. 29, 89–97.

González, A. D., Frostell, B., Carlsson-Kanyama, A., 2011. Protein efficiency per unit energy and per unit greenhouse gas emissions: Potential contribution of diet choices to climate change mitigation. Food Policy. 36, 562-570.

Granato, D., Ribeiro, J.C.B., Castro, I.A., Masson, M.L., 2010. Sensory evaluation and physicochemical optimisation of soy-based desserts using response surface methodology. Food Chem. 121, 899–906.

Gray, D.A., Auerbach, R.H., Hill, S., Wang, R., Campbell, G.M., Webb, C., South, J.B., 2000. Enrichment of oat antioxidant activity by dry milling and sieving. J. Cereal Sci. 32, 89–98.

Grieshop, C.M., Fahey, G.C., 2001. Comparison of quality characteristics of soybeans from Brazil, China, and the United States. J. Agric. Food Chem. 49, 2669–2673.

Griffith, L.D., Castell-Perez, M.E., Griffith, M.E., 1998. Effects of blend and processing method on the nutritional quality of weaning foods made from select cereals and legumes. Cereal Chem. 75, 105–112.

Gualberto, D.G., Bergman, C.J., Kazemzadeh, M., Weber, C.W., 1997. Effect of extrusion processing on the soluble and insoluble fiber, and phytic acid contents of cereal brans. Plant Foods Hum. Nutr. 51, 187–198.

Habib, H., Fazili, K., 2007. Plant protease inhibitors: a defense strategy in plants. Biotechnol. Mol. Biol. Rev. 2, 68–85.

Hager, A.-S., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., 2013. Starch properties, in vitro digestibility and sensory evaluation of fresh egg pasta produced from oat, teff and wheat flour. J. Cereal Sci. 58, 156–163.

Hager, A.-S., Taylor, J.P., Waters, D.M., Arendt, E.K., 2014. Gluten free beer – A review. Trends Food Sci. Technol. 36, 44–54.

Hager, A.-S., Wolter, A., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., Czerny, M., 2012a. Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. Eur. Food Res. Technol. 235, 333–344.

Hager, A.-S., Wolter, A., Jacob, F., Zannini, E., Arendt, E.K., 2012b. Nutritional properties and ultra-structure of commercial gluten free flours from different botanical sources compared to wheat flours. J. Cereal Sci. 56, 239–247.

Hager, A.-S., Zannini, E., Arendt, E.K., 2012a. Formulating breads for specific dietary requirements, in: Cauvain, S. (Ed.), Breadmaking. Woodhead Publishing, Cambridge, UK, pp. 711–735.

Hager, A.-S., Zannini, E., Arendt, E.K., 2012b. Gluten-free pasta - Advances in research and commercialisation. Cereal Foods World 57, 225–229.

Hallén, E., İbanoğlu, Ş., Ainsworth, P., 2004. Effect of fermented/germinated cowpea flour addition on the rheological and baking properties of wheat flour. J. Food Eng. 63, 177–184.

Hao, Y. Y., Brackett, R., 1988. Removal of aflatoxin B1 from peanut milk inoculated with Flavobacterium aurantiacum. J. Food Sci. 53, 1384-1386.

Hariadi, Y., Marandon, K., Tian, Y., Jacobsen, S.-E., Shabala, S., 2011. Ionic and osmotic relations in quinoa (*Chenopodium quinoa Willd*.) plants grown at various salinity levels. J. Exp. Bot. 62, 185–93.

Heaney, R. P., Dowell, M. S., Rafferty, K., Bierman, J., 2000. Bioavailability of the calcium in fortified soy imitation milk, with some observations on method. Am. J. Clin. Nutr. 71, 1166-1169.

Heaney, R. P., Rafferty, K., Bierman, J., 2005. Not all calcium-fortified beverages are equal. Nutr. Today. 40, 39-44.

Heiniö, R.-L., Katina, K., Wilhelmson, A., Myllymäki, O., Rajamäki, T., Latva-Kala, K., Liukkonen, K.-H., Poutanen, K., 2003. Relationship between sensory perception and flavour-active volatile compounds of germinated, sourdough fermented and native rye following the extrusion process. LWT - Food Sci. Technol. 36, 533–545.

Heiniö, R.-L., Oksman-Caldentey, K.-M., Latva-Kala, K., Lehtinen, P., Poutanen, K., 2001. Effect of drying treatment conditions on sensory profile of germinated oat. Cereal Chem. 78, 707–714.

Heiser, C.B.. J., Nelson, D.C., 1974. On the origin of the cultivated chenopods (*Chenopodium*). Genetics 78, 503–505.

Her, B.Y., Sung, H.Y., Choi, Y.S., 2005. Oligosaccharide-supplemented soy ice cream for diabetic patients : Quality characteristics and effects on blood sugar and lipids in streptozotocin-induced diabetic rats. Korean J. Nutr. 38, 663–671.

Hermann, 2007. INIA Genebank, Peru, http://commons.wikimedia.org/wiki/File:Colored_quinoa_Genebank_INIA_Juliaca.jpg (accessed 1.3.2014).

Hídvégi, M., Lásztity, R., 2003. Phytic acid content of cereals and legumes and interaction with proteins. Period. Polytech. Chem. Eng. 46, 59–64.

Hilhorst, H.W.M., Karssen, C.M., 1992. Seed dormancy and germination: the role of abscisic acid and gibberellins and the importance of hormone mutants. Plant Growth Regul. 11, 225–238.

Hinds, M. J., Beuchat, L. R., and Chinnan, M. S., 1997a. Properties of a thermal-processed beverage prepared from roasted partially defatted peanuts. International Journal of Food Science and Technology, 32(3), 203-211.

Hinds, M., Beuchat, L., and Chinnan, M., 1997b. Effects of homogenization pressure and stabilizers on some physical characteristics of a beverage prepared from partially defatted, roasted peanuts. Plant Food Hum. Nutr. 50 : 269-277.

Hinds, M., Chinnan, M., and Beuchat, L., 1997c. Particle size distribution in a heat-processed beverage prepared from roasted peanuts. Food Res. Int. 30 : 59-64.

Hischke, H., Potter, G., and Graham, W., 1968. Nutritive value of oat proteins. I. Varietal differences as measured by amino acid analysis and rat growth responses. Cereal Chem. 45 : 374-378.

House, J. D., Neufeld, J., and Leson, G., 2010. Evaluating the quality of protein from hemp seed (Cannabis sativa L.) products through the use of the protein digestibility-corrected amino acid score method. J. Agric. Food Chem. 24 : 11801-11807.

Hu, Y., Stromeck, A., Loponen, J., Lopes-Lutz, D., Schieber, A., and Gänzle, M. G., 2011. LC-MS/MS Quantification of Bioactive Angiotensin I-Converting Enzyme Inhibitory Peptides in Rye Malt Sourdoughs. J. Agric. Food. Chem. 59 : 11983-11989.

Huppes, G., Koning, A., Suh, S., Heijungs, R., Oers, L., Nielsen, P., and Guinée, J. B., 2008. Environmental Impacts of Consumption in the European Union: High-Resolution Input-Output Tables with Detailed Environmental Extensions. J. Ind. Ecol. 10 : 129-146.

Hübner, F., Schehl, B.D., Gebruers, K., Courtin, C.M., Delcour, J.A., Arendt, E.K., 2010. Influence of germination time and temperature on the properties of rye malt and rye malt based worts. J. Cereal Sci. 52, 72–79.

Hughes, G.J., Ryan, D.J., Mukherjea, R., Schasteen, C.S., 2011. Protein digestibility-corrected amino acid scores (PDCAAS) for soy protein isolates and concentrate: criteria for evaluation. J. Agric. Food Chem. 59, 12707–12.

IPCC, 2007. Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. IPCC, Geneva, Switzerland, 10.

Jackson, C. J. C., Dini, J., Lavandier, C., Rupasinghe, H., Faulkner, H., Poysa, V., Buzzell, D., DeGrandis, S., 2002. Effects of processing on the content and composition of isoflavones during manufacturing of soy beverage and tofu. Process Biochem. 37, 1117-1123.

Jacobsen, S.-E., 2003. The Worldwide Potential for Quinoa (*Chenopodium quinoa Willd.*). Food Rev. Int. 19, 167–177.

Jacobsen, S.-E., Monteros, C., Corcuera, L.J., Bravo, L.A., Christiansen, J.L., Mujica, A., 2007. Frost resistance mechanisms in quinoa (*Chenopodium quinoa Willd.*). Eur. J. Agron. 26, 471–475.

Jacobsen, S.E., Mujica, A., Ortiz, R., 2003. The Global Potential for Quinoa and Other Andean Crops. Food Rev. Int. 19, 139–148.

Jago, D., 2011. Free from foods - Mintel report. FreeFrom Allergy and Intolerance 2011; FDIN seminar. Daventry, UK.

Jiménez-Martínez, C., Hernández-Sánchez, H. And Dávila-Ortiz, G., 2003. Production of a yogurt-like product from Lupinus campestris seeds. J. Sci. Food. Agric. 83, 515–522.

Johnson, L., Suleiman, T., and Lusas, E., 1979. Sesame protein: A review and prospectus. J. Am. Oil Chem. Soc. 56, 463-468.

Juliano, B. O., Bressani, R., and Elias, L. G., 1971. Evaluation of the protein quality and milled rices differing in protein content. J. Agric. Food Chem. 19, 1028-1034.

Kaldy, M., Kasting, R., 1974. Amino acid composition and protein quality of eight faba bean cultivars. Can. J. Plant Sci. i. 54, 869-871.

Kamal-Eldin, A., Appelqvist, L.-Å., 1996. The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids 31, 671–701.

Katan, M.B., Grundy, S.M., Jones, P., Law, M., Miettinen, T., Paoletti, R., 2003. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin. Proc. 78, 965–78.

Katina, K., Liukkonen, K.-H., Kaukovirta-Norja, A., Adlercreutz, H., Heinonen, S.-M., Lampi, A.-M., Pihlava, J.-M., Poutanen, K., 2007. Fermentation-induced changes in the nutritional value of native or germinated rye. J. Cereal Sci. 46, 348–355.

Katz, K. A., Mahlberg, M. H., Honig, P. J., and Yan, A. C., 2005. Rice nightmare: Kwashiorkor in 2 Philadelphia-area infants fed Rice Dream beverage. J. Am. Acad. Dermatol. 52, 69-72.

Kaukovirta-Norja, A., Wilhelmson, A., Poutanen, K., 2004. Germination: a means to improve the functionality of oat. Agric. Food Sci.

Kim, H., Bang, J., Kim, Y., Beuchat, L., and Ryu, J. H., 2012. Reduction of Bacillus cereus spores in sikhye, a traditional Korean rice beverage, by modified tyndallization processes with and without carbon dioxide injection. Lett. Appl. Microbiol. 55, 218-223.

Kivelä, R., Pitkänen, L., Laine, P., Aseyev, V., and Sontag-Strohm, T., 2010. Influence of homogenisation on the solution properties of oat β-glucan. Food Hydrocolloid. 24 : 611-618.

Kivelä, R., Sontag-Strohm, T., Loponen, J., Tuomainen, P., and Nyström, L., 2011. Oxidative and radical mediated cleavage of β-glucan in thermal treatments. Carbohyd. Polym. 85, 645–652.

Klose, C., Mauch, A., Wunderlich, S., Thiele, F., Zarnkow, M., Jacob, F., Arendt, E.K., 2011. Brewing with 100% Oat Malt. J. Inst. Brew. 117, 411–421.

Kneepkens, C. M. F., and Meijer, Y., 2009. Clinical practice. Diagnosis and treatment of cow's milk allergy. Eur. J. Pediatr. 168, 891-896.

Konishi, Y., Hirano, S., Tsuboi, H., Wada, M., 2004. Distribution of Minerals in Quinoa (*Chenopodium quinoa Willd*.) Seeds. Biosci. Biotechnol. Biochem. 68, 231–234.

Kritchevsky, D., Chen, S.C., 2005. Phytosterols—health benefits and potential concerns: a review. Nutr. Res. 25, 413–428.

Kwok, K. I. N. C., and Niranjan, K., 1995. Review: Effect of thermal processing on soymilk. Int. J. Food Sci. Technol. 30, 263-295.

Larrea, C., Freire, W., 2002. Social inequality and child malnutrition in four Andean countries. Rev. Panam. Salud Pública 11, 356–364.

Larsson, M., Rossander-Hulthén, L., Sandström, B., Sandberg, A.S., 1996. Improved zinc and iron absorption from breakfast meals containing malted oats with reduced phytate content. Br. J. Nutr. 76, 677–88.

Lattimer, J.M., Haub, M.D., 2010. Effects of dietary fiber and its components on metabolic health. Nutrients 2, 1266–89.

Lawton, J.W., 2000. Nonfood uses of cereals, in: Kulp, K. (Ed.), Handbook of Cereal Science and Technology. CRC Press, pp. 725–740.

Leatherhead Food Research., 2011. Food Allergies and Intolerances: Consumer Perceptions and Market Opportunities for 'Free From' Foods. Leatherhead Food International, Surrey, UK.

Lee, C., and Beuchat, L. R., 1991. Changes in chemical composition and sensory qualities of peanut milk fermented with lactic acid bacteria. Int. J. Food Microbiol. 13, 273-283.

Lee, S. W., and Rhee, C., 2003. Processing suitability of a rice and pine nut (*Pinus koraiensis*) beverage. Food Hydrocolloid. 17, 379-385.

Leroy, F., and De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15, 67-78.

Li, X., Dodson, J., Zhou, X., Zhang, H., Masutomoto, R., 2007. Early cultivated wheat and broadening of agriculture in Neolithic China. The Holocene 17, 555–560.

Liener, I.E., 1970. Toxic constituents of plant foodstuffs. Proc. Nutr. Soc. 29, 56–57.

Lindahl, L., Ahldén, I., Öste, R., and Sjöholm, I., 2001. Homogeneous and stable cereal suspension. United States Patent No. 5686123.

Lindeboom, N., Chang, P.R., Falk, K.C., Tyler, R.T., 2005. Characteristics of Starch from Eight Quinoa Lines. Cereal Chem. 82, 216–222.

Liong, M.-T., Easa, A.M., Lim, P.-T., Kang, J.-Y., 2009. Survival, growth characteristics and bioactive potential of Lactobacillus acidophilus in a soy-based cream cheese. J. Sci. Food Agric. 89, 1382–1391.

Liukkonen, K.-H., Katina, K., Wilhelmsson, A., Myllymäki, O., Lampi, A.-M., Kariluoto, S., Piironen, V., Heinonen, S.-M., Nurmi, T., Adlercreutz, H., Peltoketo, A., Pihlava, J.-M., Hietaniemi, V., Poutanen, K., 2003. Process-induced changes on bioactive compounds in whole grain rye. Proc. Nutr. Soc. 62, 117–22.

Loponen, J., 2004. Angiotensin converting enzyme inhibitory peptides in Finnish cereals: a database survey. Agric Food Sci. 13, 39-45.

Loponen, J., Kanerva, P., Zhang, C., Sontag-Strohm, T., Salovaara, H., Gänzle, M.G., 2009. Prolamin hydrolysis and pentosan solubilization in germinated-rye sourdoughs determined by chromatographic and immunological methods. J. Agric. Food Chem. 57, 746–53.

Lorenz, K., 1990. Quinoa (*Chenopodium quinoa*) Starch — Physico-chemical Properties and Functional Characteristics. Starch - Stärke 42, 81–86.

Lorenz, K., Coulter, L., 1991. Quinoa flour in baked products. Plant Foods Hum. Nutr. 41, 213–223.

Maga, J.A., 1982. Phytate: its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. J. Agric. Food Chem. 30, 1–9.

Malaki Nik, A., Tosh, S., Poysa, V., Woodrow, L., and Corredig, M., 2008. Physicochemical characterization of soymilk after step-wise centrifugation. Food Res Int. 41 : 286-294.

Marcone, M.F., 1999. Biochemical and biophysical properties of plant storage proteins. Food Res. Int. 32, 79–92.

Mastebroek, H.D., Limburg, H., Gilles, T., Marvin, H.J., 2000. Occurrence of sapogenins in leaves and seeds of quinoa (*Chenopodium quinoa Willd*). J. Sci. Food Agric. 80, 152–156.

Mbithi-Mwikya, S., Van Camp, J., Yiru, Y., Huyghebaert, A., 2000. Nutrient and Antinutrient Changes in Finger Millet (Eleusine coracan) During Sprouting. LWT - Food Sci. Technol. 33, 9–14.

McSweeney, S. L., Mulvihill, D. M., O'Callaghan, D. M., 2004. The influence of pH on the heat-induced aggregation of model milk protein ingredient systems and model infant formula emulsions stabilized by milk protein ingredients. Food Hydrocolloid. 18, 109-125.

Meo, B., Freeman, G., Marconi, O., Booer, C., Perretti, G., Fantozzi, P., 2011. Behaviour of Malted Cereals and Pseudo-Cereals for Gluten-Free Beer Production. J. Inst. Brew. 117, 541–546.

Michaelsen, K. F., Hoppe, C., Roos, N., Kaestel, P., Stougaard, M., Lauritzen, L., Molgaard, C., Girma, T., and Friis, H., 2009. Choice of foods and ingredients for moderately malnourished children 6 months to 5 years of age. Food Nutr. Bull. 30, 343.

Mikkola, M., and Risku-Norja, H., 2008. Institutional consumers views of GHG emission reduction in optional milk systems within sustainability frame. 8th European IFSA Symposium, Clermont-Ferrand, France.

Millward, J., and Garnett, T., 2010. Plenary Lecture 3 Food and the planet: nutritional dilemmas of greenhouse gas emission reductions through reduced intakes of meat and dairy foods. Nutr. Soc. Proc. 69 : 103-118.

Mintel 2011. In the shadow of competition, the soy market slumps. Mintel Press Release. URL: http://www.mintel.com/press-centre/press-releases/696/in-the-shadow-of-competition-the-soy-market-slumps (accessed 7.8.2011).

Mishkin, S., 1997. Dairy sensitivity, lactose malabsorption, and elimination diets in inflammatory bowel disease. Am. J. Clin. Nutr. 65, 564-567.

Mitchell, C. R., and Mitchell, P.R., 1990. Nutritional rice milk product. United States Patent No. 4894242.

Molina, M.I., Petruccelli, S., Añón, M.C., 2004. Effect of pH and ionic strength modifications on thermal denaturation of the 11S globulin of sunflower (*Helianthus annuus*). J. Agric. Food Chem. 52, 6023–9.

Morad, M.M., Leung, H.K., Hsu, D.L., Finney, P.L., 1980. Effect of germination on physicochemical and bread-baking properties of yellow pea, lentil, and faba bean flours and starches. Cereal Chem. 57, 390–396.

Murdock, L.L., Shade, R.E., 2002. Lectins and Protease Inhibitors as Plant Defenses against Insects. J. Agric. Food Chem. 50, 6605–6611.

Mårtensson, O., Öste, R., and Holst, O, 2000. Lactic acid bacteria in an oat-based non-dairy milk substitute: fermentation characteristics and exopolysaccharide formation. LWT - Food Sci. Technol. 33 : 525-530.

Mårtensson, O., Öste, R., and Holst, O., 2002. The effect of yoghurt culture on the survival of probiotic bacteria in oat-based, non-dairy products. Food Res Int. 35 : 775-784.

Mäkinen, O.E., Arendt, E.K., 2012. Oat malt as a baking ingredient – A comparative study of the impact of oat, barley and wheat malts on bread and dough properties. J. Cereal Sci. 56, 747–753.

Nijdam, D., Rood, T., and Westhoek, H., 2012. The price of protein: Review of land use and carbon footprints from life cycle assessments of animal food products and their substitutes. Food Policy. 37, 760-770.

Nnanna, I.A., Phillips, R.D., 1990. Protein and Starch Digestibility and Flatulence Potential of Germinated Cowpeas (*Vigna unguiculata*). J. Food Sci. 55, 151–153.

Normén, L., Bryngelsson, S., Johnsson, M., Evheden, P., Ellegård, L., Brants, H., Andersson, H., Dutta, P., 2002. The Phytosterol Content of Some Cereal Foods Commonly Consumed in Sweden and in the Netherlands. J. Food Compos. Anal. 15, 693–704.

Oatly.com, 2014. Website of Oatly AB. http://www.oatly.com (accessed 25.9.2014).

Obatolu, V.A., 2002. Nutrient and sensory qualities of extruded malted or unmalted millet/soybean mixture. Food Chem. 76, 129–133.

Oksman-Caldentey, K.-M., Kaukovirta-Norja, A., Heiniö, R.-L., Kleemola, T., Mikola, M., Sontag-Strohm, T., Lehtinen, P., Pihlava, J.-M., Poutanen, K., 2001. Kauran biotekninen prosessointi uusiksi elintarvikkeiksi, in: Ryhänen, E.-L., Salo, R. (Eds.), Food Cluster Research Programme, Final Report, MTT Publications. Jokioinen, Finland, pp. 11–22.

Organic Monitor., 2005. The European market for soya and non-dairy drinks. Organic Monitor, London, UK.

Osbourn, A., 1996. Saponins and plant defence - a soap story. Trends Plant Sci. 1, 4–9.

Packaged Facts., 2012. Dairy Alternative Beverages in the U.S.: Soy Milk, Almond Milk, Rice Milk and Other Dairy Milk Alternatives. Packaged Facts, Rockville, Maryland, U.S.A.

Park, S.H., 2005. Dough and Breadmaking Properties of Wheat Flour Substituted by 10% with Germinated Quinoa Flour. Food Sci. Technol. Int. 11, 471–476.

Parnsakhorn, S.Langkapin, J., 2013. Changes in physicochemical characteristics of germinated brown rice and brown rice during storage at various temperatures. Agric. Eng. Int. 15, 293–303.

Pathomrungsiyoungul, P., Lewis, M. J., and Grandison, A. S., 2010. Effects of calcium-chelating agents and pasteurisation on certain properties of calcium-fortified soy milk. Food Chem. 118 : 808-814.

Patisaul, H. B., and Jefferson, W., 2010. The pros and cons of phytoestrogens. Front. Neuroendocrin. 31 : 400-419.

Paulsen, P. V., Welsby, D., and Huang, X. L., 2006. Ready-to-Drink soy protein nutritional beverages. In : Soy applications in foods, pp. 199-226. Riaz, M. N., Ed., Taylor & Francis, Boca Raton, Florida, U.S.A.

Pedo, I., Sgarbieri, V., and Gutkoski, L., 1999. Protein evaluation of four oat (*Avena sativa L.*) cultivars adapted for cultivation in the south of Brazil. Plant Food Hum. Nutr. 53 : 297-304.

Peñaloza, W., Davey, C.L., Hedger, J.N., Kell, D.B., 1992. Physiological studies on the solid-state quinoa tempe fermentation, using on-line measurements of fungal biomass production. J. Sci. Food Agric. 59, 227–235.

Phiarais, B.P.N., Mauch, A., Schehl, B.D., Zarnkow, M., Gastl, M., Herrmann, M., Zannini, E., Arendt, E.K., 2010. Processing of a Top Fermented Beer Brewed from 100% Buckwheat Malt with Sensory and Analytical Characterisation. J. Inst. Brew. 116, 265–274.

Phiarais, B.P.N., Schehl, B.D., Oliveira, J.C., Arendt, E.K., 2006. Use of Response Surface Methodology to Investigate the Effectiveness of Commercial Enzymes on Buckwheat Malt for Brewing Purposes. J. Inst. Brew. 112, 324–332.

Phiarais, B.P.N., Wijngaard, H.H., Arendt, E.K., 2005. The Impact of Kilning on Enzymatic Activity of Buckwheat Malt. J. Inst. Brew. 111, 290–298.

Phillips, L., Whitehead, D., and Kinsella, J., 1994. Chemical nature of proteins and polypeptides. In: Structure-Function Properties of Food Proteins, pp. 3-23. Academic Press: San Diego, CA.

Pike, P.R., Abdel-Aal, E.-S.M., McElroy, A.R., 2007. Antioxidant Activity of Oat Malt Extracts in Accelerated Corn Oil Oxidation. J. Am. Oil Chem. Soc. 84, 663–667.

Plaza, L., Ancos, B. de, Cano, P.M., 2003. Nutritional and health-related compounds in sprouts and seeds of soybean (*Glycine max*), wheat (*Triticum aestivum.L*) and alfalfa (*Medicago sativa*) treated by a new drying method. Eur. Food Res. Technol. 216, 138–144.

Pomeranz, Y., Robbins, G.S., Briggle, L.W., 1971. Amino acid composition of oat groats. J. Agric. Food Chem. 19, 536–539.

Prado, F. C., Parada, J. L., Pandey, A., Soccol, C. R., 2008. Trends in non-dairy probiotic beverages. Food Re. Int. 41, 111-123.

Prakash, C.S., 2001. The Genetically modified crop debate in the context of agricultural evolution. Plant Physiol. 126, 8–15.

Prego, I., Maldonado, S., Otegui, M., 1998. Seed structure and localization of reserves in *Chenopodium quinoa*. Ann. Bot. 481–488.

Przybylski, R., Chauhan, G., Eskin, N., 1994. Characterization of quinoa (*Chenopodium quinoa*) lipids. Food Chem. 51, 187–192.

Ramos Diaz, J.M., Kirjoranta, S., Tenitz, S., Penttilä, P.A., Serimaa, R., Lampi, A.-M., Jouppila, K., 2013. Use of amaranth, quinoa and kañiwa in extruded corn-based snacks. J. Cereal Sci. 58, 59–67.

Rafferty, K., Walters, G., Heaney, R., 2007. Calcium fortificants: overview and strategies for improving calcium nutriture of the US population. J. Food Sci. 72, R152-R158.

Ranhotra, G., Gelroth, J., Glaser, B., Lorenz, K., Johnson, D., 1993. Composition and protein nutritional quality of quinoa. Cereal Chem. 70, 303-303.

Rao, P.U., Belavady, B., 1978. Oligosaccharides in pulses: varietal differences and effects of cooking and germination. J. Agric. Food Chem. 26, 316–319.

Rayner, M., Timgren, A., Sjöö, M., Dejmek, P., 2012. Quinoa starch granules: a candidate for stabilising food-grade Pickering emulsions. J. Sci. Food Agric. 92, 1841–7.

Reddy, N.R., Sathe, S.K., Salunkhe, D.K., 1982. Phytates in legumes and cereals. Adv. Food Res. 28, 1–92.

Reilly, J. K., Lanou, A. J., Barnard, N. D., Seidl, K., Green, A. A., 2006. Acceptability of soymilk as a calcium-rich beverage in elementary school children. J. Am. Diet. Assoc. 106, 590-593.

Repo-Carrasco, R., Espinoza, C., Jacobsen, S.-E., 2003. Nutritional Value and Use of the Andean Crops Quinoa (*Chenopodium quinoa*) and Kañiwa (*Chenopodium pallidicaule*). Food Rev. Int. 19, 179–189.

Richardson, D., 1990. Food fortification. Proc. Nutr. Soc. 49, 39-50.

Ridout, C.L., Price, K.R., Dupont, M.S., Parker, M.L., Fenwick, G.R., 1991. Quinoa saponins -analysis and preliminary investigations into the effects of reduction by processing. J. Sci. Food Agric. 54, 165–176.

Robinson, N., 2014. No sign of gluten-free growth stalling. URL: http://www.foodmanufacture.co.uk/Sectors/Bakery/No-sign-of-gluten-free-growth-stalling (accessed 1.3.2014).

Rosa, M., Hilal, M., González, J.A., Prado, F.E., 2009. Low-temperature effect on enzyme activities involved in sucrose-starch partitioning in salt-stressed and salt-acclimated cotyledons of quinoa (*Chenopodium quinoa Willd*.) seedlings. Plant Physiol. Biochem. 47, 300–7.

Rosenthal, A., Deliza, R., Cabral, L., Cabral, L. C., Farias, C. A. A., Domingues, A. M., 2003. Effect of enzymatic treatment and filtration on sensory characteristics and physical stability of soymilk. Food Control. 14, 187-192.

Ruales, J., Grijalva, Y. de, Lopez-Jaramillo, P., Nair, B.M., 2002. The nutritional quality of an infant food from quinoa and its effect on the plasma level of insulin-like growth factor-1 (IGF-1) in undernourished children. Int. J. Food Sci. Nutr. 53, 143–154.

Ruales, J., Nair, B.M., 1993. Saponins, phytic acid, tannins and protease inhibitors in quinoa (*Chenopodium quinoa, Willd*) seeds. Food Chem. 48, 137–143.

Ruales, J., Nair, B.M., 1994. Effect of processing on in vitro digestibility of protein and starch in quinoa seeds. Int. J. Food Sci. Technol. 29, 449–456.

Ruiz, K.B., Biondi, S., Oses, R., Acuña-Rodríguez, I.S., Antognoni, F., Martinez-Mosqueira, E.A., Coulibaly, A., Canahua-Murillo, A., Pinto, M., Zurita-Silva, A., Bazile, D., Jacobsen, S.-E., Molina-Montenegro, M.A., 2014. Quinoa biodiversity and sustainability for food security under climate change. A review. Agron. Sustain. Dev. 34, 349–359.

Rustom, I., Lopez-Leiva, M., Nair, B., 1996. Nutritional, sensory and physicochemical properties of peanut beverage sterilized under two different UHT conditions. Food Chem. 56, 45-53.

Rustom, I., Lopez-Leiva, M., Nair, B. M., 1991. A study of factors affecting extraction of peanut (*Arachis hypogaea L.*) solids with water. Food Chem. 42, 153-165.

Rustom, I., Lopez-Leiva, M. H., Nair, B. M., 1993. Extraction of peanut solids with water-effect of the process and enzymatic hydrolysis. LWT - Food Sci. Technol. 26, 72-75.

Rustom, I., Lopez-Leiva, M. H., Nair, B. M., 1995. Effect of emulsifier type and homogenization temperature and pressure on physical properties of peanut extract. Int. J. Food Sci. Technol. 30, 773-781.

Rutherfurd, S.M., Moughan, P.J., 1998. The digestible amino acid composition of several milk proteins: Application of a new bioassay. J. Dairy Sci. 81, 909–917.

Ryan, E., Galvin, K., O'Connor, T.P., Maguire, A.R., O'Brien, N.M., 2007. Phytosterol, squalene, tocopherol content and fatty acid profile of selected seeds, grains, and legumes. Plant Foods Hum. Nutr. 62, 85–91.

Saastamoinen, M., Kumpulainen, J., Nummela, S., 1989. Genetic and environmental variation in oil content and fatty acid composition of oats. Cereal Chem. 66, 296–300.

Sahi, T., 1994. Genetics and epidemiology of adult-type hypolactasia. Scand. J. Gastroenterol. 29, 7-20.

Salovaara, H., 2004. Lactic acid bacteria in cereal-based products. In: aspectsSalminen, S., and von Wright, A., and Ouwehand, A., (Eds.), Lactic acid bacteria: Microbiological and functional, Marcel Dekker, New York, pp. 431-452.

Sandberg, A.-S., Brune, M., Carlsson, N.-G., Hallberg, L., Skoglund, E., Rossander-Hulthen, L., 1999. Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. Am J Clin Nutr 70, 240–246.

Sandberg, A. S., Carlsson, N. G., Svanberg, U., 2006. Effects of inositol tri-, tetra-, penta-, and hexaphosphates on in vitro estimation of iron availability. J. Food Sci. 54, 159-161.

Scarborough, P., Rayner, M., 2010. Nutrient density to climate impact index is an inappropriate system for ranking beverages in order of climate impact per nutritional value. Food Nutr. Res. 54, 5681.

Schaafsma, G., 2000. The protein digestibility–corrected amino acid score. J. Nutr. 130, 1865S-1867S.

Schlick, G., Bubenheim, D.L., 1996. Quinoa: candidate crop for NASA's controlled ecological life support systems. In: Janick, J. (Ed.), Progress in New Crops, ASHS Press, Alexandria, VA, pp. 632–640.

Sharma, V., Singh, R. K., Toledo, R. T., 2009. Microbial inactivation kinetics in soymilk during continuous flow high-pressure throttling. J. Food Sci. 74, M268-M275.

Schoenlechner, R., Drausinger, J., Ottenschlaeger, V., Jurackova, K., Berghofer, E., 2010. Functional properties of gluten-free pasta produced from amaranth, quinoa and buckwheat. Plant Foods Hum. Nutr. 65, 339–49.

Schonlechner, R., Linsberger, G., Kaczyk, L., Berghofer, E., 2006. Herstellung von glutenfreien Keksen aus den Pseudo-cerealien Amaranth, Quinoa und Buchweizen mit Gartenbohnen. Ernähr/Nutr 30, 101–107

Schumacher, A.B., Brandelli, A., Macedo, F.C., Pieta, L., Klug, T. V, de Jong, E. V, 2010. Chemical and sensory evaluation of dark chocolate with addition of quinoa (*Chenopodium quinoa Willd.*). J. Food Sci. Technol. 47, 202–6.

Sen, C.K., Khanna, S., Roy, S., 2006. Tocotrienols: Vitamin E beyond tocopherols. Life Sci. 78, 2088–98.

Shewry, P.R., 1999. Seed proteins, in: Shewry, P.R. (Ed.), Seed Proteins. Springer Netherlands, pp. 1–10.

Shewry, P.R., and Pandya, M.J., 1999. The 2S albumin storage proteins, in: Shewry, P.R. (Ed.), Seed Proteins. Springer Netherlands, pp. 563-686.

Silk.com, 2014. Website of the Silk® brand by The WhiteWave Foods Company. http://www.silk.com (accessed 25.9.2014).

Singh, B., Bhat, T.K., Singh, B., 2003. Potential therapeutic applications of some antinutritional plant secondary metabolites. J. Agric. Food Chem. 51, 5579–97.

Singh, J., Dartois, A., Kaur, L., 2010. Starch digestibility in food matrix: a review. Trends Food Sci. Technol. 21, 168–180.

Singh, N., Singh, J., Kaur, L., Singh Sodhi, N., Singh Gill, B., 2003. Morphological, thermal and rheological properties of starches from different botanical sources. Food Chem. 81, 219–231.

Singh, S., Gamlath, S., Wakeling, L., 2007. Nutritional aspects of food extrusion: a review. Int. J. Food Sci. Technol. 42, 916–929.

Smedman, A., Lindmark-Månsson, H., Drewnowski, A., Edman, A.K.M., 2010. Nutrient density of beverages in relation to climate impact. Food Nutr. Res. 54, 5170.

Smith, K., Mendonca, A., Jung, S., 2009. Impact of high-pressure processing on microbial shelf-life and protein stability of refrigerated soymilk. Food Microbiol. 26, 794-800.

Soetan, K., 2008. Pharmacological and other beneficial effects of antinutritional factors in plants - A review. African J. Biotechnol. 7, 4713-4721.

Sonesson, U., Davis, J., and Ziegler, F., 2010. Food production and emissions of greenhouse gases: An overview of the climate impact of different product groups. SIK-Report No 802 2010. Swedish Institute for Food and Biotechnology.

Sripriya, G., Antony, U., Chandra, T.S., 1997. Changes in carbohydrate, free amino acids, organic acids, phytate and HCl extractability of minerals during germination and fermentation of finger millet (*Eleusine coracana*). Food Chem. 58, 345–350.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., and de Haan, C., 2006. Livestock's long shadow: environmental issues and options. FAO, Rome, Italy.

Swagerty, D.L., Walling, A.D Klein, R.M., 2002. Am. Fam. Physician. 65, 1845–1850.

Tang, H., 2002. Characterization of storage starches from quinoa, barley and adzuki seeds. Carbohydr. Polym. 49, 13–22.

Tano-Debrah, K., Asiamah, K., Sakyi-Dawson, E., Budu, A., 2005. Effect of malt enzyme treatment on the nutritional and physicochemical characteristics of cowpea-peanut milk. In, Proceedings of the 1st international edible legume conference/IVth world cowpea congress, Durban, South Africa.

Tari, T.A., Annapure, U.S., Singhal, R.S., Kulkarni, P.R., 2003. Starch-based spherical aggregates: screening of small granule sized starches for entrapment of a model flavouring compound, vanillin. Carbohydr. Polym. 53, 45–51.

Tharanathan, R.., 2003. Biodegradable films and composite coatings: past, present and future. Trends Food Sci. Technol. 14, 71–78.

Theuwissen, E., Mensink, R.P., 2008. Water-soluble dietary fibers and cardiovascular disease. Physiol. Behav. 94, 285–92.

Valencia, S., Svanberg, U., Sandberg, A.-S., Ruales, J., 1999. Processing of quinoa (*Chenopodium quinoa , Willd*): effects on in vitro iron availability and phytate hydrolysis. Int. J. Food Sci. Nutr. 50, 203–211.

Valentine, J., Cowan, A.A., Marshall, A.H., 2011. Oat Breeding. In: Webster, F.H., Wood, P.J., (Eds.), Oats: chemistry and technology, AACC International, St. Paul, MN, pp. 11-30

Wang, H. J., and Murphy, P. A., 1996. Mass balance study of isoflavones during soybean processing. J. Agric. Food Chem. 44, 2377-2383.

Wansink, B., Sonka, S., Goldsmith, P., Chiriboga, J., Eren, N., 2005. Increasing the acceptance of soy-based foods. JIFAM. 17, 35-55.

Ward, S.M., 2001. A Recessive Allele Inhibiting Saponin Synthesis in Two Lines of Bolivian Quinoa (*Chenopodium quinoa Willd*.). J. Hered. 92, 83–86.

Watanabe, M., Maeda, T., Tsukahara, K., Kayahara, H., Morita, N., 2004. Application of pregerminated brown rice for breadmaking. Cereal Chem. 81, 450–455.

Weitbrecht, K., Müller, K., Leubner-Metzger, G., 2011. First off the mark: early seed germination. J. Exp. Bot. 62, 3289–309.

Wilhelmson, A., Oksman-Caldentey, K.-M., Laitila, A., Suortti, T., Kaukovirta-Norja, A., Poutanen, K., 2001. Development of a germination process for producing high β-glucan, whole grain food ingredients from oat. Cereal Chem. 78, 715–720.

Williams, J.T., Haq, N., 2002. Global research on underutilized crops: An assessment of current activities and proposals for enhanced cooperation. Bioversity International, Southampton, UK.

Wohlpart, A., Mabry, T.J., 1968. On the light requirement for betalain biogenesis. Plant Physiol. 43, 457–9.

Wolf, W. J., 1970. Soybean proteins. Their functional, chemical, and physical properties. J. Agric. Food Chem. 18, 969-976.

Wolter, A., Hager, A.-S., Zannini, E., Arendt, E.K., 2013. In vitro starch digestibility and predicted glycaemic indexes of buckwheat, oat, quinoa, sorghum, teff and commercial gluten-free bread. J. Cereal Sci. 58, 431–436.

Wood, S.G., Lawson, L.D., Fairbanks, D.J., Robison, L.R., Andersen, W.R., 1993. Seed Lipid Content and Fatty Acid Composition of Three Quinoa Cultivars. J. Food Compos. Anal. 6, 41–44.

Wood, P.J., 2010. Review: Oat and rye β-Glucan: properties and function. Cereal Chem. 87, 315-330.

Yadav, D. N., Singh, K. K., Bhowmik, S. N., and Patil, R. T., 2010. Development of peanut milk–based fermented curd. Int. J. Food Sci. Technol. 54, 2650-2658.

Yashina, S., Gubin, S., Maksimovich, S., Yashina, A., Gakhova, E., Gilichinsky, D., 2012. Regeneration of whole fertile plants from 30,000-y-old fruit tissue buried in Siberian permafrost. Proc. Natl. Acad. Sci. U.S.A. 109, 4008–13.

Yazici, F., Alvarez, V., and Hansen, P., 1997. Fermentation and Properties of Calcium-fortified Soy Milk Yogurt. J. Food Sci. 62 : 457-461.

Vähämäki, H., Kasvinen, J., 2013. Kvinoa juurtuu Suomeen Liedon ja Tarvasjoen pelloilla. YLE news. URL: http://yle.fi/uutiset/kvinoa_juurtuu_suomeen_liedon_ja_tarvasjoen_pelloilla/6827407 (accessed 3.3.14).

Zannini, E., Pontonio, E., Waters, D. M., Arendt, E. K., 2012. Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. Appl. Microbiol. Biotechnol. 93, 473-485.

Zarnkow, M., Geyer, T., Lindemann, B., Burberg, F., Back, W., Arendt, E.K., Kreisz, S., 2007. The use of response surface methodology to optimise malting conditions of quinoa (*Chenopodium quinoa l.*) as a raw material for gluten-free foods and beverages. Monatsschrift fur Brauwiss. 60, 118–126.

Zarnkow, M., Keßler, M., Back, W., Arendt, E.K., Gastl, M., 2010. Optimisation of the Mashing Procedure for 100% Malted Proso Millet (*Panicum miliaceum L.*) as a Raw Material for Gluten-free Beverages and Beers. J. Inst. Brew. 116, 141–150.

Zhang, H., Önning, G., Öste, R., Gramatkovski, E., Hulthen, L., 2007a. Improved iron bioavailability in an oat-based beverage: the combined effect of citric acid addition, dephytinization and iron supplementation. Eur. J. Nutr. 46, 95-102.

Zhang, H., Önning, G., Triantafyllou, A.Ö., Öste, R., 2007b. Nutritional properties of oat-based beverages as affected by processing and storage. J. Sci. Food Agric. 87, 2294-2301.

Zhao, Y., Martin, B.R., Weaver, C.M., 2005. Calcium bioavailability of calcium carbonate fortified soymilk is equivalent to cow's milk in young women. J. Nutr. 135, 2379-2382.

Zobel, H.F., Stephen, A.M., 2010. Starch: Structure, analysis, and application, in: Food Polysaccharides and Their Applications. CRC Press, p. 25–50.

Zulkurnain, M., Goh, M.-H., Karim, A.A., Liong, M.-T., 2008. Development of a soy-based cream cheese. J. Texture Stud. 39, 635–654.

Önning, G., Åkesson, B., Öste, R., and Lundquist, I., 1998. Effects of consumption of oat milk, soya milk, or cow's milk on plasma lipids and antioxidative capacity in healthy subjects. Ann. Nutr. Metab. 42, 211-220.

Objectives

The bitter flavour that makes the inclusion of quinoa in many products challenging, may be reduced by germination. Also, the enzyme activities that arise during germination may have functionalities in food applications. Plenty of literature covering the malting of alternative grains and pseudocereals approaches the challenge from a process optimisation point of view and use standard methods developed for barley malt. However many of these seeds are very different from traditional malting cereals both taxonomically and structurally. The aim of the first part of this thesis was to investigate the germination pattern of quinoa by following the development of hydrolytic enzyme activities and subsequent changes in starch and protein profiles during germination and early seedling growth, and to test the functionality of malted quinoa in a gluten-free product application.

The second part handles plant-based dairy-type products. The market for such products is growing, and quinoa would offer an interesting raw material because of its high quality protein. First a range of commercial plant milks was characterised, and a process for the production of quinoa milk was being developed. The high starch content of grains becomes the limiting factor in the extraction procedure, and the highest protein content achieved this way was 1%. Another approach was taken by producing a quinoa protein isolate. The functional and acid gelation properties of this isolate were studied.

Chapter 4

Amylolytic activities and starch reserve mobilization during the germination of *Chenopodium quinoa*

Anna-Sophie Hager*, Outi E. Mäkinen*, Elke K. Arendt

*co-first authorship

Abstract

Quinoa (*Chenopodium quinoa*) is a dicotyledonous plant producing perispermic seeds, which is increasingly popular in the Western world due to its nutritional value. Germination can be used in food processing to alter the sensory, nutritional and functional properties of grains, and the increased enzyme activities may have beneficial influences in food applications. Knowledge about the germination of perispermic seeds is scarce. In this study, the development of amylolytic activities and subsequent changes in sugar profiles and starch content were followed in quinoa over a period of 72 h. The seeds germinated rapidly with radicle protrusion occurring 8 h after imbibition, when the seeds had reached a moisture content of 44%. A low level of α-amylase activity was present in the embryo of non-germinating seeds, but emerged in the perisperm only after 24 h, followed by a sharp increase in activity in both tissues. An accumulation of glucose and fructose and a decrease in starch content were observed after 24 and 36 h, respectively, indicating the onset of starch reserve mobilization. Overall, the levels of amylolytic activities remained very low compared to traditional malting cereals, suggesting the unsuitability of quinoa as a source of amylases in food applications. Scanning electron microscopy, used to visualize changes in starch granule morphology, showed mainly exocorrosion, suggesting a homogenous structure of the outer layers of quinoa starch.

4.1 Introduction

Quinoa (*Chenopodium quinoa*) has been cultivated as a staple food for thousands of years in the Andean region (Abugoch, 2009), but is also increasingly popular in the Western diet. The plant produces highly nutritious seeds and has potential for production in various climates due to its wide genetic variability (Jacobsen et al., 2003). Quinoa seeds are oval shaped seeds, with a mean equivalent diameter between 1.4-2.0 mm and ranging in color from pale yellow over pink and dark red to black (Abugoch, 2009; Valencia-Chamorro, 2003). Quinoa is a dicotyledonous plant and therefore not a true cereal (monocotyledonous), but due to its starch rich seeds it is referred to as pseudocereal. The structure of the seed differs from that of cereals. Its main storage tissue is a central perisperm that is surrounded by a band-like embryo (Prego et al., 1998). The storage reserves show strict localization. Starch, the main carbohydrate reserve, is exclusively located in the perisperm, while protein and lipid bodies are found in the embryo (Prego et al., 1998). Seeds with a similar structure and reserve localization include sugar beet and amaranth (Lawrence et al., 1990; Prego et al., 1998). The embryo consists of a hypocotyl-radicle axis and two cotyledons and can make up to 60% of the seed weight. This high proportion of embryo explains the high protein content found in quinoa compared to cereal grains (Valencia-Chamorro, 2003). The endosperm is present only in the micropylar region of the seed and consists of one or two layered tissue surrounding the tip of the radicle (Prego et al., 1998).

Upon removal of the outer seed layer (i.e., pericarp) which contains saponins, quinoa can be milled into flour and used for the production of e.g. baked and extruded products (Elgeti et al., 2014). As it is not related to wheat, quinoa is suitable for people on a gluten free diet (Bergamo et al., 2011) and its inclusion into a

standard gluten-free diet has been reported to be beneficial (Lee et al., 2009). Due to the outstanding nutritional profile, the general public would also benefit from increased consumption. Quinoa contains protein with a balanced amino acid composition and is high in fiber and micronutrients, especially folate (Hager et al., 2012; Ranhotra et al., 1992).

Germination starts by the imbibition of water, and subsequent increase in respiration and protein synthesis. Hydrolytic enzymes are synthesized or activated to break down the seed storage reserves that are in turn used to nourish the growing seedling (Bewley and Black, 1994). In *sensu stricto*, germination ends with radicle protrusion and the following growth of the embryonic axis is referred to as seedling establishment. In food and malting science it is common to call the whole process including the early growth "germination" (Mikola, 2001) and the term is used in this sense throughout this publication.

The increased amylolytic activities resulting from germination are not only a prerequisite for brewing, but may have functionality in other food applications. The endogenous enzymes of germinated seeds can be used e.g. to improve the technological quality of wheat (Mäkinen and Arendt, 2012) and gluten free baked goods (Mäkinen et al., 2013), and to increase the mineral bioavailability in cereal foods (Larsson et al., 1996).

Quinoa often shows a strong grassy note that may limit its commercial applications. Germination of edible seeds modifies both the palatability and the nutritional profile of grains and is a potential means of reducing off-flavors (Alvarez-Jubete et al., 2010; Valencia et al., 1999).

Driven by their economic significance, the storage compound mobilization of barley and wheat has been thoroughly studied over the past decades (Fincher, 1989).

In contrast, the metabolism related to storage mobilization is poorly documented in perispermic seeds, such as quinoa. In such seeds, the perisperm is the unique carbohydrate source for cotyledons and embryonic axes during early developmental stages (Rosa et al., 2004). In this paper, the development of amylolytic activities and subsequent changes in sugar profile and starch content were studied to shed light on the mobilization of starch reserves of perispermic quinoa seeds. The aim of this study is to monitor the development of amylolytic activities in the perisperm and embryo of quinoa over a germination period of 72 h. In addition, changes in sugar profiles, starch content and starch granule morphology are discussed.

4.2 Materials and methods

4.2.1 Materials

Red quinoa réal (Priméal, France), dehulled and desaponified by light abrasion using spring water (personal communication with producer). All chemicals were purchased from Sigma-Alrich unless otherwise stated.

4.2.2 Germination capacity and germination

The germination capacity was tested by imbibing red quinoa réal (Priméal, France) on petri dishes with two layers of filter paper at 10, 15 and 22°C in darkness. Quinoa (150-200 seeds) was soaked in 3 % H_2O_2 for 1 min to inhibit microbial growth and germinated on petri dishes with 1 ml water at 15°C in darkness. The protruding radicle of 10 seeds was measured at different time points using a digital caliper. The germinated, abnormally germinated (in which the cotyledons emerge before the radicle) and non-germinated seeds were counted and after 30 h, and expressed as percentage from the total number of seeds. To prepare samples for

analyses, 15 g seeds were germinated in 15 ml water on large petri dishes at 15°C in darkness. The embryo was manually separated from the perisperm and samples were freeze dried. For the determination of moisture contents, sample weights were recorded before and after lyophilization. The results of all analyses were expressed on dry weight basis (dwb). Non-germinated seeds (0 h) represent dry seeds that have not been brought in contact with water.

4.2.3 Amylase activities and zymography

α-amylase activity was determined at pH 5.2 using blocked ρ-nitrophenyl-maltoheptaoside as substrate for α-amylase (K-CERA, Megazyme, Ireland). Activity of α-amylase was expressed as CU/g (Ceralpha Unit = the amount of enzyme, in the presence of excess thermostable α-glucosidase, required to release one micromole of ρ-nitrophenol from BPNPG7 in one minute under defined assay conditions). Total starch hydrolysing activity was determined as the release of reducing sugars from a starch substrate. Samples were extracted using 25 mM Tris/HCl buffer with 0.5 mM EDTA (pH 6.5) and assayed against 1% starch solution. The extent of hydrolysis was quantified using the DNS method (Miller, 1959). One unit of activity was defined as the amount of enzyme that releases reducing sugars equivalent to 1 μmol glucose per minute under the assay conditions.

For zymography, the enzymes were extracted from ground samples using a 62.5 mM Tris-HCl buffer pH 6.8 containing 10% (w/v) glycerol and 0.01% (w/v) bromophenol blue (Belay, 2001). Solids were removed by centrifugation (14 000 g x 20 min) and supernatants (25 μl perisperm or 10 μl embryo samples) were loaded into the wells of 7.5% PAGE gels with 0.6% gelatinised starch. Electrophoresis was performed according to Laemmli (LAEMMLI, 1970) under non-reducing conditions

at 7 °C (Criterion Dodeca, Bio-Rad, Richmond, CA) followed by renaturing in 2.5% Triton-X and incubation at 40 °C for 24 h (pH 5.4). After this, gels were stained using Lugol's solution and destained overnight in water. Clear bands on a dark background represent starch degradation. Alternatively, gels were stained using a modified sensitive colloidal staining protocol to visualise the protein bands (Westermeier, 2006). Molecular weight markers were run in parallel with the samples (Precision Plus Protein standards, All Blue, Bio-Rad, Richmond, CA).

4.2.4 Starch and sugar contents

Starch content was analysed using the total starch assay kit (K-TSTA; Megazyme, Ireland). Dried and ground samples were extracted in 80% EtOH for 5 min at 85°C, and centrifuged (1800 g x 10 min). The pellets were washed in EtOH and analysed. For the determination of *sugar contents*, samples (0.4 g) were extracted in H_2O (2 ml) for 20 min at room temperature. Solids were removed by centrifugation (5000 g x 10 min) and supernatants were clarified with Carrez reagents (Indyk et al., 1996) and filtered (0.45 μm). The sugar profiles were then analysed using an Agilent Infinity 1260 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a refractive index detector and a Phenomenex Rezex ROA H+ column. The mobile phase was water with a flow rate 0.6 m/min, and the analysis was run at 25°C.

4.2.5 Scanning electron microscopy

Starch granule morphology was studied using scanning electron microscopy (SEM). Dried perisperm was cut into pieces and attached on aluminium stubs with carbon tape and sputter coated with a 10 nm layer of gold/palladium (80:20) (Polaron

E5150 Sputter Coating). Samples were examined using a Jeol Scanning Electron Microscope (JSM-5510, Jeol Ltd., Tokyo, Japan) at 5 kV with a working distance of 10 mm.

4.2.6 Statistical analyses

Statistical analysis was performed using R version 3.0.2 "Frisbee Sailing" (The R Foundation for Statistical Computing, Vienna, Austria) and the package "agricolae". Analysis of variance was carried out, followed by Shapiro Wilk normality test and Tukey's honest significant difference test. Statistica 7.1 (StatSoft Software Inc., USA) was used to create boxplot shown in Figure 4.3.

4.3 Results and discussion

4.3.1 Germination capacity and radicle elongation

Germination begins with the uptake of moisture by the seed followed by the elongation of the embryonic axis or radicle. Germination capacity, i.e. the proportion of seeds capable of completing germination, was influenced by temperature and ranged from 73% to 87% (Figure 4.1). Although differences were not significant, the highest germination capacity was achieved at 15°C, and this temperature was therefore chosen for further trials. Ca. 1-2 % of the seeds showed abnormal germination, in which the cotyledons protruded instead of the radicle, eventually leading to the death of the seedling (Figure 4.2 a). The percentage of abnormally germinating seeds was low in the red quinoa investigated here, but in preliminary trials conducted with a range of commercial white quinoa samples up to 10-20% of the seeds germinated abnormally (not shown).
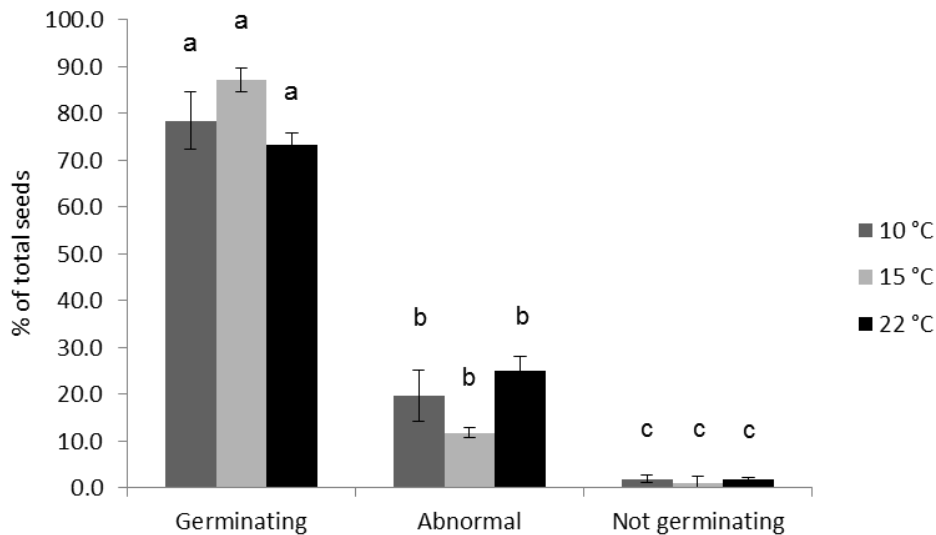
Figure 4.1. Germination capacity (%) of red quinoa germinated at different temperatures for 30 h (percentage germinated, abnormally germinated and non-germinated seeds of total seeds). Bars marked by the same letter are not significantly different (p<0.05).

Quinoa seeds exhibited rapid growth when exposed to moisture. The radicle protruded 8 hours after imbibition (hai) in 80% of seeds, and reached an average length of 1.7 ± 0.4 mm after 12 hai, and 35.0 ± 6.3 mm after 72 hai (Figure 4.3). The seeds had an average moisture content of 44% at the time of radicle protrusion (not shown). Rosa et al. (2004) reported radicle protrusion after 5 h at 25 °C and 9 h at 5 °C, showing strong temperature dependence. After radicle elongation, mobilisation of the stored starch reserves commences, up to which point sucrose and other sugars present in the grain are used as early energy sources (Bewley and Black, 1994).
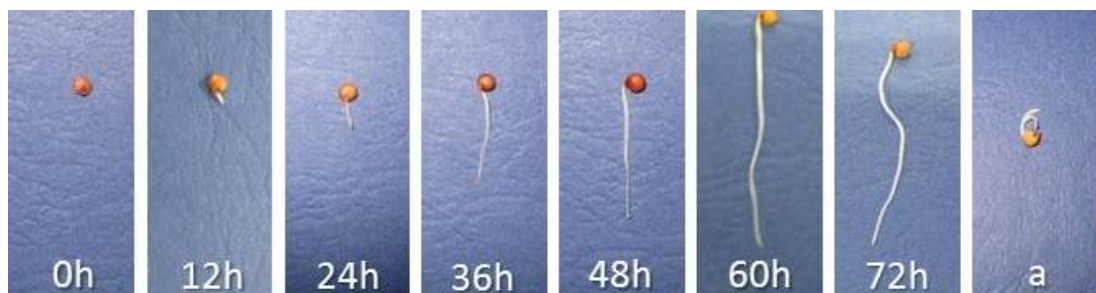

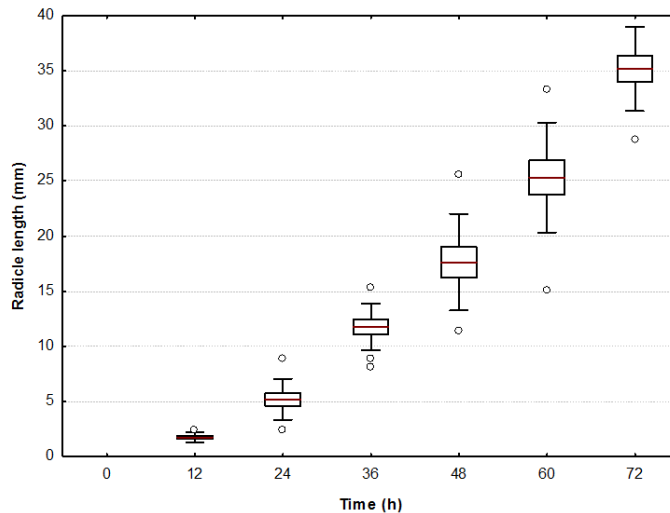**Figure 4.2.** Visual appearance of germinated red quinoa seeds as well as of an abnormally germinated seed.

Figure 4.3. Boxplot showing radicle elongation during germination of red quinoa seeds at 15 °C (ten measurements per time point).

4.3.2 Amylase activities

The mobilization of starch starts by the hydrolysis of amylose and amylopectin in starch granules into glucose by α-amylase (EC 3.2.1.1), debranching enzyme (EC 3.2.1.41), β-amylase (EC 3.2.1.2) and α-glucosidase (EC 3.2.1.20). Glucose in turn is converted into sucrose and transported to the site of requirement (Bewley and Black, 1994). Total starch hydrolysing activity (42 U/g) was present already in the non-germinating seeds (Fig. 4.4 a). A dip to a value of 29 U/g was observed after 24 hai, after which the activity started increasing and reached 53 U/g after 72 h. It is important to note that the use of a non-specific starch substrate gives an indication of the total starch hydrolytic activity that could be the results of the action of more than one enzyme simultaneously, rather than pure α- or β-amylase for instance (Agu and Palmer, 1997; Bassinello et al., 2002).

In non-germinating dry seeds, the perisperm showed no of α-amylase activity, whereas a low level of activity could be detected in the embryo (Fig.4.4 b). A sharp increase in activity occurred in both tissues after 24 hai. The maximum recorded

value was reached after 60 hai (1.8 CU/g) in the embryo, after which the activity started to decline. The perisperm α-amylase activity kept increasing until the end of the observation period, with a value of 3.29 CU/g after 72 hai. According to a previous report where germination was carried out at a higher temperature of 25°C, α-amylase activity in quinoa perisperm peaked between 12 and 48 h after imbibition, after which it declined sharply (Rosa et al., 2004). Other previous reports regarding the α-amylase activity of germinating quinoa include an increase after 12 h (germination at 22°C) (Atwell et al., 1988) and no increase in 24 h germinated quinoa (Mäkinen et al., 2013). Zarnkow et al. (2007) optimized the malting conditions for quinoa using response surface methodology. Tested germination times ranged from 5 to 7 d, of which 5 d at 15 °C was the optimum. The resulting malt showed good proteolytic modification when mashed, but the α-amylase activity fell below the limit of detection, leading to a somewhat low extract (55%) and residual mash viscosity
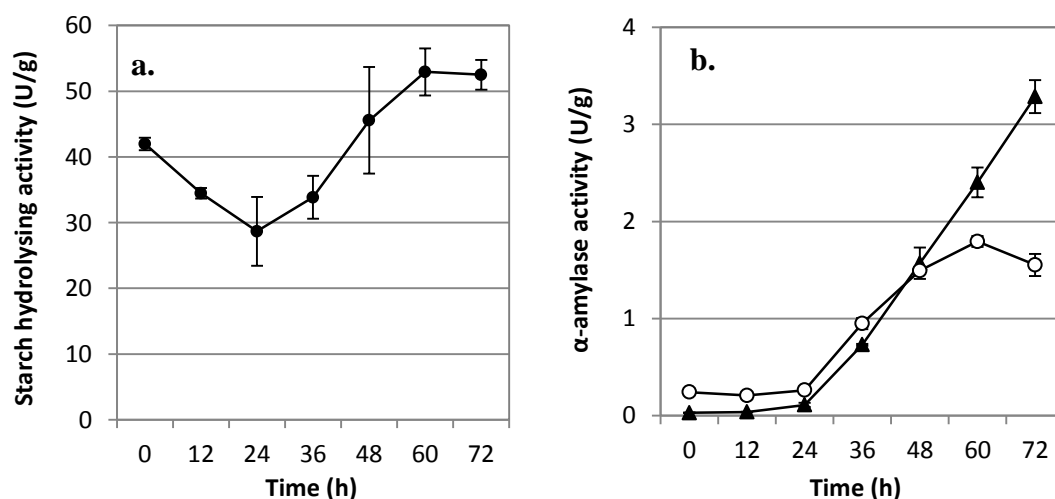

Figure 4.4. (a) Total starch hydrolysing activity in the whole seed (●) and **(b)** α-amylase activity in the embryo (■) and perisperm (▲). Means ± standard deviations. Values labelled with the same letter are not significantly different (p<0.05).

Zymography was performed using starch-containing acrylamide gels that revealed amylase activity as light bands after staining (Figure 4.5 a). Two distinct bands of activity were observed on zymograms, and corresponding protein bands were found on the SDS page gels at ~90 kDa and ~140 kDa (Figure 4.5 b). The 90 kDa activity was present in the perisperm tissue 24 hai and increased in intensity over time, whereas the 140 kDa activity appeared after 72 hai. Both activities were visible in the embryo tissue already 24 hai after imbibition. The activities increased over time, showing very bright bands after 48 hai and 72 hai in the embryo.
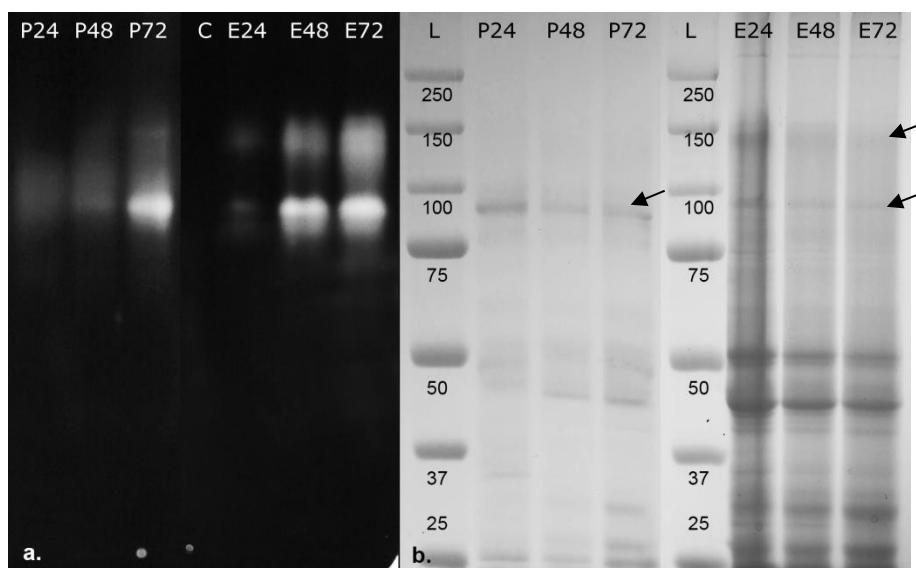

Figure 4.5. Zymograms (a) and SDS-PAGE gels (b) from perisperm (P) and embryo (E) samples after 24 h (P/E24), 48 h (P/E48) and 72 h (P/E72) of germination. A heated sample was used as a negative control (C).

The observed pattern may suggest that the embryo has a role in the synthesis of the α-amylases that are then transported to the starchy perisperm. In sugar beet, which is also a perispermic seed, α-amylase accumulates in the perisperm of an imbibed seed but not in an isolated perisperm, indicating that the embryo is involved in the enzyme accumulation (Catusse et al., 2012). It has been proposed that the

structure in which the perisperm is surrounded by a curled embryo, as in quinoa and sugar beet seeds, suggests that the embryo may act both as a secretory (synthesis and transport of enzymes) and an absorption system (taking up metabolites) (Lawrence et al., 1990).

4.3.3 Starch content and sugar profiles

The initial starch content in a non-germinating seed was 52.0 ± 1.0 % dwb (Fig. 4.6 a). The content started to decrease 36 hai after imbibition, down to 39.5 ± 0.1 % dwb after 72 hai. The decrease appeared to follow the sharp increase in α-amylase activity 24 h after imbibition (Fig. 4.4 b). The development of sucrose, fructose and glucose levels are shown in Fig. 4.6 b. The non-germinating seed contained 2.60 ± 0.24 % dwb of sucrose, 1.73 ± 0.29 % dwb of glucose and 0.08 ± 0.03 % dwb of fructose. After 24 hai, the contents of glucose and fructose started to increase to 13.4 % and 6.9 % after 72 hai, respectively. The sucrose content decreased slightly from 5.2 to 3.4 % dwb during the first 24 hai, possibly being used as an energy source, but hereupon the level remained constant. No information on the sugar profiles in the quinoa perisperm is available, but glucose and fructose contents increased in both cotyledons and radicles during the first 24 h, while sucrose has been found only in the cotyledons (Rosa et al., 2004).

4.3.4 Starch granule morphology

Starch is located in the perisperm of quinoa and can exist as simple units or as spherical aggregates. Quinoa starch granules have a diameter in the range of 0.4-2.0 μm (Valencia-Chamorro, 2003) and granules are angular in shape. The appearance of erosion channels during germination and malting of buckwheat, barley and millet
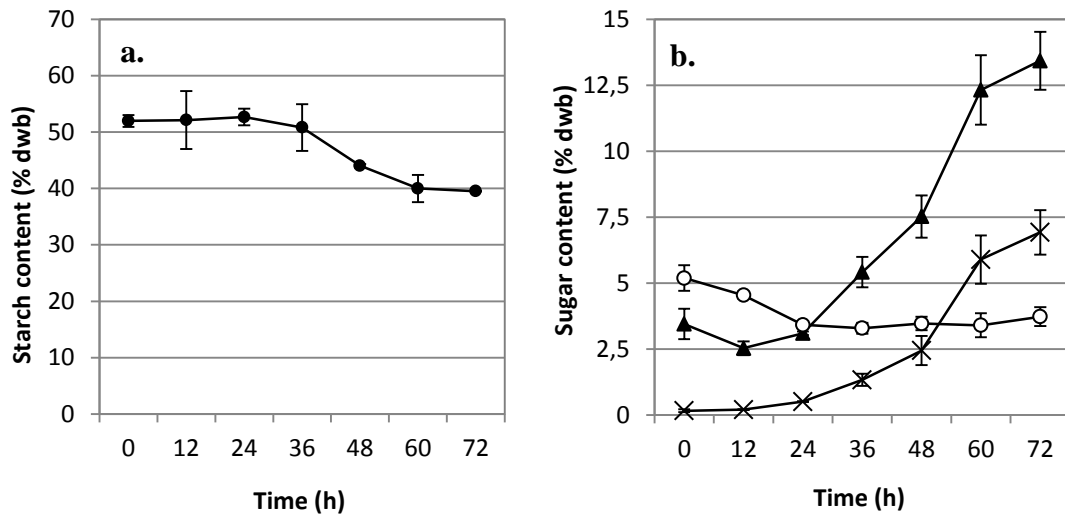
Figure 4.6. Starch (a) and sugar (b) contents of whole quinoa seeds over 72 h germination. Symbols: glucose (▲), fructose (×) and sucrose (○). Means ± standard deviations.

was documented previously (Wijngaard et al., 2007; Zarnkow et al., 2007). To investigate ultrastructural changes during germination and early seedling development of quinoa, starch granules found both in the center of the perisperm and the edges facing the embryo were studied using scanning electron microscopy. No changes in granules were observed in the center of the perisperm (not shown). Also the granules located in the outer perisperm did not show erosion channels during the first 48 hai, but some tunnels appeared 72 h after imbibition (arrows in Fig. 4.7). The fact that mainly exocorrosion was observed indicates a homogenous structure of the outer layers of quinoa starch (Tang and Watanabe, 2002).
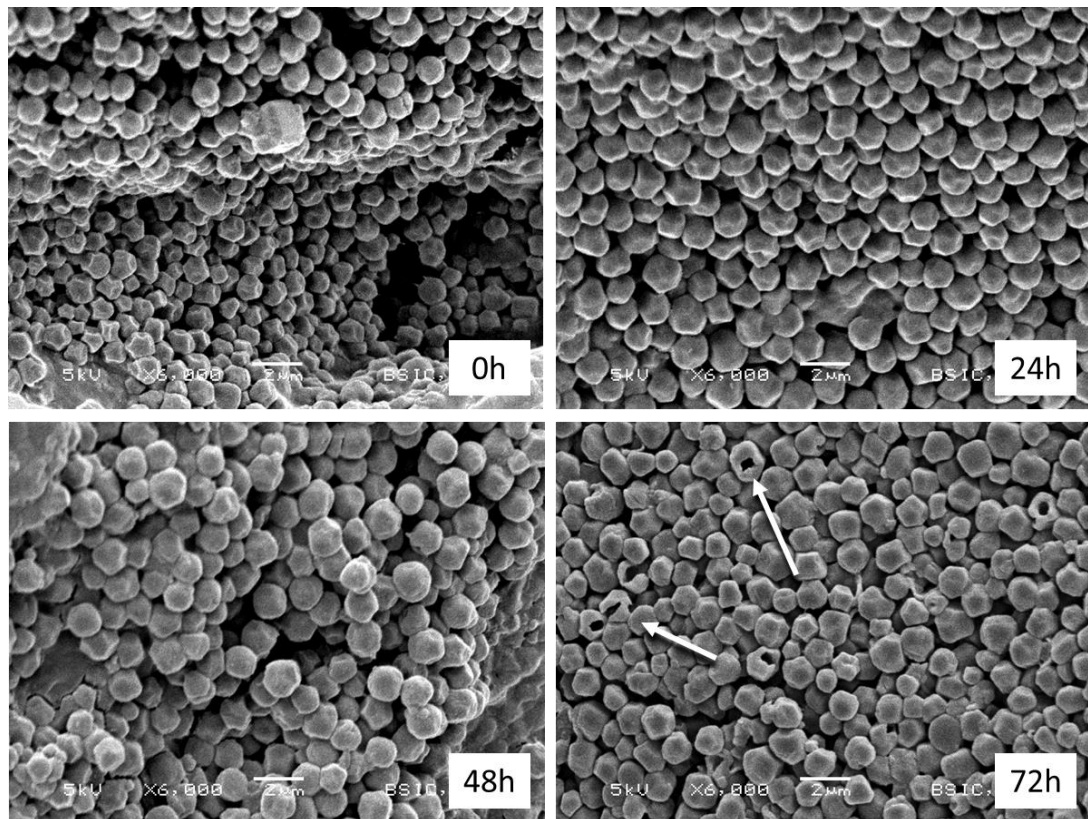
Figure 4.7. Scanning electron micrographs of starch granules obtained from the quinoa perisperm after 0, 24, 48 and 72 hours of germination. Arrows are pointing to erosion channels. Scale bar represents 2 μm.

4.4 Conclusion

The storage compound mobilisation of barley and wheat ("true cereals") has been thoroughly studied over the past decades driven by their economic significance (Fincher, 1989). This publication adds to the limited knowledge available on quinoa germination. The results suggest that the embryo is involved in the accumulation of α-amylase in the starchy perisperm of quinoa in a similar manner as previously reported for other perispermic seeds. Major changes in the α-amylase activity and monosaccharide concentrations occurred 24 h after imbibition, indicating the onset of starch mobilization during germination.

Oftentimes in cereal science and industry the knowledge and procedures used in barley malting are applied directly to other seeds. This study clearly shows, that in the case of quinoa, this is not suitable. Germination capacities were determined at

10°C, 15°C and 22°C, and the lowest number of ungerminated or abnormally germinated seeds were found at 15°C. Quinoa is a fast growing seed and radicles were already measureable at 12 hours. The increased enzyme activities of germinated seeds like wheat or barley are not only a prerequisite for brewing, but have functionality in other cereal applications. The suitability of quinoa as a gluten-free alternative to barley or wheat malts in regards to enzyme activities, but beer can be produced with the aid of brewing enzymes. As indicated by increased enzyme activity as well as decreased starch content, this study showed that the total starch hydrolysing capacity is significantly lower than that e.g. for barley (approximately 180 U/g barley malt in the study of Yaldagard and Mortazavi (2008), compared to about 30-60 U/g quinoa in this study. Activity of α-amylase starts to increase only after significant radicle growth and the use of this material would therefore mean high malting losses.

For future work it would be interesting to investigate the influence of the germination process as performed in this study on the palatability of quinoa, as this very nutritious grain but may be a source of a "grassy/bitter" flavour when used in product formulations.

Acknowledgments

References

Abugoch James, L., E., 2009. Quinoa (*Chenopodium quinoa Willd.*): Composition, chemistry, nutritional, and functional properties. Adv. Food Nutr. Res. 58, 1–31.

Agu, R.C., Palmer, G.H., 1997. The effect of temperature on the modification of sorghum and barley during malting. Process Biochem. 32, 501–507.

Alvarez-Jubete, L., Wijngaard, H., Arendt, E.K., Gallagher, E., 2010. Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. Food Chem. 119, 770–778.

Atwell, W.A., Hyldon, R.G., Godfrey, P.D., 1988. Germinated quinoa flour to reduce the viscosity of starchy foods. Cereal Chem. 65, 508–509.

Bassinello, P.Z., Cordenunsi, B.R., Lajolo, F.M., 2002. Amylolytic Activity in Fruits: Comparison of Different Substrates and Methods Using Banana as Model. J. Agric. Food Chem. 50, 5781–5786.

Belay, G., 2001. Zymogram patterns of a-amylase isozymes in Ethiopian tetraploid wheat landraces : insight into their evolutionary history and evidence for gene flow. Genet. Resour. Crop Ev. 48, 507-512.

Bergamo, P., Maurano, F., Mazzarella, G., Iaquinto, G., Vocca, I., Rivelli, A.R., De Falco, E., Gianfrani, C., Rossi, M., 2011. Immunological evaluation of the alcohol-soluble protein fraction from gluten-free grains in relation to celiac disease. Mol. Nutr. Food Res. 55, 1266–70.

Bewley, J.D., Black, M., 1994. Germination, structure and composition, in: Seeds - Physiology of development and germination. Plenum Press, New York, pp. 1–33.

Catusse, J., Job, C., Job, D., 2012. Proteomics Reveals A Potential Role of the Perisperm in Starch Remobilization During Sugarbeet Seed Germination, in: Agrawal, G.K., Rakwal, R. (Eds.), Seed Development: OMICS Technologies toward Improvement of Seed Quality and Crop Yield. Springer Netherlands, Dordrecht, pp. 27–41.

Elgeti, D., Nordlohne, S.D., Föste, M., Besl, M., Linden, M.H., Heinz, V., Jekle, M., Becker, T., 2014. Volume and texture improvement of gluten-free bread using quinoa white flour. J. Cereal Sci. 59, 41–47.

Fincher, G.B., 1989. Molecular and Cellular Biology Associated with Endosperm Mobilization in Germinating Cereal Grains. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 305–346.

Hager, A.-S., Wolter, A., Jacob, F., Zannini, E., Arendt, E.K., 2012. Nutritional properties and ultra-structure of commercial gluten free flours from different botanical sources compared to wheat flours. J. Cereal Sci. 56, 239–247.

Indyk, H.E., Edwards, M.J., Woollardb, D.C., 1996. High performance liquid chromatographic analysis of lactose-hydrolysed milk. Food Chem. 57, 575–580.

Jacobsen, S.E., Mujica, A., Ortiz, R., 2003. The global potential for quinoa and other Andean crops. Food Rev. Int. 19, 139–148.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Larsson, M., Rossander-Hulthén, L., Sandström, B., Sandberg, A.S., 1996. Improved zinc and iron absorption from breakfast meals containing malted oats with reduced phytate content. Br. J. Nutr. 76, 677–88.

Lawrence, D.M., Halmer, P., Bowles, D.J., 1990. Mobilisation of storage reserves during germination and early seedling growth of sugar beet. Physiol. Plant. 78, 421–429.

Lee, A.R., Ng, D.L., Dave, E., Ciaccio, E.J., Green, P.H.R., 2009. The effect of substituting alternative grains in the diet on the nutritional profile of the gluten-free diet. J. Hum. Nutr. Diet. 22, 359–63.

Mäkinen, O.E., Arendt, E.K., 2012. Oat malt as a baking ingredient – A comparative study of the impact of oat, barley and wheat malts on bread and dough properties. J. Cereal Sci. 56, 747–753.

Mäkinen, O.E., Zannini, E., Arendt, E.K., 2013. Germination of oat and quinoa and evaluation of the malts as gluten free baking ingredients. Plant Foods Hum. Nutr. 68, 90–5.

Mikola, M., 2001. Electrophoretic studies on the endoproteinases of oat grain. PhD thesis, University of Helsinki, Finland.

Miller, G.L., 1959. Use of dinitrosalicylic acid Reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.

Prego, I., Maldonado, S., Otegui, M., 1998. Seed structure and localization of reserves in *Chenopodium quinoa*. Ann. Bot. 481–488.

Ranhotra, G.S., Gelroth, J.A., Glaser, B.K., Lorenz, K.J., Johnson, D.L., 1992. Composition and protein nutritional quality of quinoa. Cereal Chem. 70, 303-305.

Rosa, M., Hilal, M., González, J.A., Prado, F.E., 2004. Changes in soluble carbohydrates and related enzymes induced by low temperature during early developmental stages of quinoa (*Chenopodium quinoa*) seedlings. J. Plant Physiol. 161, 683–9.

Tang H, Watanabe K, M.T., 2002. Characterization of storage starches from quinoa, barley and adzuki seeds. Carbohydr. Polym. 49, 13–22.

Valencia, S., Svanberg, U., Sandberg, A.-S., Ruales, J., 1999. Processing of quinoa (*Chenopodium quinoa , Willd*): effects on in vitro iron availability and phytate hydrolysis. Int. J. Food Sci. Nutr. 50, 203–211.

Valencia-Chamorro, S.., 2003. Quinoa, In: Caballero, B (Ed.), Encyclopedia of Food Science and Nutrition. Academic Press, Amsterdam, pp. 4895–4902.

Westermeier, R., 2006. Sensitive, quantitative, and fast modifications for Coomassie Blue staining of polyacrylamide gels. Proteomics 6 Suppl 2, 61–4.

Wijngaard H.H., Renzetti S., and E.K.A., 2007. Microstructure of Buckwheat and Barley During Malting Observed by Confocal Scanning Laser Microscopy and Scanning Electron Microscopy. J Inst Brew 113, 34–41.

Yaldagard M, Mortazavi SA., T.F., 2008. Influence of ultrasonic stimulation on the germination of barley seed and its alpha-amylase activity. African J. Biotechnol. 7, 2465–2471.

Zarnkow M, Mauch A, Back W, Arendt EK, K.S., 2007. Proso millet (*Panicum miliaceum L.*): An evaluation of the microstructural changes in the endosperm during the malting process by using scanning-electron and confocal laser microscopy. J. Inst. Brew. 113, 355–364.

Zarnkow, M., Geyer, T., Lindemann, B., Burberg, F., Back, W., Arendt, E.K., Kreisz, S., 2007. The use of response surface methodology to optimise malting conditions of quinoa (*Chenopodium quinoa*) as a raw material for gluten-free foods and beverages. Monatsschrift fur Brauwiss. 60, 118–126.

Chapter 5

Localisation and development of proteolytic activities in quinoa (*Chenopodium quinoa*) seeds during germination and early seedling growth

Outi E. Mäkinen, Anna-Sophie Hager, Elke K. Arendt

Abstract

Quinoa (*Chenopodium quinoa*) is an Andean seed crop used in a similar manner to cereals. Its seeds are perispermic with an endosperm present only in the micropylar region. The storage globulins are mainly deposited in the embryo. In this study, the development of proteolytic activities and protein profiles were studied in these tissues during germination and early growth. The endosperm was highly proteolytic with a 60-fold activity compared to the embryo. This endosperm activity consisted 100 % of cysteine-proteases, and increased until 24 hours after imbibition (hai). The activities found in the embryo consisted of aspartic, serine and metallo-proteases, but remained nearly unchanged over the course of growth. The bulk of seed proteins were found in the embryo globulin fraction with electrophoretic mobilities corresponding to 49 and 57 kDa. These bands started fading 48 hai. The presence of abscisic acid led to a lower proteolytic activity in the endosperm, and increased the percentage of abnormally germinating seeds. The protease activities found in the endosperm are not likely to be significant for storage protein mobilisation as the majority of the storage proteins are located in the embryo. These data suggests they may have a role in endosperm weakening during germination.

5.1. Introduction

Quinoa (*Chenopodium quinoa*) is a dicotyledonous seed crop with high resistance to adverse abiotic factors including drought, soil salinity and frost (Bonifacio, 2003; Jacobsen et al., 2003). It is native to the Andean region, where it has been cultivated for over 5000 years (Abugoch, 2009). Quinoa was the most important seed crop in the pre-Columbian times, but the consumption nearly vanished after the Spanish conquest, and it remained the food of Quechua and Aymara peoples in rural mountain areas (Abugoch, 2009). In the recent years, quinoa has attracted renewed interest due to its high nutritional value and absence of proteins that cannot be tolerated by individuals with the celiac disease (Abugoch, 2009; Ranhotra et al., 1992). This has led to a steep increase in demand, with a nearly 5-fold increase in production since 1970 (FAOSTAT, 2014; Jacobsen, 2003).

Quinoa seeds are flat and spherical with a mean diameter of 1.4-1.6 mm (Abugoch, 2009). They are composed of a large central perisperm and a peripheral embryo. The endosperm is present only in the micropylar region of the seed as a cap surrounding the radicle tip (Prego et al., 1998). The deposition of storage is highly compartmentalised: starch is located in the perisperm, while protein and lipid bodies are found in the embryo and endosperm (Prego et al., 1998). Quinoa storage proteins are a legumin type globulin (chenopodin) with a sedimentation coefficient of 11S, and high-cysteine globulin with a sedimentation coefficient of 2S (Brinegar and Goundan, 1993; Brinegar et al., 1996). Native chenopodin is a hexamer of subunits, that each consist of an acidic (32-39 kDa) and basic (22-23 kDa) polypeptides (Brinegar and Goundan, 1993; Müntz, 1996).

Germination starts by water uptake and ends when the radicle emerges through the surrounding layers. The following growth is referred to as early seedling

establishment (Bewley and Black, 1994a). In the seeds of some species, the micropylar endosperm cap controls the germination by acting as a physical barrier that prevents radicle protrusion (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006). The weakening of the micropylar endosperm has been reported to occur during early germination by autolysis caused by cell-wall modifying proteins, cell-wall polysaccharide degradation, and protein degradation (Morris et al. 2011, Finch-Savage and Leubner-Metzger, 2006). Abscisic acid (ABA), a plant hormone that regulates the induction and maintenance of dormancy, delays the onset of endosperm weakening (Morris et al., 2011).

Dry, ungerminated quinoa seeds have a very high proteolytic activity, comparable to that of malted barley. Additionally, the total activity does not markedly change over 24 h germination period when analysed in the whole seed (Mäkinen et al., 2013). In most seeds, storage protein mobilisation is initiated by *de novo* synthesised endopeptidases that render the proteins into a more soluble form and available for further degradation by another proteolytic enzyme (Müntz, 1996). For example, the activity is very low in ungerminated cereals and starts to increase only after 2-5 d after exposure to water (Brijs et al., 2002; Wrobel and Jones, 1992).

To shed light on the physiological role of the peculiar pattern of proteolytic activities in quinoa, the development of these activities and changes in protein profiles were studied separately in the perisperm, embryo and endosperm tissues during germination and seedling establishment.

5.2. Materials and methods

5.2.1 Materials

Red quinoa réal (Priméal, France) was desaponified by light abrasion using spring water (personal communication with producer). Protein content of the whole seed was 12.15%. Chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise stated.

5.2.2 Germination and separation of tissues

Quinoa seeds (2 g) were imbibed with distilled water (6 ml) and grown in petri dishes on two layers of filter paper in darkness at 15 °C. The water uptake of the seeds was measured by choosing 20 seeds that were dried gently with tissue, weighed and placed back in petri dishes. The influence of abscisic acid (ABA) on germination power was tested with 10 and 50 μM ABA in identical conditions. For analyses, samples were collected at different time points and the endosperms and embryos were separated from perisperms using forceps. Samples were immediately frozen and lyophilised.

5.2.3 Synthetisis of azogelatin

Azogelatin was prepared according to the method of Jones et al (1998). Solution A was prepared by dissolving 20 g porcine skin gelatin (300 bloom) in 275 ml H_2O containing 4 g $NaHCO_3$ and heated until dissolved. Solution B was prepared by subsequently dissolving 0.4 g NaOH; 1.73 g sulfanilic acid; 0.69 g $NaNO_2$; 3.33 ml 6 M HCl in 30 ml H_2O. Solution B was stirred until cloudy, 0.8 g NaOH was added and the solution was combined with solution A under stirring. The mixture

was dialysed (MW cut-off 12 400) against 3 x 4 l sodium azide (0.01%) and lyophilised.

5.2.4 Extraction of proteolytic enzymes

Proteases from endosperm, perisperm and embryo of 50 seeds were extracted in 0.05 M phosphate buffer (pH5.0) with 2 mM L-cysteine for 1 h at 4 °C. Perisperms were homogenised in the buffer using a mortar and pestle, embryos were homogenised using a high-shear dispenser (Ultra-Turrax T10, IKA-Werke GmbH & Co. KG, Staufen, Germany) before extraction. Endosperms were extracted as is, and homogenised using Ultra-Turrax only after the extraction, as considerable amount of sample got caught in the dispersion head. Solids were separated by centrifugation (15 000 g x 15 min), and supernatants were filtered (0.45 μm). Extracts were analysed immediately for in solution proteolytic activities.

5.2.5 Assay of proteolytic activities

Proteolytic activities of endosperms, perisperms and embryos excised from quinoa seeds at different stages of growth were analysed at pH 2.5-7.0 as follows. Extracts (30 μl) were mixed with 30 μl 0.1 M phosphate buffer of reaction pH. Aqueous azogelatin solution was mixed with 0.2 M phosphate buffer of reaction pH (1:1), to a final azogelatin concentration of 2%, and 67.5 μl of this solution was added in the sample. Samples were incubated for 30 min at 40 °C, and reaction was stopped by adding 112.5 μl cold trichloroacetic acid (TCA) (25%). Precipitated substrate was removed by centrifugation (10 000 g x 10 min), and 200 μl of supernatants were transferred in the wells of a flat-bottomed 96 well plates. The absorbance was read at 450 nm (Multiscan FC, Thermo Fisher scientific, Vantaa,

Finland). General proteolytic activities were expressed as increase in absorbance compared to a reaction blank under experimental conditions. The reaction blank was prepared by adding TCA before reaction. A unit of activity (U) was defined as the increase in absorbance under experimental conditions.

5.2.6 Effect of class-specific inhibitors on protease activity

The effect of class-specific inhibitors on gelatin hydrolysing activity was determined at optimum pH of each tissue. The following inhibitors were used at concentrations chosen based on preliminary trials: 20 μm E-64 (cysteine (Cys) protease inhibitor), 20 μm pepstatin A (Pep-A; aspartic (Asp) protease inhibitor), 1 mM phenylmethanesulfonyl fluoride (PMSF; serine (Ser) protease inhibitor) and 10 mM o-phenanthroline (o-phen; metallo (Met) protease inhibitor). Extracts (30 μl) prepared as described under *2.3.2* were incubated with each inhibitor (1.25 μl) for 5 min and the proteolytic activities were analysed as previously described, and the inhibition was expressed against a control without the inhibitor [Eq. 1].

$$Inhibition(\%) = A_{control} - \left(\frac{A_{sample}}{A_{control}}\right) \times 100 \qquad \text{[Eq. 1]}$$

5.2.7 SDS-PAGE

Tissues were extracted sequentially in dH$_2$O (albumin fraction) and 0.05 M Tris-HCl buffer (pH 9.0) with 0.5 M NaCl (globulin fraction), both containing 10 μl/ml protease inhibitor cocktail (P9599; Sigma-Aldrich). Perisperm samples were ground using mortar and pestle in extraction solution, embryos were homogenised before and after extraction using a high-shear disperser, and endosperms were homogenised only after extraction. The extraction was performed at 5 °C for 60 min, followed by centrifugation (2500 g x 5 min at 4 °C). Protein contents of the extracts

were determined using the Bradford assay, and samples were diluted in water or extraction buffer, and sample loading buffer (yielding final concentrations of 2% SDS, 4% glycerol, 2.5 mM DTT and 0.01% coomassie blue in 0.05 M Tris-HCl, pH 6.8). Each well of a 12.5 % polyacrylamide gel (10 x 10 cm) was loaded with 10 µg protein, and electrophoresis was performed at 7 °C according to Laemmli (1970). Gels were stained using a modified sensitive colloidal staining protocol (Westermeier, 2006). Molecular weight markers were run in parallel with the samples (Precision Plus Protein standards, All Blue, Bio-Rad, Richmond, CA). Gels were scanned and bands were quantified by densitometry (ImageJ 1.47v, National Institutes of Health, USA). Band densities were expressed relative to the corresponding bands from ungerminated seeds (0 h).

5.2.8 Scanning electron microscopy

Whole seeds and endosperm caps excised from ungerminated and 24 hai seeds were lyophilised, attached on aluminium stubs with conductive carbon cement (Leit-C, Neubauer Chemikalien, Münster, Germany), and sputter coated with a 5 nm layer of gold/palladium (80:20) (Polaron E5150 Sputter Coating). Samples were examined using a Jeol Scanning Electron Microscope (JSM-5510, Jeol Ltd., Tokyo, Japan) at an acceleration voltage of 7 kV and a working distance of 20 mm.

5.2.9 Statistical analyses

All analyses were carried out at least in triplicate. Means were compared using one way analysis of variance (ANOVA) and Tukey's post hoc test using Statistica 12 (StatSoft, Tulsa, OK, USA). The level of significance was determined at $p < 0.05$.

5.3 Results

5.3.1 Water uptake, germination and the influence of ABA

The majority of seeds had completed germination (defined as ruptured endosperm) between 6 and 10 hours after imbibition (hai), when the water content of the seeds increased from 41 to 45 % (Figure 5.1 a). The seeds germinated in two steps, where the rupture of the seed coat and endosperm occurred separately approx. 2 h apart (Figure 5.2 a-b). The extent of endosperm rupture varied, ranging from the formation of a hole through which the radicle continued to grow (Figure 5.2 d), to complete splitting of the tissue on one side (Figure 5.2 e-f).
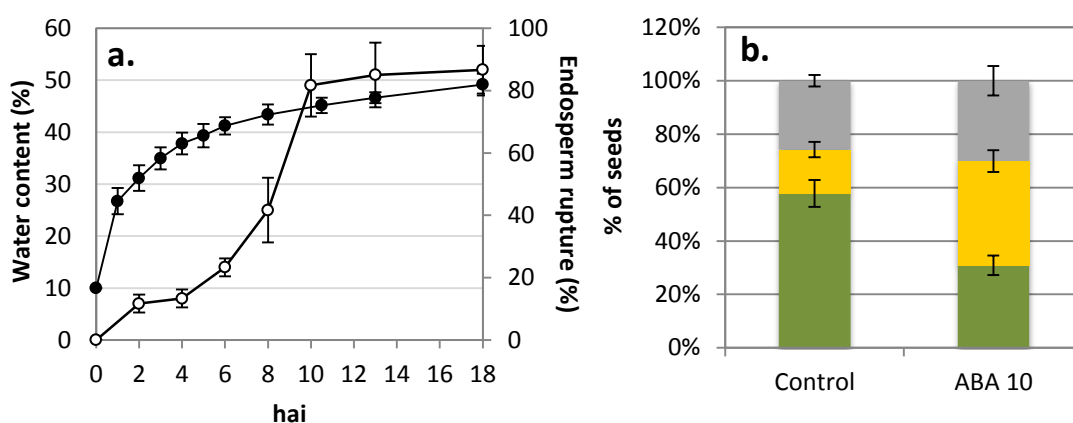

Figure 5.1. Time course of water uptake (●) and germination (○) (a), and the influence of ABA (10 μM) on the occurrence of ruptured endosperm and testa (green), ruptured testa only (yellow) and no radicle expansion (grey) 22 hai (b).

The presence of abscisic acid (ABA) led to a near 2.5-fold increase in the percentage of seeds that exhibited ruptured testa only, but an intact endosperm 22 hai (Figure 5.1 b). ABA did not significantly influence the percentage of seeds not showing visible changes ("non-germinating"). Higher ABA concentration (50 μM) had an impact similar to 10 μM after 22 h (not shown). When the seeds that exhibited only testa rupture were germinated for another 12 h (total 34 h), endosperm
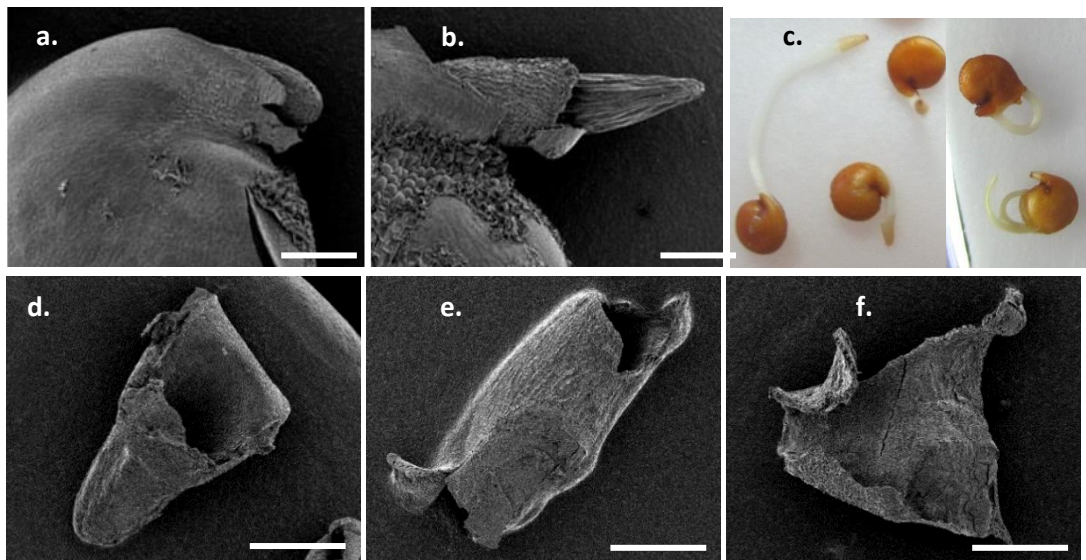
Figure 5.2. SEM images of ruptured seed coat (a) and endosperm (b) in germinating quinoa seeds. Abnormal germination in the presence of ABA (c). The appearance of an intact endosperm of a non-germinated seed (d) ruptured endosperms excised from 24 hai seeds (e-f). Bars 500 μm.

rupture had occurred in 33 and 39% of the seeds in the absence and presence of ABA, respectively (not shown). Additionally, 32.5 % of the ABA treated seeds showed radicle elongation despite an intact endosperm. In this case, the endosperm was either torn off the seed but still around the tip of the growing radicle, or still connected to the seed, causing the radicle-hypocotyl axis to burst out the seed with the radicle tip and cotyledons still attached (Figure 5.2 c). A higher ABA concentration (50 μM) decreased the number of seeds exhibiting this behaviour, with most seeds remaining unchanged between 22 and 34 h. Overall, ABA appeared to inhibit or delay the endosperm rupture, but did not stop the embryo from growing.

5.3.2 Proteolytic activities

Proteolytic activities of endosperms, perisperms and embryos excised from quinoa seeds at different stages of growth are presented in Figures 5.3a-c as a function of pH. Activity was found at a pH range 3.0-6.0 in endo- and perisperms with optima at pH 4.0. The activities increased until 24 hai in endo- and perisperms,

after which they declined. A low level of activity at a higher pH range, between 6.0 and 6.5, arose 24 hai in the endosperm, and 48 hai in the perisperm (Figure 5.3 a-b). In the embryo, the activity shifted towards higher pH, with most activity found between pH 3.5 and 6.5, with an optimum between 4.5 and 5.0 (Figure. 5.3 c). In contrast to the other tissues, nearly no changes in the level of activity were found in the embryo except a slight increase in activity between 12 and 24 hai despite the increase in total mass of the tissue over the course of growth.

When the activities were expressed per mass, it was clear that the endosperm was highly proteolytic, with a maximum activity of 116.6 U/g, compared to the maxima of perisperm (4.1 U/g) and embryo (2.0 U/g) (not shown). The endosperm made up 29 % of the total activity in ungerminated seeds (0 hai), which increased to 40 % by 24 hai.

As ABA appeared to inhibit or delay the endosperm rupture, the influence of 10 µM ABA on the proteolytic activity found in the endosperm was tested after 24 hai. The activity was significantly lower in the presence of ABA (Figure 5.3 d), and comparable to the level of activity found after 12 h in the absence of ABA (Figure 5.3 a). Hence, it appears that the high proteolytic activity in the endosperm tissue is at least partly related to endosperm weakening.

5.3.3 Effect of class-specific inhibitors

Class-specific protease inhibitors were used to evaluate the activities in each tissue. The presence of E-64 (Cys) led to a full inhibition of proteolytic activity in endo- and perisperms (Table 5.1). Also, chelation of metal ions with *o*-phenanthroline inhibited the activity by 68 and 56% in endo- and perisperms,
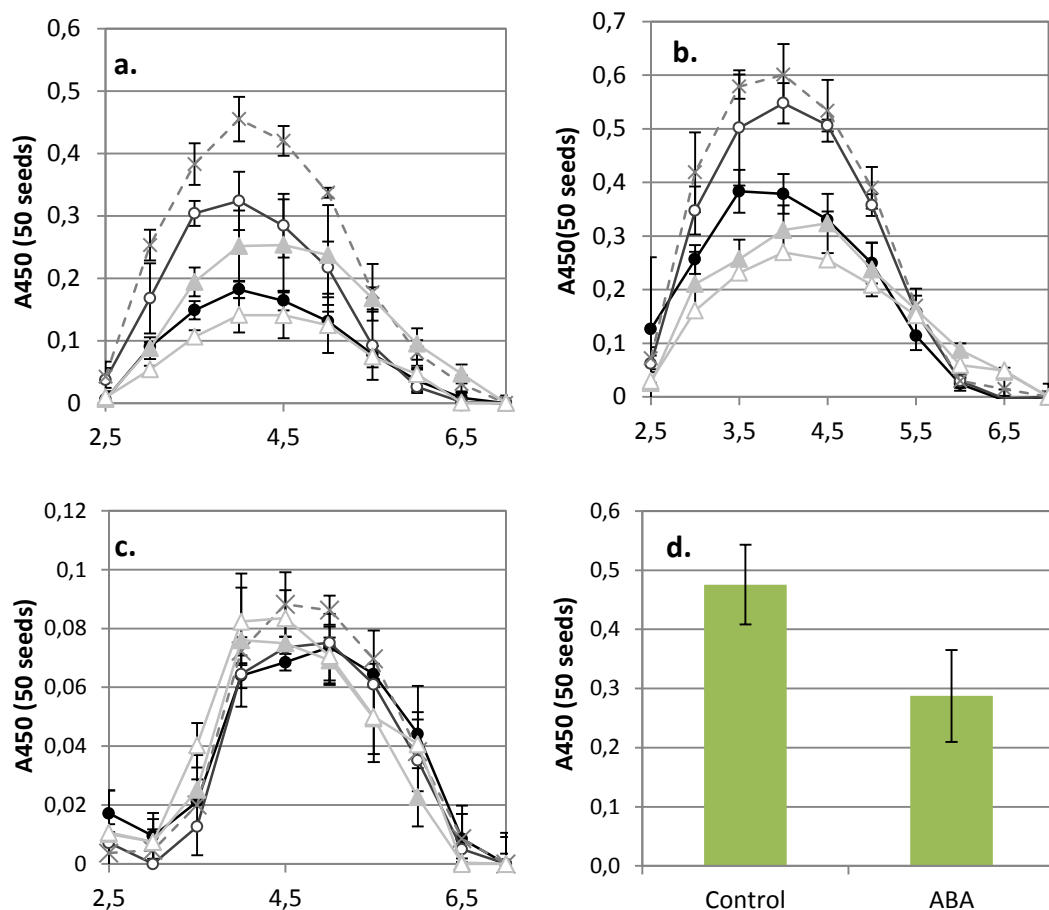
Figure 5.3. General proteolytic activities of quinoa endosperm (a), perisperm (b) and embryo (c) as a function of pH 0 hai (—●—), 12 hai (—○—), 24 h (—✳—), 48 hai (—▲—) and 72 hai (—△—), and in the endosperm after 24 h in the absence and presence of 10 μM ABA (d).

Cys protease activity remained unchanged. Low levels of inhibition were achieved with Pep-A (Asp) and PMSF (Ser) in ungerminated endosperm, and in the perisperm 72 hai.

In the ungerminated embryo, proteolytic activity was inhibited by Pep-A (41.4 %), PMSF (27.4%) and *o*-phen (81.3 %), indicating the presence of Asp, Ser and metallo protease activities, but it was completely absent of Cys protease activity. However, 72 hai Cys protease activity emerged (13.6 % inhibition by E-64), while the other activities did not change over the course of germination. Overall, the activities in endo- and perisperms showed very similar patterns, but a different set on proteolytic activities was present in the embryo.

Table 5.1. Inhibition (as % of the activity without inhibition) by class-specific inhibitors.

Tissue	hai	Inhibitor			
		E-64	Pep-A	PMSF	*o*-phen
Endosperm	0	101.2 ± 1.58 a	10.5 ± 2.24 a	5.5 ± 3.49 a	68.0 ± 4.45 a
	24	100.7 ± 0.43 a	1.3 ± 2.91 b	0.8 ± 2.47 a	54.9 ± 2.47 b
	48	101.3 ± 2.33 a	-3.1 ± 2.82 b	-1.0 ± 2.86 a	57.9 ± 5.50 b
	72	99.2 ± 0.38 a	-0.7 ± 5.39 b	2.8 ± 4.34 a	54.6 ± 3.82 b
Perisperm	0	99.4 ± 0.86 a	-1.1 ± 0.75 c	1.9 ± 0.64 b	56.0 ± 2.18 b
	24	98.8 ± 1.73 a	0.7 ± 3.38 bc	4.2 ± 1.66 b	56.6 ± 3.75 b
	48	100.5 ± 2.48 a	5.9 ± 2.01 b	9.9 ± 4.46 a	58.9 ± 2.62 b
	72	100.7 ± 3.84 a	13.2 ± 5.16 a	12.8 ± 1.54 a	68.1 ± 2.34 a
Embryo	0	-2.5 ± 5.19 b	41.4 ± 5.46 a	27.4 ± 4.61 ab	81.3 ± 5.78 a
	24	1.4 ± 4.48 b	39.0 ± 3.51 a	27.2 ± 1.75 b	71.0 ± 7.20 a
	48	3.7 ± 4.58 ab	46.4 ± 1.40 a	35.9 ± 4.67 a	74.3 ± 2.01 a
	72	13.6 ± 3.84 a	46.3 ± 2.61 a	35.5 ± 1.36 a	72.9 ± 1.50 a

Values within a column within that tissue that share a superscript are not significantly different from one another ($p < 0.05$).

5.3.4 Changes in protein profiles

The majority of seed proteins was found in the embryo globulin (GL) fraction (Figure. 5.4 a). Under non-reducing conditions, two bands corresponding to chenopodin subunits with molecular weights of 49 and 57 kDa were observed. These proteins dissociated into pairs of acidic (28 and 34 kDa) and basic (17 and 19 kDa) chains in the presence of a reducing agent (DTT). The molecular weights were slightly lower than previously reported (32-39 and 22-23 kDa) (Brinegar and Goundan, 1993). Also a 50 kDa protein was found in the GL fraction under reducing conditions by Brinegar and Goundan (1993). They suggested that this protein may be an 11S subunit precursor before the post-translational separation of the acidic and

basic chains. The 49 and 57 kDa proteins showing the same behavior under reducing conditions were also found in the endosperm decreased to 0.95 and 0.96 after 48 h and 0.84 and 0.88 after 72 h for 49 and 57 kDa bands, respectively. In the endosperm, changes were visible already after 24 h. The 57 kDa protein was degraded faster: the relative density was 0.49 after 24 h, and the whole band has disappeared after 48 h, while the 49 kDa band was still visible after 48 h with a relative density of 0.31.
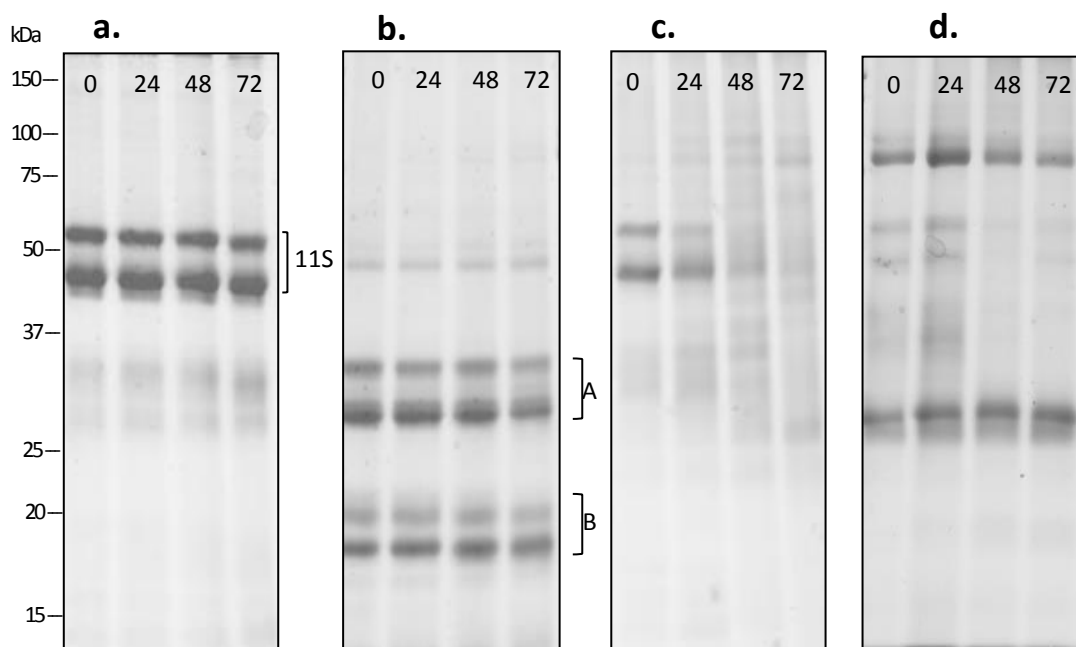

Figure 5.4. SDS-PAGE profiles of globulin fractions of embryo under non-reducing (a) and reducing (b) conditions, and endosperm (c) and perisperm (d) under non-reducing conditions. Numbers over each lane indicates hours after imbibition. Chenopodin subunits (11 S), and acidic (A) and basic (B) polypeptides and are indicated.

5.4. Discussion

Quinoa germinated very fast, with the majority of seeds showing radicle protrusion 6-10 h after exposure to water. Very fast germination (<24 h) is a trait found in some seeds from high-stress habitats, most of them belonging in the former *Chenopodiaceae* family (Parsons, 2012). The ability to exploit favourable conditions by fast germinating rapidly may be a means of survival in these environments (Gutterman, 1972; Parsons, 2012).

The application of exogenous ABA inhibited or delayed endosperm rupture but did not stop the embryo from growing, leading to a high occurrence of seeds showing significant radicle elongation despite intact endosperms. The presence of ABA also reduced the proteolytic activity found in the endosperm after 24 h by 40%. These observations suggest that ABA may be involved in the control of endosperm weakening, but does not prevent the seed from germinating alone at least when added during imbibition. ABA has been found to delay the endosperm weakening in cress (*Lepidium sativum)* and *Arabidopsis thaliana*, playing at least a partial role in the mediation of radicle protrusion (Müller et al., 2006).

Experiments with class-specific protease inhibitors showed that the endosperm activity consisted 100 % of Cys proteases, of which 68 % were also inhibited by a metallo protease inhibitor, indicating the requirement of metal ions for these enzymes. The embryo was absent of Cys activity until 72 hai, before which its activity consisted of Asp, Ser and Met protease activities. Storage protein mobilisation from the native protein to amino acids is a result of sequential activity of various enzymes (Bewley and Black, 1994b). The general pattern in dicotyledonous seeds is the initiation of globulin mobilisation by a *de novo* synthesised Cys protease (proteinase A), followed by further degradation by another

Cys protease (proteinase B) and carboxypeptidases (Bewley and Black, 1994b; Müntz, 1996). Some reports of triggering proteases from other classes exist: in soybean (*Glycine max*), a Ser protease initiates the degradation of β-conglycinin subunits, and a Met protease has been found to perform limited proteolysis on buckwheat *(Fagopyrum esculentum)* 13S globulin (Belozersky et al., 1990; Qi et al., 1992). Storage protein mobilisation in the cotyledons and endospermic storage tissues generally occurs after radicle protrusion, but stored proteases start globulin mobilisation in the embryonic axes and cotyledons of vetch (*Vicia sativa*) already during germination (Schlereth et al., 2001).

It is notable that the class-specific protease activities remained relatively unchanged over the course of 72 h. The endosperm activities increased and peaked 24 hai, but no significant changes in the total activity or the influence of class-specific inhibitors was observed in embryo proteases except the emergence of a low level of Cys protease activity between 48 and 72 hai before. Before 48 hai, the embryo was absent of Cys proteases. Whether this activity is relevant for storage globulin mobilisation remains unanswered. Although degradation of 11S subunits in the GL fraction was not obvious before 72 hai, the fading of the corresponding bands in the AL fraction 48 hai indicate occurrence of proteolysis earlier in the embryonic tissues. Storage globulin mobilisation in amaranth (*Amaranthus hypochondriacus)* seeds is initiated by limited proteolysis leading to changes in charge immediately after the completion of germination (Aphalo et al., 2009). In vetch (*Vicia sativa*), stored proteases trigger the globulin mobilisation, but the bulk of the proteolysis is performed by *de novo* synthesised proteases (Müntz, 2001; Schlereth et al., 2001). The absence of significant changes in the protease activities in the quinoa embryo do not indicate major synthesis of proteases. However it has to be noted that activities

measured in tissue extracts may not reflect *in planta* activity, as the storage proteins may be protected from premature degradation by maintaining the enzymes inactive, a structural feature in the protein that inhibits proteolysis, or compartmentation that prevents the enzyme from contact with its substrate (Bewley and Black, 1994a; Müntz, 1996; van der Hoorn et al., 2004). For example, a vacuolar Cys proteinase that hydrolyses storage proteins in legumes is processed into a mature form at pH values <5.8, which occurs only after the pH has decreases from 6 to 5.5 during germination, preventing its activity at the pH of the protein bodies of ungerminated seeds (Okamoto et al., 1999).

Although embryo is the major protein storage tissue in quinoa, protein bodies are present also in the endosperm (Prego et al., 1998). Extraction of protein from endosperms at different stages of growth showed rapid degradation of the 11S subunit bands. It may be assumed, that the high Cys protease activity found in the endosperm is responsible for the hydrolysis of these proteins. In a study on cress, the incubation of excised endosperm caps with Asp, Ser and Cys protease inhibitors each decreased tissue autolysis by 30 % compared to control, and the inhibition of the 26S proteasome led to a complete inhibition of the endosperm autolysis (Morris et al., 2011). Each class of studied proteases had an effect, implying that the degradation of numerous protein targets is necessary for endosperm autolysis in this particular seed. Müller et al. (2010) reported Asp protease accumulation in the endosperm cap of germinating cress during endosperm weakening. The authors suggested that the enzyme serves a non-nutritional function in the control of germination.

To conclude, the high endosperm protease activity is not likely to be significant in terms of storage protein mobilisation because of the bulk of storage proteins is located in the embryo. The negative influence of ABA on the occurrence

of endosperm rupture and the development of proteolytic activity in the endosperm suggests that protein degradation is involved in endosperm weakening. The total proteolytic activity in the embryo remained unchanged over the course of 72 h, evoking the question whether the key proteases responsible for embryonic storage protein mobilisation are already stored in the dry ungerminated seed.

Acknowledgments

References

Abugoch James, L., E., 2009. Quinoa (*Chenopodium quinoa Willd.):* Composition, chemistry, nutritional, and functional properties. Adv. Food Nutr. Res. 58, 1–31.

Aphalo, P., Martínez, E.N., Añón, M.C., 2009. Structural modifications of Amaranth proteins during germination. Protein J. 28, 131–8.

Belozersky M., A., Dunaevsky Y., E., Voskoboynikova N., E., 1990. Isolation and properties of a metalloproteinase from buckwheat (*Fagopyrum esculentum*) seeds. Biochem. J. 15, 677–682.

Bewley, J.D., 1997. Breaking down the walls - a role for endo-β-mannanase in release from seed dormancy? Trends Plant Sci. 2, 464–469.

Bewley, J.D., Black, M., 1994a. Germination, structure and composition, in: Seeds - Physiology of development and germination. Plenum Press, New York, pp. 1–33.

Bewley, J.D., Black, M., 1994b. Mobilization of stored seed reserves, in: Seeds - Physiology of Development and Germination. Plenum Press, New York, pp. 293–343.

Bonifacio, A., 2003. Chenopodium Sp.: Genetic resources, ethnobotany, and geographic distribution. Food Rev. Int. 19, 1–7.

Brijs, K., Trogh, I., Jones, B.L., Delcour, J.A., 2002. Proteolytic enzymes in germinating rye grains. Cereal Chem. 79, 423–428.

Brinegar, C., Goundan, S., 1993. Isolation and characterization of chenopodin, the 11S seed storage protein of quinoa (*Chenopodium quinoa*). J. Agric. Food Chem. 41.

Brinegar, C., Sine, B., Nwokocha, L., 1996. High-cysteine 2S seed storage proteins from quinoa (*Chenopodium quinoa*). J. Agric. Food Chem. 44, 1621–1623.

FAOSTAT, 2014. Agricultural statistics database. Word Agric. Inf. Cent., Rome, Italy.

Finch-Savage, W.E., Leubner-Metzger, G., 2006. Seed dormancy and the control of germination. New Phytol. 171, 501–23.

Gutterman, Y., 1972. Delayed seed dispersal and rapid germination as survival mechanisms of the desert plant *Blepharis persica (Burm.) kuntze*. Oecologia 10, 145–149.

Jacobsen, S.-E., 2003. The worldwide potential for quinoa (*Chenopodium quinoa Willd*.). Food Rev. Int. 19, 167–177.

Jacobsen, S.-E., Mujica, A., Jensen, C.R., 2003. The resistance of quinoa (*Chenopodium quinoa Willd*.) to adverse abiotic factors. Food Rev. Int. 19, 99–109.

Mäkinen, O.E., Zannini, E., Arendt, E.K., 2013. Germination of oat and quinoa and evaluation of the malts as gluten free baking ingredients. Plant Foods Hum. Nutr. 68, 90–5.

Morris, K.D., Linkies, A., Müller, K., Oracz, K., Wang, X., Lynn, J.R., Leubner-Metzger, G., Finch-Savage, W.E., 2011. Regulation of seed germination in the close *Arabidopsis* relative Lepidium sativum: a global tissue-specific transcript analysis. Plant Physiol.

Müller, K., Job, C., Belghazi, M., Job, D., Leubner-Metzger, G., 2010. Proteomics reveal tissue-specific features of the cress (*Lepidium sativum L.*) endosperm cap proteome and its hormone-induced changes during seed germination. Proteomics 10, 406–16.

Müller, K., Tintelnot, S., Leubner-Metzger, G., 2006. Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and Arabidopsis thaliana. Plant Cell Physiol. 47, 864–77.

Müntz, K., 2001. Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth. J. Exp. Bot. 52, 1741–1752.

Müntz, K., 1996. Proteases and proteolytic cleavage of storage proteins in developing and germinating dicotyledonous seeds. J. Exp. Bot. 47, 605–622.

Okamoto, T., Yuki, A., Mitsuhashi, N., Mimamikawa, T., 1999. Asparaginyl endopeptidase (VmPE-1) and autocatalytic processing synergistically activate the vacuolar cysteine proteinase (SH-EP). Eur. J. Biochem. 264, 223–232.

Parsons, R.F., 2012. Incidence and ecology of very fast germination. Seed Sci. Res. 22, 161–167.

Prego, I., Maldonado, S., Otegui, M., 1998. Seed structure and localization of reserves in *Chenopodium quinoa*. Ann. Bot. 481–488.

Qi, X., Wilson, K.A., Tan-Wilson, A.L., 1992. Characterization of the major protease involved in the soybean - conglycinin storage protein mobilization. Plant Physiol. 99, 725–733.

Ranhotra, G.S., Gelroth, J.A., Glaser, B.K., Lorenz, K.J., Johnson, D.L., 1992. Composition and protein nutritional quality of quinoa. Cereal Chem. 70, 303-305.

Schlereth, A., Standhardt, D., Mock, H.-P., Müntz, K., 2001. Stored cysteine proteinases start globulin mobilization in protein bodies of embryonic axes and

cotyledons during vetch (*Vicia sativa L.*) seed germination. Planta. 212, 718–727.

Van der Hoorn, R.A.L., Leeuwenburgh, M.A., Bogyo, M., Joosten, M.H.A.J., Peck, S.C., 2004. Activity profiling of papain-like cysteine proteases in plants. Plant Physiol. 135, 1170–8.

Westermeier, R., 2006. Sensitive, quantitative, and fast modifications for Coomassie Blue staining of polyacrylamide gels. Proteomics 6 Suppl 2, 61–4.

Wrobel, R., Jones, B.L., 1992. Appearance of endoproteolytic enzymes during the germination of barley. Plant Physiol. 100, 1508–1516.

Chapter 6

Germination of oat and quinoa and evaluation of the malts as gluten free baking ingredients

Outi E. Mäkinen, Emanuele Zannini, Elke K. Arendt

Abstract

Germination can be used to improve the sensory and nutritional properties of cereal and pseudocereal grains. Oat and quinoa are rich in minerals, vitamins and fibre while quinoa also contains high amounts of protein of a high nutritional value. In this study, oat and quinoa malts were produced and incorporated in a rice and potato based gluten free formulation. Germination of oat led to a drastic increase of α-amylase activity from 0.3 to 48 U/g, and minor increases in proteolytic and lipolytic activities. Little change was observed in quinoa except a decrease in proteolytic activity from 9.6 to 6.9 U/g. Oat malt addition decreased batter viscosities at both proofing temperature and during heating. These changes led to a decrease in bread density from 0.59 to 0.5 g/ml and the formation of a more open crumb, but overdosing of oat malt deteriorated the product as a result of excessive amylolysis during baking. Quinoa malt had no significant effect on the baking properties due to low α-amylase activity. Despite showing a very different impact on the bread quality, both malts influenced the electrophoretic patterns of rice flour protein similarly. This suggests that malt induced proteolysis does not influence the technological properties of a complex gluten free formulation.

6.1 Introduction

Celiac disease (CD) is an immune-mediated enteropathy with a worldwide prevalence of 1%, triggered by wheat gluten and related proteins in rye and barley (Catassi and Fasano, 2008). The only treatment is the complete exclusion of these proteins from the diet (Catassi and Fasano, 2008). Uncontaminated oat is well tolerated by most CD patients and the consumption of "pure oat" is recommended by many national coeliac associations, while quinoa is considered safe (Bergamo et al., 2011; Butzner, 2011; Kemppainen et al., 2007). Commercial gluten free breads are often produced using ingredients low in minerals and fibre (Hager et al., 2011). The inclusion of oat and quinoa in standard gluten free diets has been found to improve the nutrient profile, increasing especially the intake of fibre, iron, folate and protein (Lee et al., 2009).

Oat (*Avena sativa*) is a cereal rich in minerals, vitamins and compounds with antioxidant activities, and it contains protein high in lysine (Lásztity, 1998). Also its main cell wall constituent, $(1\rightarrow3)$, $(1\rightarrow4)$-β-glucan, carries an FDA and EFSA approved health claim on a cholesterol lowering effect (Wood, 2010). Quinoa (*Chenopodium quinoa*) is a pseudocereal indigenous to the Andean region, where it has been used as a staple food for thousands of years (Repo-Carrasco et al., 2003). Quinoa seeds contain high amounts of vitamins, minerals and protein with a good digestibility and a balanced amino acid profile (Repo-Carrasco et al., 2003; Ruales and Nair, 1992). Due to its excellent nutritional value and a potential for production in various climates (incl. Europe), quinoa has been declared as one of the humanity's most promising crops by the Food and Agriculture Organisation of the United Nations (FAO, 2011; Jacobsen et al., 2003).

During malting the grains are soaked, germinated and subjected to a heat treatment to end the metabolic processes and to develop aroma and flavour. During the germination step the storage compounds of the seed are being mobilised by a variety of synthesised and activated enzymes, resulting in an improved protein digestibility and mineral bioavailability (Kaukovirta-Norja et al., 2004; Valencia et al., 1999). The metabolic processes occurring in the germinating seeds also lead to an increase in antioxidant activity and the formation of secondary metabolites with possible bioactivities (Kaukovirta-Norja et al., 2004; Kim et al., 2012).

The aim of this study was to germinate oat and quinoa and evaluate their suitability for use in gluten free baking. In addition to influencing the nutritional profile of gluten free products, the malt enzymes may influence the technological quality of the products: barley and wheat malts are used in the industry as natural dough conditioners in wheat based products. Fortification of wheat bread with unconventional malted grains has been studied previously on malted quinoa, oat, sorghum and brown rice (Hugo et al., 2000; Mäkinen and Arendt, 2012; Park and Morita, 2005; Watanabe et al., 2004) but to the author's knowledge no work on gluten free systems has been published.

6.2 Materials and methods

6.2.1 Malting

Oat (variety Lutz, Germany) was steeped, germinated at 15 °C for 5 d and subsequently kilned in three stages (35 °C, 50 °C and 60 °C) in a malting machine (Joe White Malting Systems, Perth, Australia). Commercial white quinoa (variety unknown, Bolivia) was steeped for 5 h, germinated at 15 °C for 24 h and kilned in four stages (45°C, 50°C, 55°C and 65°C). Before use the rootlets and protruding

cotyledons were removed by hand, and the malts were ground to pass a 0.25 mm sieve.

6.2.2 Enzyme activities of malts

α- and β-amylase activities were determined by Ceralpha and Betamyl-3 methods (Megazyme, Wicklow, Ireland). *Proteolytic activities* were determined from malts extracted in 0.05 M acetate buffer containing 2 mM L-cysteine (pH 5.0) at a ratio of 1:3 for 30 min at 5 °C. Solids were removed by centrifugation (10 000 g x 15 min) and samples assayed against 1.4% (w/v) azocasein in 0.2 M sodium acetate buffer for 1 h. The reaction was stopped with 10% trichloroacetic acid and the samples were centrifuged. The supernatant was mixed with 0.5 M NaOH (1:1) and absorbance at 440 nm measured after 20 min. *Lipase activities* were determined using the dough method (Matlashewski et al., 1982) by incubating defatted malt samples in a mixture of Tris-HCl buffer (0.05 M, pH 7.5), 9.8 % (w/w) glyceryl trioleate and 1% Triton-X for 60 min as described in detail previously (Mäkinen and Arendt, 2012). The reaction was stopped with 1 M HCl. The free fatty acids were extracted in 2,2,4-trimethylpentane, quantified using the copper soap method against an oleic acid standard curve (Kwon and Rhee, 1986).

6.2.3 Malt protease induced changes in ingredients using lab-on-a-chip capillary electrophoresis

The effect of malt proteases on whey protein isolate (WPI) and rice flour proteins were studied by incubating each ingredient with 5% malts in 0.2 M acetate buffer (pH 5.4) at 30°C for 24 h, followed by lyophilisation. Ground samples (rice flour 20 mg; WPI 10 mg) were extracted in 1 ml buffer (5 M urea, 50 mM DTT and 2% (w/v) SDS in 0.1 M Tris-HCl; pH 8.8) for 2 h, solids removed by centrifugation

(15 000 g x 15 min) and supernatants were diluted (rice flour 1:1 and WPI 1:9). The protein profiles were analysed using a Protein80 kit with a molecular weight range of 5-80 kDa (Bioanalyzer, Agilent Technologies, Palo Alto, USA) under reducing conditions using reagents and standards provided by the manufacturer. For result evaluation the raw data was rescaled to match the height of the upper marker when necessary.

6.2.4 Batter properties

Starch pasting properties of rice flour and potato starch mixtures (1:1) with oat and quinoa malts were determined using the Rapid Visco Analyzer (General Pasting Method, AACC 76-21). The densities of the batters was measured by transferring 30 g batter in a 100 ml measuring cylinder immediately after mixing and recording the volume before and after 30 min proofing at 30 °C.

For rheological measurements, batters excluding yeast were mixed for 70 s with Glutomatic (Falling Number AB, Huddinge, Sweden), incubated in a proofer (30°C) for 30 min and then mounted on a controlled stress rheometer (MCR301, Anton Paar GmbH, Austria) with a cross-hatched parallel plate geometry (50 mm; gap 2 mm). A frequency sweep at a 0.01% strain was performed for angular frequencies (ω) 0.628-62.8 s^{-1} followed by a viscosity measurement for shear rates 0.6-5 s^{-1}. The complex moduli (G*) values from the frequency sweep were fitted using a weak gel model (Eq. 1) (Gabriele et al., 2001).

$$G^*_{(\omega)} = A_F \omega^{1/z}$$
Eq. 1

The effect of the malt enzymes on the viscosities of 0.3% xanthan and hydroxypropylmethylcellulose (HPMC) solutions was studied by incubating the samples with malt extracts (extracted for 15 min in 0.04 M acetate buffer (pH 4.6);

1:3 extraction ratio) for 30 min at 30 °C and measuring the viscosities between shear

rates 1-50 s^{-1}. All measurements were performed at 30 °C.

6.2.5 Baking and bread properties

A previously published formulation was used for the baking trials (Table 6.1) (Nunes

et al., 2008). The yeast (2%; Puratos, Belgium) was activated by dissolving it in 30˚C

tap water (90% on flour basis) and the suspension was added to pre-mixed dry

ingredients: rice flour (50%; Doves Farm Foods Ltd, UK), potato starch (50%;

Doves Farm Foods Ltd, UK), whey protein isolate (10%; Glanbia, Ireland), vegetable

oil (6%; Homestead, Ireland), sugar (2%; Siucra, Ireland), salt (2%; Glacia British

Salt Ltd., UK), xanthan gum (0.3%; Keltrol F; CP Kelko, Atlanta, U.S.A), HPMC

(0.3%; Metolose NE-4000, Harke, Germany) and the ground malts, and mixed for 2

min using a Kenwood Chef (Kenwood Manufacturing Co. Ltd., UK). Batter was

proofed in tins for 30 min (30˚C; RH 85%) and the loaves were baked for 45 min at

190 °C in a deck oven (MIWE, Arnstein, Germany).

Table 6.1. Recipes used in the baking trials.

Ingredient	Baker's % (%)	Control (g)	0.5% malt (g)	0.75% malt (g)	1% malt (g)	2% malt (g)	2.5% malt (g)	5% malt (g)
Rice flour	50	300	298.5	297.75	297	294	292.5	285
Potato starch	50	300	298.5	297.75	297	294	292.5	285
Malt	0	0	3	4.5	6	12	15	30
WPI	10	60	60	60	60	60	60	60
Vegetable oil	6	36	36	36	36	36	36	36
Sugar	2	12	12	12	12	12	12	12
Salt	2	12	12	12	12	12	12	12
Dry yeast	2	12	12	12	12	12	12	12
Xanthan gum	0.3	1.8	1.8	1.8	1.8	1.8	1.8	1.8
HPMC	0.3	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Water	90	540	540	540	540	540	540	540

Dough yield 212.6

The volume of the loaves was measured with VolScan Profiler (Stable Micro Systems, Surrey, UK) and the bread density calculated by dividing the loaf weight by the loaf volume. The crumb hardness was measured using a TAXT2i texture analyser (Stable Micro Systems, Surrey, UK) by compressing a 25 mm slice to 50% of its original height with a 20 mm aluminium probe. Hardness was defined as the maximum force during compression. Crumb grain was evaluated by image analysis using a C-cell Imaging System and software (Calibre Control International Ltd., UK). The parameters used were cell diameter, wall thickness and number of cells/cm^2 calculated from the number of cells and slice area.

6.2.6 Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM)

SEM samples were prepared by placing a small drop of batter on stubs (Agar Scientific, plain stubs 10mm x 10mm) and immersing the stub in liquid nitrogen after leavening (30 min at 30 °C) or baking at 190 °C. The frozen samples were fractured and lyophilised immediately. Dry samples were mounted on SEM stubs and sputter coated with a 5 μm layer of 80:20 gold-palladium and examined with a JEOL Scanning Electron Microscope (JSM-5510, Jeol Ltd., Tokyo, Japan) at 5 kV and working distance of 20 mm. For CLSM, batters were prepared with a Glutomatic a 1:3 mixture of 0.1% Rhodamin B and 50% Calcofluor white (Sigma-Aldrich) as dough liquid, incubated (30 min; 30 °C) and examined with a FV300 confocal laser-scanning system mounted on an Olympus IX80 inverted microscope with a 20x dry objective (Olympus, Germany), using λ_{ex} = 405 and 543 nm. Bread pieces were stained for 5 min in the Rhodamin B and Calcofluor mixture described above,

followed by 10 s in 0.3% FITC (Sigma-Aldrich) in acetone, and rinsed with H_2O. The samples were examined using $\lambda_{ex} = 405$, 488 and 543 nm.

6.2.7 Statistical analysis

All analyses were performed at least in triplicates and means were compared using one way analysis of variance with Tukey post-hoc test at a significance level of $p < 0.05$. All statistical analyses were performed using Statistica 7.1 (Statsoft, USA). Model fitting for rheological data was performed using Origin 7.5 (Originlab Corporation, Northampton, USA).

6.3 Results and discussion

6.3.1 Malt enzyme activities

The enzyme activities of the malts are given in Table 6.2. α- and β-amylase activities of oat increased from 0.3 to 48 and 0.5 to 2.3 U/g during malting, respectively. In quinoa malt, the amylolytic activities remained nearly unchanged before and after germination (< 1 U/g). This trend was reflected in the effect of added malts on the RVA peak viscosities: a 0.5% oat malt addition led to a viscosity loss of 18.5% and 56% with 2% oat malt (Table 6.3). Quinoa malt decreased the peak viscosity only little: 2.5% and 5% quinoa malt additions decreased the viscosities by 4.7% and 5.2%, respectively. A starch paste viscosity (Amylograph) loss of 18% as a result of adding 5% 12 h germinated quinoa in wheat flour has been reported previously (Atwell et al., 1988). The low amylolytic activities observed in this study may be caused by varietal differences or poor germination performance due to post-harvest processing.

Table 6.2. Enzyme activities of oat and quinoa before and after malting (U/g).

Parameter	α-amylase	β-amylase	Lipase	Protease
Oat	0.29 ± 0.07 b	0.5 ± 0.05 b	27.7 ± 4.8 b	2.13 ± 1.0 c
Oat malt	47.7 ± 2.0 a	2.3 ± 0.23 a	31.3 ± 3.3 a	5.86 ± 0.6 b
Quinoa	0.09 ± 0.05 b	0.5 ± 0.16 b	N/A	9.6 ± 1.1 a
Quinoa malt	0.08 ± 0.01 b	0.7 ± 0.28 b	1.3 ± 0.1 c	6.9 ± 1.2 b

Lipase activity of unmalted oat was high, and malting only slightly increased it (Table 6.2). No lipase activity was detected in unmalted quinoa, but a low level of activity (1.3 U/g) appeared as a result of malting. The protease activity of oat nearly tripled to 5.68 U/g during malting, but decreased from 9.6 to 6.9 U/g in quinoa. Compared to barley and wheat malts, both malts produced in this study were high in proteolytic activities (Mäkinen and Arendt, 2012).

6.3.2 Malt protease induced changes in ingredients

The electropherogramms of the WPI samples revealed two peaks at 15 kDa and 25 kDa, but malt proteases had no visible effect on either of the peaks (not shown). The electropherogramms of rice flour protein (Figure 6.1) show major peaks at molecular weights 10-15 kDa (a prolamin), at 21 kDa and a triple peak at 36-39 kDa (corresponding to oryzenin subunits) and two peaks at 55 and 59 kDa (Moroni et al., 2011). After 24 h, all peaks except the last one (59 kDa) showed a decrease in samples digested with both oat (Figure 6.1a) and quinoa malts (Figure 6.1b). No differences between the oat malt and quinoa malt digested samples were observable.

Table 6.3. Properties of batters with oat (O) and quinoa (Q) malts.

Parameter	RVA		Rheology		
	Peak viscosity (RVU)	Viscosity loss (%) [a]	Viscosity (Pas) [b]	A_f [c]	z [c]
Control	363 ± 2.2 a	0.0	33.5 ± 2.6 a	812 ± 98 a	6.36 ± 0.2 a
O 0.5%	296 ± 2.8 c	18.5	33.2 ± 2.5 a	765 ± 125 ab	6.08 ± 0.2 a
O 0.75%	256 ± 3.1 d	29.5	33.1 ± 2.2 a	746 ± 17 ab	6.32 ± 0.0 a
O 1%	225 ± 3.2 e	37.9	30.5 ± 5.0 ab	602 ± 62 bc	6.14 ± 0.2 a
O 2%	159 ± 8.3 f	56.2	25.4 ± 1.5 b	503 ± 23 c	6.15 ± 0.5 a
Q 1%	356 ± 3.4 ab	1.8	33.6 ± 4.0 a	821 ± 60 a	6.89 ± 0.5 a
Q 2.5%	346 ± 12.4 b	4.7	31.0 ± 2.6 a	871 ± 59 a	6.80 ± 0.4 a
Q 5%	344 ± 0.9 b	5.2	29.8 ± 3.4 ab	824 ± 78 a	6.73 ± 0.4 a

[a] Peak viscosity$_{sample}$ / Peak viscosity$_{control}$ * 100
[b] Apparent viscosity at $\gamma = 5$ s^{-1}
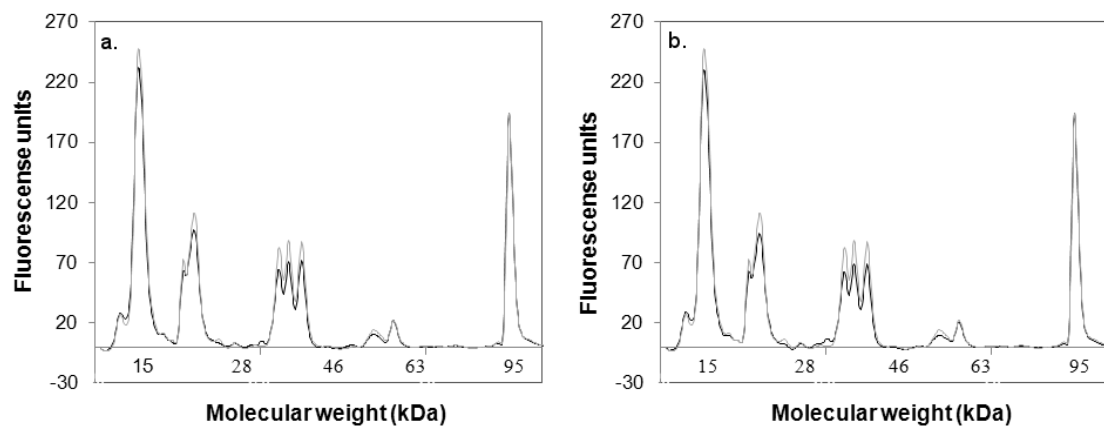[c] r^2 was > 0.95 for all samples

Figure 6.1. Electropherogramms of rice protein digested with oat malt (a. black); quinoa malt (b. black) and without malt (a-b. grey) for 24 h. Data from three runs averaged.

6.3.3 Batter rheology

Only 2% oat malt decreased batter viscosities significantly (Table 6.2). The results from the small strain oscillation measurements were interpreted by fitting the data in a weak gel model, yielding parameters A_F and z. The z value is an interaction factor indicating the amount of interactions in the food network, while A_F can be interpreted as the strength of the interactions (Gabriele et al., 2001). As a result of oat malt addition, the A_F values decreased, while z values remained nearly unchanged. This suggests that the amount of interactions remained the same while only their strength was weakened as a result of oat malt enzyme action on the batter components, as opposed to the effect of sourdough fermentation that decreases both factors (Moroni et al., 2011). The viscosities of 0.3% xanthan gum and HPMC solutions were 1.4 and 0.01 Pas at 5 s^{-1}, respectively, and not influenced by incubation with malt enzyme extracts (not shown).

As starch is still in its granular state at the proofing temperature and not prone to amylolysis, the main contributors to the rheological properties of the batters were

xanthan gum and proteins (endogenous and ingredient derived). As oat malt had no influence on the viscosity of a xanthan gum solution, it would appear that the viscosity decreasing effect would be due to proteolysis, as reported in previous studies (Renzetti and Arendt, 2009a, 2009b). However, quinoa malt had a proteolytic activity comparable to oat malt and their effects on the electrophoretic pattern of protein containing ingredients are identical. The impact of proteolysis on gluten free batter and bread properties depends strongly on the matrix (Renzetti and Arendt, 2009b). A possible explanation for the apparent lack of significance of the proteolytic activities may be the dominant effect of two ingredients with strong foaming properties, HPMC and WPI.

6.3.4 Bread properties

Loaves with varying levels of oat (0.5; 0.75; 1 and 2%) and quinoa (1%; 2.5% and 5%) malts were baked in addition to a control bread without malt. The addition of oat malt decreased the bread densities (Table 6.4), translating to higher loaf volumes, but a 2% addition led to a formation of large holes in the centre of the crumb (Figure 6.2). There were no significant differences in batter densities between the control and batters with oat malt. Quinoa malt addition had no impact on bread or batter densities even at an addition level of 5%. Image analysis showed that oat malt addition gave a more open crumb with fewer and larger cells with thicker walls. The no. cells/cm^2 decreased from 80.8 to 63.9 in the bread baked with 0.75% oat malt, still resulting in an even crumb. The cause for the lower values in the breads with 1 and 2% oat malt is the forming of large holes due to excessive cell coalescence in the centre of the loaf. Quinoa malt had no significant effect on the crumb grain.

Batter densities showed no differences but the densities of baked breads decreased with increasing oat malt level. It would thus appear that the changes leading to a higher loaf volume and more open crumb grain occurred during baking. RVA results indicated a drastic drop in peak viscosity as a result of oat malt α-amylase action, which has a major impact on the stability of the gas cells. The lack of impact of quinoa malt on any bread properties is probably due to a very low α-amylase activity. This also suggests that proteolytic activity had little role in the properties of the formulation used in this study, as both malts contained high protease activities.

Malt additions had no influence on crumb hardness (not shown). A crumb softening effect in rice bread using a maltogenic α-amylase has been reported before (Gujral et al., 2003). Possibly the early inactivation of malt α-amylases (75-80 °C) makes them inefficient as crumb softening enzymes. Lipases alter the polarity of lipids that may contribute to the stability of the gas cell walls (Primo-Martín et al., 2006).
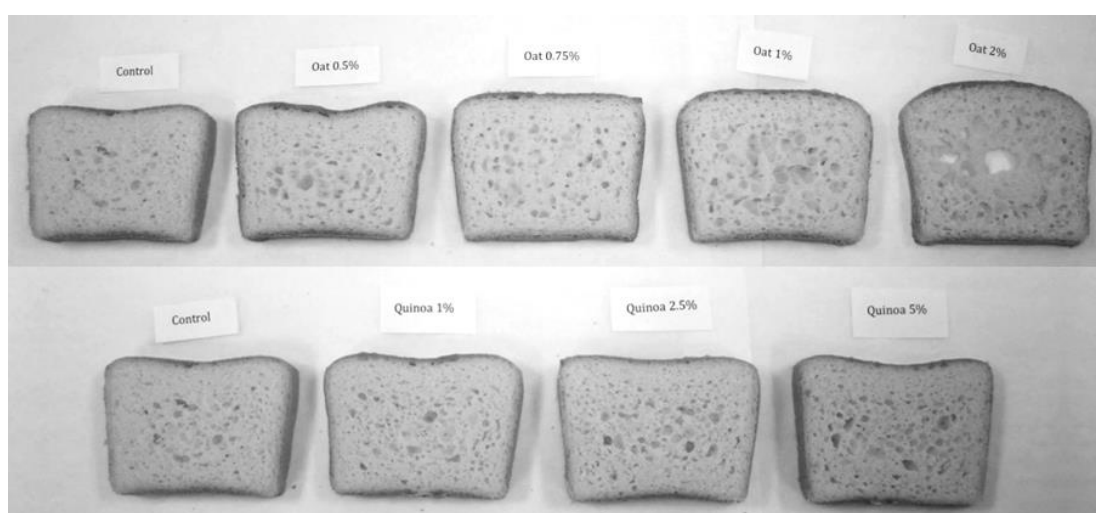

Figure 6.2. Photos of bread slices with added oat (1st row) and quinoa (2nd row)

Table 6.4. Bread and batter densities and crumb properties with oat (O) and quinoa (Q) malts.

Parameter	Batter density (g/ml)	Bread density (g/ml)	No. cells/cm^2	Cell diameter (mm)	Wall thickness (mm)
Control	0.69 ± 0.03 a	0.59 ± 0.02 a	80.8 ± 7.5 a	1.55 ± 0.21 a	0.44 ± 0.01 a
O 0.5%	0.66 ± 0.04 a	0.58 ± 0.04 ab	71.4 ± 8.6 b	1.89 ± 0.35 b	0.48 ± 0.03 b
O 0.75%	0.67 ± 0.03 a	0.54 ± 0.03 bc	63.9 ± 5.8 cd	2.27 ± 0.26 c	0.51 ± 0.01 c
O 1%	0.69 ± 0.04 a	0.53 ± 0.02 c	64.7 ± 3.9 c	2.28 ± 0.18 c	0.51 ± 0.01 c
O 2%	0.67 ± 0.02 a	0.50 ± 0.01 d	56.0 ± 4.1 d	2.63 ± 0.25 d	0.50 ± 0.01 d
Q 1%	0.70 ± 0.03 a	0.60 ± 0.02 a	79.0 ± 6.4 ab	1.45 ± 0.13 a	0.43 ± 0.01 a
Q 2.5%	0.70 ± 0.02 a	0.58 ± 0.04 a	79.2 ± 9.5 ab	1.55 ± 0.31 a	0.44 ± 0.02 a
Q 5%	0.69 ± 0.02 a	0.58 ± 0.04 a	78.8 ± 8.9 ab	1.57 ± 0.26 a	0.45 ± 0.02 ab

Monoglycerides have been reported to increase the volume and increase the cell size in the crumb when added in the same formulation used in this study (Nunes et al., 2008). Lipases may thus influence the bread properties, but their role was not confirmed in this study.

6.3.5 Microscopy

CLSM micrographs of bread crumbs (Figure 6.3d-f) show a dominating matrix of gelatinised starch visualised with FITC, surrounded by discontinuous networks of protein and hydrocolloids. The protein matrix consists of larger aggregates in the control bread compared to the ones baked with malts. CLSM micrographs of the batters and SEM micrographs of batters and breads reveal no visible differences upon malt addition (Figure 6.3a-c; g-l).

6.3.6 Conclusion

Oat malt produced from pure oats may be used to improve the volume and crumb grain of gluten free bread at levels <1%, but overdosing may deteriorate the crumb. Quinoa malt had no effect on the baking quality and germinating quinoa for bakery products may not be feasible, unless improvements in the palatability and nutritional properties are desired. The key parameter to altered technological properties was α-amylase activity. Malt induced proteolysis did not influence the properties of a gluten free formulation at least in the presence of other ingredients with strong foaming properties such as WPI and HPMC.
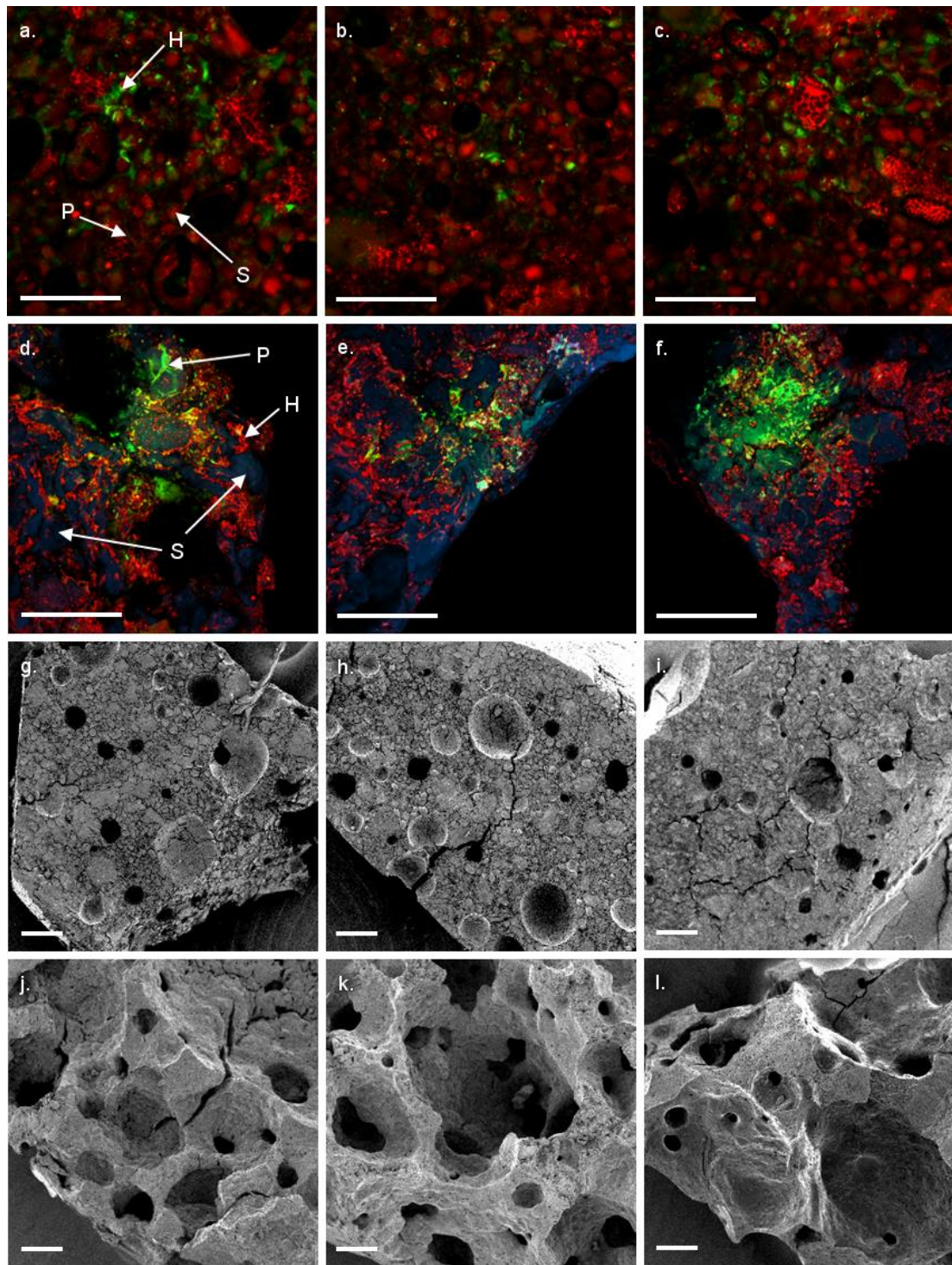
Figure 6.3. CLSM micrographs of of batters after proofing (a-c) and bread crumbs (d-f) and SEM micrographs of batters after proofing (g-i) and bread crumbs (j-l). Letters indicating protein (P), starch (S) and hydrocolloids (H); Bars 200 μm.

Acknowledgments

References

Atwell, W.A., Hyldon, R.G., Godfrey, P.D., 1988. Germinated quinoa flour to reduce the viscosity of starchy foods. Cereal Chem. 65, 508–509.

Bergamo, P., Maurano, F., Mazzarella, G., Iaquinto, G., Vocca, I., Rivelli, A.R., De Falco, E., Gianfrani, C., Rossi, M., 2011. Immunological evaluation of the alcohol-soluble protein fraction from gluten-free grains in relation to celiac disease. Mol. Nutr. Food Res. 55, 1266–70.

Butzner, J.D., 2011. Pure oats and the gluten-free diet: are they safe? JPEN. J. Parenter. Enteral Nutr. 35, 447–8.

Catassi, C., Fasano, A., 2008. Celiac disease. Curr. Opin. Gastroenterol. 24, 687–691.

FAO, 2011. Quinoa: An ancient crop to contribute to world food security. FAO Regional Office for Latin America and the Caribbean, Santiago, Chile.

Gabriele, D., de Cindio, B., D'Antona, P., 2001. A weak gel model for foods. Rheol. Acta 40, 120–127.

Gujral, H.S., Haros, M., Rosell, C.M., 2003. Starch hydrolyzing enzymes for retarding the staling of rice bread. Cereal Chem. 80, 750–754.

Hager, A.-S., Axel, C., Arendt, E.K., 2011. Status of carbohydrates and dietary fiber in gluten-free diets. Cereal Foods World 56, 109–114.

Hugo, L.F., Rooney, L.W., Taylor, J.R.N., 2000. Malted sorghum as a functional ingredient in composite bread. Cereal Chem. 77, 428–432.

Jacobsen, S.E., Mujica, A., Ortiz, R., 2003. The global potential for quinoa and other Andean crops. Food Rev. Int. 19, 139–148.

Kaukovirta-Norja, A., Wilhelmson, A., Poutanen, K., 2004. Germination: a means to improve the functionality of oat. Agric. Food Sci. 13, 100-112.

Kemppainen, T., Janatuinen, E., Holm, K., Kosma, V.-M., Heikkinen, M., Mäki, M., Laurila, K., Uusitupa, M., Julkunen, R., 2007. No observed local immunological

response at cell level after five years of oats in adult coeliac disease. Scand. J. Gastroenterol. 42, 54–9.

Kim, D.-K., Jeong, S.C., Gorinstein, S., Chon, S.-U., 2012. Total polyphenols, antioxidant and antiproliferative activities of different extracts in mungbean seeds and sprouts. Plant Foods Hum. Nutr. 67, 71–5.

Kwon, D.Y., Rhee, J.S., 1986. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. J. Am. Oil Chem. Soc. 63, 89–92.

Lásztity, R., 1998. Oat grain – a wonderful reservoir of natural nutrients and biologically active substances. Food Rev. Int. 14, 99–119.

Lee, A.R., Ng, D.L., Dave, E., Ciaccio, E.J., Green, P.H.R., 2009. The effect of substituting alternative grains in the diet on the nutritional profile of the gluten-free diet. J. Hum. Nutr. Diet. 22, 359–63.

Mäkinen, O.E., Arendt, E.K., 2012. Oat malt as a baking ingredient – A comparative study of the impact of oat, barley and wheat malts on bread and dough properties. J. Cereal Sci. 56, 747–753.

Matlashewski, G., Urquhart, A., Sahasrabudhe, M., Altosaar, I., 1982. Lipase activity in oat flour suspensions and soluble extracts. Cereal Chem. 59, 418–422

Moroni, A. V., Dal Bello, F., Zannini, E., Arendt, E.K., 2011. Impact of sourdough on buckwheat flour, batter and bread: Biochemical, rheological and textural insights. J. Cereal Sci. 54, 195–202.

Nunes, M.H.B., Moore, M.M., Ryan, L.A.M., Arendt, E.K., 2008. Impact of emulsifiers on the quality and rheological properties of gluten-free breads and batters. Eur. Food Res. Technol. 228, 633–642.

Park, S.H., Morita, N., 2005. Dough and breadmaking properties of wheat flour substituted by 10% with germinated quinoa flour. Food Sci. Technol. Int. 11, 471–476.

Primo-Martín, C., Hamer, R.J., Jongh, H.H.J., 2006. Surface layer properties of dough liquor components: Are they key parameters in gas retention in bread dough? Food Biophys. 1, 83–93.

Renzetti, S., Arendt, E.K., 2009a. Effect of protease treatment on the baking quality of brown rice bread: From textural and rheological properties to biochemistry and microstructure. J. Cereal Sci. 50, 22–28.

Renzetti, S., Arendt, E.K., 2009b. Effects of oxidase and protease treatments on the breadmaking functionality of a range of gluten-free flours. Eur. Food Res. Technol. 229, 307–317.

Repo-Carrasco, R., Espinoza, C., Jacobsen, S.-E., 2003. Nutritional value and use of the Andean crops quinoa (*Chenopodium quinoa*) and Kañiwa (*Chenopodium pallidicaule*). Food Rev. Int. 19, 179–189.

Ruales, J., Nair, B.M., 1992. Nutritional quality of the protein in quinoa (*Chenopodium quinoa, Willd*) seeds. Plant Foods Hum. Nutr. 42, 1–11.

Valencia, S., Svanberg, U., Sandberg, A.-S., Ruales, J., 1999. Processing of quinoa (*Chenopodium quinoa , Willd*): effects on in vitro iron availability and phytate hydrolysis. Int. J. Food Sci. Nutr. 50, 203–211.

Watanabe, M., Maeda, T., Tsukahara, K., Kayahara, H., Morita, N., 2004. Application of pregerminated brown rice for breadmaking. Cereal Chem. 81, 450–455.

Wood, P.J., 2010. Review: Oat and Rye β-Glucan: Properties and Function. Cereal Chem. 87, 315–330.

Chapter 7

Physicochemical and acid gelation properties of commercial UHT-treated plant-based milk substitutes and lactose free bovine milk

Outi E. Mäkinen, Therese Uniacke-Lowe, James A. O'Mahony, Elke K. Arendt

Abstract

Physicochemical and acid gelation properties of commercial soy, oat, quinoa, rice and lactose-free bovine milks were studied. The separation profiles were determined using a LUMiSizer dispersion analyzer. Soy, rice and quinoa milks formed both cream and sediment layers, while oat milk sedimented but did not cream. Bovine milk was very stable to separation at 1000 rpm (30 min) followed by 3000 rpm (60 min), while all plant milks separated at varying rates; rice and oat milks being the most unstable products. Particle sizes in plant-based milk substitutes, expressed as volume mean diameters ($d_{4.3}$), ranged from 0.55 μm (soy) to 2.08 μm (quinoa) while the average size in bovine milk was 0.52 μm. Particles of plant-based milk substitutes were significantly more polydisperse compared to those of bovine milk. Upon acidification with glucono-δ-lactone (GDL), bovine, soy and quinoa milks formed structured gels with maximum storage moduli of 262, 187 and 105 Pa, respectively. In addition to soy products currently on the market, quinoa may have potential in dairy-type food applications.

7.1 Introduction

Plant-based milk substitutes are water soluble extracts of legumes, oil seeds, cereals or pseudocereals that resemble bovine milk in appearance. These products are used to replace bovine milk in the diet by an increasing number of consumers for medical reasons (e.g., lactose intolerance, cow's milk allergy) or as a lifestyle choice (Mäkinen, Wanhalinna, Zannini, & Arendt, 2013). Soy products dominate the market, but products based on other plant materials, e.g., coconut endosperm and oat, almond, rice, hemp and quinoa seeds are also available (Mäkinen et al., 2013). Plant-based milk substitutes are generally manufactured by the extraction of plant material in water, removal of solids, and product formulation, followed by homogenization and heat-treatment. The resulting products are suspensions of extracted and disintegrated plant material and other ingredients such as oils and hydrocolloids, if used (Durand, Franks, & Hosken, 2003; Mäkinen et al., 2013). Such liquid systems may separate during storage either by sedimentation, creaming, or syneresis, which is detrimental to the product quality. The separating particles can be denatured and aggregated protein particles, oil droplets or cellular fragments (van Vliet & Walstra, 1989). Plant storage proteins are often oligomeric: they are composed of two or more subunits that in turn consist of a number of polypeptide chains (Bewley and Black, 1994). The native storage proteins of seeds may be considered as a complex of individual proteins held together by a combination of intermolecular disulphide bonds, hydrogen bonding, ionic bonding and hydrophobic interactions rather than a single protein (Bewley & Black, 1994). Generally, proteins having a tertiary structure are more susceptible to heat denaturation and subsequent aggregation upon intermolecular disulphide and hydrophobic bonding, leading to decreased solubility (Morr, 1979).

Bovine milk contains 3.0-3.7% protein of which ca. 80% is casein and the remaining 20% is whey protein. The casein fraction exists in the form of micelles that play a crucial role in the physicochemical stability of milk. The hydrophilic C-terminal region of κ-casein or 'brush' protrudes from the surface of the micelles and sterically stabilizes them against aggregation (de Kruif & Zhulina, 1996). Coagulation of casein micelles can occur only following collapse of the brush, which occurs on acidification of milk or during the rennet-induced coagulation of milk (de Kruif & Zhulina, 1996). Caseins have very little secondary or tertiary structure and contain no disulphide bridges, making them relatively stable to thermal processing which can cause denaturation and aggregation of plant proteins (Morr, 1979; Singh, 2004). Whey proteins on the other hand, are sensitive to unfolding at temperatures above 60°C, which renders them susceptible to protein-protein interactions on thermal processing. Heat-induced denaturation of β-lactoglobulin results in its disulfide-mediated binding with κ-casein, consequently affecting the properties of the casein micelles, e.g., this may promote the aggregation of micelles at low pH (Singh, 2004).

Some proteins are capable of gelling when heat-induced particle aggregates form a continuous, three-dimensional network structure upon lowering of pH or addition of salt. Glucono-δ-lactone (GDL) can be used to replicate acid production on microbial fermentation, as it converts to gluconic acid in water and slowly releases H^{+} over time gradually decreasing the pH of milk (Lucey, 2002). This approach has been widely used to study the gelation of bovine milk proteins (Lucey, 2002; van Marle & Zoon, 1995) and soy proteins by several authors (Kuipers, Alting, & Gruppen, 2007; Grygorczyk & Corredig, 2013).

The physicochemical and acid gelation properties of commercial ultra-high temperature-treated (UHT) plant-based milk substitutes, as well as their hedonic response among consumers was studied and compared to lactose free UHT-treated bovine milk. Commercial products were chosen to represent the products as the consumer would purchase them.

7.2 Materials and methods

7.2.1 Samples

The samples were commercial UHT-treated lactose-free bovine (full fat), soy, oat, quinoa and rice milks purchased from a local health store. The oat milk contained added calcium in the form of calcium carbonate and calcium phosphate. The samples were stored at 4°C and used within 3-5 days of opening the packaging, as preliminary work showed that the viscosity of soy and quinoa milks increased gradually during storage: from 7.15 to 19.5 and 116 mPas after 11 and 14 d storage, respectively, for soy milk, and from 23.8 to 236 mPas after 21 d for quinoa milk.

7.2.2. Composition and hedonic response

The total nitrogen content of the samples was analysed using the Kjeldahl method (MEBAK 1.5.2.1). Nitrogen to protein conversion factor of 6.38 was used for bovine milk, 5.75 for rice milk and 5.95 for all other samples, based on the different proportions of amino acid residues to amino acid nitrogen, as well as the amount of non-protein nitrogen, that vary between plant species (Fujihara, Sasaki, Aoyagi, & Sugahara, 2008). The ash was analysed by incineration in a muffle furnace: 10 g of sample was pre-heated in crucibles for 1 h at 100°C and ashed for 4 h at 600°C. The dishes were cooled to room temperature and weighed. Fat content

was determined using the Gerber method. For sugar analysis, samples were first clarified using Carrez reagents. Samples (2.5 ml) were diluted with ca. 15 ml warm water in 25 ml volumetric flasks and Carrez 1 and 2 reagents (2.5 ml each) were added sequentially. Extracts were diluted to 25 ml, centrifuged (5000 g x 5 min) and filtered (0.45 μm). Sugar profiles were analysed using an Infinity 1260 HPLC system equipped with an refractive index detector (Agilent Technologies, Palo Alto, CA) and a 300 x 7.8 mm Rezex ROA H$^+$ column (Phenomenex, Torrance, CA, USA). Water was used as the mobile phase at a flow rate of 0.6 ml/min at 25°C. Sucrose, maltose, glucose and fructose were quantified using standard curves (Sigma-Aldrich, St Louis, MO). Maltose and sucrose peaks overlap when analysed with Rezex H$^+$ column, but only the sucrose peak splits into two peaks corresponding to glucose and fructose when analysed at 65°C. To distinguish between sucrose and maltose, samples were analysed at 25 and 65°C, and only if the peak was still present at 65°C, was it quantified as maltose.

A consumer panel consisting of students and staff who consume bovine or plant milks at least monthly was recruited from University College Cork, Ireland (n=62, 76% female, mean age 23.3 years, S.D.=4.4 years). The refrigerated products (40 ml) were served in sensory laboratory booths in a randomized order in covered transparent plastic cups with three-digit codes, and rated using a 9-point hedonic scale (appearance, smell, mouthfeel, flavour, overall; like extremely - dislike extremely). After testing, the panellists filled out a questionnaire on demographic information, beverage consumption habits and motivators for plant milk consumption, as well as the food neophobia scale (FNS) questionnaire (Pliner & Hobden, 1992).

7.2.3. Physicochemical properties

Viscosity of the plant milk substitutes was measured using a controlled stress rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a 75 mm cone and plate geometry at a shear rate (γ) range of 0.5-100 s^{-1}. Experimental data were fitted to the Ostwald de Waele equation (Eq. 1), yielding the consistency coefficient (K) and the power law index (n). The measurements were carried out at 10°C. The apparent viscosity measured at 10 s^{-1} is referred to as "viscosity" throughout this paper.

$$\eta = K(\dot{\gamma})^{n-1} \qquad \text{[Eq. 1]}$$

Separation rates of the plant milk substitutes were analyzed with an analytical centrifuge (LUMiSizer®; LUM GmbH, Berlin, Germany). Samples were subjected to centrifugal force, while near-infrared light illuminated the entire sample cell to measure the intensity of transmitted light as a function of time and position over the entire sample length. The separation rate was expressed as the slope of the integrated transmission–time plots determined at 1000 rpm for 30 min and followed by 3000 rpm for 60 min s at 24°C.

The particle size distribution of the milk substitutes was determined using a static laser light diffraction unit equipped with a 300 RF lens (reverse Fourier; range 0.05 to 880 μm) and a He-Ne laser light source (633 nm) using a polydisperse optical analysis model (Mastersizer, Malvern Instruments Ltd, Worcestershire, UK) equipped with a small volume sample presentation unit. Samples were applied to the instrument using the small volume sample presentation unit (MSXL 5), with distilled water as dispersion medium. For bovine milk, a refractive index of 1.342 was used (Singh, McCarthy, & Lucey, 1997). The refractive indices of plant milks were

measured using a hand-held refractometer (Atago R5000, Atago, Tokyo, Japan). The values ranged from 1.338 to 1.340, but the measurements were not consistent due to the opaque nature of the samples. An average value of 1.339 from these measurements was used for all plant milks. The refractive index of the dispersant (water) was 1.333. The target for laser obscuration was ~15%. The following values were reported: volume mean diameter ($d_{4.3}$) surface to volume weighed mean diameter ($d_{3.2}$), the arithmetic mean diameters below which 10, 50 and 90% of particles have diameters lower than (D(v,0.1), D(v,0.5), D(v,0.9)), and the span as a measure of the width of the distributions (Eq. 2).

$$Span = \frac{D(v, 0.9) - D(v, 0.1)}{D(v, 0.5)}$$

[Eq. 2]

The solubility of proteins in the beverages as affected by pH was determined by adjusting the pH from 3.0 to 8.0 at 0.5 unit intervals using 0.1-1 M HCl or NaOH. The samples were incubated at 4°C overnight. The pH was re-adjusted before measurements in case it had drifted. Samples were centrifuged at 5000 g x 30 min. The protein contents of the supernatants were analyzed using the Bradford assay, and by Kjeldahl in untreated products. The results were expressed as % of the protein content of the supernatant of the untreated product (Eq. 3).

$$Solubility = \frac{\text{Protein content in supernatant}}{\text{Protein content in product}} * 100$$

[Eq. 3]

7.2.4 Rheological changes during acidification

For the acidification trials, concentrations of GDL sufficient to reduce the pH of the samples below 5 (plant milks) or 4.5 (bovine milk) within 120 min were used,

as these pH values were within the range of minimum protein solubility for each sample. These corresponded to 1.2, 0.6, 2.5, 0.2 and 1% GDL for soy, quinoa, bovine, rice and oat milks, respectively.

Rheological changes during the acidification of milks were determined by small deformation oscillatory measurements using an AR-G2 controlled stress rheometer (Waters TA Instruments, Leatherhead, Surrey, UK) equipped with a Peltier concentric cylinder (aluminium conical rotor, 42.01 mm (h) x 28.02 mm (d)) at a constant temperature of 30°C. Each milk (25 ml) was pre-warmed at $30°C$ for 15 min, GDL was added to each sample as described above and the sample was immediately placed in the pre-heated cup. Simultaneously, the pH of sub-samples were monitored continuously under quiescent conditions at 30°C. The storage (G′) and loss (G″) moduli and loss tangent (tan $\delta = G''/G'$) were recorded at an angular frequency (ω) of 0.6283 rad s^{-1} and a shear strain of 0.01 (within the linear viscoelastic range) for 150 min. Each sample was analyzed in at least triplicate.

To obtain further information about the properties of the final bovine, soy and quinoa milk gels, a frequency sweep in the frequency range 0.1-100 rad s^{-1} and a fixed strain of 0.1 % was performed after 150 min acidification. G′, G″ and complex dynamic viscosity η* [Eq. 4] were recorded and the mechanical spectra of the gels (frequency-dependence of G′, G″ and η*) were plotted.

$$\eta^* = \frac{(G'^2 + G''^2)^{\frac{1}{2}}}{\omega} \qquad \text{[Eq. 4]}$$

To compare the viscoelastic properties of acidified bovine, quinoa and soy milk gels creep tests were carried out using the AR-G2 rheometer with concentric cylinder geometry described above. Milk samples were prepared by heating 25 mL

of each sample to $30^{\circ}C$ in a waterbath for 15 min. GDL was added as described above and the sample stirred vigorously for 1 min before pouring into the preheated concentric cylinder of the rheometer. Creep-recovery experiments were carried out 150 min after the addition of GDL by applying a shear stress (τ) for 5 min, with measurement of the resulting strain (γ) followed by a 5 min recovery phase after removal of the stress. The applied stresses used were increazed by 2-fold increments, from 1.6 to 102.4 Pa. For all samples, creep-recovery curves are reported as compliance, *J,* where, $J = \gamma / \tau$.

The difference in the resistance of acidified bovine, quinoa and soy milk gels to fracture and in their extent of deformation in response to lower stresses was analyzed by plotting maximum strain values reached at the end of the initial 5 min period against the applied stress.

7.2.5 Microscopy

Milk samples were prepared for confocal laser scanning microscopy by adding 70 µl of 0.1% Rhodamine B (aq) in 5 ml sample. When analysing acidified samples, GDL was added in the samples before staining, a drop of the mixture was placed between cover slips and incubated at 30°C (RH = 85%) for an hour before microscopic observation. All samples were examined with an FV300 confocal laser scanning system mounted on an Olympus IX80 inverted microscope with a 40x dry objective (Olympus, Germany), using λ_{exc} = 543 nm and a 560-600 nm emission filter. Unstained samples were also observed using only transmitted light.

For transmission electron microscopy (TEM) samples of bovine, soy and quinoa milk before, and 150 min after acidification with GDL, were fixed with electron microscopy (EM)-grade, aqueous gluteraldahyde in a ratio of 9:1, v/v,

milk:gluteraldehyde to a final gluteraldehyde concentration of 25 g L^{-1} by the addition of 100 µL of 250 g L^{-1} gluteraldehyde to 900 µL sample in an Eppendorf tube. Ten µL of each fixed sample was mixed with 10 µL of 20 g L^{-1} phosphotungstic acid (PTA), pH 7.2, and held for 5 min. A Formvar-coated copper grid (400 mesh; Agar Scientific, Stansted, UK) was immersed in the mixture for 5 min after which it was dabbed around the periphery with filter paper to remove excess sample. The sample was allowed to air-dry at room temperature under a clean up-turned Petri-dish and was imaged the following day.

7.2.6 Statistical analyses

Statistical analysis of data was carried out using Minitab release 16 (Minitab Inc., State College, PA, USA). Initially the data for all parameters measured were examined for normality using the Anderson-Darling normality test at a significance level of 0.1, i.e., when calculated p values were found to be < 0.1, it was assumed that the data were not normally distributed. The Kruskal-Wallis non-parametric alternative to one-way analysis of variance was used to make inferences about the equality of medians between the samples. If the p values from the Kruskal-Wallis test was found to be greater than a pre-determined α-level (0.05), it was concluded that none of the treatment effects (samples) were significant and population medians were equal. Where p values were less than or equal to 0.05, a difference in population medians was apparent and rank values were examined to ascertain which samples were different. All analyses were carried out in triplicate. In the case of rheological analyses, the data presented are the results from a single analysis for clarity. The coefficient of variation was < 5% for data points.

7.3 Results and discussion

7.3.1 Composition and hedonic response

The compositional data of the plant milk substitutes is given in Table 7.1. The protein contents ranged from 0.07% of rice milk to 3.32% for bovine milk. Only the protein content of soy milk was close to bovine milk at 2.95% protein. Bovine and quinoa milks were high in fat at 3.50% and 2.40%, respectively, while rice and oat milks contained <1% fat. Bovine and soy milks contained ca. 0.7% ash. Rice and quinoa milks were lower in ash at 0.18% and 0.22%, respectively, but oat milk had values closer to bovine milk at 0.48% ash, as it contained added calcium. The main sugar was maltose in oat and rice milks, fructose in quinoa milk, and sucrose and fructose in soy milk (Table 1). The sugars may originate from a starch hydrolysis step during processing, or from additional ingredients, such as apple concentrate (soy) and agave syrup (quinoa). Only lactose hydrolysis products glucose and galactose were detected in bovine milk.

Plant milk substitutes are often used to replace bovine milk in an individual's diet. In addition to containing high quality protein, bovine milk is an important source of calcium, iodine, vitamin B_{12} and riboflavin, and population groups with low milk intakes often have a poor status for these nutrients (Millward & Garnett, 2010). The beverages studied varied greatly in protein contents, with only soy milk being comparable to bovine milk in terms of protein content. Considerable deviations from the nutritional composition of the product being substituted may be harmful; cases of kwashiorkor, a protein-energy malnutrition typical for areas of famine, have been reported in Western countries as a result of using rice milk as a weaning food (Carvalho, Kenney, Carrington, & Hall, 2001).

Table 7.1. Composition (%) and consumer hedonic ratings of bovine milk and plant-based milk substitutes. Hedonic ratings were evaluated on a 9-point hedonic scale.

	Bovine	Soy	Oat	Quinoa	Rice
Protein	3.32 ± 0.11	2.95 ± 0.07	0.78 ± 0.13	1.26 ± 0.03	0.07 ± 0.02
Fat	3.50 ± 0.05	1.72 ± 0.03	0.28 ± 0.03	2.40 ± 0.10	0.87 ± 0.12
Ash	0.67 ± 0.01	0.71 ± 0.02	0.48 ± 0.02	0.22 ± 0.01	0.13 ± 0.01
Sugars[1]					
Mal	-	-	2.75	0.92	4.88
Suc	-	0.91	-	-	-
Glu	2.06	0.45	-	0.22	0.11
Fru	-	1.02		1.9	-
Gal	2.55	-	-	-	-
Hedonic					
Appearance	7.30 ± 1.35	4.84 ± 1.94	3.41 ± 1.66	5.49 ± 1.91	4.95 ± 1.87
Smell	5.20 ± 1.82	4.18 ± 1.92	4.30 ± 1.27	3.07 ± 2.15	4.30 ± 1.56
Mouthfeel	6.49 ± 1.79	5.54 ± 1.61	5.16 ± 1.57	4.62 ± 2.23	505 ± 1.91
Flavour	5.79 ± 2.44	4.79 ± 1.86	4.82 ± 1.91	3.07 ± 2.33	4.34 ± 2.06
Overall	5.9 ± 2.21	4.87 ± 1.78	4.74 ± 1.85	3.23 ± 2.32	4.52 ± 1.94

[1] Standard deviations < 0.008 (%)

The values for compositional data were significantly different between samples (Kruskal-Wallis test; p<0.05).

The hedonic ratings were the highest for bovine milk samples across all attributes, while quinoa milk was the least liked (Table 7.1). Soy, oat and rice milks received similar scores in all attributes except appearance which was very low for oat milk, possibly due to yellowish colour. The overall liking ranged from 4.5 to 4.9 ("neither like or dislike") for the aforementioned samples, which was slightly lower than for bovine milk (5.79 "like slightly"). Quinoa milk scored very low (3, "dislike moderately") in the attributes smell, flavour and overall. When observing the results from the Kruskal-Wallis test, rice, oat and soy had average rank values close to the overall rank (smell, flavour and overall), indicating similarity, while bovine and quinoa milks deviated from it. Bovine milk had rank values furthest from the overall rank in all attributes, showing that it received very different ratings in all attributes

compared to the plant based samples. Although bovine milk got the highest hedonic ratings, the flavour attribute scored between "neither like or dislike" and "like slightly". The bovine milk product used in this study was lactose free, as it is likely to be used by the same consumer segment as plant milk substitutes. The lower values may be caused by the sweetness of lactose hydrolysis products glucose and galactose, and the deterioration of the flavour as a result of UHT treatment. Quinoa milk was generally disliked, possibly due to its strong nutty flavour/aroma atypical of a milk-like product. The hedonic ratings did not correlate with demographic data or the food neophobia score. 73% of panellists stated they would consume more plant milk substitutes if the products had proven health benefits, 86% if the products tasted better, 68% if they had allergies and 43% if the products were proven to be an environmentally friendly option.

7.3.2. Physicochemical properties

The viscosities of the beverages studied were between 2.02 and 7.15 mPas for all samples except quinoa milk which had a significantly higher viscosity of 23.8 mPas (Table 7.2). Bovine and soy milks showed slight pseudoplastic behaviour with flow indices of 0.80 and 0.83, respectively, while quinoa milk showed considerable pseudoplasticity with a flow index of 0.45. The solubility of proteins in all plant milk substitutes decreased to their minimum between pH 5.0 and 6.0, while the minimum solubility was reached at pH 4.5 in bovine milk (Figure 7.1). Bovine milk showed nearly 100% protein solubility at pH 7.0 and above, but the maximum solubility was lower in plant milks: the corresponding values were 78.6% in soy milk, 65% in quinoa milk and as low as 11.8% rice milk. Oat milk protein was nearly insoluble.
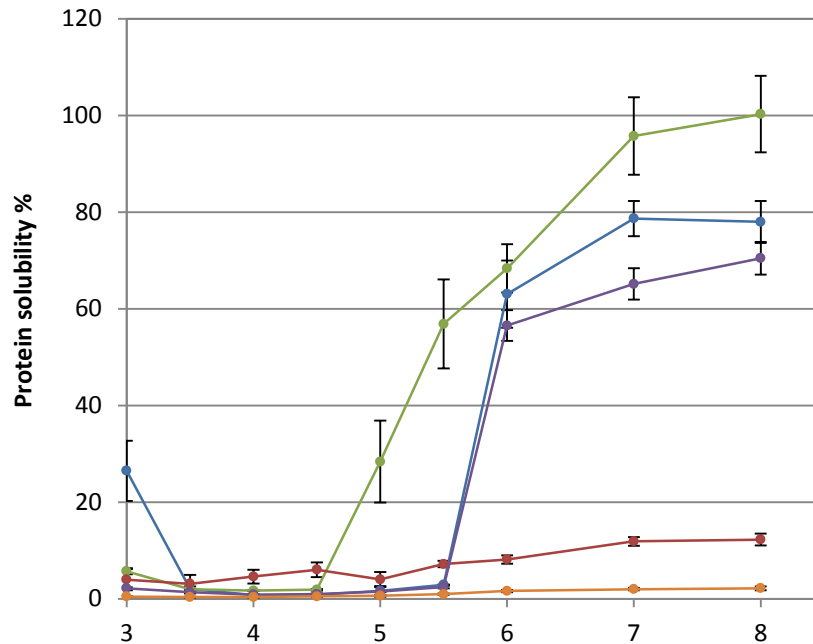
Figure 7.1. Protein solubility of bovine (green), soy (blue) and quinoa (purple), rice (red), and oat milks (orange) as a function of pH. The arrows indicate the initial pH of the product.

Numerical data describing the particle size distributions of the beverages are presented in Table 7.2. The surface to volume average diameters ($d_{3.2}$) ranged from 0.39 µm for soy milk to 0.49 µm for oat milk. Volume mean diameters ($d_{4.3}$) showed larger variation: bovine and soy milks had low values (0.52 and 0.55 µm), but oat and quinoa milks had diameters of 1.51 µm and 2.08 µm, respectively. The $d_{3.2}$ parameter is sensitive to smaller particles, whereas $d_{4.3}$ is a better indicator of the presence of larger particles or aggregates (Surh, Decker, & McClements, 2006). Span values were used to indicate the width of the distributions. Bovine and soy milks had low span values of 1.31 and 1.59 µm, respectively, whereas oat milk had a span value of 4.99, indicating the presence of particles with broader size distribution. The similarity of bovine and soy milks compared to the other samples is evident from the Kruskal-Wallis test results: the ranks of these samples deviated the most from the overall rank in all particle size parameters ($p<0.05$). Bovine milk contains casein

micelles and fat globules. The size of the fat globules is strongly influenced by treatments: they are decreased from ca. 4 μm to 0.6 μm as a result of homogenisation, and UHT-treatment decreases the diameter further to 0.3 μm (Walstra et al. 2006). Casein micelles are much smaller, ranging from 0.04 to 0.3 μm, but they can be aggregated in UHT-treated milk (Walstra et al. 1999; Donato & Guyomarc'h, 2009).

The light microscopy images support the particle size data (Figure 7.2). Bovine milk appeared to consist of small, uniformly sized particles when observed using light microscopy (Figure 7.2 a). In TEM micrographs, these particles appeared to be slightly clustered casein micelles around lighter fat globules (Figure 7.2 a insert). Soy milk contained a similar population of small particles in addition to larger fragments of varying diameters. Also, the smaller particles appeared to have the tendency to cluster, which can be seen clearly in the TEM image (Figure 7.2 b; TEM as insert). Soy milk particles have previously been shown to exist as aggregated structures with a "chain-like" morphology, and some lipid globules visible in the aggregates (Ringgeberg et al., 2012). The particles in oat milk existed as large aggregates of varying sizes (Figure 7.2 c), supported by the large particle sizes and wide distributions from the particle size measurements. Quinoa milk contained a population of small, homogenous, tightly-packed particles, and whole large cellular fragments were also found in the samples (Figure 7.2 d). Particles in rice milk were heterogeneous in size, while no aggregates or larger fragments could be observed (Figure 7.2 e).
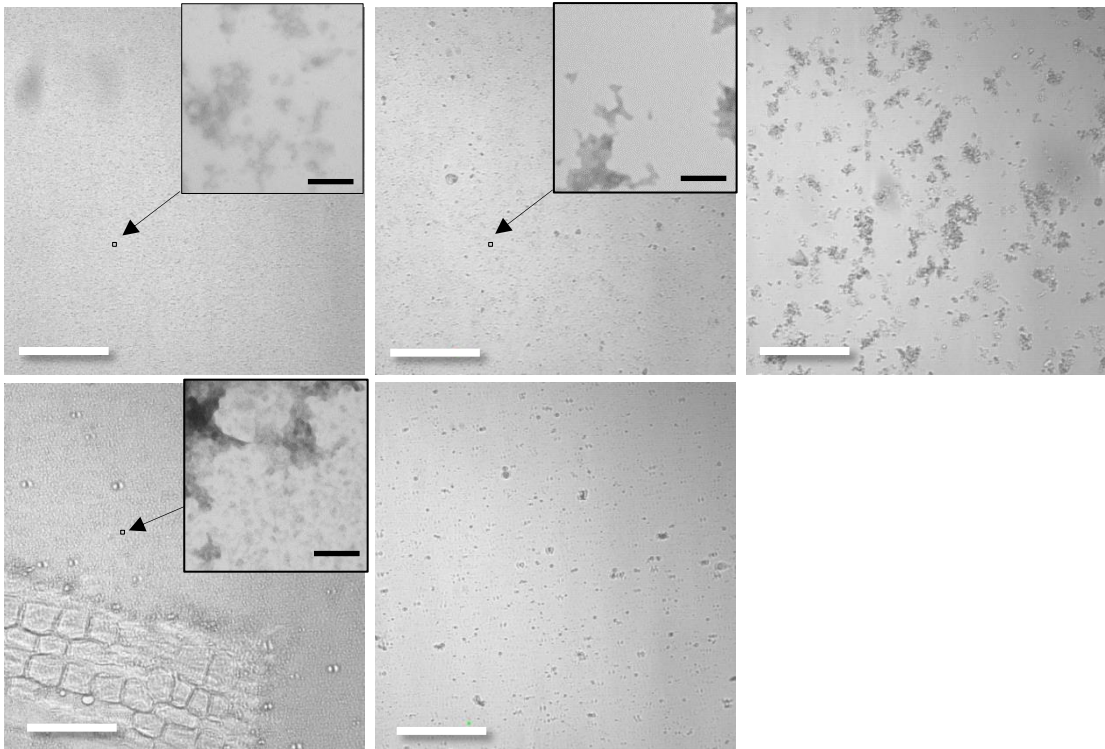
Figure 7.2. Light microscopy images of bovine (a), soy (b), oat (c), quinoa (d) and rice (e) milks, and transmission electron micrographs of bovine (a), soy (b) and quinoa (d) milks as inserts. Scale bars 50 μm (white) and 250 nm (black).

The rates of separation showed large variation between the products (Table 7.2). Bovine milk was very stable with a separation rate of 1.58 %/h. Soy and quinoa milks also showed considerable stability with values <10 %/h, but oat and rice milks separated rapidly. Soy, quinoa and rice milks formed both sediment and cream layers, while oat milk sedimented leaving a very clear serum. In samples stored at 5°C, sedimentation in oat milk and creaming in the rice milk sample were clearly observable after 24 h (Figure 7.2; marked with arrows).

Table 7.2. Rheological and particle size properties of bovine milk and plant-based milk substitutes.

	Apparent viscosity (mPas)	Flow index	Separation rate (%/h)	$d_{3.2}$ (µm) [a]	$d_{4.3}$ (µm) [b]	Span (µm)	pH	Storage modulus G′ (Pa)	Loss tangent tan δ
Bovine	3.57 ± 1.21	0.80 ± 0.07	1.58 ± 0.03	0.42 ± 0.01	0.52 ± 0.01	1.31 ± 0.00	6.83	262.0± 3.42	0.22 ± 0.00
Soy	7.15 ± 0.62	0.83 ± 0.05	9.95 ± 0.30	0.39 ± 0.01	0.55 ± 0.01	1.59 ± 0.01	6.80	186.5 ± 6.68	0.15 ± 0.01
Oat	6.09 ± 0.12	0.98 ± 0.02	49.6 ± 1.58	0.49 ± 0.02	1.51 ± 0.03	4.99 ± 0.28	7.14	< 1	2.64 ± 0.02
Quinoa	23.8 ± 4.72	0.45 ± 0.03	8.52 ± 0.37	0.46 ± 0.01	2.08 ± 0.01	2.14 ± 0.19	6.40	104.6 ± 0.59	0.15 ± 0.00
Rice	2.02 ± 0.11	0.97 ± 0.04	55.7 ± 0.15	0.47 ± 0.01	0.81 ± 0.04	2.74 ± 0.03	7.47	< 1	2.87 ± 0.01

[a] $d_{3.2}$ Surface to volume weighed mean diameter (Sauter mean diameter)
[b] $d_{4.3}$ Volume mean diameter

After 5 d, the cream layer in the rice milk sample had further separated from the serum. Creaming has been reported to be the main mode of destabilisation in bovine and soy milks in a previous study on commercial products, while oat and rice milks that contained large particles and less fat sedimented (Durand et al., 2003). In addition to the properties of the raw material itself, separation rate and mechanism depend on the processing (e.g., heat-treatment) and other constituents of the product (e.g., stabilisers), and are likely to vary between products and manufacturers.
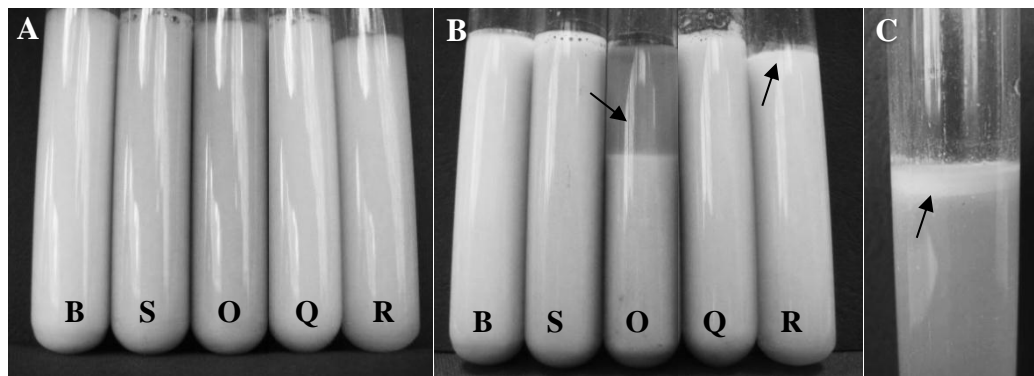
Figure 7.2 Bovine (B), soy (S), oat (O), quinoa (Q) and rice (R) milks immediately after shaking and opening the package (a) and after 24 h storage (b), and rice milk after 5 d storage at 5°C (c).

Sedimentation or creaming velocity is dependent on the density difference between the particle and the continuous phase, the particle size, and viscosity of the continuous phase (van Vliet & Walstra, 1989). Oat and rice milks were highly unstable with separation rates ranging from 50 to 55 %/h. Oat milks had the largest particles and the highest span indicating a wide distribution in particle sizes. Combined with a low viscosity and protein solubility, these attributes yielded an unstable product. The physicochemical properties of soy milk were the closest to bovine milk, as has been reported previously (Durand et al., 2003). They had very similar volume mean particle diameters, but the larger particles present in soy milk

widened the particle size distribution slightly. Generally, the stability of plant milks correlates with their particle sizes (Durand et al., 2003). In this study, quinoa milk was an exception to this as it had the highest volume mean particle diameter caused by the dominance of very large particles, but had a low separation rate. The viscosity of quinoa milk was much higher than the other beverages, which retards the sedimentation or creaming of the large particles (van Vliet & Walstra, 1989).

TEM images of bovine, soy and quinoa milks showed clustering of particles. UHT-treatment of bovine milk has been reported to cause interactions between denatured whey proteins and casein micelles, leading to an increase in particle size and age gelation during longterm storage (3-18 months) (Datta and Deeth, 2001). It has been reported that low temperature storage of UHT-treated bovine milk may accelerate the age gelation process (Andrews, Brooker, & Hobbs, 1977). Increased protein aggregation as a result of UHT induced protein denaturation also occurs in soy milk (Cruz et al., 2007 The $d_{3.2}$ values of bovine and soy milk were similar to previously reported for UHT-treated commercial products (Durand et al., 2003) but 3-fold higher compared to soy milks prepared in a pilot plant (Malaki Nik, Tosh, Poysa, Woodrow, & Corredig, 2008). Malaki Nik et al. (2008) reported a decrease in particle sizes after heating, and further decrease after homogenisation. The authors concluded that although heating decreases the solubility of 7S subunits, the larger 11S subunits are disrupted, leading to a narrower particle size distribution and smaller particles overall. Heating to high temperatures may however alter the behaviour of the proteins during storage. Quinoa milk showed similar changes, and both light microscopy and TEM revealed the presence of a matrix of tightly packed particles and aggregates. Some beverages are weak gels with continuous network structures that stabilise the system from separation. An example of this includes a

cocoa milk system which is a fluid with a weak network consisting of protein aggregates and cocoa particles in the presence of κ-carrageenan (Boomgaard, Vliet, & Hooydonk, 1987). Such networks stabilise the system if the yield stress is greater than the sedimentation stress (van Vliet & Walstra, 1989).

7.3.3 Rheological changes during acidification

The influence of acidification by GDL on the storage modulus (G′) of bovine, quinoa, soy, rice and oat milk is shown in Figure 7.4 a, and the time course of pH decrease in Figure 7.4 b. Generally, the gel point observed during small amplitude oscillatory rheology is regarded as the point where the storage modulus (G′) and the loss modulus (G″) cross-over or when G′ increases to ~ 1 Pa. The G′ of bovine milk increased sharply when the pH decreased to 5.4 at ~ 30 min after GDL addition and a final G′ of ~ 261 Pa was recorded after 150 min. The G′ of soy and quinoa milks started to increase at pH values below 6.2 and 6.4, respectively. The final G′ values after 150 min acidification were 186 Pa for soy and 104 Pa for quinoa. No increase in G′ was observed for either acidified rice or oat milk during 150 min after GDL addition, even though the pH decreased to 3.99 and 4.11, respectively. Oat proteins are capable of heat gelation at high concentrations, but oat-based milk-type suspensions do not form gel-like structures when acidified, and the production of a yoghurt-type product requires the presence of thickeners (Mårtensson, Öste, & Holst, 2000; Nieto-Nieto, Wang, Ozimek, & Chen, 2014). The final G′ values recorded decreased with decreasing protein content, in the order bovine > soy > quinoa.
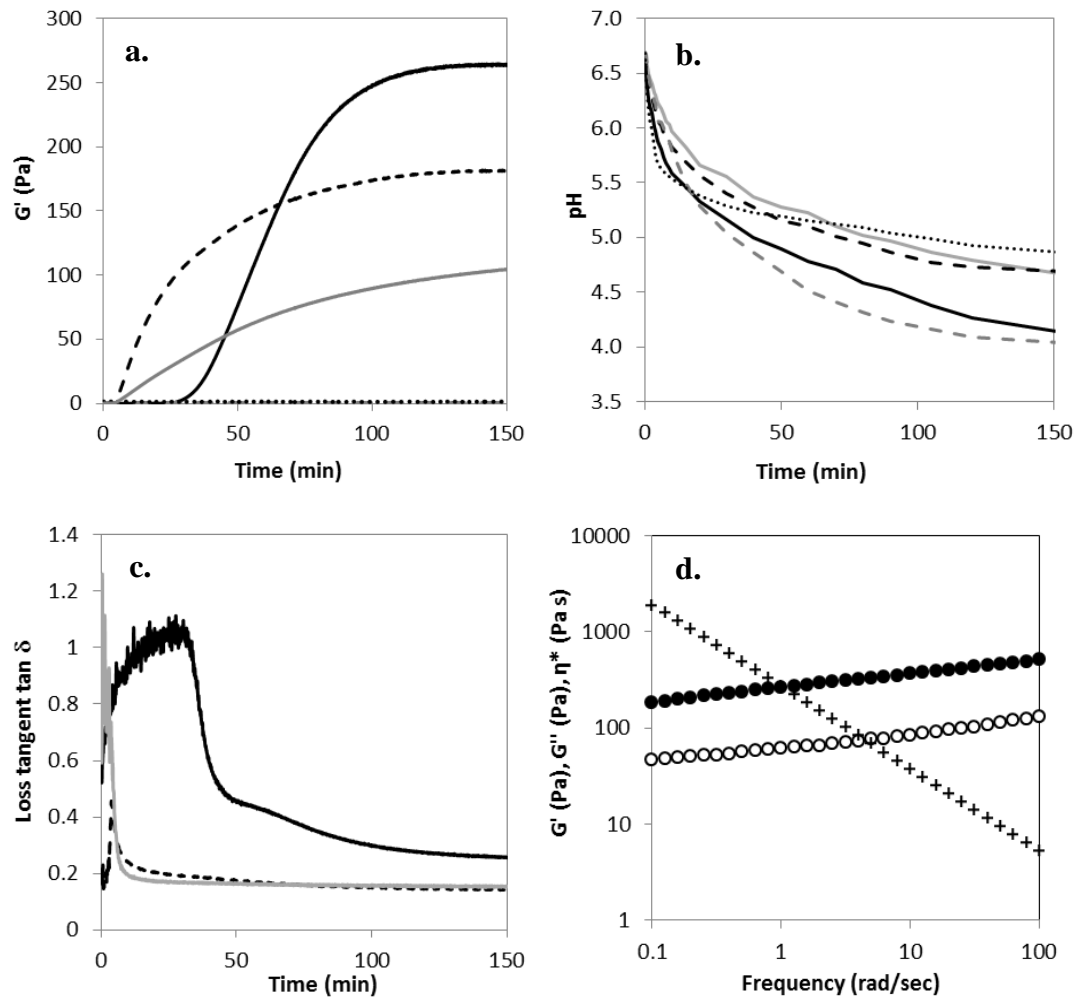
Figure 7.4. Effect of acidification with glucono-δ-lactone (GDL) on the storage modulus (G′) (A), and pH (B) and loss tangent (tan δ) (C) of bovine (solid black), soy (dashed black), quinoa (grey), rice (dashed grey) and oat (dotted black) milks, and the mechanical spectrum (D) of acidified bovine milk showing the frequency dependence of G' (●), G" (○) and η* (+).

Rheological data were supported by the microscopic observations which showed a clear structural change from liquid to solid in acidified bovine and soy milk samples (Figure 7.5). Quinoa milk showed aggregated structures before acidification that became more structured and 'gel-like' as a result of the pH reduction. Oat milk particles appeared to form larger aggregates, but no network structures could be observed.
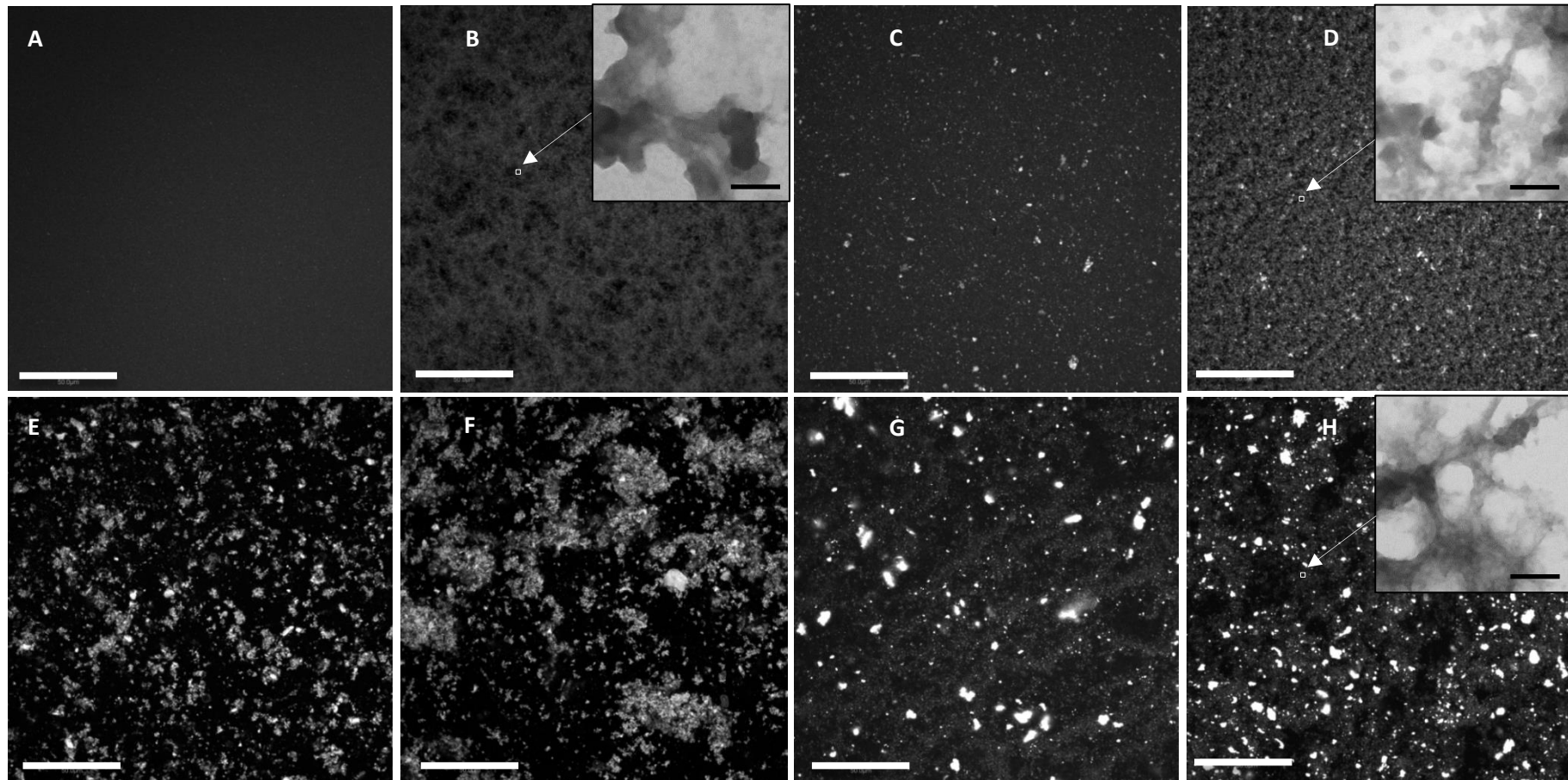
Figure 7.5. Confocal laser scanning micrographs of bovine (a-b), soy (c-d), oat (e-f) and quinoa (g-h) milks before and after acidification. Scale bars 50 μm (white) and 250 nm (black).

In acid-induced gels from bovine milk, casein is the dominant structural component and it is heterogeneously arranged as three-dimensional aggregates of strands and linkage sites (Roefs & Walstra, 1986). The principal factors governing acid milk gel formation are the concentration of casein, pH, temperature and ionic strength (Roefs & van Vliet, 1990). The final G′ value of 264 Pa for acidified UHT-treated bovine milk was similar in this study to values of 300-450 Pa reported by Lucey et al. (2000) for bovine milk acidified with GDL following heat treatment at $\geq$ 80°C for 15 min. Gels made from non-heat-treated bovine milk have lower G′ values (typically < 20 Pa), because the compact clusters of aggregated casein particles formed by the extensive rearrangement of particles during gel formation do not entirely cross-link as the gel network is formed (Lucey, 2002). In heated milk samples, denatured whey proteins associated with casein micelles interact with each other and act as bridging material which increases the number and strength of bonds between protein particles (Lucey, 2002). In unheated bovine milk, gelation occurs at pH ~ 4.9, whereas pre-heated milk gels at pH ~ 5.2-5.4, as heat-denatured whey proteins associated with κ-casein at the micelle surface have higher isoelectric points (Lucey, 2004). Acid-induced gels formed from preheated milk therefore develop a firm texture due to disulphide bridging which leads to increased cross-linking through the gel network with high whey retention capacity (Lucey, 2004).

In soy milk the protein structures are very complex due to subunit structures and heterogeneity (Mohamed & Xu, 2003). Heat treatment of soy milk causes protein denaturation and exposes reactive amino acid groups usually buried in the core of the proteins (Doi, 1993), which results in an increase in surface hydrophobicity and leads to protein aggregation via non-covalent interactions and some disulphide interchange (Malaki Nik et al., 2008). Aggregation of soy proteins

occurs on addition of salt or acid, probably due to a reduction in electrostatic interaction with aggregation occurring via hydrophobic interactions (Kohyama & Nishinari, 1993). The decreased charge on soy proteins due to acidification is reported to be a prerequisite for aggregation which is primarily driven by hydrogen bridging (Ringgenberg, 2011). The G′ of soy milk started to increase below pH 6.2, having reached the final G′ at pH 4.7. Previous reports for the pH at the onset of gelation for soy proteins include 6.3 and 5.90 (Grygorczyk & Corredig, 2013; Kuipers et al., 2007). This is close ot the isoelectric point of glycinin (6.4), which indicates that the gelation is a result os short-range interactions (Grygorczyk & Corredig, 2013; Thanh & Shibasaki, 1976).

Statistical analysis showed that loss tangent (tan δ) values were significantly different for all milks (Table 7.2) and median rank values indicated soy and quinoa milk gels were similar. For those samples that clearly gelled, i.e., bovine, quinoa and soy milk, tan δ values as a function of time were plotted (Figure 7.4 c). Tan δ is related to the spatial distribution and the number of protein-protein bonds formed during the gelation of milk and may be regarded as a good indicator of the type and strength of bonds between protein particles (Roefs, 1986). The result for bovine milk is in agreement with the data reported by Lucey et al. (2000) where tan δ decreased at gelation and continued to decrease as the milk gel aged. Also the maximum in the tan δ values just after the onset of gelation of acidified bovine milk was observed in this study, which is in agreement with the results of Lucey et al. (2000) for the acidification of preheated bovine milk. An increase in tan δ at the onset of gelation has been reported in gels formed by microbially acidified high heat-treated milk (Rönnegård & Dejmek, 1993; van Marle & Zoon, 1995) and in gels formed from heated bovine milk acidified with GDL (Lucey et al., 2000). Tan δ values for both

soy and quinoa milk followed a similar pattern with a very sharp decrease in values at the onset of gelation compared to the pattern seen for bovine milk which could be attributed to the presence of protein aggregates seen in the TEM micrographs (Figure 7.5) which quickened the gelation process considerably. This decrease in tan δ during gelation is a general and well-understood phenomenon: initially, intermolecular associations will create aggregates or clusters which are free to move independently through the solvent, but offer greater resistance to flow than any individual protein molecules; this causes an increase in G'' and therefore in tan δ. As the extent of association increases, protein clusters will link together in a continuous network, which causes a substantial increase in the solid-like, elastic character and a reduction in the viscous response causing a rapid and sharp decrease in tan δ. The lower tan δ values recorded for both soy and quinoa milk compared to bovine milk 150 min after GDL addition could indicate that they contain a significant amount of material that does not contribute to gel formation under acidic conditions.

7.3.4 Rheological properties of gels

Mechanical spectra displaying the frequency dependence of G', G'' and η^* were plotted for acidified bovine, soy and quinoa milks (the spectrum of bovine milk shown in Figure 7.4d). All samples showed gel-like character with $G' >> G''$ and a linear reduction in log η^* with increasing log ω (the spectrum of bovine milk shown as an example in Figure 7.4d). For bovine milk, the maximum G' after 150 min acidification was 264 Pa and the sample displayed typical gel-like behaviour as the frequency of oscillation was increased. The separation between G' and G'' was considerably smaller than in typical, self-supporting biopolymer gels and the frequency dependence of both moduli showed a slight increase as the frequency of

oscillation increased indicating some weakness of the gel structure (Ross-Murphy, 1983). Similarily, gels formed from acidified soy and quinoa milk showed slight frequency-dependence of both G′ and G″ and in the case of quinoa milk both moduli were lower than those of either soy or bovine milk. The higher moduli recorded for both bovine and soy milk indicate more intermolecular association between protein particles compared to quinoa milk which is consistent with the higher protein contents in both bovine and soy milk (Table 7.1). In a true viscoelastic gel, the dynamic moduli are totally independent of frequency (ω) and in a typical biopolymer gel, G′ (the solid-like response) exceeds G″ (the liquid-like response) by an order of magnitude and a linear relationship exists between log η^* and log ω with a slope of ~ -1 (Ross-Murphy, 1983). To form an 'ideal' gel, permanent covalent bonding is required and the dynamic moduli are independent of frequency, but in a typical protein gel, some frequency dependence is always observed and log G′ *vs.* log ω has a slope which is greater than zero but typically is < 0.1 (Ross-Murphy, 1983).

The creep curves recorded for acidified bovine, quinoa and soy milk are shown in Figure 7.6a-c. In the case of bovine milk, the strains generated in response to the stresses, the compliance (*J*) curves, were characteristic of a gel network with some elasticity. In a true viscoelastic gel, the compliance curves would superimpose closely and the irrecoverable strain would be similar for each applied stress. For the weak gel formed from bovine milk in this study, some irrecoverable deformation of the sample occurred for all stress levels and increased as the stress increased. At an applied stress of 102.4 Pa, the compliance increased dramatically at the start of the creep period and the gel network fractured and flowed when a finite 'yield stress' value was exceeded. Yielding of sample occurred instantaneously at 12.5 Pa for acidified quinoa milk and at 25.6 Pa for soy milk. More overlap of compliance

versus time curves was observed in the case of soy milk (Figure 7.6 b) compared to those of either bovine or quinoa, indicating quite an elastic protein network although application of a stress of 25.6 Pa fractured this structure easily. Acidified quinoa milk had the sharpest increase in strain/compliance on application of each stress with a significant increase over time possibly due to more rearrangement of the protein network as it was stressed compared to those of bovine and soy gels.
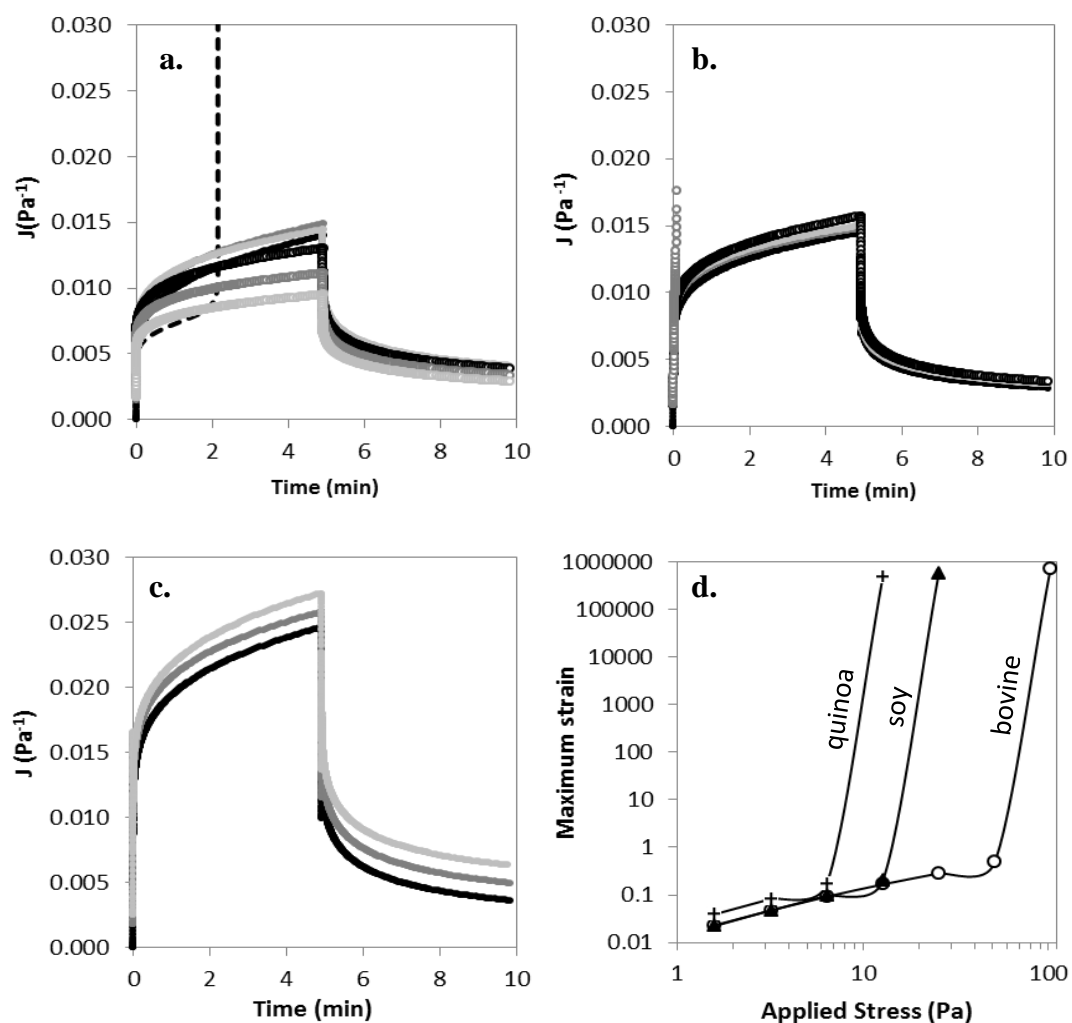

Figure 7.6. Creep-recovery curves recorded at 30°C for acidified bovine (a), soy (b) and quinoa (c) milks and variation of maximum strain after 5 min creep (d) for acidified bovine (○), soy (▲) and quinoa milks (+). In a-c, the curves show the variation of compliance (J) in response to applied stress values of 1.6 (black solid), 3.2 (grey solid), 6.4 (light grey solid), 12.8 (black open), 25.6 (grey open), 51.2 (light grey open) or 102.4 Pa (dashed line).

The differences in resistance to fracture and in the extent of deformation in response to applied stresses of acidified bovine, quinoa and soy milk gels was illustrated by plotting, double-logarithmically, the maximum strain reached at the end of the 5 min period of applied stress (Figure 7.6d). The gel formed from bovine milk was substantially stronger than that formed from either quinoa or soy milk and fractured at a higher stress value presumably due to the higher protein content of bovine milk compared to that of quinoa or soy milk (Table 7.1). The strength of protein-protein bonds, the number of bonds per cross-sectional area of a protein strand, the relaxation times for the network bonds and the orientation of the strands in the protein matrix all contribute to the yield properties of gels (Vliet, Dijk, Zoon, & Walstra, 1991). Despite having a protein content quite similar to bovine milk, the result here would suggest that a proportion of the protein in soy milk is not actually incorporated fully in the gel formed after 150 min acidification.

7.4 Conclusion

To date, soy proteins have been widely utilized for their functional properties and the tendency to form gels (Kinsella, 1979). Quinoa proteins and their hydrolyzates have been studied for their functional properties, and they also form mechanically resistant films with chitosan (Abugoch, Romero, Tapia, Silva, & Rivera, 2008; Valenzuela, Abugoch, Tapia, & Gamboa, 2013). This study shows for the first time the ability of quinoa proteins to form gel structures on acidification. Due to its balanced amino acid profile and a high biological value (Ranhotra, Gelroth, Glaser, Lorenz, & Johnson, 1992), quinoa protein may be an interesting raw material to study further.

Acknowledgments

References

Abugoch, L. E., Romero, N., Tapia, C. A., Silva, J., Rivera, M., 2008. Study of some physicochemical and functional properties of quinoa (*Chenopodium quinoa Willd.*) protein isolates. J. Agric. Food Chem. 56, 4745–50.

Andrews, A. T., Brooker, B. E., Hobbs, D. G., 1977. Properties of aseptically packed ultra-heat-treated milk: Electron microscopic examination of changes occurring during storage. J. Dairy Res. 44, 283.

Bewley, J. D., Black, M., 1994. Germination, structure and composition. In: Seeds - Physiology of development and germination. New York: Plenum Press. pp, 1–33.

Boomgaard, T., Vliet, T., Hooydonk, A. C. M., 1987. Physical stability of chocolate milk. Int. J. Food Sci. Technol. 22, 279–291.

Carvalho, N. F., Kenney, R. D., Carrington, P. H., Hall, D. E., 2001. Severe nutritional deficiencies in toddlers resulting from health food milk alternatives. Pediatrics. 107, e46–e46.

Cruz, N., Capellas, M., Hernández, M., Trujillo, A. J., Guamis, B., Ferragut, V., 2007. Ultra high pressure homogenization of soymilk: Microbiological, physicochemical and microstructural characteristics. Food Res. Int. 40, 725–732.

De Kruif, C. G., Zhulina, E. B., 1996. κ-casein as a polyelectrolyte brush on the surface of casein micelles. Colloid Surf. A. 117, 151–159.

Doi, E., 1993. Gels and gelling of globular proteins. Trends Food Sci. Technol. 4, 1–5.

Donato, L., Guyomarc'h, F., 2009. Formation and properties of the whey protein/κ-casein complexes in heated skim milk – A review. Dairy Sci. Technol. 89, 3–29.

Durand, A., Franks, G. V, Hosken, R. W., 2003. Particle sizes and stability of UHT bovine, cereal and grain milks. Food Hydrocoll. 17(5), 671–678.

Fujihara, S., Sasaki, H., Aoyagi, Y., Sugahara, T., 2008. Nitrogen-to-protein conversion factors for some cereal products in Japan. J. Food Sci. 73, C204–9.

Grygorczyk, A., Corredig, M., 2013. Acid induced gelation of soymilk, comparison between gels prepared with lactic acid bacteria and glucono-δ-lactone. Food Chem. 141, 1716–1721.

Millward, J.D., Garnett, T., 2010. Plenary Lecture 3 Food and the planet: nutritional dilemmas of greenhouse gas emission reductions through reduced intakes of meat and dairy foods. Proc. Nutr. Soc. 69, 103–118.

Kinsella, J. E., 1979. Functional properties of soy proteins. J. Am. Oil Chem. Soc. 56, 242–258.

Kohyama, K., & Nishinari, K., 1993. Rheological studies on the gelation process of soybean 7S and 11S proteins in the presence of glucono-delta-lactone. J. Agric. Food Chem. 41, 8–14.

Kuipers, B. J. H., Alting, A. C., Gruppen, H., 2007. Comparison of the aggregation behavior of soy and bovine whey protein hydrolysates. Biotechnol. Adv. 25, 606–610.

Lucey, J. A., Tamehana, M., Singh, H., Munro, P. A., 2000. Rheological properties of milk gels formed by a combination of rennet and glucono-delta-lactone. J. Dairy Res. 67, 415–27.

Lucey, J.A., 2002. Formation and Physical Properties of Milk Protein Gels. J. Dairy Sci. 85, 281–294.

Lucey, John A., 2004. Cultured dairy products: an overview of their gelation and t exture properties. Int. J. Dairy Technol. 57, 77–84.

Mäkinen, O. E., Wanhalinna, V., Zannini, E., Arendt, E. K., 2013. Foods for special dietary needs: Non-dairy plant based milk substitutes and fermented dairy type products. Crit. Rev. Food Sci. Nutr. *in press*.

Malaki Nik, A., Tosh, S., Poysa, V., Woodrow, L, Corredig, M., 2008. Physicochemical characterization of soymilk after step-wise centrifugation. Food Res. Int. 41, 286–294.

Mårtensson, O., Öste, R., Holst, O., 2000. Lactic Acid Bacteria in an Oat-based Non-dairy Milk Substitute: Fermentation Characteristics and Exopolysaccharide Formation. LWT - Food Sci. Technol. 33, 525–530.

Mohamed, A., Xu, J., 2003. Effect of ionic strength and pH on the thermal and rheological properties of soy protein–amylopectin blend. Food Chem. 83, 227–236.

Morr, C. V., 1979. Technical problems and opportunities in using vegetable proteins in dairy products. J. Am. Oil Chem. Soc. 56, 383–385.

Nieto-Nieto, T. V., Wang, Y. X., Ozimek, L., Chen, L., 2014. Effects of partial hydrolysis on structure and gelling properties of oat globular proteins. Food Res. Int. 55, 418–425.

Pliner, P., Hobden, K., 1992. Development of a scale to measure the trait of food neophobia in humans. Appetite. 19, 105–120.

Ranhotra, G.S., Gelroth, J.A., Glaser, B.K., Lorenz, K.J., Johnson, D.L., 1992. Composition and protein nutritional quality of quinoa. Cereal Chem. 70, 303-305.

Ringgenberg, E., 2011. The physico-chemical characterization of soymilk particles and gelation properties of acid-induced soymilk gels, as a function of soymilk protein concentration. MSc thesis. University of Guelph, Canada.

Roefs, S.P.F.M., van Vliet, T., 1990. Structure of acid casein gels. II, Dynamic measurements and type of interaction forces. Colloids Surf. 50, 161–175.

Roefs, S.P.F.M., 1986. Structure of acid casein gels: a study of gels formed after acidification in the cold . PhD thesis. Wageningen University, Netherlands.

Rönnegård, E., Dejmek, P., 1993. Development and breakdown of structure in yoghurt studied by oscillatory rheological measurements. Le Lait. 73, 371–379.

Ross-Murphy, S.B., 1983. Rheological methods. In: H. W. S. Chan (Ed.), Biophysical methods in food research. Critical reports on applied chemistry. Society of Chemical Industry, London, UK, pp. 195–290.

Singh, H., McCarthy, O. J., Lucey, J. A., 1997. Physico-chemical properties of milk. In P. F. Fox (Ed.), Advanced Dairy Chemistry Vol. 3: Lactose, Water, Salts and Vitamins. Springer, Boston, MA, pp. 469–518.

Singh, H., 2004. Heat stability of milk. Int. J. Dairy Technol. 57, 111–119.

Surh, J., Decker, E. A., McClements, D. J., 2006. Properties and stability of oil-in-water emulsions stabilized by fish gelatin. Food Hydrocoll. 20, 596–606.

Thanh, V. H., & Shibasaki, K., 1976. Major proteins of soybean seeds. A straightforward fractionation and their characterization. J Agric Food Chem. 24, 1117-1121.

Valenzuela, C., Abugoch, L., Tapia, C., Gamboa, A., 2013. Effect of alkaline extraction on the structure of the protein of quinoa (*Chenopodium quinoa Willd.*) and its influence on film formation. Int. J. Food Sci. Technol. 48, 843–849.

Van Marle, M. E., Zoon, P., 1995. Permeability and rheological properties of microbially and chemically acidified skim-milk gels. Neth. Milk Dairy J. 49, 47–65.

Van Vliet, T., Walstra, P., 1989. Weak particle networks. In R. D. Bee, P. Richmond, & J. Mingings (Eds.), Food Colloids. Royal Society of Chemistry, London, UK, pp. 206–217.

Van Vliet, T., Dijk, H. J. M., Zoon, P., Walstra, P., 1991. Relation between syneresis and rheological properties of particle gels. Colloid Polym. Sci. 269, 620–627.

Walstra, P., Wouters, J. T., & Geurts, T. J. 2010. Colloidal particles of milk. In: Dairy science and technology. CRC Press, Boca Raton, FL, USA. pp, 109-157.

Chapter 8

Functional and acid gelation properties of quinoa protein isolate: influence of heat-denaturation pH

Outi E. Mäkinen, Elke K. Arendt

Submitted manuscript

Abstract

Heat-denaturation of quinoa protein isolate (QPI) at alkali pH and its influence on protein functionality were investigated. Heating QPI at pH 8.5 led to increased surface hydrophobicity and decreases in free and bound sulfhydryl group contents. Heating at pH 10.5 caused a lesser degree of changes in sulfhydryls and surface hydrophobicity, and the resulting solutions showed drastically increased solubility and emulsifying activity. SDS PAGE revealed the presence of large aggregates only in the sample heated at pH 8.5, suggesting that any aggregates present in the sample heated at pH 10.5 were non-covalently bound and disintegrated in the presence of SDS. Reducing conditions partially dissolved the aggregates in the pH 8.5 heated sample, indicating the occurrence of disulphide linking, but caused no major alterations in the separation pattern of the sample heated at pH 10.5. Denaturation pH influenced the acid gelation properties greatly. Solutions heated at pH 8.5 formed a coarse coagulum with maximum G' of 5 Pa. Heat-induced denaturation at 10.5 enabled the proteins to form a finer and regularly structured gel with a maximum G' of 1140 Pa. Particle size analysis showed that the pH 10.5 heated sample contained very small particles (0.1-1.0 μm, that readily aggregated (22-140 μm) when pH was lowered to 5.5. When the sample was heated at pH 8.5, large aggregates (26-500 μm) were present in addition to the <1 μm particles. Differences in the nature of aggregates formed during heating may explain the dramatic variance in gelation properties.

8.1 Introduction

Quinoa is a dicotyledonous crop native to the Andean region. The plant produces starchy seeds that have been used as a food staple for thousands of years by the Quechua and Ayamara peoples (Abugoch, 2009). Demand for quinoa has increased in Europe and North America of late, due to its remarkable nutritional properties. Quinoa protein in particular has received attention because of its excellent nutritional quality: it has a high digestibility and a balanced amino acid profile. It is notably high in lysine, the limiting amino acid in cereals (Abugoch, 2009; Ranhotra, Gelroth, Glaser, Lorenz, & Johnson, 1992).

In addition to their nutritional relevance, proteins often provide and stabilise the structural and textural properties of foods (Foegeding & Davis, 2011). The chemical and physical properties that affect the behaviour of the protein in a food system are referred to as functional properties. These properties largely determine the usefulness of proteins in food systems (Kinsella, 1982). Proteins in foods are seldom in their native state. Structural transitions, i.e., unfolding in solution or at an interface, are associated with food applications due to heating, changes in pH or ionic strength and the application of mechanical force (Foegeding & Davis, 2011). Heat-denaturation of proteins may lead to loss of solubility and other functional properties, but it is also often a prerequisite for gel formation (Hermansson, 1986; Nakai & Li-Chen, 1989).

A gel structure is formed if intermolecular cross-linking occurs in a manner that leads to the development of a continuous network that exhibits elastic behaviour (Foegeding & Davis, 2011; Hermansson, 1979). A critical balance between attractive and repulsive forces must occur: if attractive forces dominate, a random coagulum

unable to hold water forms, and excessive repulsive forces prevent gelation altogether (Hermansson, 1979; Kinsella, Rector, & Phillips, 1994).

Cold gelation of proteins is a two-step process, e.g. during yoghurt or tofu making. First, a solution of proteins is heated at a pH distant from the isoelectric point (pI), resulting in the formation of soluble aggregates. Gelation is induced by reducing the electrostatic repulsion by lowering the pH towards pI or by salt addition, which leads to association of the soluble aggregates into a continuous network structure (Alting et al., 2004).

As is common in dicotyledonous seeds, quinoa storage proteins are found in the globulin fraction. Two protein fractions, a legumin type globulin (11S) and a globulin with sedimentation coefficient of 2S have been found in quinoa (Brinegar & Goundan, 1993; Brinegar, Sine, & Nwokocha, 1996). The storage proteins are deposited in protein storage vacuoles located in the embryo and endosperm of the quinoa seed (Prego, Maldonado, & Otegui, 1998). The embryo is a band-like tissue surrounding the perisperm, contributing ca. 30% of the seed weight. It can be separated from the perisperm by milling and sieving (Ando et al., 2002).

In this study, we used an embryo-enriched milling fraction for the production of a protein isolate. We then investigated the alteration of chemical properties (sulfhydryl groups, hydrophobicity, deamidation, molecular weight and particle size) as a result of heat-denaturation at alkali pH, as preliminary results showed that a high pH alters the properties significantly, and the effects of these changes on the functional and acid gelation properties of protein solutions.

8.2 Materials and Methods

8.2.1 Fractionation and QPI production

8.2.1.1 Quinoa fractionation

White quinoa (Real, Bolivia) was kindly fractionated by Buehler AG into flour (perisperm), bran (testa) and pollard (embryo enriched). Pollard had the highest protein content and was used for the production of quinoa protein isolate (QPI). The fraction was defatted with petroleum ether (3x) for 8 h and air dried.

8.2.1.2 Production and heat-processing of quinoa protein isolate

Quinoa protein isolates were produced using a procedure modified from (Abugoch, Romero, Tapia, Silva, & Rivera, 2008). Defatted quinoa pollard was ground with coffee grinder and dispersed in 0.1 M Tris-HCl buffer (pH 9.0) with 0.5 M NaCl (10% w/v). The suspension was extracted at room temperature for 2 h under shaking and the solids were removed by centrifugation (9000 g x 20 min). The supernatants were adjusted to pH 5 with 6 M HCl and centrifuged at 4°C (9000 g x 20 min). The pellet was suspended in distilled water (1:3 w/v), neutralised using 6 M NaOH, homogenised with an Ultra-Turrax disperser (IKA-Werke GmbH and Co., Staufen, KG, Germany) for 1 min and freeze dried. The dried isolate was ground with a ball mill (Tissuelyzer II, Qiagen, Valencia, CA). For analyses, QPI was dispersed in water and left under gentle shaking for 24-48 h. Sodium azide (0.02%) was used for microbial control. Samples that were heat denatured were pH adjusted to 8.5 or 10.5, and heated for 15 min at 100°C in sealed tubes. The 'native' sample was not heated before analyses.

8.2.2 Compositional analysis

The total nitrogen content of the samples was analysed using the Kjeldahl method (MEBAK 1.5.2.1). A nitrogen to protein conversion factor of 5.95 was used. Moisture content was determined by oven drying for 1 h at 103°C. Amino acid composition was analysed after hydrolysis by ion chromatography using ninhydrin post-column derivatization (EEC, 2009).

8.2.3. Heat-induced changes in QPI solutions

8.2.3.1 Sulfhydryl groups

Free and total sulfhydryl groups were determined with Ellman's reagent (TNB, 5,5'-dithio-bis-(2-nitrobenzoic acid). Sample (62.5 µl) was diluted with 1.312 ml 0.1 M phosphate buffer (pH 8.0) containing 2 mM EDTA, and 25 µl TNB (0.4%) was added. The absorbance (412 nm) was measured after 15 min incubation. Total sulfhydryl groups were analysed as free sulfhydryl groups, but in the presence of 6 M urea and 0.5% SDS. The sulfhydryl group concentration was calculated from the molar extinction coefficient of TNB,

$$c_{SH} = \frac{A_{412nm}}{bE}$$

[Eq. 1]

where b is cuvette path length and E = 14,150 $M^{-1}cm^{-1}$.

8.2.3.2 Surface hydrophobicity

Surface hydrophobicity was measured using 1-anilino-8-naphthalene sulfonate (ANS) as a hydrophobic probe (Hayakawa & Nakai, 1985). Protein solutions were diluted to a concentration series ranging from 0.0006% to 0.004% with 0.01 M phosphate buffer (pH 8.0). ANS (10 µl) was added to 2 ml sample, and

the fluorescence intensity was measured immediately with a spectrofluorometer (SFM25, Bio-Tek Kontron Instruments, Zürich, Switzerland). The excitation and emission wavelengths were 390 and 470 nm, respectively. The slope of relative fluorescence intensity and protein concentration (x1000) was used as a measure of surface hydrophobicity (S_0).

8.2.3.3 Degree of deamidation

Heated samples were dialysed (cut-off 12 400kDa) against water overnight, and lyophilised. Dry samples (100 mg) were then digested by heating in 2 N H_2SO_4 (750 µl) for 4 h, filtered (0.2 µm), and neutralised with 2 N NaOH. The content of released ammonia was measured using the Berthelot method (Maita, Parsons, & Lalli, 1984). The degree of deamidation was expressed as the ratio of ammonia released from the heated protein samples to that of the unheated control (%).

8.2.3.4 SDS-PAGE

Protein solutions were analysed under non-reducing conditions using a 4-20% gradient gel (Bio-Rad, Richmond, CA). Samples were diluted in sample loading buffer, yielding final concentrations of 2% SDS, 4% glycerol, 0.01% bromophenol blue in 0.05 M Tris-HCl, pH 6.8, 45 µg protein was loaded and electrophoresis was performed according to Laemmli (1970). Gels were stained using a modified sensitive colloidal staining protocol (Westermeier, 2006). Molecular weight markers were run in parallel with the samples (Precision Plus Protein standards, All Blue, Bio-Rad, Richmond, CA).

8.2.4 Physicochemical and emulsifying properties of QPI after treatments

8.2.4.1 Solubility

A pH series was prepared by adjusting the pH of a 2% QPI solution to 3.0-9.5 at 0.5 increments, and diluting each sample to a final concentration of 1%. The samples were refrigerated overnight and re-adjusted before analysis when necessary. Solubility was determined after centrifugation (10 000 g x 15 min). Protein content of the supernatants were analysed using the Bradford assay. The results are expressed as % of the protein content of the supernatant of the solution:

$$Solubility\ \% = \frac{Protein\ in\ supernatant\ (\frac{mg}{ml})}{Initial\ protein\ (\frac{mg}{ml})} * 100\% \qquad [Eq.\ 1]$$

8.2.4.2. Viscosity

Viscosity of 1% QPI solutions was measured using a rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a 50 mm cone (2°) and plate. The measurement was performed at 20°C from shear rate 0.5 to 50 s^{-1}, and the apparent viscosity at 20 s^{-1} was reported. If a sediment was formed in the sample, only the supernatant was used for the measurement.

8.2.4.3 Emulsifying activity index and emulsion stability

Emulsifying properties were using the turbidity based method by (Pearce & Kinsella, 1978) with slight modifications. A 2 ml sample of QPI solution (0.4%) was mixed with 0.5 ml sunflower oil and homogenised using an Ultra-Turrax T10 equipped with a S10N-10G dispersing element (Ika-Labortechnik, Janke and Kunkel GmbH, Staufen). An aliquote was collected from the bottom of the tube immediately (A_0) and after 10 min (A_{10}), and diluted 200-fold in 0.1% SDS solution. The

absorbance (500 nm) was read. The emulsifying activity index (EAI) was calculated using the corrected equation by (Cameron, Weber, Idziak, Neufeld, & Cooper, 1991):

$$EAI = {2T}/{C\phi}$$ [Eq. 2]

Where T is turbidity (2.303*A_0/l), C the concentration of the protein solution (mg/ml), ϕ the oil fraction (0.2) and l the cuvette path length. Emulsion stability (ES) was calculated as:

$$ES = {A_{10}}/{A_0}$$ [Eq. 3]

8.2.4.4. Particle size distribution

Particle size distribution was determined using a static laser light diffraction unit equipped with a 300 RF lens (reverse Fourier; range 0.05 to 880 μm) and a He-Ne laser light source (633 nm) using a polydisperse optical analysis model (Mastersizer, Malvern Instruments Ltd, Worcestershire, UK) equipped with a small volume sample presentation unit. Samples were applied to the instrument using the small volume sample presentation unit (MSXL 5), with distilled water adjusted to sample pH as dispersion medium. A refractive index of 1.339 from was used for the dispersed phase, and 1.333 for the dispersant (water). The target for laser obscuration was ~15%.

8.2.5 Gelation properties

8.2.5.1 Rheological changes during acidification

The acid gelation properties were measured by adding glucono-δ-lactone (GDL, 0.33 mg per mg protein) and performing a time sweep (f= 1 Hz; γ= 0.05%; T= 30°C) immediately using a rheometer (MCR301, Anton Paar GmbH, Austria). The geometry used was a 50 mm cone and plate with a 0.8 mm gap. In addition to the samples studied, as QPI solution heated at pH 9.5 was acidified because there was a large difference in properties between the samples heated at pH 8.5 and 10.5.

8.2.5.2. Confocal laser scanning microscopy (CLSM)

Samples were prepared for microscopy by acidifying as decribed in Chapter 7, but 10 µl 0.1% Rhodamine B (aq) was added in the solution before the GDL). A drop of the mixture was placed between cover slips, using pieces of cover slips as spacers. The samples were incubated in a plastic box containing wet cotton wool at 30°C for the time required for the G' to level off in the rheological measurements. All samples were observed using a FV300 confocal laser-scanning system (Olympus, Germany) mounted on an Olympus IX80 inverted microscope, with and excitation wavelength of 543 nm and a 560-600 nm emission filter.

8.2.6. Statistical analyses

All analyses were carried out at least in triplicate. Means were compared using one way analysis of variance (ANOVA) using Statistica 12 (StatSoft, Tulsa, OK, USA). The level of significance was determined at $p < 0.05$.

8.3 Results and discussion

8.3.1 Composition of QPI

Quinoa pollard, the embryo enriched milling fraction, was used for the production of QPI. The whole quinoa seed contained 12.7 % protein (as is) and pollard 23.9 %. After defatting, the protein content increased to 27.4 %. QPI produced from pollard had a protein content of 91.3 % (dry basis), and from the whole quinoa 64.3 % (not shown). The main amino acids in QPI were glutamic acid (13.8 mol%), aspartic acid (10.3 mol%), glycine (9.02 mol%), leucine (8.67 mol%) (Table 8.1). The values were very similar to pollard, indicating no major loss in amino acids as a result of extraction. The main differences were a 2.26% decrease in glycine and 1.21 and 0.96% increases in arginine and isoleucine, respectively. The lysine content remained nearly unchanged. The overall amino acid composition was very similar to that of chenopodin (Brinegar & Goundan, 1993).

8.3.2 pH-dependent heat-induced changes in QPI solutions

The native structure of 11S type globulins is stabilised by electrostatic and hydrophobic interactions, and hydrogen bonding (Marcone, 1999). Disulfide bridges are found as interchain disulfides connecting the acidic and basic polypeptides, or intrachain disulfides stabilising the tertiary structure (Wolff 1993). These bonds can be disrupted by heating, leaving them available for the formation of new disulfide bonds (Wolff 1993).

The quantity of free SH groups was 10.9 umol/g protein in QPI before heating (Figure 8.1a). The concentration decreased to 6.2 umol/g protein when heated at pH 8.5, and 8.8 umol/g protein when heated at pH 10.5. Also, the total SH group content decreased upon heating at both pH values. Heating leads to increased

exposure of buried SH groups, that can react further, leading to reductions in both free and total SH groups. Both occurences are favoured by high pH (Monahan, German, &Kinsella, 1995). SH group content also decreases during heating via β-elimination of cysteine (Whitaker & Feeney, 1983). At pH 8.5, 10% of SH and disulfides were lost during 15 min heating in soy protein (Wang & Damodaran, 1990). As the reaction rate is directly proportional to OH^- concentration, the thermal destruction of these groups is assumed to be significantly higher at pH 10.5 (Whitaker & Feeney, 1983).

Table 8.1. Amino acid composition of quinoa protein isolate compared to those of pollard, whole quinoa and literature values of chenopodin (11S) and 2S globulins.

mol%	QPI	Pollard	Whole quinoa	11S[a]	2S[b]
Nonpolar					
Alanine	7.04	7.77	7.38	5.3	1.4
Proline	4.92	5.66	4.53	4.9	4.5
Phenylalanine	4.08	3.77	4.12	4.0	1.2
Glycine	9.02	11.3	11.1	8.7	7.4
Isoleucine	5.47	4.50	4.85	4.9	1.3
Leucine	8.67	8.07	8.02	7.4	2.2
Valine	6.51	6.28	6.65	6.8	0.7
Polar/charged					
Glutamic acid	13.8	13.7	13.7	14.8	30.8
Arginine	7.97	6.77	6.92	9.7	15.2
Histidine	2.13	2.80	2.75	3.0	7.6
Lysine	5.43	5.67	6.06	3.2	0.9
Polar/uncharged					
Aspartic acid	10.3	9.99	10.1	10.5	5.3
Serine	7.08	6.23	6.52	8.9	2.0
Tyrosin	2.77	2.22	2.26	2.9	2.9
Threonine	4.80	5.28	5.12	3.9	0.4

[a] Brinegar & Goundan. 1993. J Agric Food Chem 41. 41 (2), 182–185.
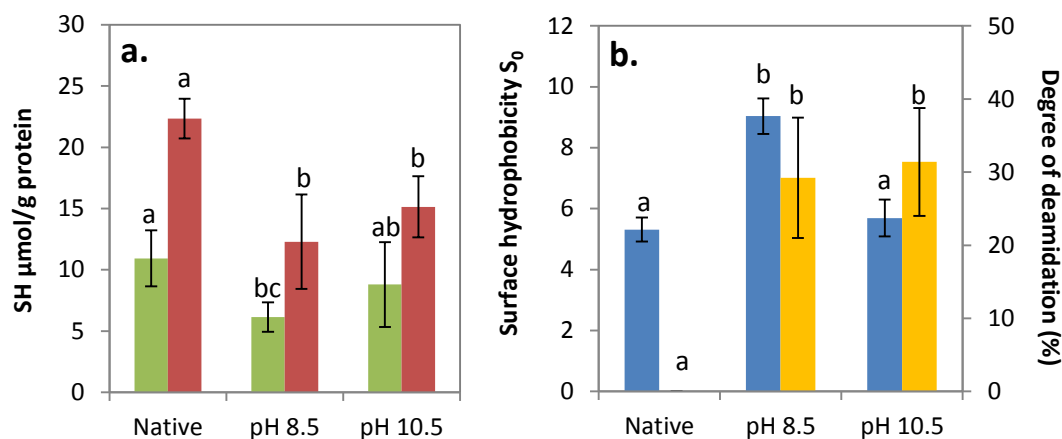[b] Brinegar, Sine & Nwokocha. 1996. J Agric Food Chem. 44(7), 1621–1623.

Figure 8.1. Free (green) and total (red) sulfhydryl groups (a), and surface hydrophobicity (blue) and degree of deamidation (yellow) (b) before and after heating at pH 8.5 and 10.5.

Heating at pH 8.5 also caused a nearly 2-fold increase in surface hydrophobicity (S_0), while the difference between native and pH 10.5 heated sample was not significant (Figure 8.1b). The higher S_0 indicates exposure of hydrophobic amino acid residues as a result of protein unfolding. As this occurs during heating and at alkaline pH alone (Monahan et al., 1995), the relatively low S_0 of the pH 10.5 heated sample is suprising. A similar observation has however been reported previously: when whey proteins were heated at pH 9.0, S_0 increased steadily, but decreased sharply after the temperature exceeded 60°C at pH 11.0 (Monahan et al., 1995).

Deamidation, the conversion of amide groups of glutamine and asparagine into acid groups, alters the solubility and surface properties drastically. It occurs at acidic (< 3.0) or basic (>8.0) pH, and during heating (Riha, Izzo, Zhang, & Ho, 1996). The degree of deamidation was ~30% after heat-treatments, with no differences between pH (Figure 8.1b).

SDS PAGE gels of non-heated samples showed major bands at 45 and 55 kDa, corresponding to 11S subunits (Figure 8.2a). In the presence of DTT, the

subunits have partially dissociated into two acidic and two basic polypeptides with molecular weights of 27-30 and 18-20 kDa (Figure 8.2b). After heating at pH 8.5, the 11S subunits had disintegrated, leaving polypeptides with MW ~42 and ~30 kDa as major bands. The latter may be an acidic polypeptide, but no bands corresponding to basic polypeptides were present. When heated at pH 10.5, it appears that the 11S subunits have dissociated into acidic and basic polypeptides, but the bands are fuzzy, indicating heterogeneity in size. Aggregates can be seen on the top of the gel in the sample heated at pH 8.5, as well as entities with MW >250 kDa. Under reducing conditions, the intensity of the aggregate bands has reduced significantly, and the >250 kDa band has smeared all the way down to 50 kDa. Disulfide bonds appear to be at least partially involved in the aggregation occurring during heating at pH 8.5. In the sample heated at pH 10.5, the presence of DTT did not largely influence the protein profile.
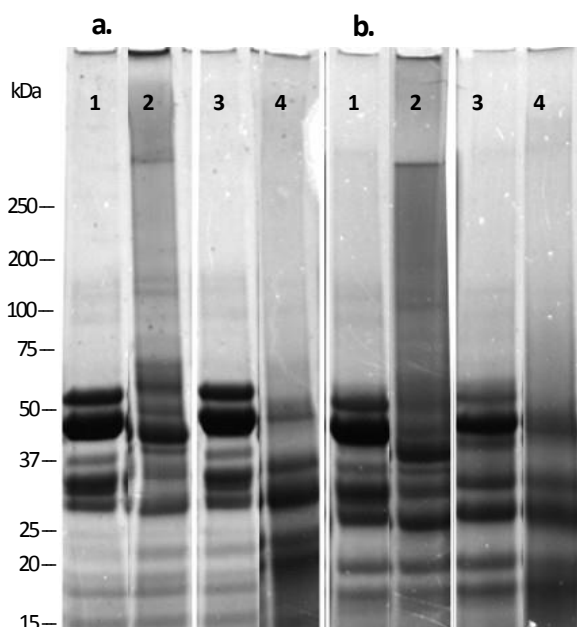

Figure 8.2. SDS PAGE under non-reducing (a) and reducing (b) conditions. Lanes 1, pH 8.5 non-heated; lane 2, pH 8.5 heated; lane 3, pH 10.5 non-heated; lane 4, pH 10.5 heated.

The subunits of oligomeric globulins dissociate during heating. Also, cleavage of the disulfide bonds occurs, releasing the acidic and basic subunits. After heating at 100°C, soy glycinin is not found in 11S form, but as dissociation products (S4) and aggregates (Wolf & Tamura, 1969). The basic polypeptides have a low solubility at pH 5.0-9.0 and aggregate readily during heating at this pH range (German, Damodaran, & Kinsella, 1982). It is possible that, when heated at pH 10.5, all the polypeptides remain soluble.

8.3.3 Physicochemical and emulsifying properties of QPI after treatments

Solubility depends on the composition, size and surface net charge of the protein The net charge is pH-dependent and dictates the extent of attractive and repulsive electrostatic forces (Kinsella, 1982). The solubility of native and heated QPI was determined as a function of pH (Figure 8.3a). The solubility was strongly pH dependant, with a maximum solubility of 37-75% and 45-95% at pH 3.0-3.5 and 8.0-9.0, respectively. QPI was essentially insoluble between pH 5.0 and 6.5. The shape of the curve was not altered by heat-treatments, but heating at pH 10.5 increased the solubility at low and high pH by 30-40%, while heating at pH 8.5 decreased it slightly at high pH when compared to the native sample ($p<0.05$).

When in solution, native proteins are usually folded with their polar amino acid residues exposed to the aqueous phase and the hydrophobic residues buried in the core of the structure (Kinsella, 1982). Unfolding of the protein due to e.g. heating or pH extremes can expose the hydrophobic amino acid residues and decrease the solubility of the protein (Marcone, 1999; Voutsinas, Cheung, & Nakai, 1983). The rather low overall solubility of the unheated protein is likely to result from the pH extremes or freeze-drying step during the extraction procedure. Abugoch et al.

(2008) acquired a very different solubility curve for a QPI isolated under similar conditions, but at ionic strength of 0.5. The solubility remained high (80-95%) between pH 4.5 and 11.0 and very low between pH 3.0-4.0 instead of the U-shaped curve obtained in this study.

Viscosity of the solutions was highest at low pH, and appeared to peak at pH 4.5 in all samples (Figure 8.3b). At pH 5.5 and above, the viscosity remained low ($\leq$ 2 mPas). Solutions heated at pH 8.5 had lower viscosity at low pH, while samples heated at pH 10.5 had slightly higher viscosity at high pH compared to the rest
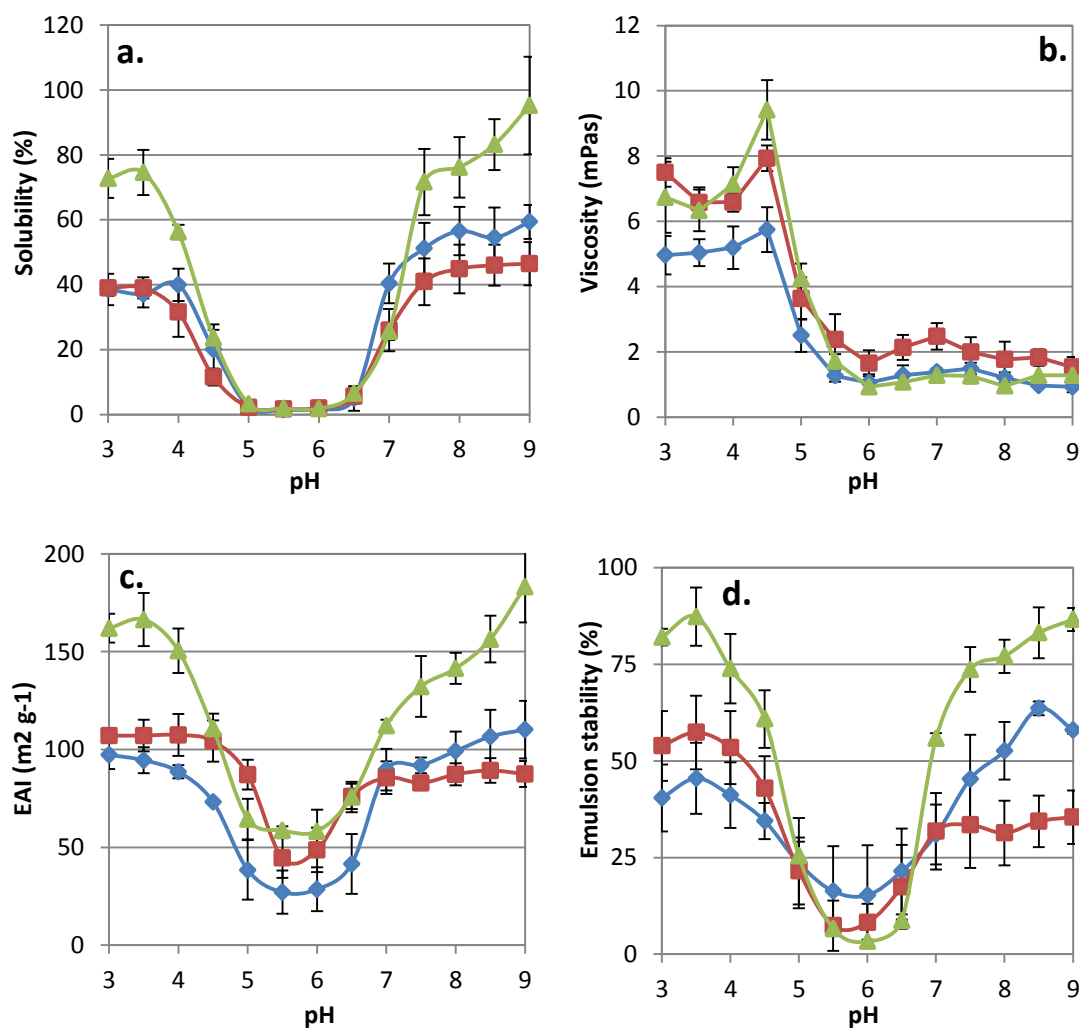
Figure 8.3. Solubility (a), viscosity (b), emulsifying activity index (c), and emulsion stability (d) of native (red), denatured at pH 8.5 (red) and denatured at pH 10.5 (green) QPI as a function of pH.

(p<0.05). In a model system, the viscosity of protein solutions should be inversely related to solubility because increased intermolecular interactions would be expected to increase the viscosity (Schneppf, 1992; Shen, 1981). However viscosity behaviour is complex and influenced by other factors such as conformation and exposure of hydrophobic groups, making more fundamental interpretations in complex systems challenging (Schneppf, 1992; Shen, 1981). The viscosity of soy protein as a function of pH gives a similarly shaped curve as solubility, which was the case below neutral pH in this study (Shen, 1981).

Emulsifying activity index (EAI) (Figure 8.3c) and emulsion stability (Figure 8.3d) figures were nearly identical to the solubility curves: EAI and emulsion stability had their minimum between pH 5.0 and 6.5 in all samples. Heating at pH 10.5 increased the values at low and high pH, and heating at pH 8.5 increased them slightly at pH 7.5-9.0, compared to the native sample (p<0.05). The values cannot be compared directly to literature as the outcome depends on the means of emulsifying, but as a comparison, the EAI for bovine serum albumin, a good emulsifier was measured in this study. The EAI was 172 m^2g^{-1} at pH 8.0 with this experimental setup. The corresponding values were 87.3 and 141 m^2g^{-1} for native and pH 10.5 heated QPI, respectively.

Proteins stabilise emulsions by adsorbing and partially unfolding on the oil-water interface, hence forming a viscoelastic layer that prevents coalescence (Wilde, 2000). Generally, heating increases the surface hydrophobicity of proteins, but this does not always improve the emulsifying properties because of a loss in solubility (Nakai & Li-Chen, 1989). Many native plant proteins have poor interfacial properties because of their quaternary structure and compact tertiary structures (Gruener & Ismond, 1997; Liu, Lee, & Damodaran, 1999). Dissociation of the subunits of soy

protein decreases the molecular weight and increases surface hydrophobicity and molecular flexibility, leading to an increased ability to adsorb on the interface (Qi, Hettiarachchy, & Kalapathy, 1997; Wagner & Guéguen, 1999; Withana-Gamage & Wanasundara, 2012).

Particle size distributions were determined in heated and non-heated solutions at pH 3.5 (Figure 8.4a; very acidic pH, high solubility), pH 5.5 (Figure 8.4b; acidic pH, insoluble) and pH 8.0 (Figure 8.4c; alkali pH, high solubility). Three distinct populations of particles were present with diameters ca. 0.1-1.0 µm, 1-10 µm and 35-350 µm, the population size depending on pH and heat-treatment. When the particle sizes were measured at pH of high protein solubility (3.5 and 8.0), native and pH 8.5 heated samples showed similar profiles, but pH 8.5 contained more large particles. In the sample heated at pH 10.5, the particle size distribution was monomodal with only small particles present (Figure 8.4a and c). At minimum solubility (pH 5.5), all samples larger aggregated particles, but the sizes varied: the particles were in the range of 50-500 in native samples, 26-500 in pH 8.5 heated samples and 22-140 µm in pH 10.5 heated samples (Figure 8.4 b). It appears that heating QPI at pH 10.5 leads an increased dissociation and/or hydrolysis of the protein into small particles. These particles readily assemble into large aggregates at pH close to the isoelectric point (pI). Larger aggregates (22-140 µm) exist in the pH 10.5 heated sample sample at pH with minimum solubility, although these were not visible in the comb of the SDS PAGE gel unlike in the case of the pH 8.5 treated sample. These aggregates are either small enough to enter the gel, or they are disintegrated by SDS, indicating non-covalent forces.
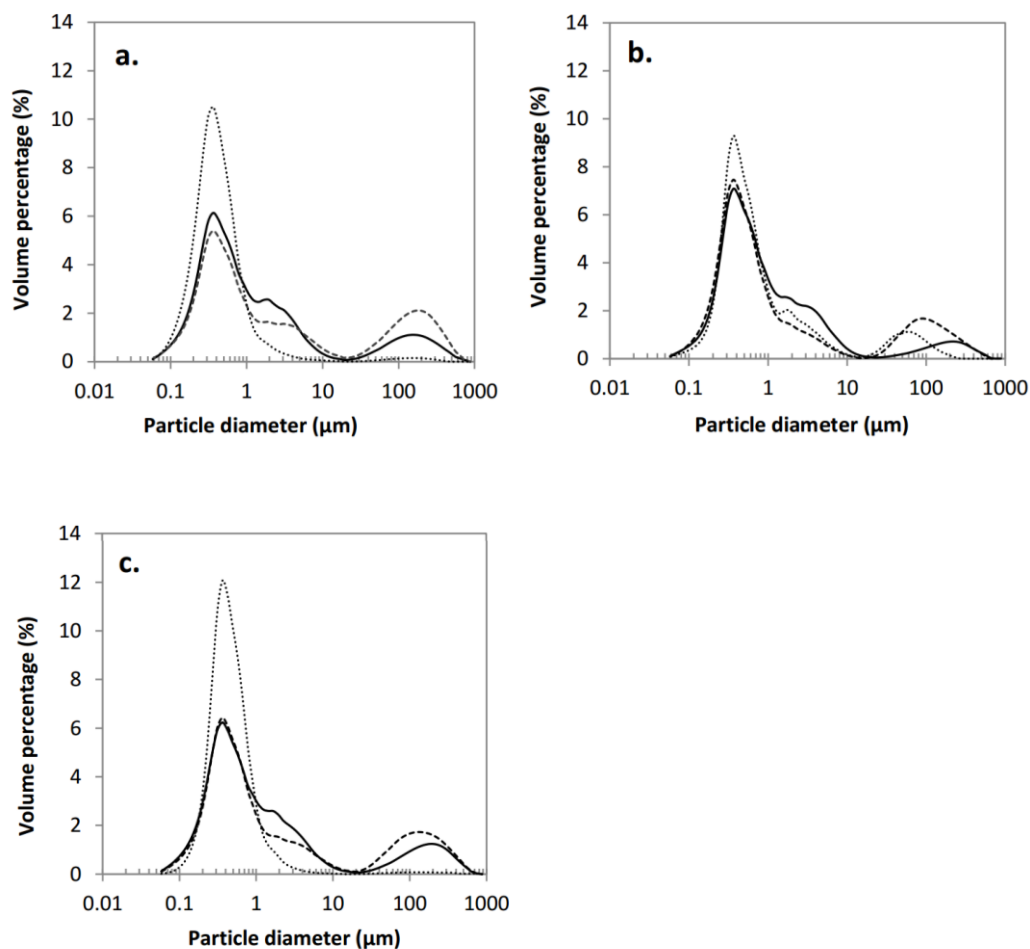
Figure 8.4. Particle size distributions of native (solid) and denatured at pH 8.5 (dashed) and pH 10.5 (dotted) QPI solutions at pH 3.5 (a), 5.5 (b) and pH 8.0 (c).

8.3.4 Acid gelation of QPI solutions

QPI solutions (5%) were heat-treated at pH 7.5, 8.5, 9.5 and 10.5 and acidified using glucono-δ-lactone (GDL). The storage moduli (G') were recorded simultaneously (Figure 8.5). An increase in G' occurred in samples heated at pH 8.5 and above, but not at 7.5 or a non-heated solution (not shown). The maximum G' reached during the acidification depended very strongly on the pH at which the solution was heated: the final G' values were 5 Pa for pH 8.5, 20 Pa for pH 9.5 and 1140 Pa for pH 10.5 (Figure 8.5 a-b). The G' was very dependent on sample

preparation, and, for example, high shear mixing of the protein solution before acidification resulted in gels with a G' < 100 Pa.
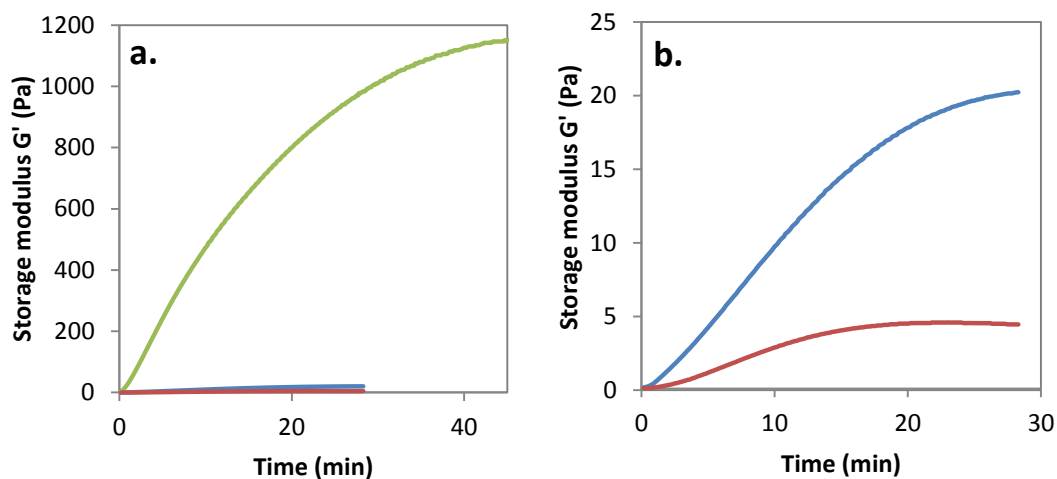

Figure 8.5. Development of storage moduli (G') during the acidification of 5% QPI solutions heated at pH 8.5 (red), 9.5 (blue) and 10.5 (green). Figure b is a magnification of the lower Pa range of figure a.

When observing the gels using a confocal laser scanning microscope, it is evident that the structures of gels from solutions heated at pH 8.5 and 9.5 were coarse and they appeared to consist of irregular clusters of aggregated particles (Figure 6a-b). The gel formed from the pH 10.5 sample on the other has a fine and regular structure and a smaller pore size (Figure 6c).

The degree of aggregation determines the type of gel that is formed (Hermansson, 1986). Renkema, Lakemond, de Jongh, Gruppen, & van Vliet (2000) observed that a fine-stranded gel was formed when soy protein was heat-gelled at a neutral pH, whereas acidic pH led to the formation of a coarse, granulated gel because the proteins were aggregated to a larger extent. The authors hypothesised that only the precipitated protein in the solution is incorporated in the gel, and the

higher proportion of soluble protein at neutral pH explains the finer structure (Renkema et al., 2000).
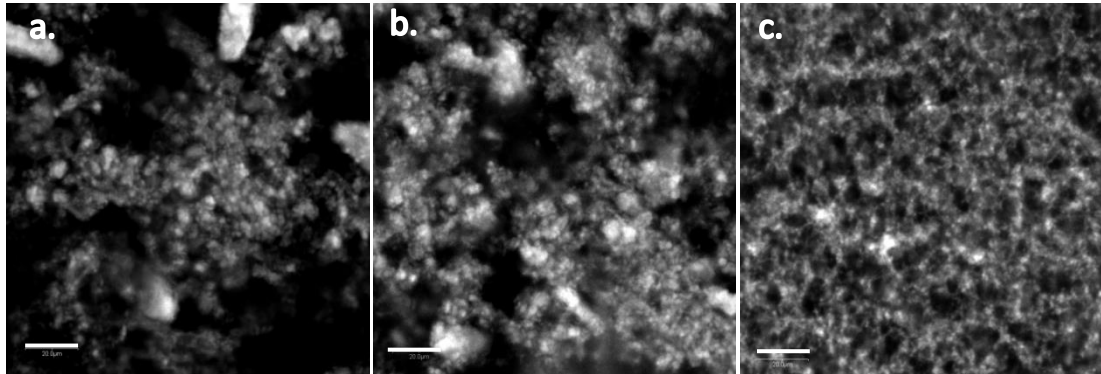

Figure 8.6.CLSM images of acidified QPI heated at different pH 8.5 (a), 9.5 (b) and 10.5 (c). Bars 20 µm.

The pH 10.5 heated sample was marked by lower surface hydrophobicity and higher solubility than the sample treated at pH 8.5. Gel electrophoresis showed the apparent absence of larger disulphide-bound aggregates that were found in the sample heated at pH 8.5, but particle size data (Figure 8.5) revealed the presence of aggregates of 30-200 µm diameters. These particles however appear be soluble to a large extent. If aggregation is suppressed during unfolding, the resulting gel network will have a finer structure and higher elasticity than when random aggregation and unfolding occur simultaneously (Hermansson, 1979). The solubility of the basic polypeptide increases above 9, below which it readily aggregates during heating (German et al., 1982). Possibly, in the sample heated at pH 10.5, the basic polypeptides remain soluble after dissociation and the formation of protein-protein interactions occurs largely only during acidification. Also hydrolysis may increase the gelation properties of proteins. For example in the case of whey proteins, extensive hydrolysis (>DH=18%) leads to the formation of a strong gel (Doucet,

Gauthier, & Foegeding, 2001), and similar behavior has been reported for soy glycinin (Kuipers & Gruppen, 2008).

8.4 Conclusions

The present study shows that the pH at which heat-denaturation occurs influences the functional and structure forming properties of QPI strongly. Heating QPI at pH 8.5 led to an increase in surface hydrophobicity and subsequent reduction of solubility and viscosity, whereas heat-denaturation at pH 10.5 increased the solubility and emulsifying activity strongly. Also the acid gelation properties were dramatically altered: the maximum G' of an acidified QPI denatured at pH 10.5 was 1140 Pa compared to 5-20 Pa of proteins denatured at lower pH. We propose the following: formation of random disulphide bound aggregates occurred during heating at pH 8.5. When the repulsive forces were brought down by acidification, these aggregates formed a coarse coagulum with a low G'. When heating at pH 10.5, the high solubility of basic polypeptides and increased surface charge retarded aggregate formation and, when this system was acidified, the soluble aggregates cross-linked in a manner leading to a fine and regular network structure.

Acknowledgments

References

Abugoch James, L., E., 2009. Quinoa (*Chenopodium quinoa Willd.*): Composition, chemistry, nutritional, and functional properties. Adv. Food Nutr. Res. 58, 1–31.

Abugoch, L. E., Romero, N., Tapia, C. A., Silva, J., Rivera, M., 2008. Study of some physicochemical and functional properties of quinoa (*Chenopodium quinoa Willd*) protein isolates. J. Agric. Food Chem. 56, 4745–50.

Alting, A. C., Weijers, M., de Hoog, E. H. A., van de Pijpekamp, A. M., Cohen Stuart, M. A., Hamer, R. J., Visschers, R. W., 2004. Acid-induced cold gelation of globular proteins: effects of protein aggregate characteristics and disulfide bonding on rheological properties. J. Agric. Food Chem. 52, 623–31.

Ando, H., Chen, Y.-C., Tang, H., Shimizu, M., Watanabe, K., Mitsunaga, T., 2002. Food components in fractions of quinoa seed. Food Sci. Technol. Res. 8, 80–84.

Brinegar, C., Goundan, S., 1993. Isolation and characterization of chenopodin, the 11S seed storage protein of quinoa (*Chenopodium quinoa.* J. Agric. Food Chem. 41, 182–185.

Brinegar, C., Sine, B., Nwokocha, L., 1996. High-cysteine 2S seed storage proteins from quinoa (*Chenopodium quinoa*). J. Agric. Food Chem. 44, 1621–1623.

Cameron, D. R., Weber, M. E., Idziak, E. S., Neufeld, R. J., Cooper, D. G., 1991. Determination of interfacial areas in emulsions using turbidimetric and droplet size data: correction of the formula for emulsifying activity index. J. Agric. Food Chem. 39, 655–659.

Doucet, D., Gauthier, S. F., Foegeding, E. A., 2001. Rheological characterization of a gel formed during extensive enzymatic hydrolysis. J. Food Sci. 66, 711–715.

EEC, 2009. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Official Journal of the European Union L 054, 26.2.2009, p.1.

Foegeding, E. A., Davis, J. P., 2011. Food protein functionality: A comprehensive approach. Food Hydrocoll. 25, 1853–1864.

German, B., Damodaran, S., Kinsella, J. E., 1982. Thermal dissociation and association behavior of soy proteins. J. Agric. Food Chem. 30, 807–811.

Gruener, L., Ismond, M. A. H., 1997. Effects of acetylation and succinylation on the functional properties of the canola 12S globulin. Food Chem. 60, 513–520.

Hayakawa, S., Nakai, S., 1985. Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. J. Food Sci. 50, 486–491.

Hermansson, A. M., 1986. Soy protein gelation. J. Am. Oil Chem. Soc. 63, 658–666.

Hermansson, A.-M., 1979. Aggregation and denaturation involved in gel formation - Chapter 5. In: Pour-El, A. (Ed.), Functionality and Protein Structure. American Chemical Society, Washington D.C., U.S.A. pp, 281-304

Kinsella, J. E., 1982. Relationships between structure and functional properties of food proteins. In: Fox, P.F., Condon, J.J. (Eds.), Food Proteins 1. Applied Science Publishers Ltd, Barking, UK. pp. 51–103

Kinsella, J. E., Rector, D. J., Phillips, L. G., 1994. Physicochemical properties of proteins: Texturization via gelation, glass and film formation. In: Yada, R.Y., Jackman, R.L., Smith, J.L. (Eds.), Protein Structure-Function Relationships in Foods. Springer US, Boston, M.A., U.S.A. pp, 1-21.

Kuipers, B. J. H., Gruppen, H., 2008. Identification of strong aggregating regions in soy glycinin upon enzymatic hydrolysis. J. Agric. Food Chem. 56, 3818–27.

Liu, M., Lee, D.-S., Damodaran, S., 1999. Emulsifying properties of acidic subunits of soy 11S globulin. J. Agric Food Chem. 47, 4970–4975.

Maita, Y., Parsons, T. R., Lalli, C. M., 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Press, Oxford, U.k.

Marcone, M. F., 1999. Biochemical and biophysical properties of plant storage proteins. Food Res. Int. 32, 79–92.

Monahan, F. J., German, J. B., Kinsella, J. E., 1995. Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. J. Agric. Food Chem. 43, 46–52.

Nakai, S., Li-Chen, E., 1989. Effects of heating on protein functionality. In: Phillips, R.D., Finley, J.W. (Eds.), Protein Quality and the Effects of Processing. Dekker, New York, U.S.A. pp 125−144.

Pearce, K. N., Kinsella, J. E., 1978. Emulsifying properties of proteins: evaluation of a turbidimetric technique. J. Agric. Food Chem. 26, 716-723.

Prego, I., Maldonado, S., Otegui, M., 1998. Seed structure and localization of reserves in *Chenopodium quinoa*. Ann. Bot. 82, 481–488.

Qi, M., Hettiarachchy, N. S., Kalapathy, U., 1997. Solubility and emulsifying properties of soy protein isolates modified by pancreatin. J. Food Sci. 62, 1110–1115.

Ranhotra, G.S., Gelroth, J.A., Glaser, B.K., Lorenz, K.J., Johnson, D.L., 1992. Composition and protein nutritional quality of quinoa. Cereal Chem. 70, 303-305.

Renkema, J. M., Lakemond, C. M., de Jongh, H. H., Gruppen, H., van Vliet, T., 2000. The effect of pH on heat denaturation and gel forming properties of soy proteins. J. Biotechn. 79, 223–230.

Riha, W.E., Izzo, H.V, Zhang, J., Ho, C.T., 1996. Nonenzymatic deamidation of food proteins. Crit. Rev. Food Sci. Nutr. 36, 225–55.

Schneppf, M. I., 1992. Protein-water interactions. In: Hudson, B.J.F. (Ed.), Biochemistry of Food Proteins. Springer US, Boston, M.A. pp. 1–33

Shen, J.L., 1981. Solubility and viscosity. In: Cherry, J. (Ed.), Protein functionality in foods. American Chemical Society, Washington D.C. pp, 89-109.

Voutsinas, L.P., Cheung, E., Nakai, S., 1983. Relationships of hydrophobicity to emulsifying properties of heat denatured proteins. J. Food Sci. 48, 26–32.

Wagner, J.R., Guéguen, J., 1999. Surface functional properties of native, acid-treated, and reduced soy glycinin. 2: Emulsifying properties. J. Agric. Food Chem. 47, 2181–2187.

Wang, C.H., Damodaran, S., 1990. Thermal destruction of cysteine and cystine residues of soy protein under conditions of gelation. J. Food Sci. 55, 1077–1080.

Whitaker, J.R., Feeney, R.E., 1983. Chemical and physical modification of proteins by the hydroxide ion. Crit. Rev. Food Sci. Nutr. 19, 173–212.

Wilde, P., 2000. Interfaces: their role in foam and emulsion behaviour. Curr. Opin. Colloid Interface Sci. 5, 176–181.

Withana-Gamage, T.S., Wanasundara, J.P.D., 2012. Molecular modelling for investigating structure–function relationships of soy glycinin. Trends Food Sci. Technol. 28(2), 153–167.

Wolf, W.J., Tamura, T., 1969. Heat denaturation of soybean IIS protein. Cereal Chem. 46, 331–344.

Chapter 9

Heat-denaturation and aggregation of quinoa (*Chenopodium quinoa*) globulins: influence of pH

Outi E. Mäkinen, Peter Koehler, Elke, K. Arendt

Abstract

The influence of heating (100°C; 0-15 min) on the molecular weight, protein unfolding and secondary structure of quinoa globulins was studied at pH 6.5 (very low solubility), 8.5 and 10.5 (high solubility). The mechanism of denaturation and aggregation varied with pH. Heating triggered the disruption of the disulphide bonds connecting the acidic and basic chains of the chenopodin subunits at pH 8.5 and 10.5, but these remained intact at pH 6.5. Large aggregates unable to enter a 4% SDS-PAGE gel were formed at pH 6.5 and 8.5, and were mostly dissolved under reducing conditions. Heating at pH 10.5 lead to a rapid dissociation of the native chenopodin and the disruption of the subunits, but no SDS-insoluble aggregates were formed. No major changes in secondary structure occurred during a 15 min heating, but an increase in hydrophobicity indicated unfolding of the tertiary structure in all samples.

9.1 Introduction

The relevance of plant-derived proteins in human nutrition has been predicted to increase in the future, e.g. in the form of meat and dairy substitutes (Aiking, 2011; Day, 2013). Increasing the knowledge about the physical properties, structure-function relationship and processing behaviour of plant proteins is fundamental for the development of desirable products (Aiking, 2011; Kinsella, 1982). Quinoa (*Chenopodium quinoa*) is a dicotyledonous seed crop native to the Andean region, where it has been used as a food staple for thousands of years (Abugoch James, L., 2009). Quinoa seeds contain 12-16% protein with a balanced amino acid profile and a protein efficiency ratio equal to casein (Abugoch James, L., 2009; Ranhotra et al., 1992). The protein storage vacuoles are primarily located in the embryonic tissue that surrounds the seed (Prego et al., 1998). This tissue can be readily separated by milling into a fraction that contains 23% protein (Ando et al., 2002; Mäkinen and Arendt, 2014), leaving the starchy perisperm available for other applications (Elgeti et al., 2014; Rayner et al., 2012).

The main protein present in quinoa protein isolated by alkali extraction and isoelectric precipitation is chenopodin, an 11S globulin (Mäkinen and Arendt, 2014; Brinegar et al., 1993). Chenopodin consists of ~49 and 57 kDa subunits (AB-11S) that are associated into a hexamer by non-covalent interactions (Brinegar and Goundan, 1993; Casey, 1999; Mäkinen et al., 2014). Each subunit consists of acidic (~28 and 34 kDa; A) and basic (~17 and 19 kDa; B) chains, that are linked by a disulphide bond (Mäkinen et al., 2014). These proteins have their isoelectric point between pH 5.0 and 6.5, where they exhibit minimum solubility (Mäkinen and Arendt, 2014).

The physical and physicochemical properties of proteins dictate their potential use (Kinsella, 1982). Previous work has shown that the denaturation pH drastically influences the properties of quinoa globulins. Heating quinoa protein isolate at alkali pH (10.5) improved its solubility and emulsifying activity, and also formed a gel with a regular structure and high storage modulus upon acidification (Mäkinen and Arendt, 2014). Mildly alkali denaturation pH (8.5-9.5) led to the formation of a weak coagulum, while protein denatured at a neutral pH formed no structures (Mäkinen and Arendt, 2014). The differences were linked to the higher solubility of the polypeptides released from the chenopodin subunits during heating that were able to arrange into a fine gel network upon acidification. Heating at lower pH led to the formation of large aggregates, that formed merely a randomly aggregated coagulum with no gel-like properties (Hermansson, 1979; Mäkinen and Arendt, 2014). Improved emulsifying properties have also been reported for soy protein subjected to alkaline pH-shifting (Jiang et al., 2009).

The aim of this study was to investigate the influence of pH on the structural changes occurring during the heat-induced denaturation of quinoa globulins, that may be related to altered functionality. Protein solutions (1%) were subjected to heating at pH 6.5 (very low solubility), 8.5 and 10.5 (high solubility), and changes in molecular weight and aggregation, free sulfhydryl groups, surface hydrophobicity and secondary structure were monitored as a function of heating time.

9.2 Materials and Methods

9.2.1 Heat processing of protein solutions

Quinoa globulins were isolated by alkaline extraction and isoelecetric precipitation as described in previous work (Mäkinen and Arendt, 2014). The freeze dried protein isolate was dispersed in water and left under gentle shaking for 24-48 h. Insoluble protein was removed by filtering through filter paper. Sodium azide (0.02%) was used for microbial control. The pH of solutions was adjusted with NaOH and HCl, and heat-treatments were carried out in sealed tubes in a boiling water bath.

9.2.2 Free sulfhydryl groups

Free sulfhydryl groups were determined with Ellman's reagent (TNB, 5,5'-dithio-bis-(2-nitrobenzoic acid). Sample (62.5 µl) was diluted with 1.312 ml 0.1 M phosphate buffer (pH 8.0) containing 2 mM EDTA, and 25 µl TNB (0.4%) was added. The absorbance (412 nm) was measured after 15 min incubation. The sulfhydryl group concentration was calculated from the molar extinction coefficient of TNB,

$$c_{SH} = \frac{A_{412nm}}{bE} \qquad \text{[Eq. 1]}$$

where b is cuvette path length and E = 14,150 $M^{-1}cm^{-1}$.

9.2.3 Surface hydrophobicity

Surface hydrophobicity was measured using 1-anilino-8-naphthalene sulfonate (ANS) as a hydrophobic probe (Hayakawa & Nakai, 1985). Protein

solutions were diluted to a concentration series ranging from 0.0006% to 0.004% with 0.01 M phosphate buffer (pH 8.0). ANS (10 µl) was added to 2 ml sample, and the fluorescence intensity was measured immediately with a spectrofluorometer (SFM25, Bio-Tek Kontron Instruments, Zürich, Switzerland). The excitation and emission wavelengths were 390 and 470 nm, respectively. The slope of relative fluorescence intensity and protein concentration (x1000) was used as a measure of surface hydrophobicity (S_0).

9.2.4 HPLC analysis of proteins

For HPLC analysis, samples were filtered (0.45 µl) and separated by reverse phase (RP) to study changes in the hydrophobic nature of the samples, and size exclusion (SEC) for information on molecular weight. Both analyses were carried out using an Infinity 1260 HPLC system equipped with a UV detector (Agilent Technologies, Palo Alto, CA). UV detection was at 210 nm, and a reference wavelength of 360 nm was used.

A C18 column (Agilent Technologies, Palo Alto, CA) was used for RP-HPLC, with eluents A: water + 0.1% TFA, and B: acetonitrile + 0.1% TFA. Two linear gradients were applied at a flow rate of 03 ml/min: 0-5 min 10-30% B, and 5-25 min 40-60% B, followed by a 15 min equilibration period before the next run.

The SEC analysis was carried out using two columns with exclusion limits of 500-150 000 and 5 000-1 250 000 (SEC-5 150 and 300Å, Agilent Technologies, Palo Alto, CA), and 0.1 M phosphate buffer (pH 7.0) with 0.1 M NaCl as a mobile phase at 1 ml/min. A calibration curve was constructed using aprotinin, cytochrome c, ribonuclease A, carbonic anhydrase, hen egg albumin, bovine serum albumin, γ-globulin, catalase and thyroglobulin (6.5-670 kDa) (Sigma-Aldrich).

9.2.5 SDS-PAGE

Protein solutions were analysed under non-reducing conditions using a 4-20% gradient gel (Bio-Rad, Richmond, CA). Samples were diluted in sample loading buffer, yielding final concentrations of 2% SDS, 4% glycerol, 0.01% bromophenol blue in 0.05 M Tris-HCl, pH 6.8, 45 µg protein was loaded and electrophoresis was performed according to Laemmli (1970). Gels were stained using a modified sensitive colloidal staining protocol (Westermeier, 2006). Molecular weight markers were run in parallel with the samples (Precision Plus Protein standards, All Blue, Bio-Rad, Richmond, CA).

9.2.6 Circular dichroism

Far-UV circular dichroism (CD) measurements of protein solutions (1 mg/ml) were carried out in the range of 180-260 nm using Chirascan (Applied Photophysics, Leatherhead, UK) with a path length of 0.1 mm. The spectra of three scans were averaged and a 5-point smoothing algorithm was applied after correction for the water baseline.

9.2.7 Statistical analyses

All analyses were carried out in triplicate. Where applicable, means were compared using one way analysis of variance (ANOVA) using Sigmaplot 11.0 (Systat Software Inc., USA). The level of significance was determined at $P < 0.05$.

9.3 Results

9.3.1 Changes in molecular weight (MW)

Size exclusion chromatograms of protein solutions before and after various heating times are presented in Figure 9.1. Non-heated samples (solid black line) showed a major peak corresponding to 250 kDa. At pH 6.5 and 8.5, a small peak with a MW of 456 kDa was also present (Figure 9.1a-b). At pH 10.5, the 250 kDa peak was partly dissociated into smaller fragments with MW in the range of ca. 10-60 kDa before
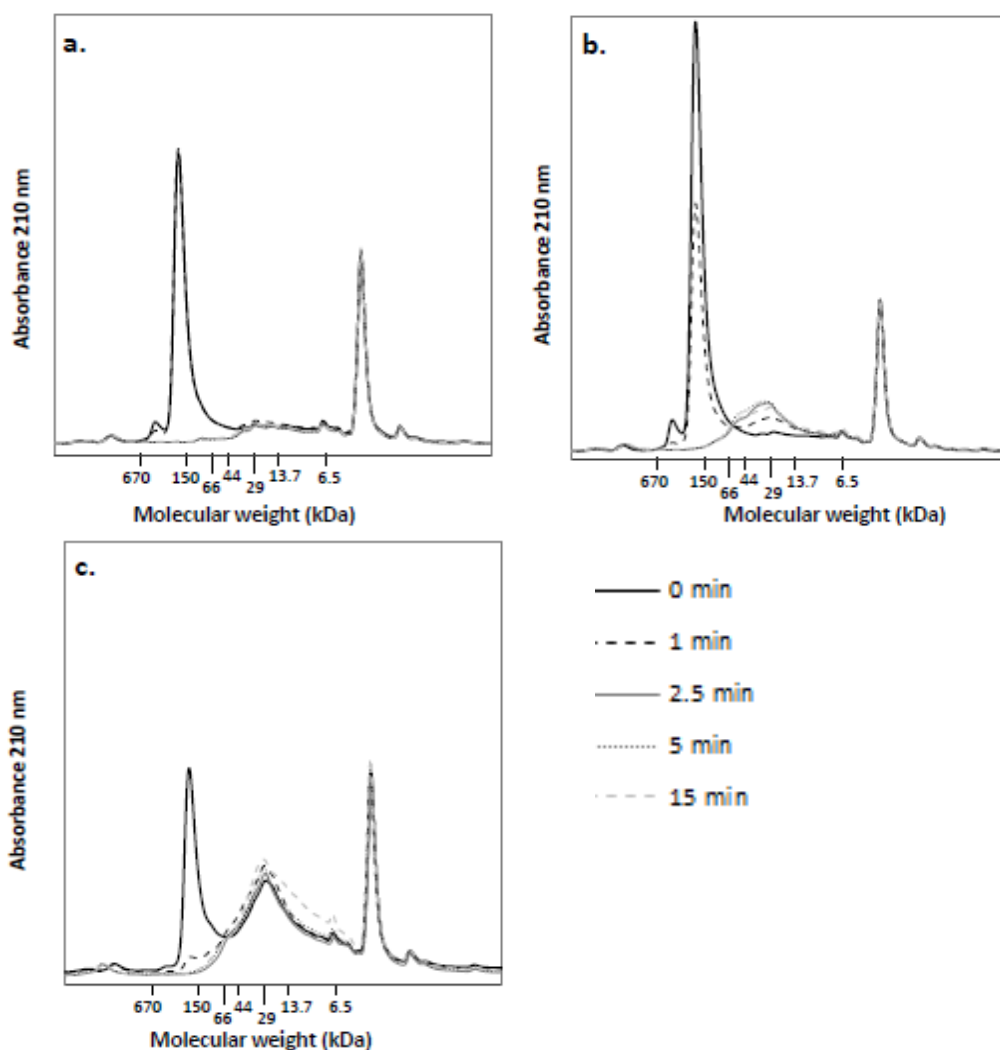

Figure 9.1. Size exclusion chromatography elution profiles of samples heated at pH 6.5 (a), 8.5 (b) and 10.5 (c).

heating (Figure 9.1c). This occurred as a result of heating at pH 6.5 and 8.5, but the low MW peaks were either smaller (pH 8.5) or completely absent (pH 6.5), indicating the formation of aggregates with diameters larger than 0.2 μm, that were caught in the syringe filter before analysis.

The MW of 250 kDa is considerably lower than previously reported; according to Brinegar and Goundan (1993), native chenopodin has a MW of 320 kDa, and 11S globulins from a number of dicotyledonous species all fall within a relatively narrow range of 300-370 kDa (Marcone, 1999). To see whether this difference was due to alteration of the proteins during isolation, especially isoelectric precipitation, the globulin fraction extracted from excised quinoa embryos was also analyzed. An identical SEC elution profile to the non-heated samples was obtained, showing that the MW had not been affected by the isolation procedure (not shown), and the difference to literature values is likely to be caused by calibration. The relationship between retention time and MW is non-linear at higher MW, and even small shifts in retention times result in large differences in MW. This could possibly be improved by using more standards > 150 kDa, but the availability of high MW standards is limited.

SDS-PAGE gels of the samples were run under non-reducing (Figure 9.2a) and reducing (Figure 9.2b) conditions. The presence of SDS disrupts non-covalent bonds, enabling the observation of dissociated subunits. The major proteins in unheated samples were two bands found at ~45 and 55 kDa, and three other bands at ~30-35 kDa. The former correspond to chenopodin subunits (AB-11S), and have been previously observed in the globulin fraction of quinoa embryo (Brinegar and Goundan, 1993; Mäkinen and Arendt, 2014). These subunits consist of an acidic (A)

and a basic (B) chain, connected by a disulfide bond, which can be disrupted in the presence of reducing agents or as a result of heating.
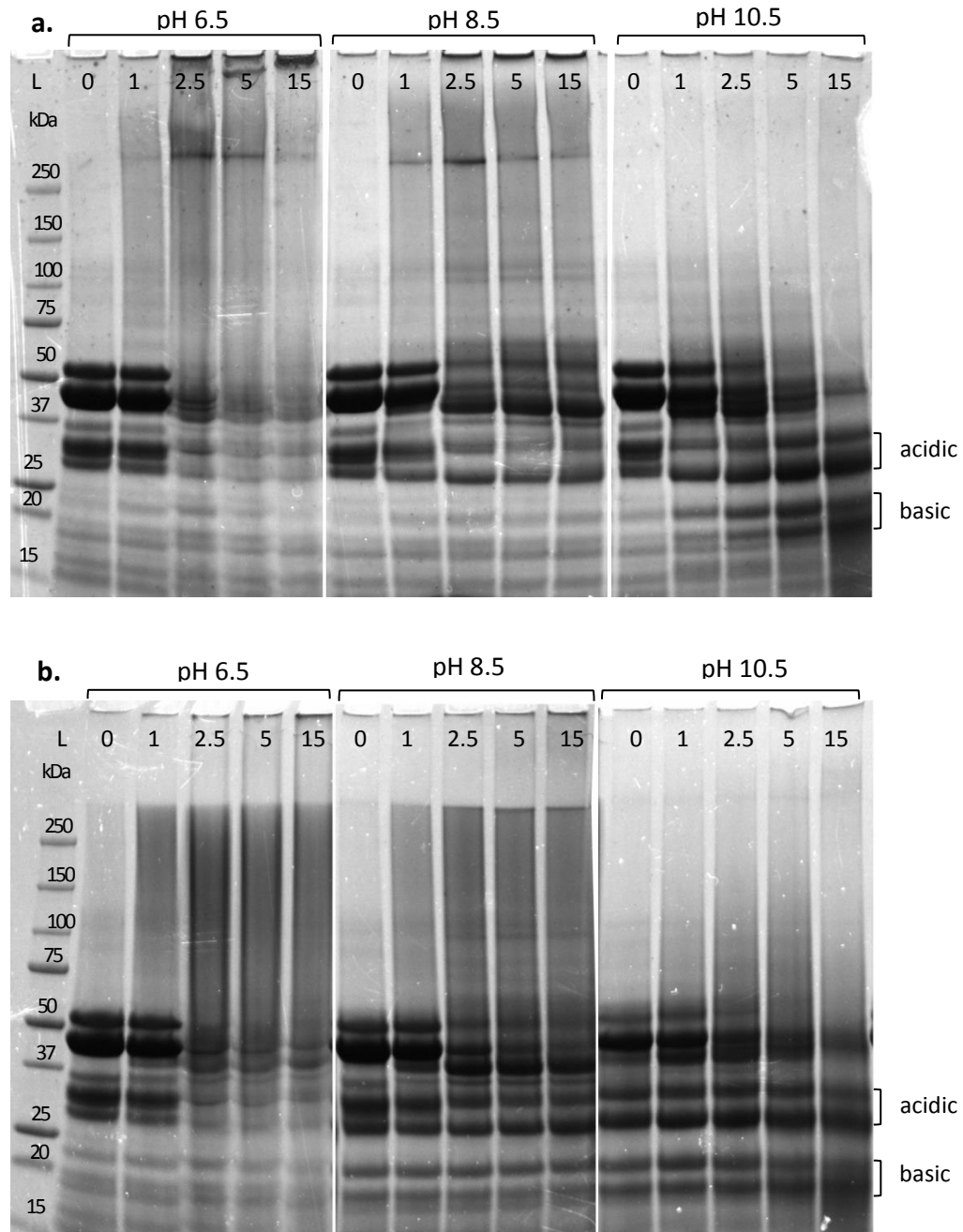

Figure 9.2. SDS-PAGE gels of samples heated for varying times at pH 6.5, 8.5 and 10.5 as analysed under non-reducing (a) and reducing (b) conditions.

The AB-11S bands had faded considerably after 5 min heating and nearly disappeared after 15 min of heating at pH 6.5 and 10.5. At pH 6.5 and 8.5, aggregates appeared as a result of heating just above 250 kDa and in the wells, unable to enter the gel. The gel concentration was 4%, indicating an aggregate MW larger than million Da (Utsumi et al., 1984). The >250 kDa aggregates faded after 15 min heating, possibly polymerizing further. The aggregates were largely dissociated under reducing conditions, indicating disulfide bonding as the polymerization mechanism (Figure 9.2b). In the samples heated at pH 10.5, no aggregates were found. The disulfide bonds connecting the acidic and basic chains were gradually disrupted, giving rise to bands at 18 and 21 kDa and 30 and 35 kDa. These bands were present under reducing conditions in samples heated at pH 8.5, but not at pH 6.5. It appears that the aggregates are formed of intact subunits at pH 6.5, and both intact and dissociated subunits at pH 8.5. Additionally, the bands in the sample heated at pH 10.5 for 15 min were fuzzy, suggesting some degree of hydrolysis of the acidic and basic chains.

9.3.2 Free sulfhydryl groups and surface hydrophobicity

The concentration of free sulfhydryl (SH) groups increased from 10.9 to 17.7 and 16.8 μmol/g protein at pH 8.5 and 10.5, respectively during the first 5 min of heating (Figure 9.3a). This was likely to result from the disruption of the disulfide (SS) bonds connecting the acidic and basic subunits seen in the SDS-PAGE gels (Figure 9.2a), as well as the exposure of buried SH groups due to unfolding. When heated further, the SH group concentration decreased below the starting level at both
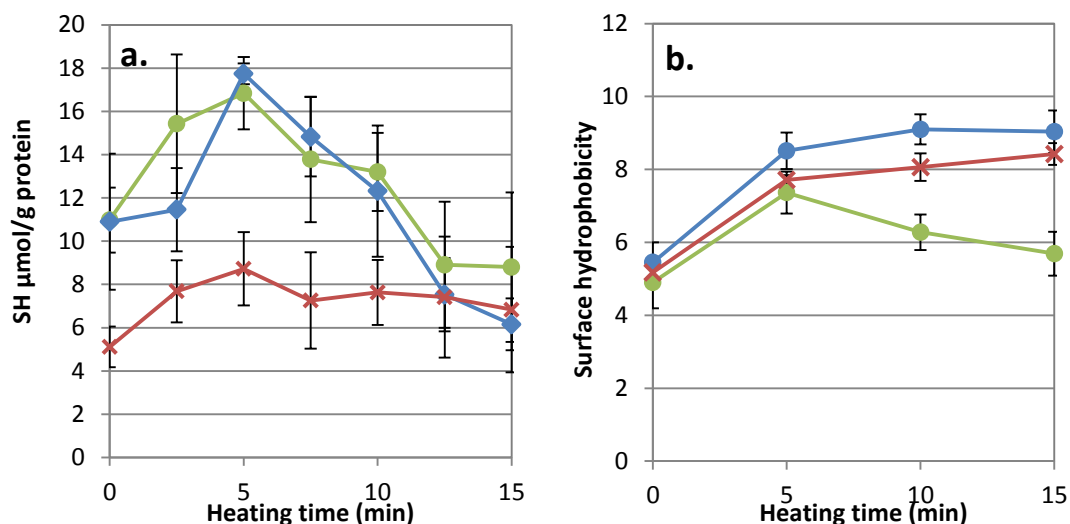
Figure 9.3. Development of free sulfhydryl groups (a) and surface hydrophobicity (b) during heating at pH 6.5 (red), 8.5 (blue) and 10.5 (green).

pH values. At pH 6.5, the quantity of free SH groups was low and increased only slightly from 5.1 to 8.7 μmol/g protein. ANS binding experiments showed that the surface hydrophobicity (S_0) increased in all samples during the first 5 min (Figure 9.3b). This indicates changes in tertiary structure related to unfolding and subsequent exposure of hydrophobic amino acid residues (Marcone et al., 1998). Also, the dissociation of the subunits may contribute to surface hydrophobicity due to the exposure of the hydrophobic areas that are stacked facing each other in the native protein (Adachi et al., 2003). Interestingly, similar degree of hydrophobic exposure occurredat pH 6.5 despite the apparent lack of disruption of SS bonds. When the samples were heated further, S_0 increased slightly at pH 6.5 and 8.6, but decreased nearly back to its value before heating at pH 10.5.

9.3.3 Reverse phase chromatography (RP-HPLC)

Peak regions on the RP-HPLC elution profiles of samples were grouped in four categories (P1-P4) with P1 being least hydrophobic and P4 most hydrophobic (Figure 9.4). The area beneath P3 and P4 decrease in all samples as a result of

heating, but the main differences occur in P1 and P2. Heating at pH 6.5 led to a decrease in P1 and P2 due to aggregation, but the shape of the peaks remained similar (Figure 9.4a). At pH 8.5, a shift from P2 to P1 becomes evident after 5 min heating (Figure 9.4b), whereas at pH 10.5, P2 disappears leaving a large peak in P1 as the major fraction (Figure 9.4c). In contrary to ANS-binding results, the amount of more hydrophilic proteins appeared to increase during heating at pH 10.5, and to some extent at pH 8.5.
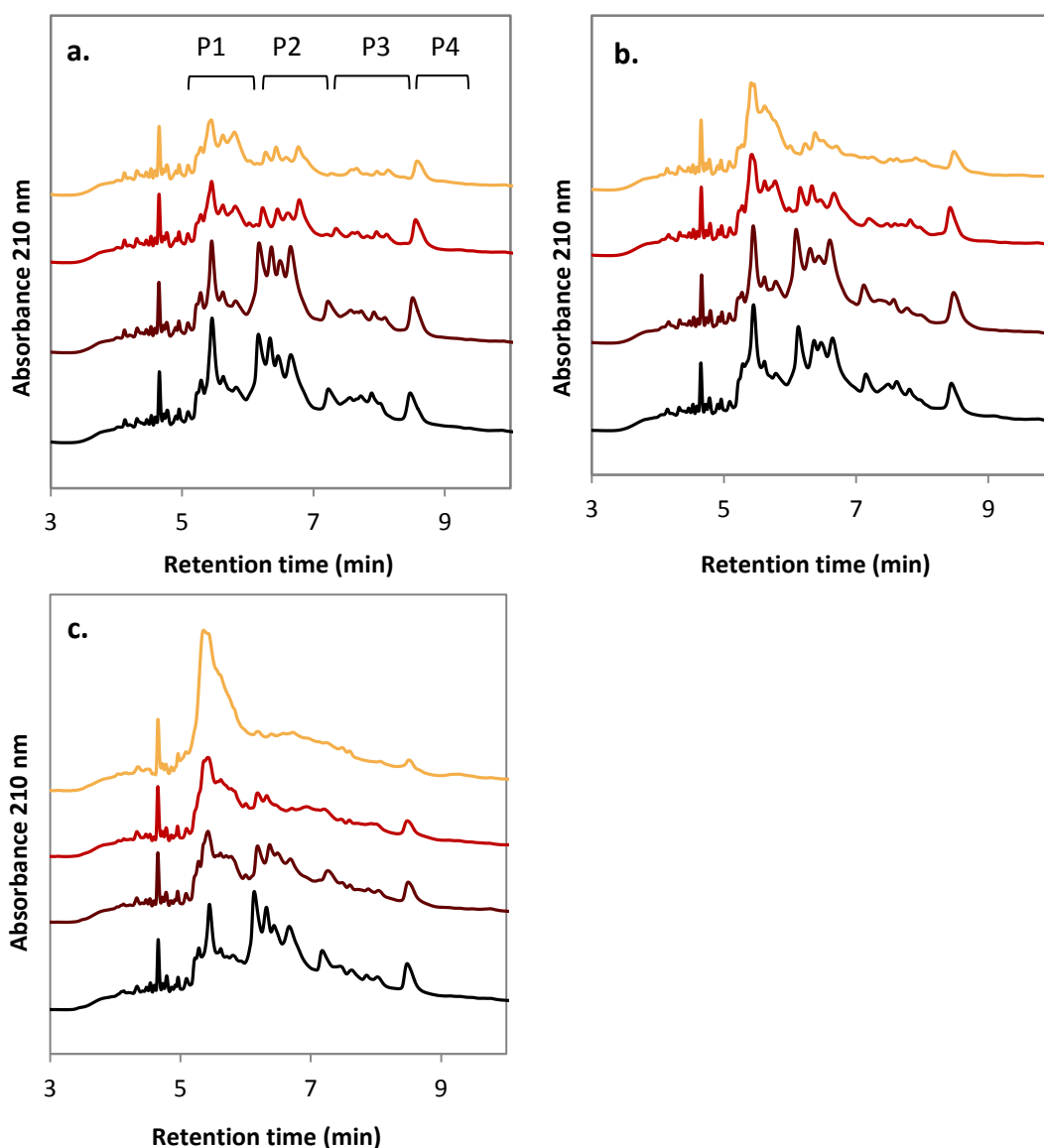

Figure 9.4. Reverse phase chromatograms of samples heated at pH 6.5 (a), 8.5 (b) and 10.5 (c) for 0 min (black), 2.5 min (wine), 5 min (red) or 15 min (orange).

9.3.4 Circular dichroism (CD)

CD spectroscopic measurements were performed at the far UV range to gain information about the secondary structure of the proteins as affected by heating. All spectra exhibited a strong positive peak at 190-195 nm, and a broad negative peak in the region of with a minimum at 208 nm in unheated samples at pH 6.5 and 8.5, and 207 nm at pH 10.5 (Figure 9.5). At pH 8.5, this peak did not change, but at pH 6.5 the negative maximum was attenuated in samples heated for 5 and 15 min, leaving a broad peak ranging from 208 to 226 nm (Figure 9.5a). This atypical spectral shape may be caused by the turbidity of these two samples, that aggregated during heating (Kim et al., 2004). In the samples at pH 10.5, the negative peak was shifted towards lower wavelength and peaked at 204 nm after 15 min heating (Figure 9.5c). This indicates a slight conversion of α-helix and β-sheet to aperiodic structure (Prabakaran and Damodaran, 1997). Overall, the changes in spectra were minor, i.e. the secondary structures of the native protein were mostly retained (Tani et al., 1995).
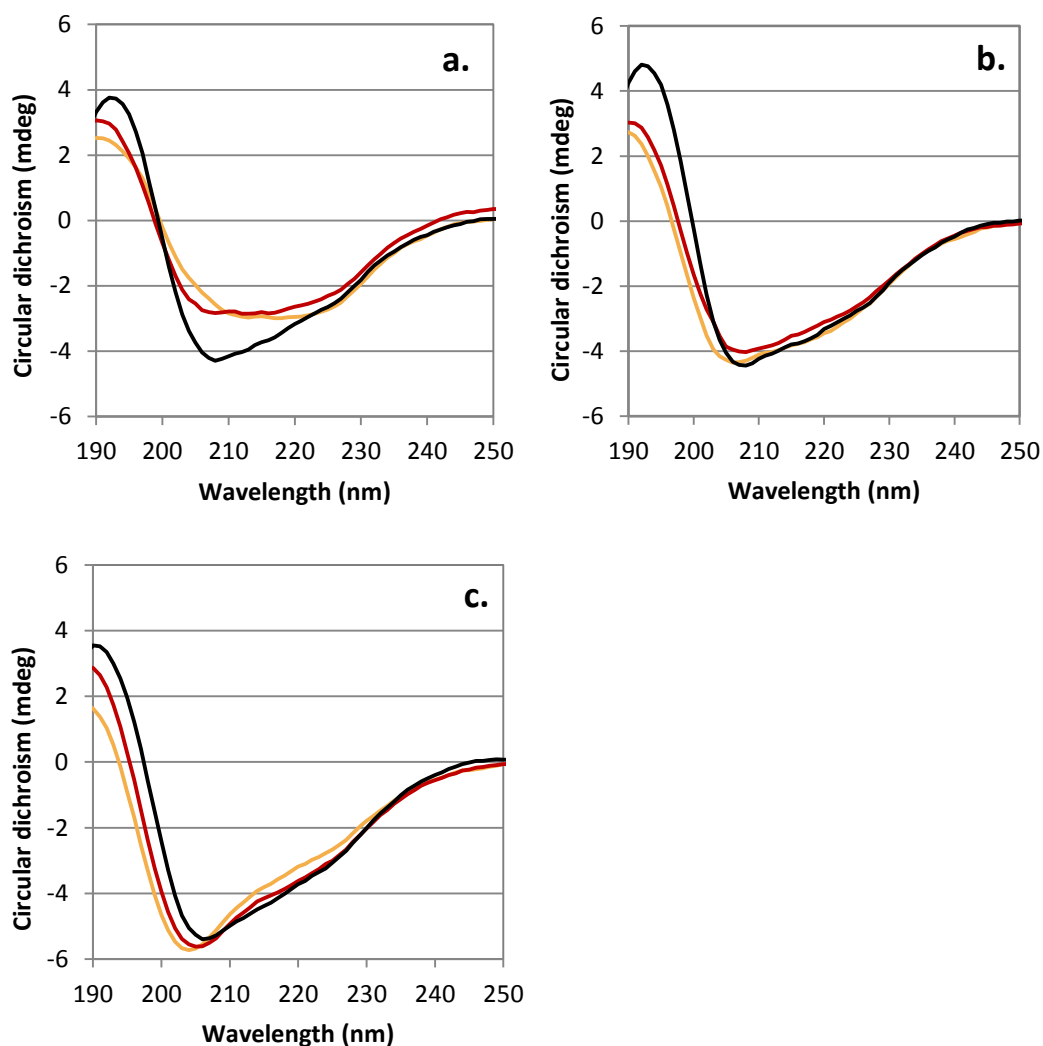
Figure 9.5. Circular dichroism spectra recorded after 0 min (black), 5 min (red) and 15 min (orange) heating at pH 6.5 (a), pH 8.5 (b) and pH 10.5 (c).

9.4 Discussion

pH influences the conformation of proteins greatly, as the ionization of charged amino acid residues is pH dependent. The surface charge distribution impacts the interactions of proteins with the solvent and each other. The further the pH is from the isoelectric point, the greater the electrostatic repulsion and subsequently, the solubility of the protein, while little repulsion promotes aggregation (Creighton, 1993). Also, pH extremes advance unfolding and decrease the thermal stability of proteins (Creighton, 1993; Petruccelli and Añón, 1996).

The main storage globulin in quinoa is chenopodin, an 11S type globulin (Brinegar and Goundan, 1993). The native structure of these proteins is a hexamer of ~50 kDa subunits associated by hydrogen bonding, and electrostatic and hydrophobic interactions (Marcone, 1999). The 11S subunit (AB-11S) consist of a small basic chain (B) and a larger acidic chain (A) that are connected by a single disulfide bridge. Heating can disrupt these bonds, leaving them available for cross-linking by the formation of new disulfide bonds (Schurer et al., 2007; Wolf, 1993).

Heating at pH 8.5 and 10.5 led to the dissociation of native chenopodin oligomer. This occurred especially rapidly, in less than a minute, at pH 10.5. Further heating disrupted the disulfide bond connecting the acidic and basic chains. This was also observed as an increase in the quantity of free SH groups during the first 5 min of heating, after which the number declined. At pH 8.5, these chains aggregated into disulfide bound species accompanied by a reduction in the quantity of free SH groups. At pH 10.5 the acidic and basic chains remained soluble, but the quantity of free SH groups decreased nevertheless. Cysteine and cysteine residues are destroyed by heating under neutral and alkali pH via β-elimination (Wang and Damodaran, 1990). β-elimination is catalyzed by the hydroxyl ion, leading to an increased rate of destruction at higher pH. It is also influenced by the ionization of the sulfhydryl group (pK = 8.8) (Wang and Damodaran, 1990; Whitaker and Feeney, 1983). This reaction may however carry a food safety concerns: dehydroalanine is formed as result of β-elimination, and can react further with other amino acids e.g. into lysine alanine and ornithoalanine (Friedman, 1999; Whitaker and Feeney, 1983). These amino acids have been reported to impair the digestibility of proteins, and dietary lysinoalanine has been associated with changes in kidney cells in rats (Friedman, 1999).

Thermally induced aggregation of partially unfolded proteins can occur either by disulfide crosslinking of newly available SH groups, or non-covalently to counter the increased hydrophobic exposure (Visschers and de Jongh, 2005). While aggregation was clearly disulfide-mediated at pH 8.5, the latter mechanism would explain the decrease in S_0 after the initial increase observed at pH 10.5. These aggregates would not be expected to be visible in during electrophoresis, as non-covalent bonds are disrupted in the presence of SDS. In soy glycinin, the basic chains have been shown to aggregate readily once dissociated between pH 6.5-8.0 (German et al., 1982). The solubility of basic subunits increases at alkali pH, reaching its maximum above pH 9.0 (German et al., 1982). The high solubility of the basic chains at pH 10.5 as well as the destruction of sulfhydryl groups may explain the lack of SDS-insoluble aggregate formation.

At pH 6.5, aggregation occurred without the dissociation of the subunits. (Renkema et al., 2000) suggested different heat denaturing mechanisms for soy glycinin at varying pH. At an acidic pH (3.8), the disulfide bonds between the acidic and basic subunits were not disrupted, while at pH 7.6 they were. This greatly influenced the properties of the formed heat-set gels: at pH 3.8 the gel was coarse and granular, but fine and smooth with a lower G' at pH 7.6. The higher gel strength of the coarse gel was likely to result from stronger aggregation due to more protein-protein interactions (Renkema et al., 2000). In previous work, quinoa globulins heated below 8.5 only aggregated but did not form gel like structures when acidified, while a homogenous acid gel with a high G' was formed from protein denatured at alkali pH (10.5) (Mäkinen and Arendt, 2014). This behavior is likely to vary greatly between different proteins. More aggregation does not necessarily mean stronger gelation, as gel formation is dictated by the balance of attractive and repulsive forces:

if attractive forces dominate, a randomly aggregated coagulum unable to trap water may be formed (Hermansson, 1979; Kinsella et al., 1994). In fact, suppressing the degree of aggregation of egg white proteins with SDS makes the resulting gel structure finer and more uniform (Handa et al., 1998). Also the size of the particles formed during denaturation influence the acid gelation properties. Sonication of soy protein isolate decreases the particle size and surface hydrophobicity, and leads to the formation of small, soluble particles. When acidified, these particles form a stronger and more uniform gel compared to non-sonicated protein with larger particles (Hu et al., 2013).

Heating up to 15 min did not considerably influence the secondary structure at any pH studied. Changes in S_0 and RP-HPLC elution patterns however indicated unfolding in all samples, and the disruption of the tertiary structure was evident in samples heated at pH 8.5 and 10.5. Numerous globular proteins, including oligomeric seed globulins, have been shown to take a partially unfolded state, "molten globule" (Hirose, 1993; Marcone et al., 1997; Tani et al., 1995). This structure is clearly distinguished from both native and fully unfolded structures, and is characterized by native-like secondary structure, some exposed hydrophobic regions, and a compact but slightly loosened state rather than a random coil (Tani et al., 1995). The molten globule state may explain the behavior of proteins when exhibiting functionality such as emulsifying or gelation (Hirose, 1993). It is worth noting that the change in S_0 was similar in all samples despite the resistance of disulfide bonds to heating at pH 6.5. This suggests that the changes in tertiary structure are likely to be different when heated at pH 6.5, because of the intact disulfide bonds. Partially unfolded protein that retains disulfide bonds is more

compact, as cross-links of any kind decrease the conformational flexibility (Creighton, 1993).

These results demonstrate that, while the secondary structure was retained and some unfolding occurred in all conditions studied, the denaturation and aggregation mechanisms of quinoa globulins are strongly pH-dependent. Heat-induced disruption of SS bonds was prevented by heating at a pH close to the isoelectric point, but this lead to rapid aggregation. Mildly and highly alkaline pH (8.5 and 10.5) disrupted the SS bonds, but while the free SH groups appeared to be involved in aggregation at pH 8.5, in a highly alkaline environment these groups were possibly destroyed, altering the behavior of the proteins. Overall, processing conditions of quinoa globulins largely influence their functionality.

Acknowledgments

References

Abugoch James, L., E., 2009. Quinoa (*Chenopodium quinoa Willd.*): Composition, chemistry, nutritional, and functional properties. Adv. Food Nutr. Res. 58, 1–31.

Adachi, M., Kanamori, J., Masuda, T., Yagasaki, K., Kitamura, K., Mikami, B., Utsumi, S., 2003. Crystal structure of soybean 11S globulin: glycinin A3B4 homohexamer. Proc. Natl. Acad. Sci. 100, 7395–7400.

Aiking, H., 2011. Future protein supply. Trends Food Sci. Technol. 22, 112–120.

Ando, H., Chen, Y.-C., Tang, H., Shimizu, M., Watanabe, K., Mitsunaga, T., 2002. Food Components in Fractions of Quinoa Seed. Food Sci. Technol. Res. 8, 80–84.

Brinegar, C., Goundan, S., 1993. Isolation and characterization of chenopodin, the 11S seed storage protein of quinoa (*Chenopodium quinoa*). J. Agric. Food Chem. 41, 182–185.

Casey, R., 1999. Distribution and some properties of seed globulins,. In: Shewry, P. (Ed.), Seed Proteins. Springer Netherlands, N.L. pp, 159–169.

Creighton, T.E., 1993. Proteins in solution and in membranes, in: Proteins: Structures and Molecular Properties. W.H. Freeman and Company, New York, U.S.A. pp. 261 –325.

Day, L., 2013. Proteins from land plants – Potential resources for human nutrition and food security. Trends Food Sci. Technol. 32, 25–42.

Elgeti, D., Nordlohne, S.D., Föste, M., Besl, M., Linden, M.H., Heinz, V., Jekle, M., Becker, T., 2014. Volume and texture improvement of gluten-free bread using quinoa white flour. J. Cereal Sci. 59, 41–47.

Friedman, M., 1999. Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins. J. Agric. Food Chem. 47, 1295–1319.

German, B., Damodaran, S., Kinsella, J.E., 1982. Thermal dissociation and association behavior of soy proteins. J. Agric. Food Chem. 30, 807–811.

Handa, A., Takahashi, K., Kuroda, N., Froning, G.W., 1998. Heat-induced egg white gels as affected by pH. J. Food Sci. 63, 403–407.

Hermansson, A.-M., 1979. Aggregation and Denaturation Involved in Gel Formation - Chapter 5. In: Pour-El, A. (Ed.), Functionality and Protein Structure. American Chemical Society, Washington D.C., U.S.A. pp, 281-304

Hirose, M., 1993. Molten globule state of food proteins. Trends Food Sci. Technol. 4, 48–51.

Hu, H., Fan, X., Zhou, Z., Xu, X., Fan, G., Wang, L., Huang, X., Pan, S., Zhu, L., 2013. Acid-induced gelation behavior of soybean protein isolate with high intensity ultrasonic pre-treatments. Ultrason. Sonochem. 20, 187–95.

Jiang, J., Chen, J., Xiong, Y.L., 2009. Structural and emulsifying properties of soy protein isolate subjected to acid and alkaline pH-shifting processes. J. Agric. Food Chem. 57, 7576–83.

Kim, K.S., Kim, S., Yang, H.J., Kwon, D.Y., 2004. Changes of glycinin conformation due to pH, heat and salt determined by differential scanning calorimetry and circular dichroism. Int. J. Food Sci. Technol. 39, 385–393.

Kinsella, J.E., 1982. Relationships between structure and functional properties of food proteins, in: Fox, P.F., Condon, J.J. (Eds.), Food Proteins 1. Applied Science Publishers Ltd, Barking, UK, pp. 51–103.

Kinsella, J.E., Rector, D.J., Phillips, L.G., 1994. Physicochemical properties of proteins: Texturization via gelation, glass and film formation, in: Yada, R.Y., Jackman, R.L., Smith, J.L. (Eds.), Protein Structure-Function Relationships in Foods. Springer US, Boston, MA, pp. 1–21.

Mäkinen, O.E., Arendt, E.K., 2014. Functional and acid gelation properties of quinoa protein isolate: influence of heat-denaturation pH. *Manuscript.*

Mäkinen, O.E., Hager, A.-S., Arendt, E.K., 2014. Localisation and development of proteolytic activities in quinoa (*Chenopodium quinoa*) seeds during germination and early seedling establishment. J. Cereal Sci. *In press*.

Marcone, M.F., 1999. Biochemical and biophysical properties of plant storage proteins. Food Res. Int. 32, 79–92.

Marcone, M.F., Kakuda, Y., Yada, R.Y., 1998. Salt-soluble seed globulins of dicotyledonous and monocotyledonous plants II. Structural characterization. Food Chem. 63, 265–274.

Marcone, M.F., Yada, R.Y., Kakuda, Y., 1997. Evidence for a molten globule state in an oligomeric plant protein. Food Chem. 60, 623–631. doi:10.1016/S0308-8146(97)00043-5

Petruccelli, S., Añón, M.C., 1996. pH-induced modifications in the thermal stability of soybean protein isolates. J. Agric. Food Chem. 44, 3005–3009.

Prabakaran, S., Damodaran, S., 1997. Thermal unfolding of β-lactoglobulin: Characterization of initial unfolding events responsible for heat-induced aggregation. J. Agric. Food Chem. 45, 4303–4308.

Prego, I., Maldonado, S., Otegui, M., 1998. Seed Structure and Localization of Reserves in *Chenopodium quinoa*. Ann. Bot. 82, 481–488.

Ranhotra, G.S., Gelroth, J.A., Glaser, B.K., Lorenz, K.J., Johnson, D.L., 1992. Composition and protein nutritional quality of quinoa. Cereal Chem. 70, 303-305.

Rayner, M., Timgren, A., Sjöö, M., Dejmek, P., 2012. Quinoa starch granules: a candidate for stabilising food-grade Pickering emulsions. J. Sci. Food Agric. 92, 1841–7.

Renkema, J.M.., Lakemond, C.M.., de Jongh, H.H.., Gruppen, H., van Vliet, T., 2000. The effect of pH on heat denaturation and gel forming properties of soy proteins. J. Biotechnol. 79, 223–230.

Schurer, F., Kieffer, R., Wieser, H., Koehler, P., 2007. Effect of hydrostatic pressure and temperature on the chemical and functional properties of wheat gluten II. Studies on the influence of additives. J. Cereal Sci. 46, 39–48.

Tani, F., Murata, M., Higasa, T., Goto, M., Kitabatake, N., Doi, E., 1995. Molten globule state of protein molecules in heat-induced transparent food gels. J. Agric. Food Chem. 43, 2325–2331.

Utsumi, S., Damodaran, S., Kinsella, J.E., 1984. Heat-induced interactions between soybean proteins: preferential association of 11S basic subunits and .beta. subunits of 7S. J. Agric. Food Chem. 32, 1406–1412.

Visschers, R.W., de Jongh, H.H.J., 2005. Disulphide bond formation in food protein aggregation and gelation. Biotechnol. Adv. 23, 75–80.

Wang, C.H., Damodaran, S., 1990. Thermal Destruction of Cysteine and Cystine Residues of Soy Protein under Conditions of Gelation. J. Food Sci. 55, 1077–1080.

Whitaker, J.R., Feeney, R.E., 1983. Chemical and physical modification of proteins by the hydroxide ion. Crit. Rev. Food Sci. Nutr. 19, 173–212.

Wolf, W.J., 1993. Sulfhydryl content of glycinin: effect of reducing agents. J. Agric. Food Chem. 41, 168–176.

Chapter 10

General discussion

10.1 Germination of quinoa and its application

Quinoa seeds are perispermic with highly compartmentalised storage reserves. The main starch reserve is located in the perisperm, and the protein and lipid bodies are found in the embryonic tissues. An endosperm is present only in the micropylar region, surrounding the tip of the radicle (Prego et al., 1998). Quinoa germinates very fast after being exposed to water. The majority of the seeds showed radicle protrusion 6-10 h after imbibition at 15°C, after the seeds had reached a moisture level between 41 and 45% (Mäkinen et al., 2014a/Chapter 5). The radicle also expanded rapidly, reaching a length of 5 mm and 35 mm after 24 and 72 h, respectively (Hager et al., 2014/Chapter 4). Very fast germination (<24 h) is a trait found in some seeds from high-stress habitats. Most of the known species belong in the former *Chenopodiaceae* family, like quinoa (Parsons, 2012).

A dry, non-germinating quinoa seed is characterised by a very low α-amylase activity, undetectable lipolytic activity and a very high proteolytic activity (Mäkinen et al., 2013/Chapter 6). The proteolytic activity is comparable to the level found in malted barley, that arises only after 3-5 d of germination (Mäkinen and Arendt, 2012). When the enzyme activities were measured separately in the anatomical parts of the seeds, it was observed that a low level of α-amylase activity was present in the embryo of non-germinating seeds, but emerged in the perisperm only after 24 h. After this point, a sharp increase in activity in both tissues occurred (Hager et al., 2014/Chapter 4). The overall level of the activity however remained extremely low compared to traditional malting cereals barley and wheat, or even oat (Mäkinen and Arendt, 2012).

Quinoa seeds have an endosperm only in the micropylar region. In some seeds, this tissue controls dormancy and germination by acting as a physical barrier

that prevents the radicle from penetrating from protruding (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006). In order for germination to occur, the mechanical strength of the micropylar endosperm cap has to be weakened by autolysis of cell-wall polysaccharides and proteins (Morris et al. 2011, Finch-Savage and Leubner-Metzger, 2006). The endosperm dissected from dry, non-germinating quinoa seeds was highly proteolytic with a 60-fold activity compared to the embryo (Mäkinen et al., 2014a/Chapter 5). This is likely to be one reason for the extremely high proteolytic activity observed for the whole seeds before germination (Mäkinen et al., 2013/Chapter 6). The proteolytic activity in the endosperm increased until 24 hai after which it declined. In the embryo where the protein storage vacuoles are mainly found, both the quality and quantity of proteolytic activities remained nearly unchanged over the course of germination (Mäkinen et al., 2014a/Chapter 5).

It has to be noted that activities measured in homogenised tissue extracts may not reflect *in planta* activity, because the enzyme might be inactive under the physiological conditions in the seed at that stage of development, or simply not contact with the substrate due to compartmentation (Bewley and Black, 1994a; Müntz, 1996; van der Hoorn et al., 2004). Still, the high proteolytic activity in the endosperm is not necessarily related to storage protein mobilisation because the storage proteins are mainly located in the embryo. Instead, the results suggest these activities may have a role in endosperm weakening and thus, the regulation of germination. This is also supported by the observed influence abscisic acid (ABA), a plant hormone that regulates the induction and maintenance of dormancy: the presence of ABA led to a lower proteolytic activity in the endosperm after 24 h, and increased the occurrence of abnormally germinated seeds, where endosperm rupture did not occur (Mäkinen et al., 2014a/Chapter 5).

In true cereals (monocotyledonous seed crops), proteolytic enzymes are generally either absent or very low in non-germinated seeds, and arise after 2-3 h of germination in e.g. barley, rye and oat (Brijs et al., 2002; Wrobel and Jones, 1992; Mikola et al. 2002). These studies also show that seeds that are taxonomically and anatomically distant from conventional malting cereals may show very different patterns in enzyme activity development during germination. The use of standard methods in malting and brewing research that have been developed for barley should be done with caution when dealing with different grains.

Malted barley and wheat were widely used for the adjustment for α-amylase activity in baking flours before the rise of commercial fungal amylases (Briggs, 1998). They are also used in some baked goods as sources for starch-degrading enzymes to increase the volume and shelf-life in baked goods, or to alter the rheological properties of dough due to proteolysis (Mäkinen and Arendt, 2012). Similar use in a gluten-free bread formulation was tested with malted quinoa (24 h) and oat (Mäkinen et al., 2013/Chapter 6). These two malts had similar proteolytic activities but the α-amylase activity of oat malt was vastly higher than that of quinoa malt. Oat malt improved the volume and crumb grain of the bread but quinoa malt had no influence of any of the parameters. As the two malts had similar proteolytic activities, it appeared that α-amylase was the key to the improved bread quality.

Generally, proteolytic enzymes have a favourable influence on gluten-free bread because proteolysis enhances the foaming properties of proteins (Renzetti and Arendt, 2009a, 2009b). The recipe used in this study however contained whey protein isolate (WPI), a protein mixture with a very high foaming capacity. The influence of WPI may have hidden any effect from quinoa or oat malt proteases (Mäkinen et al., 2013/Chapter 6). Also, as the proteolytic activity was very high in

nongerminated seeds and did not increase dramatically, germination may not be necessary if proteolytic activity is desired for a technological purpose. Also it has to be considered that in addition to the amylolytic activities remaining low, the radicle has reached a considerable length by the time the activities start to increase. In terms of food applications, this would mean a high malting loss if the radicles are removed, as is the practise with malts. This would suggest that germinating quinoa for an amylase source for food applications would not be feasible.

10.2 Plant milks and quinoa protein properties

Plant-based milk substitutes ("plant milks") are suspensions of dissolved and disintegrated plant material that resemble cow's milk in appearance. They are manufactured by extracting the plant material in water, separating the liquid and formulating the final product, or alternatively reconstituted from protein isolates and other ingredients (Debruyne, 2006; Mäkinen et al., 2014b/Chapter 2). This base product can be further processed into other dairy type product e.g. the manufacture of a yoghurt-type product by fermentation. A market overview (Table 2.8) showed that commercially available plant milks vary remarkably in their nutritional profiles, especially in terms of protein content that was very low in most products (Mäkinen et al., 2014b/Chapter 2). Also, the physicochemical properties of these products varied; the particles are generally larger and more polydisperse compared to cow's milk, which leads to higher sedimentation rates (Mäkinen et al., 2015/Chapter 7). Upon acidification with glucono-δ-lactone (GDL), cow's milk and beverages made from soy and quinoa formed structured gels.

When grains that are high in starch are used as raw material, starch forms a thick slurry when the mixture is heated above the gelatinisation temperature (55-65

°C). To prevent this in the further processing steps, starch has to be gelatinised and liquefied with α-amylase or a malt enzyme extract (Mitchell and Mitchell, 1990; Tano-Debrah et al., 2005). Starch gelatinisation limits the flour-to-water ratio that can be used for milk extraction, and thus the protein content of the final product. In order to explore the possibility of the reconstitution approach to achieve a beverage with a higher protein content, a protein isolate was prepared and studied for functional and acid gelation properties.

A milling fraction enriched in embryo that had a protein content of 27.4% after defatting was used for protein extraction. The resulting protein isolate (QPI) had a protein content of 91.3% and an amino acid composition similar to that of chenopodin (11S). Also the electrophoretic profile showed clearly that the main proteins present were 11S type globulins (Mäkinen and Arendt, 2014c/Chapter 8). The study showed that the heat-denaturation pH influences the functional and acid gelation properties of QPI strongly. Heating QPI at pH 8.5 increased the surface hydrophobicity and subsequently reduced solubility and viscosity, whereas heat-denaturation at pH 10.5 increased the solubility and emulsifying activity strongly. Solutions heated at pH 8.5 formed a coarse coagulum with maximum G' of 5 Pa when acidified with GDL. Heat-induced denaturation at 10.5 enabled the proteins to form a finer and regularly structured strong gel with a maximum G' > 1000 Pa. A possible explanation for this large variation in behaviour is the differences in the nature of aggregates that are formed during heating.

When the changes occurring during denaturation at various pH was studied in more detail, it was confirmed that the mechanism of denaturation and aggregation indeed varied with pH. Heating triggered the disruption of the disulphide bonds connecting the acidic and basic chains of the chenopodin subunits at pH 8.5 and 10.5,

but these remained intact at pH 6.5. At pH 6.5 and 8.5, rapid formation of mainly disulphide-bound aggregates occurred, but the proteins appeared to remain soluble at pH 10.5 (Mäkinen et al., 2014d/Chapter 9). However the quantity of free sulfhydryl groups decreased during heating also at pH 10.5 but due to the apparent lack of disulphide-driven aggregation, it is possible that these groups were destroyed at highly alkali pH (Wang and Damodaran, 1990).

Coarse and granular appearance of a gel indicates strong aggregation due to more protein-protein interactions (Renkema et al., 2000). More aggregation does not necessarily mean stronger gelation, as gel formation depends on the balance of attractive and repulsive forces: if attractive forces dominate, a randomly aggregated coagulum unable to trap water may be formed (Hermansson, 1979; Kinsella et al., 1994). Also the suppression of aggregation during denaturation enables the formation of a fine network compared to a system where unfolding and random aggregation occur simultaneously (Hermansson, 1979). Both the higher solubility of the proteins and lack of disulfide bonding may retard aggregation. When this system is acidified, the particles are cross-linked in a manner that leads to the formation of a fine and regular network structure.

All in all, processing conditions of quinoa proteins influence their functionality strongly. Native quinoa protein isolate has a rather low solubility and does not gel upon acidification, but these factors can be enhanced by modification. Highly alkaline processing however may carry health risks due to the potential formation of "unnatural amino acids" such as lysinoalanine. The formation of these amino acids as well as alternative ways to modify the functionality of proteins require further studies. These results show that quinoa protein can form structures and act as a decent emulsifier under certain conditions. Further studies are needed to get more

detailed information about the gelation mechanism and the required pre-treatments. Also other means of structural modification should be explored, e.g., enzymatic cross-linking and limited hydrolysis. Quinoa protein has a balanced amino acid profile and a high biological value (Ranhotra et al., 1992), which is exceptional in the plant kingdom. Because of this, quinoa would make an interesting raw material for gelled or emulsified dairy-type products.

10.3 Future outlook

It would be interesting to study how changes occurring during germination influence the functional and gelation properties of proteins. Elkhalifa and Bernhardt (2010) observed and increase in solubility, emulsifying activity and stability, and foaming capacity of sorghum flour as a result of germination. There would however be some practical considerations: the protein content of an isolate produced out of whole seeds is rather low at 64%. A high protein content isolate (91%) was only obtained when defatted embryo-rich milling fraction was used for the extraction procedure (Mäkinen et al., 2014c/Chapter 8). This milling fraction can be produced from dry, non-germinated seeds but the embryo elongates rapidly during germination and becomes very fragile when the germinated seeds are dried. The application of milling and sieving techniques are most likely not suitable for such material. Other approaches do however exist if a high protein isolate is desired: a crude isolate can be purified with other methods such as ultrafiltration and ion exchange (Moure et al., 2006; Tzeng et al., 1988).

Because the different tissues of quinoa have a very different composition, fractionation offers interesting potential. Although quinoa is not a protein crop *per se*, the embryo-enriched milling fraction has a significant protein content. Both the

sustainability and economic feasibility of isolating protein from quinoa depends on the other uses of non-protein fractions (Aiking, 2011). For example, the starchy perisperm fraction appears to be well suitable for the production of gluten-free bread (Elgeti et al., 2014). Also, the very small diameter and unimodal size distribution of quinoa starch offers some applications, such as the stabilisation of Pickering (particle-stabilised) emulsions in food, cosmetic or pharmaceutical applications, or microencapsulation of e.g. flavouring (Rayner et al., 2012; Tari et al., 2003).

References

Aiking, H., 2011. Future protein supply. Trends Food Sci. Technol. 22, 112–120.

Briggs, D.E., 1998. Malts and Malting. Blackie Academic and Professional, London, UK.

Brijs, K., Trogh, I., Jones, B.L., Delcour, J.A., 2002. Proteolytic Enzymes in Germinating Rye Grains. Cereal Chem. 79, 423–428.

Debruyne, I., 2006. Soy base extract: soymilk and dairy alternatives, in: Riaz, M.N. (Ed.), Soy Applications in Foods. Taylor & Francis, Boca Raton, Florida, U.S.A, pp. 111–134.

Elgeti, D., Nordlohne, S.D., Föste, M., Besl, M., Linden, M.H., Heinz, V., Jekle, M., Becker, T., 2014. Volume and texture improvement of gluten-free bread using quinoa white flour. J. Cereal Sci. 59, 41–47.

Elkhalifa, A.E.O., Bernhardt, R., 2010. Influence of grain germination on functional properties of sorghum flour. Food Chem. 121, 387–392.

Hager, A.-S., Makinen, O.E., Arendt, E.K., 2014. Amylolytic activities and starch reserve mobilization during the germination and early seedling establishment in quinoa. Eur. Food Res. Technol, *in press*.

Hermansson, A.-M., 1979. Aggregation and Denaturation Involved in Gel Formation - Chapter 5. In: Pour-El, A. (Ed.), Functionality and Protein Structure. American Chemical Society, Washington D.C., U.S.A. pp, 281-304

Kinsella, J.E., Rector, D.J., Phillips, L.G., 1994. Physicochemical properties of proteins: Texturization via gelation, glass and film formation, in: Yada, R.Y., Jackman, R.L., Smith, J.L. (Eds.), Protein Structure-Function Relationships in Foods. Springer US, Boston, MA, pp. 1–21.

Mäkinen, O.E., Arendt, E.K., 2012. Oat malt as a baking ingredient – A comparative study of the impact of oat, barley and wheat malts on bread and dough properties. J. Cereal Sci. 56, 747–753.

Mäkinen, O.E., Zannini, E., Arendt, E.K., 2013. Germination of oat and quinoa and evaluation of the malts as gluten free baking ingredients. Plant Foods Hum. Nutr. 68, 90–5.

Mäkinen, O.E., Hager, A.., Arendt, E.K., 2014a. Localisation and development of proteolytic activities in quinoa (*Chenopodium quinoa*) seeds during germination and early seedling establishment. J. Cereal Sci, *in press*/Chapter 5.

Mäkinen, O.E., Wanhalinna, V., Zannini, E., Arendt, E.K., 2014b. Foods for special dietary needs: Non-dairy plant based milk substitutes and fermented dairy type products. Crit. Rev. Food Sci. Nutr, *In press*.

Mäkinen, O.E., Uniacke-Lowe, T., O'Mahony, J.A., Arendt, E.K., 2015. Physicochemical and acid gelation properties of commercial UHT-treated plant-based milk substitutes and lactose free bovine milk. Food Chem. 168, 630–638. Chapter 7.

Mäkinen, O.E., Arendt, E.K., 2014c. Functional and acid gelation properties of quinoa protein isolate: influence of heat-denaturation pH. *Manuscript*/Chapter 8.

Mäkinen, O.E., Koehler, P., Arendt, E.K., 2014d. Heat-denaturation and aggregation of quinoa (*Chenopodium quinoa*) globulins: influence of pH. *Manuscript*/Chapter 9.

Mitchell, C.R., Mitchell, P.R., 1990. Nutritional rice milk product. United States Patent No. 4894242.

Moure, A., Domínguez, H., Parajó, J.C., 2006. Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. Process Biochem. 41, 447–456.

Prego, I., Maldonado, S., Otegui, M., 1998. Seed Structure and Localization of Reserves in *Chenopodium quinoa*. Ann. Bot. 481–488.

Rayner, M., Timgren, A., Sjöö, M., Dejmek, P., 2012. Quinoa starch granules: a candidate for stabilising food-grade Pickering emulsions. J. Sci. Food Agric. 92, 1841–7.

Renkema, J.M.., Lakemond, C.M.., de Jongh, H.H.., Gruppen, H., van Vliet, T., 2000. The effect of pH on heat denaturation and gel forming properties of soy proteins. J. Biotechnol. 79, 223–230.

Renzetti, S., Arendt, E.K., 2009a. Effect of protease treatment on the baking quality of brown rice bread: From textural and rheological properties to biochemistry and microstructure. J. Cereal Sci. 50, 22–28.

Renzetti, S., Arendt, E.K., 2009b. Effects of oxidase and protease treatments on the breadmaking functionality of a range of gluten-free flours. Eur. Food Res. Technol. 229, 307–317.

Tano-Debrah, K., Asiamah, K., Sakyi-Dawson, E., Budu, A., 2005. Effect of malt enzyme treatment on the nutritional and physicochemical characteristics of cowpea-peanut milk., in: Proceedings of the 1st International Edible Legume conference/IVth World Cowpea Congress. Durban, South Africa.

Tari, T.A., Annapure, U.S., Singhal, R.S., Kulkarni, P.R., 2003. Starch-based spherical aggregates: screening of small granule sized starches for entrapment of a model flavouring compound, vanillin. Carbohydr. Polym. 53, 45–51.

Tzeng, Y.-M., Diosady, L.L., Rubin, L.J., 1988. Preparation of Rapeseed Protein Isolate by Sodium Hexametaphosphate Extraction, Ultrafiltration, Diafiltration, and Ion-Exchange. J. Food Sci. 53, 1537–1541.

Wang, C.H., Damodaran, S., 1990. Thermal Destruction of Cysteine and Cystine Residues of Soy Protein under Conditions of Gelation. J. Food Sci. 55, 1077–1080.

Wrobel, R., Jones, B.L., 1992. Appearance of Endoproteolytic Enzymes during the Germination of Barley. Plant Physiol. 100, 1508–1516.

Appendix

Publications, abstracts and awards

Peer-reviewed publications

Mäkinen, O.E, Arendt, E.K. Non-brewing applications of malted cereals, pseudocereals and legumes – A review. *Submitted manuscript.*

Mäkinen, O.E., Koehler, P. and Arendt, E.K. 2014. Heat-denaturation and aggregation of quinoa (*Chenopodium quinoa*) globulins: influence of pH. *Submitted manuscript.*

Mäkinen, O.E. and Arendt, E.K. 2014. Functional and acid gelation properties of quinoa protein isolate: influence of denaturation and pH. *Submitted manuscript.*

Mäkinen, O.E., Uniacke-Lowe, T., O'Mahony, J.A., Arendt, E.K., 2015. Physicochemical and acid gelation properties of commercial UHT-treated plant-based milk substitutes and lactose free bovine milk. Food Chem. 168, 630–638.

Mäkinen, O.E., Hager, A-S., Water, D.M. and Arendt, E.K. 2013. Localisation and development of proteolytic activities in germinating quinoa (*Chenopodium quinoa*) seeds. J. Cereal Sci, *in press.*

Hager, A-S., **Mäkinen, O.**E.*, Arendt, E.K. 2013. Amylolytic activities and starch reserve mobilization in germinating *Chenopodium quinoa*. Eur. Food Sci. Technol. 239, 621-627.

Mäkinen, O.E., Wanhalinna, V., Zannini, E. and Arendt, E. 2013. Foods for special dietary needs: Non-dairy plant based milk substitutes and fermented dairy type products. Critical Reviews in Food Science and Nutrition, *in press*.

Mäkinen, O.E., Zannini, E. and Arendt, E. 2013. Germination of oat and quinoa and evaluation of the malts as gluten free baking ingredients. Plant Food Hum. Nutr. 68, 90-95.

Conference abstracts

Mäkinen O.E., Hager A-S. and Arendt E.K. 2013. Hydrolytic enzyme activities and the mobilisation of starch and protein reserves in Chenopodium quinoa. *42nd Annual Food Research Conference, Dublin, Ireland, June 2013 (oral presentation).* Best oral presentation (runner-up) prize.

Mäkinen, O.E., Uniacke-Lowe, T., O'Mahony, J.A. and Arendt, E.K. 2013. Physical stability and acid gelation properties of plant-based milk substitutes. *C&E Spring Meeting, Leuven, Belgium, May 2013 (oral presentation).*

Mäkinen O.E., Hager A-S. and Arendt E.K. 2013. Mobilisation of starch and protein reserves and the development of related enzyme activities in Chenopodium quinoa. *3rd international Symposium on Gluten Free, Vienna, Austria, June 2013. Poster presentation.*

Mäkinen O.E., Hager A-S. and Arendt E.K. 2013. Investigation on the mobilisation of starch and protein reserves and the development of related enzyme activities in Chenopodium quinoa. *12th European Young Cereal Scientists and Technologists Workshop, Nottingham, United Kingdom, April 2013. Oral presentation.*

Mäkinen, O.E., Oliveira, P. and Arendt, E. 2012. Use of confocal laser scanning and scanning electron microscopy in cereal science. Proceedings of the Microscopic Society of Ireland Annual Symposium. August, 29-31st. *Poster presentation.*

Oral presentations delivered

Mäkinen O.E., Hager A-S. and Arendt E.K. 2013. Hydrolytic enzyme activities and the mobilisation of starch and protein reserves in Chenopodium quinoa. *42nd Annual Food Research Conference, Dublin, Ireland, June 2013 (oral presentation).* Best oral presentation (runner-up) prize.

Mäkinen, O.E., Uniacke-Lowe, T., O'Mahony, J.A. and Arendt, E.K. 2013. Physical stability and acid gelation properties of plant-based milk substitutes. *C&E Spring Meeting, Leuven, Belgium, May 2013 (oral presentation).*

Mäkinen O.E., Hager A-S. and Arendt E.K. 2013. Investigation on the mobilisation of starch and protein reserves and the development of related enzyme activities in Chenopodium quinoa. *12th European Young Cereal Scientists and Technologists Workshop, Nottingham, United Kingdom, April 2013. Oral presentation.*

Mäkinen, O.E. and Arendt, E. 2012. Malted oat as an ingredient in wheat and gluten-free baking. 11th European Young Cereal Scientists and Technologists Workshop. May, 9-11th, Barcelona, Spain. *Oral presentation*.

Awards

Best oral presentation award (runner-up), 42nd Annual Food Research Conference June 2013, Taegasc, Dublin, Ireland