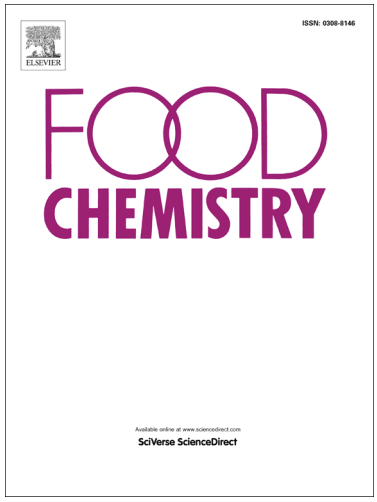
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Characterization of protein hydrolysates from blue whiting (*Micromesistius poutassou*) and their application in beverage fortification

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Key Words: fish protein hydrolysates; health ingredients; functional properties; amino acids; anti-oxidant.

Abstract

Enzymatic hydrolysis of fish proteins has been employed as a principle method for converting under-utilised fish into valuable products for the pharmaceutical and health food industries. In this study, six commercial enzymes were tested for their ability to make fish protein hydrolysate powders from whole blue whiting. The chemical and functional properties of these powders were compared. The powders all had high solubility (>80%) across a wide pH in water and their solubility improved further within a vitamin-tea beverage matrix (>85%). Varying degrees of anti-oxidant properties were recorded for the powders using three model systems (DPPH, ferrous chelating and reducing power). This study demonstrates how commercial enzymes can be used for the extraction and alteration of protein from a low value source to produce highly digestible, low molecular weight peptide powders that could be used as a fortifying health ingredient, especially in beverages.

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1. Introduction

The demand for protein is growing; driven by a rising population, changing food preferences and a growing recognition of the importance of protein as a key ingredient for health and nutrition. Dietary protein supplementation is becoming popular, especially for people on restrictive diets, athletes and elderly people. Dairy and soy are the main sources of protein used in nutritional beverage products thus far, with whey protein concentrate at 80% protein being the most widely used (Galaz, 2013). However, fish is an excellent source of protein. It has higher protein content than most terrestrial animals and proven satiating effects (Egerton, Culloty, Whooley, Stanton & Ross, 2017). Furthermore, fish proteins are highly digestible and have excellent essential amino acid (EAA) profiles that closely approximate human dietary requirements as set by the World Health Organisation (WHO). Many smaller fish species, e.g. blue whiting, *Micromesistius poutassou*; boarfish, *Capros aper;* and sprat, *Sprattus sprattus*, are currently considered undesirable for direct human consumption. These fish, often caught in vast numbers, are most commonly used in animal feeds or fertilisers. However, they represent an untapped resource of functional and health ingredients for human nutrition (Thorkelsson & Kristinsson, 2009).

Enzymatic hydrolysis of fish proteins has been employed as a principle method for converting under-utilised fish into valuable products for the pharmaceutical and health food industries (Chalamaiah, Rao, Rao & Jyothirmayi, 2010). This process can create products that provide enhanced bioactivity, nutrition and physicochemical properties beyond that of the parent protein (Ryan, Ross, Bolton, Fitzgerald & Stanton, 2011). The production of fish protein hydrolysates (FPH) has been widely studied and research shows that the properties of the hydrolysates are highly dependent on the hydrolytic process and reaction conditions, as well as the specific substrates and enzymes used (Chalamaiah, Hemalatha & Jyothirmayi, 2012; Kristinsson & Rasco, 2000a). Previous studies of FPH have shown that they can contribute to water holding, emulsification and texture

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properties when added to food (Halim, Yusof & Sarbon, 2016). Enhanced solubility is a frequently reported and valued property of FPH (Benjakul, Yarnpakdee, Senphan, Halldorsdottir & Kristinsson, 2014). Bioactive properties such as anti-oxidation, anti-bacterial, anti-hypertension and anti-proliferation are also frequently reported (Lee, Qian & Kim, 2010; Picot et al., 2006; Song, Wei, Zhang & Wang, 2011; Thiansilakul, Benjakul & Shahidi, 2007). Anti-oxidant properties can be an important feature for food preservation, as well as providing potential health benefits (Mendis, Rajapakse & Kim, 2005; Phanturat, Benjakul, Visessanguan & Roytrakul, 2010).

Recently, some studies have gone beyond producing and characterizing FPH, and have tested their applicability to fortify foods. Mohamed, Sulieman, Soliman and Bassiuny (2014) used carp and shark fish protein concentrate to increase protein content of biscuits without negatively affecting acceptability in sensory analyses. Similarly positive results were provided by a sensory panelist tasting cheese sticks and biscuits fortified with tilapia FPH up to 15 percent (Ariyani & Widyasari, 2000). Fish and roe PH have been successfully used to stabilize emulsions and protect against oxidation, especially with fish oil-water emulsions (Ghelichi, Sørensen, García-Moreno, Hajfathalian & Jacobsen, 2017; Jónsdóttir et al., 2016). These properties have been harnessed in the production of catfish sausages which resulted in finer fat globules and retardation of lipid oxidation during 12 days of storage (Intarasirisawat, Benjakul, Visessanguan & Wu, 2014). The possibility of fortifying beverages with FPH is also beginning to be investigated. Galactose-gelatin hydrolysate from unicorn leatherjacket skin was used to fortify instant coffee brew, creating a source of anti-oxidant without negatively effecting sensory characteristics (Karnjanapratum & Benjakul, 2017). While Chuaychan, Benjakul and Sae-Leaw (2017) have analyzed the effects of different spray drying temperatures on the properties of gelatin hydrolysate from spotted golden goatfish scales with the aim of fortifying apple juice.

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Blue whiting are fish caught in extensive shoals around the world. However, these fish are used primarily for fish meal and are not considered palatable for direct human consumption. There have been few studies on producing hydrolysates from blue whiting but they have presented results that show excellent potential for adding value to this resource. Hydrolysates made from isolated proteins from headed and gutted blue whiting with Alcalase 2.4 L were bioactive with up to 75% angiotensin I-converting enzyme (ACE) inhibitory activities (Geirsdottir et al., 2011). In another study it has been shown *in vitro* and subsequently *in vivo*, that blue whiting protein hydrolysates, produced using a mix of commercial enzymes, have satiating effects (Cudennec, Fouchereau-Peron, Ferry, Duclos & Ravallec, 2012). Powders with properties such as these would be considered desirable as functional ingredients in food products.

This study adds to the research on blue whiting protein hydrolysates (BWPH). It aims to compare the applicability of six commercial enzymes for the production of BWPH. The objective is to characterize the peptide powders, comparing their chemical, functional and sensory properties and to assess their potential use in fortifying a vitamin tea beverage.

2. Materials and methods

2.1 Material

Blue whiting (*Micromesistius poutassou)* was kindly donated by Biomarine Ingredients Ireland Ltd. The fish were caught in spring 2016 off the north-west coast of Ireland. They were transported frozen, on ice to the laboratory in March 2016 and were stored at -20^{o}C until required. Whole fish were homogenized (Robot Coupe blixer 2 commercial food processor, Stephens Catering Equipment Co. Ltd., Ireland) to create a uniform mince. The mince was stored in aliquots at -20^{o}C and defrosted as required prior to hydrolysis.

2.2 Chemicals and Reagents

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The enzymes Alcalase® 2.4L (endoproteinase from *Bacillus licheniformis*), Protamex® (endoproteinase from *Bacillus sp.*), Savinase® 16L (endoproteinase from *Bacillus sp.*), Neutrase® 0.8L (endoproteinase from *Bacillus amyloliquefaciens*), Flavourzyme® 500L (endoproteinase and exopeptidase from *Aspergillus oryzae*) and Papain (endoproteinase extracted from latex of the plant *Carica papaya*) were purchased from Sigma-Aldrich Ireland Ltd. and were of a food grade standard. All the chemicals used in different analyses were at least of analytical grade.

2.3 Enzyme Activity Assay

To evaluate and standardize the general proteolytic activity of the six enzymes, a synthetic substrate, Azocoll™ (Calbiochem-Novabiochem, La Jolla, CA) was used with some modifications (Chavira, Burnett & Hageman, 1984; Kristinsson & Rasco, 2000b). The assay was performed for the six enzymes at their optimum pH and temperature as outlined in the literature (Alcalase® 2.4L: 50 ^{o}C, pH 8; Protamex®: 55 ^{o}C, pH 7.5; Savinase® 16L: 50 ^{o}C, pH 9; Neutrase® 0.8L: 50 ^{o}C, pH 6.5; Flavourzyme® 500L: 50 ^{o}C, pH 7; Papain: 65 ^{o}C, pH 6.5). To begin, Azocoll (75 mg) was mixed with 0.1 M sodium phosphate buffer (pH 7.5) and left to stand for one hour before centrifuging (27670 *g*, 40^{o}C, two min) and removing supernatant. The step was repeated once more. This removed excess small azo-dye labeled collagen peptides that can interfere with the assay. Sodium phosphate buffer (1.5 mL), adjusted to optimum pH for each enzyme, was added to the Azocoll and heated for five min. Enzyme (10 µL) was added to the assay tubes at five levels of increasing concentration. After mixing, the tubes were incubated at optimum temperature for 15 min. The reaction was terminated by putting the tubes on ice for five min. The suspension was then filtered (Sarstedt Filtropur S 0.45) and the filtrate absorbance measured at 520 nm using a CARY 1 UV–vis spectrophotometer (Alto, CA). The absorbance was plotted against enzyme quantity used for each assay. The results were expressed as Azocoll units (AzU) per gram (g) of enzyme preparation, where one AzU is defined as

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the amount of enzyme producing absorption of 0.1 at 520 nm under the conditions described above.

2.4 Enzymatic Hydrolysis

The hydrolysis reaction was performed using pH-stat apparatus (Titrando 842, Metrohm) equipped with Tiamo 1.1 software. Mince (100 g) was mixed with distilled water (1:2 w/v). The reaction pH and temperature were set at optimum for each enzyme (outlined in section *2.3 Enzyme Activity Assay*) and the pH was kept constant using 1 N NaOH. Enzyme quantities equating to 39054 AzU, which corresponded to 0.1 mL of Alcalase, were used in the hydrolysis and the solution was stirred at 300 rpm throughout reaction. Aliquots (3 mL) of the reaction mixture were removed at 10 intermittent time points (see Figure 1a). Following enzyme denaturation (20 min at 90^{o}C) 50 µL of the aliquots were added to 950 µL of 1% sodium dodecyl sulfate (SDS) solution and stored at -20^{o}C until required for analysis. The soluble protein fractions of the main samples were isolated by centrifugation (4566 *g,* 0^{o}C, 20 min) and filtration (Whatman paper No. 2) and stored at -20^{o}C until required for freeze drying. The five resulting blue whiting soluble protein hydrolysate (BWSPH) powders are hence forth referred to by the name of their producing enzyme, e.g. powder of Alcalase 2.4L is referred to as Alcalase.

2.5 *Degree of Hydrolysis*

The trinitrobenzenesulfonic acid (TNBS) method was used with some modifications to calculate the degree of hydrolysis (DH). BWSPH time point samples, diluted in 1% SDS solution (0.15 mL) were added to tubes containing sodium phosphate buffer (1.0 mL, pH 8.2). A 5% TNBS solution (1.0 mL) was added; each tube was capped, mixed and incubated (60 min at 50^{o}C). The reaction was terminated by the addition of 0.1 N HCl (2 mL). Absorbance values were measured at 340nm using a CARY 1 UV–vis spectrophotometer (Alto, CA). Results were calculated using a standard curve

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prepared using L-leucine and the same methods. DH (%) was calculated using the following equation:

$$DH\% = \frac{AN_2 - AN_1}{Npb} \times 100$$

Where AN_1 and AN_2 are the amino nitrogen content of the protein substrate before and after hydrolysis, respectively, and *Npb* is the nitrogen content of the peptide bonds in the protein substrate; taken as 123.3.

2.6 Proximate Composition

Proximate composition was determined for whole blue whiting and the BWSPH produced. Moisture content was determined gravimetrically after drying at 105 °C for 24 hours. Ash content was measured by incinerating the samples overnight in a furnace at 550 °C. Total nitrogen content was determined by the macro-Kjeldahl method and crude protein content was estimated by multiplying total nitrogen content by 6.25. Lipid content was determined gravimetrically after extraction following a modified Bligh and Dyer method (Smedes, 1999). Protein and fat analyses were performed in duplicate.

2.7 Total and Free Amino Acids

Free amino acid content was determined for the BWSPH according to McDermott et al. (2016). Amino acids were quantified using a Jeol JLC-500/V amino acid analyzer (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column. For total amino acid content of BWSPH, proteins were hydrolyzed in 6N HCl at 110°C for 23 hours and the resulting hydrolysates analyzed as per free amino acids method.

2.8 Molecular Weight Distribution Profile

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Liquid BWSPH time point samples were diluted in distilled water (1:10 v/v) and filtered (Sarstedt Filtropur S 0.2). Molecular weight distributions were determined by size exclusion gel permeation chromatography using a high-performance liquid chromatography system (Waters Corporation, 34 Maple St, Milford, Massachusetts, USA) and a UV detector. Samples were injected (20 µL) on a TSK2000 SW (300x7.5mm) and a TSK G2000swxl column in series (300x 7.8mm, Tosu Hass, Japan) using 30% (v/v) acetonitrile with 0.1% trifluoroacetic acid buffer, at a flow rate of 1 mL/min, as the eluent. Data were collected and analyzed using a Waters Empower 3 software package. The column was calibrated with standard proteins dissolved at 1 mg/mL in distilled water and elutes were monitored at 214 nm. The percentage of individual protein fractions was calculated using the area under the peak of each component divided by the total area.

2.9 Functional Properties

2.9.1 Solubility

Nitrogen solubility was initially determined in distilled water over a range of pH values (2, 4, 6, 8, 10) as described elsewhere (Sila et al., 2014). BWSPH (200 mg) were suspended in distilled water (30 mL) and mixed for 30 min at room temperature prior to centrifugation (4566 *g*, 20^{o}C, 20 min) and filtered (No.2 Whatman filter paper). The nitrogen content of the resulting supernatant was determined using the Kjeldahl method and nitrogen solubility was calculated as follows:

$$Nitrogen\ solubility\ (\%) = \frac{N_1}{N_0} \times 100$$

Where N_1 = supernatant nitrogen concentration and N_0 = sample nitrogen concentration.

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Subsequently nitrogen solubility of the BWSPH was determined in a vitamin-tea beverage following the method above. The pH of the beverage was measured. All analyses were performed in duplicate.

2.9.2 Emulsifying properties

The emulsion activity index (EAI) and emulsion stability index (ESI) of BWSPH were determined with some modifications (Sila et al., 2014). BWSPH were reconstituted in distilled water (15 mL) at 0.5%, 1% and 2% w/v. Olive oil (5 mL) was homogenized with the BWSPH solution with a Tissue Tearor (Biospec Products, Model 985370) for one min at room temperature. Aliquots (50 µL) of the emulsion were taken from the bottom of the conical flask directly after the homogenization and again 10 min later and diluted 100-fold in 0.1% (w/v) SDS solution. The new mixture was mixed for 10 sec and the absorbance was measured at 500 nm using a CARY 1 UV–vis spectrophotometer (Alto, CA). The EAI and ESI were calculated as follows:

$$EAI(m^2 g^{-1}) = \frac{2 \times 2.303 \times A_0}{0.25 \times protein\ weight\ (g)} \qquad ESI(min) = \frac{\Delta A}{A_0} \times t$$

Where A = absorbance, ΔA = (A_0 - A_{10}) and t = 10 min.

2.9.3 Oil binding capacity

To analysis the oil binding capacity (OBC) of BWSPH, an aliquot (0.5 g) was weighed in a centrifuge tube and the combined weight (sample + tube) noted. Olive oil (10 mL) was added to the tube and kept at room temperature for one hour, mixing every 15 min for 5 sec. The mixture was then centrifuged (2,000 *g*, 20^{o}C, 25 min), the supernatant decanted and the tube and sample re-weighed. OBC was calculated as the weight of the contents of the tube after draining divided by the weight of the BWSPH sample, and expressed as the % weight of dried BWSPH.

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2.10 Antioxidant Properties

2.10.1 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity assay

The radical-scavenging activity of BWSPH was determined using DPPH based on previous methods (Wu, Chen & Shiau, 2003). Briefly, 2.0 mL of sample (0.5, 1.5, 3, 6, 12, 18 mg/mL) was mixed with 2.0 mL of 0.15% (w/v) DPPH dissolved in 95% ethanol. The mixture was then kept at room temperature in the dark (30 min) before the reduction of DPPH radical was measured at 517 nm using a CARY 1 UV–vis spectrophotometer (Alto, CA). Distilled water was used as the negative control and butylated hydroxytoluene (BHT) (10 mM) was used as a positive control. The DPPH radical-scavenging activity was calculated as follows:

$$Scavenging\ ability\ (\%) = \left(A_c - \frac{A_1 - A_0}{A_c}\right)$$

Where A_c is the absorbance of the negative control, A_1 is the absorbance of the sample mixed with the reagents and A_0 is the absorbance of the diluted samples without reagents.

2.10.2 Ferrous chelating assay

The Fe^{2+} chelating activity of BWSPH was determined according to Wang, Jónsdóttir and Ólafsdóttir (2009). An aliquot (500 µL) of sample (0.5, 1.5, 3, 6, 12, 18 mg/mL) was mixed with 50 µL of 2 mM Iron (II) chloride and 1.6 mL of distilled water. After incubating at room temperature for 15 min, 100 µL of 5mM ferrozine was added and the mixture was left to stand for a further 10 min after-which the absorbance was measured at 562 nm (CARY 1 UV–vis spectrophotometer; Alto, CA). Distilled water was used as the negative control and EDTA (100 µM) was used as a positive control.

$$Chelating\ activity\ (\%) = \left(A_c - \frac{A_1 - A_0}{A_c}\right)$$

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Where A_c is the absorbance of the negative control, A_1 is the absorbance of the sample mixed with the reagents and A_0 is the absorbance of the diluted samples without reagents.

2.10.3 Reducing power assay

Reducing power was measured according to the methods of Gringer, Osman, Nielsen, Undeland and Baron (2014). Diluted samples (0.5, 1.5, 3, 6, 9, 12 mg/mL) were centrifuged (14,000 *g*, 3 min). The supernatant (200 µL) was then mixed with 200 µL of 0.2 M phosphate buffer (pH 6.6) and 200 µL of 1% potassium ferricyanide and incubated for 20 min at 50°C. Subsequently, 200 µL of 10% (w/v) trichloroacetic acid was added, mixed and centrifuged (10,000 *g*, 10 min). In a microplate, 100 µL of the upper supernatant was mixed with 100 µL of distilled water and 20 µL of 0.1% (w/v) ferric chloride. The microplate was incubated for 10 min at room temperature before the absorbance was measured at 700 nm using Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT). Distilled water was used as the negative control and ascorbic acid (500 µM) was used as a positive control. The absorbance of the diluted samples, without reagents, was also measured and results were taken as the difference between the absorbance of the sample mixed with the reagents and the absorbance of the diluted samples without reagents. Results were expressed as OD_{700}.

2.11 Color

Aliquots of BWSPH were diluted (200 mg/mL and 1000 mg/mL) and color measurements were taken from the solutions in sealed glass bottom petri dishes (µ-Dish 35 mm; ibidi GmbH). Three repeated measures of L* (lightness), a* (red-green color), and b*(yellow-blue color) values were taken at random locations across the lid of the petri dishes using a Minolta Chroma-Meter CR-400 (Mason Technology Ltd., Dublin, Ireland). The mean of three repeated measures was calculated and used as a unit for one replicate.

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2.12 Statistical Analysis

All analyses were performed in triplicate unless otherwise stated. Statistical tests were performed using Microsoft Excel® and the SPSS® computer programs (SPSS Statistical Software, Inc., Chicago, Ill.). All data were submitted to Analysis of Variance (ANOVA) and pairwise comparisons were conducted by 'Tukey's test'. Significance level was determined at the 95% probability level.

3. Results and discussions

3.1 Enzyme Activity Assay

An activity assay was performed before enzymatic hydrolysis as a method of estimating the quantity of each enzyme needed to obtain a uniform level of proteolytic activity. It also acted to assess the economic costs relating to enzyme activity. The results showed that Alcalase 2.4L (390541.06 AzU/ mL), Protamex (250110.80 AzU/ mL) and Savinase 16L (177813.67 AzU/ mL) had over 100-times greater activity than Neutrase 0.8L (1394.74 AzU/ mL), Papain (1225.08 AzU/ mL) and Flavourzyme 500L (6176.00 AzU/mL). The activity level recorded for Alcalase 2.4L is within the same magnitude as reported elsewhere (Kristinsson & Rasco, 2000c). Flavourzyme was also analyzed by Kristinsson and Rasco (2000c) who reported an activity 10 times greater than what was recorded here. Although this difference is noteworthy, they worked with Flavourzyme 1000L rather than Flavourzyme 500L and the assays were performed under different pH and temperature values. Papain had the lowest activity level and a considerably higher price (60.84 €/mL) compared to the microbial enzyme products (1.66 – 2.21 €/mL). This made it unsuitable for commercial hydrolysate production and consequentially was not used in further experiments for this study. All other enzymes analyzed were of a microbial source. Microbial derived enzymes have become prolific and now account for approximately 40% of the total worldwide enzyme sales (Rao, Tanksale, Ghatge & Deshpande, 1998). This is because microbes can be cultivated rapidly, in limited space and are easily

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manipulated to produce large quantities of desirable enzymes. The results of this assay support this market trend.

3.2 Degree of Hydrolysis

DH is defined as the percentage of peptide bonds cleaved and it is the standard parameter commonly used to monitor and compare the level of proteolysis. The TNBS method was favored over the other two common methods, the pH-stat and OPA methods, to calculate DH because previous studies have shown that it provides the most accurate results for a wide range of conditions (Spellman, McEvoy, O'Cuinn & FitzGerald, 2003).

Based on the TNBS method, the hydrolysis curves of whole blue whiting after 3 hours of reaction time with the five enzymes (at volumes dictated by the precluding activity assay) are shown in Figure 1a. Statistically significant differences in DH were found between the enzymes (F (4, 10) = 92.88, $p < 0.001$). Flavourzyme produced a significantly higher DH compared to the other four enzymes (41.47 ± 0.76%; $p < 0.001$). Neutrase (22.78 ± 2.62%) produced the second highest DH but was only considered significantly higher than that of Protamex which had the lowest final DH (16.43 ± 1.61%; $p < 0.05$). Comparing the DH across the three hours, an initial rapid cleaving stage was observed in the first 20 min followed by a decreasing rate of hydrolysis. This is an archetypical reaction commonly observed (Gbogouri, Linder, Fanni & Parmentier, 2004; Kristinsson & Rasco, 2000c). Flavourzyme, however, continued to have greater increases in DH until 90 min of hydrolysis, after which it also began to level off, resulting in powder with distinctive traits as discussed below.

Variation in enzymatic activity between the initial assay and the fish hydrolysis highlights the unique protease-protein interaction that occurs and similar results, using Azocoll as a standardizing reagent, have been reported elsewhere (Kristinsson & Rasco, 2000b).

3.3 Proximate Composition

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The proximate compositions of the freeze-dried powders were determined and compared to that of whole blue whiting (Figure 1b). Protein content significantly increased in all the BWSPH powders in comparison to the starting material (17.23 ± 1.46%; F (5, 6) = 637.47, $p < 0.001$). Protamex had the highest protein content (76.84 ± 0.96%). The protein content of the powder produced with Neutrase 0.8L had the lowest protein content (40.74 ± 0.18%). A protein yield of below 50% was considered unsuitable for purpose and consequentially this powder was not included in further analyses in this study. Moisture content remained similar to starting material for Alcalase, Protamex and Neutrase, with a significant increase and decrease in the Flavourzyme and Savinase BWSPH, respectively. The BWSPH produced with Flavourzyme 500L was found to contain 19.96 ± 0.05% moisture after freeze-drying. This powder had high moisture sorption behavior. This is likely a result of a large number of available water-binding sites associated with the high degree of hydrolysis and subsequent increased number of lower molecular weight peptides (Figure 2). Previous studies have also found that hydrolysates with high proportions of lower molecular weight peptides have increased moisture sorption behavior (Hogan & O'Callaghan, 2013).

3.4 Molecular Weight Distribution Profile

The protein molecular weight distribution of blue whiting prior to enzymatic hydrolysis and the four remaining BWSPH were analyzed by high performance liquid chromatography. The blue whiting starting material was composed of 43.20 ± 3.15% protein > 20 kDa whereas all the BWSPH powders were reduced to smaller peptides with at least 50% of each powder composed of proteins ≤ 0.5 kDa (Figure 2). In agreement with the DH results, Flavourzyme produced the powder with the greatest proportion of small peptides (< 1 kDa). These results suggest that hydrolysis with Flavourzyme yielded a high proportion of shorter (di- and tri-) peptides and free amino acids.

The key characteristics of protein quality for health are digestibility and amino acid composition. The body absorbs dietary proteins in the form of single amino acids and di- and

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tripeptides. Di- and tripeptides are absorbed more rapidly than that of an equivalent amount of free amino acids (Clemente, 2000). Larger intact peptides (≥ tetra-peptides) require additional brush-border hydrolysis to be broken down into their smaller counterparts before absorption (Grimble & Silk, 1986). This extra hydrolysis step has an important rate limiting effect on absorption rates. From the molecular weight composition of the four BWSPH it is expected they would all have a fast digestion and absorption rate.

3.5 Total and Free Amino Acids

As outlined above, for protein quality the second key characteristic to consider is amino acid composition. Therefore, the total and free amino acid compositions of the four BWSPH were determined (Table 1). The three serine proteases; Alcalase 2.4L, Protamex and Savinase 16L all produced powders with very similar free amino acid profiles. This is unsurprising as they did not differ significantly in the degree of hydrolysis that they underwent and the enzymes are all endoproteases with a common reaction mechanism consisting of a catalytic triad of serine, aspartate and histidine. Flavourzyme 500L includes endoproteases and exopeptidases. It breaks the bonds between hydrophobic amino acids at the N terminal of peptide chains. Thus, the use of this enzyme inevitably results in high numbers of free amino acids.

Of the 20 amino acids that are active in the body nine of them are EAA which the body cannot produce and therefore need to be acquired in the diet. The UN's WHO has provided a recommended daily allowance of these EAA (Table 1). The four BWSPH produced in this study contain 15.90 to 30.85 g of EAA in 100g of powder. Of the four BWSPH, Flavourzyme had a ratio of total EAA close to that recommended by the WHO (Table 1). Within the EAA, the branched chain amino acids (BCAA; valine, leucine and isoleucine), and in particular leucine, stimulate protein synthesis (Kimball & Jefferson, 2006). Flavourzyme BWSPH has a slightly lower level of this amino acid compared to the recommended amount, which may moderate protein synthesis, whereas the

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three other BWSPH provide notably higher amounts of these BCAA which could work to stimulate higher levels of protein synthesis.

Functional food ingredients can be added to products for physicochemical or nutritional benefits. They are ideally bland and colorless, therefore providing minimum impact on the intended sensory characteristics of the food. Since production began, fish hydrolysates have been associated with undesirable sensory properties. The most commonly reported flavors are brothy, fishy and bitter (Nilsang, Lertsiri, Suphantharika & Assavanig, 2005). Proteins, especially free amino acids, can play an important role in flavor (Imm & Lee, 1999). Hydrolysis alters the composition of proteins, exposing and releasing interior hydrophobic amino acids. The main amino acids that create a bitter taste are tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine and valine, in decreasing order (Lindsay, 2007; Nilsang et al., 2005). These include the three BCAA that are highly sought after for protein synthesis and the three AA with aromatic rings. The three serine endoproteases produced powders with between 25.7 – 27.2 g/100g of these bittering amino acids (Table 1), while Flavourzyme BWSPH had slightly fewer (9.6g/100g powder; Table 1). The amino acids alanine, aspartic acid, serine, methionine and glutamic acid have been associated with umami flavors (Imm & Lee, 1999; Maehashi, Matsuzaki, Yamamoto & Udaka, 1999), while threonine, glycine, serine, proline and alanine are important sweet taste amino acids (Imm & Lee, 1999; Shen, Guo, Dai & Zhang, 2012). When considering the total amino acid composition of the BWSPH; umami is likely to be the strongest flavor experienced during consumption (Table 1).

3.6 Functional Properties of BWSPH

Protein hydrolysate functionality is influenced by the amino acid composition and sequence as well as the size of peptides, and the charge and distribution of charges of the molecules. The

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degree of hydrolysis and percentage of free amino acids are known to significantly alter the functional properties of protein powders (Chalamaiah et al., 2013). Functional properties of proteins can be broadly divided into three groups, relating to molecular characteristics; (1) hydration properties; (2) protein surface-related properties; and (3) hydrodynamic/rheological properties (Damodaran, 2008). Solubility, emulsification, foaming, viscosity, water binding, gelation, elasticity and flavor/fat binding are all important functions of proteins for the food industry.

3.6.1 Nitrogen solubility

Solubility is often considered the most important physicochemical property for protein hydrolysates, especially when being considered in terms of beverage fortification. Solubility of proteins is influenced by pH, ionic strength, temperature, type of salts, and protein conformation (Damodaran, 2008). The food industry demands high solubility across a wide range of pH. In this study, nitrogen solubility in water was found to be above 80% for all BWSPH across the pH levels (2 – 10). There was no significant difference in solubility between the pH levels (F (4, 19) = 3.649, p = 0.023; Figure 3a). Similar levels of solubility have been recorded for other fish protein hydrolysates (Klompong, Benjakul, Kantachote & Shahidi, 2007). Alcalase, Protamex and Savinase had statistically similar results across all pH values tested while Flavourzyme was less soluble. Protein solubility is the thermodynamic manifestation of the equilibrium between protein–protein and protein–water interactions. The amino acids present and their sequences within peptides largely determine moisture-absorption and water dynamics (Yang, Liu, Zhang, Lin & Chen, 2017). The reduced solubility of Flavourzyme was a predictable result of the higher water content of this powder. A greater proportion of these proteins were small peptides and free amino acids which, due to their hydrophilic nature, were bound to water molecules before the nitrogen solubility analysis. However, all the BWSPH had high solubility which would assist the production of an attractive appearance and smooth mouth-feel to any food or beverage product.

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For beverage fortification, good solubility at low pH ranges is crucial. Carbonated and fruit drinks tend to be acidic by nature. At low pH the charge on the weakly acidic and basic side-chains of amino acids are influenced and become less soluble with possible precipitation (Gbogouri et al., 2004). The results of the solubility test showed that all the BWSPH had excellent solubility at low pH (2 and 4). A beverage matrix will, however, have other compounds in it which may promote or impede solubility. The vitamin-tea beverage in which the BWSPH were dissolved had a pH of 3.32. All the BWSPH displayed excellent solubility within the beverage (> 85%) and there was no significant difference in solubility between the powders (F (3, 4) = 11.627, p = 0.019; Figure 3b).

3.6.2 Emulsifying properties

EAI (m^2g^{-1}) and ESI (min) of the BWSPH at different concentrations of powder (0.5%, 1%, 2%) are shown in Table 2. All the BWSPH had reduced EAI as their concentration increased. The difference among the BWSPH was most significant at the 0.5% concentration, with Flavourzyme (70.25 ± 1.76 m^2g^{-1}) having the highest EAI and Alcalase (37.81 ± 0.68 m^2g^{-1}) having the lowest (F (1, 12) = 826.30, p < 0.001). The ESI differed significantly depending on concentration as well as between BWSPH. Similarly to the EAI, the difference in ESI between BWSPH was largest at 0.5% concentration (F (3, 24) = 213.06, p< 0.001). Protamex had the highest ESI, recorded at 1% and 2% concentration (7.82 ± 0.17 min; 7.57 ±0.47 min, respectively). Emulsification occurs during homogenization when proteins are absorbed to the surface of oil droplets as they are formed, creating a membrane which prevents them from consolidating (Sila et al., 2014). In low concentration solutions, proteins adsorb at the oil-water interface by diffusion, whereas, at high concentrations the activation energy barrier prevents diffusion and the proteins tend to accumulate in the aqueous phase (Thiansilakul et al., 2007). In line with this, the results of this study found that higher concentrations of protein prevented as many oil droplets from being preserved.

3.6.3 Oil binding capacity

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Oil binding occurs through physical entrapment. For this reason, the higher the bulk density of the protein the greater the OBC (Tanuja, Viji, Zynudheen & Joshy, 2012). The OBC of all four BWSPH was low, though Savinase had a statistically higher value compared to the other three powders (F (3, 8) = 17.73, $P < 0.05$; Table 2). Other studies investigating the functional properties of fish protein hydrolysates have reported higher OBC than those found here (Geirsdottir et al., 2011; Jemil et al., 2014). The OBC reported in this study are comparable to the capacity of soy protein (Geirsdottir et al.). The oil binding capacity of proteins will affect functional characteristics as well as taste of the final product. It is considered an important attribute for proteins that are to be used in the meat and confectionery industry.

3.6.4 Color

Color is an important part of the sensory experience of food and beverage consumption, contributing to the quality and appeal of product to the consumer. The color of fish protein hydrolysates will depend on the composition of raw material, enzymes used and the conditions of hydrolysis. Color analysis of the four BWSPH in solution was performed at 1% and 10% concentration (w/v). Color is reported in terms of lightness (L*), red-green color (a*) and yellow-blue color (b*) as described by the Lighting International Commission. There were statistically significant differences between the colors of specific BWSPH on all axes at 10% concentration. However, once diluted to 1% concentration, the only statistically significant differences that remained were on the yellow-blue axis. Superficially all the solutions were light yellow in color at both concentrations. On analysis, Protamex (49.05 ± 0.36) was significantly lighter than Alcalase, Savinase and Flavourzyme at 10% concentration (F (4, 10) = 57.12, $P < 0.01$). At the same concentration, on the green – red spectrum, Flavourzyme was on the red side of neutral which was significantly different to the other BWSPH, which were all on the green side of neutral (F (4, 10) = 110.55, $P < 0.01$). Finally, Flavourzyme (11.72 ± 2.69) was significantly more yellow than the BWSPH,

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apart from Protamex at 10% concentration (F (4, 10) = 19.47, P < 0.01). These results are in line with other studies that have shown that increased hydrolysis causes darkening through enzymatic browning (Jemil et al., 2014).

3.6.5 Antioxidant Properties of BWSPH

Antioxidants are important in health as well as food preservation. Food and pharmaceutical industries often use synthetic antioxidants in their products, however; there is an interest for finding safe and potent natural antioxidants. In this study, we compared the anti-oxidant ability of the BWSPH using three different assays; DPPH free radical scavenging, reducing power assay and ferrous chelating ability.

Using the DPPH assay, significant differences in the scavenging ability of all BWSPH were found (F (3, 48) = 838.37, p < 0.001). Differences in scavenging ability became most significant at concentrations of 3.0 mg/mL and above, at which Flavourzyme had a significantly greater scavenging ability compared to the other BWSPH (p < 0.001) until at 18 mg/mL, where Alcalase also displayed 100% scavenging ability (Figure 4a). Protamex had significantly higher reducing power compared to the other BWSPH (F (4, 60) = 909.4, p < 0.001; Figure 4b). Flavourzyme had the second highest reducing power, while Alcalase and Savinase were significantly lower statistically (F (4, 60) = 909.4, p < 0.001). These three BWSPH showed a linear increase in reducing power, while Protamex leveled off after 6 mg/mL concentration (Figure 4b). Finally, in the ferrous chelating ability assay the antioxidant ability of all the BWSPH increased in a similar concentration dependent manner. However, statistically significant differences in chelating ability were found between all BWSPH except for Flavourzyme and Alcalase (F (3, 48) = 51.29, p < 0.001). When the BWSPH were tested at the highest concentration (18 mg/mL) they all displayed similar ferrous chelating ability (Figure 4c).

Different mechanisms of oxidation occur in the three assays, as outlined elsewhere (Thiansilakul et al., 2007). Therefore, it is understandable that antioxidant ability varied in the

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BWSPH between assays. Taking the three assays together, however, Flavourzyme appeared to have the greatest overall antioxidant ability. Previous studies have reported that proteins with a higher DH and lower molecular weight peptides interact more effectively with radicals to interfere with the oxidation process (Klompong et al., 2007).

4. Conclusions

In summary, this study has shown that whole blue whiting fish are a suitable source of highly nutritious protein. Through hydrolysis, the use of commercial enzymes allowed for the extraction and alteration of protein from a low value source to produce highly digestible, low molecular weight protein powders that have EAA ratios close to what is required for human protein synthesis. Producing powders with a high DH (>15%) allowed for very high solubility but concurrently reduced other functional properties. All the powders showed anti-oxidant properties which will provide benefits for food preservation as well as an extra dimension to a health ingredient. Flavourzyme 500L produced significantly more free amino acids which potentially provide properties that are less desirable in terms of absorption speed but may improve flavor. The BWSPH had mild coloring and combined with a commercial test beverage matrix excellently. Further research will aim to test the palatability of the supplemented beverage with a sensory panel and investigate the shelf life characteristics.

Abbreviations used

BHT – Butylated hydroxytoluene

BWSPH – Blue whiting soluble protein hydrolysate

DH – Degree of hydrolysis

DPPH – 1, 1-diphenyl-2-picrylhydrazyl

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EAA – Essential amino acids

EAI – Emulsion activity index

EDTA – Ethylenediaminetetraacetic acid

ESI – Emulsion stability index

FAA – Free amino acids

OBC – Oil-binding capacity

SDS – Sodium dodecyl sulfate

TAA – Total amino acids

TNBS – Trinitrobenzenesulfonic acid

WHO – World Health Organisation

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Conflict of interest

This study was conducted under an Irish Research Council's Enterprise Partnership funding scheme, and was thereby part-funded by Biomarine Ingredients Ireland Ltd., a marine-biotech company that produces marine ingredients utilising boarfish and blue whiting as raw material.

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References

Ariyani, F., & Widyasari, H. (2000). Fortification of fish protein hydrolysates powder into snacks for children. *Journal Penelitian Perikanan Indonesia, 4*(3-4), 111-119.

Benjakul, S., Yarnpakdee, S., Senphan, T., Halldorsdottir, S. M., & Kristinsson, H. G. (2014). Fish protein hydrolysates: production, bioactivities and applications. In H. G. Kristinsson (Ed.), *Antioxidants and Functional Components in Aquatic Foods* (1st Ed., pp. 237-283). Reykjavik, Iceland: John Wiley & Sons.

Chalamaiah, M., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. *Food Chemistry, 135*(4), 3020-3038.

Chalamaiah, M., Jyothirmayi, T., Bhaskarachary, K., Vajreswari, A., Hemalatha, R., & Kumar, B. D. (2013). Chemical composition, molecular mass distribution and antioxidant capacity of rohu (Labeo rohita) roe (egg) protein hydrolysates prepared by gastrointestinal proteases. *Food Research International, 52*, 221–229.

Chalamaiah, M., Rao, G. N., Rao, D. G., & Jyothirmayi, T. (2010). Protein hydrolysates from meriga (Cirrhinus mrigala) egg and evaluation of their functional properties. *Food Chemistry, 120*(3), 652-657.

Chavira, R., Burnett, T. J., & Hageman, J. H. (1984). Assaying Proteinases with Azocoll. *Analytical Biochemistry, 136*, 446-450.

Chuaychan, S., Benjakul, S., & Sae-Leaw, T. (2017). Gelatin hydrolysate powder from the scales of spotted golden goatfish: Effect of drying conditions and juice fortification. *Drying Technology*, 1-9.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science & Technology, 11*(7), 254-262.

Cudennec, B., Fouchereau-Peron, M., Ferry, F., Duclos, E., & Ravallec, R. (2012). In vitro and in vivo evidence for a satiating effect of fish protein hydrolysate obtained from blue whiting (Micromesistius poutassou) muscle. *Journal of Functional Foods, 4*(1), 271-277.

Damodaran, S. (2008). *Amino acids, peptides, and proteins* (Vol. 4). Boca Raton, FL: CRC Press.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R. P. (2017). Boarfish (Capros aper): review of a new capture fishery and its valorization potential. *ICES Journal of Marine Science*, *74*(8), 2059-2068.

Galaz, G. A. (2013). Chapter 20. An overview on the history of sports nutrition beverages. In D. Bagchi, S. Nair & C. K. Sen (Eds.), *Nutrition and enhanced sports performance: muscle building, endurance, and strength* (pp. 205-210). San Diego, California: Academic Press.

Gbogouri, G. A., Linder, M., Fanni, J., & Parmentier, M. (2004). Influence of Hydrolysis Degree on the Functional Properties of Salmon Byproducts Hydrolysates. *Journal of Food Science, 69*(8), 615-622.

Geirsdottir, M., Sigurgisladottir, S., Hamaguchi, P. Y., Thorkelsson, G., Johannsson, R., Kristinsson, H. G., & Kristjansson, M. M. (2011). Enzymatic hydrolysis of blue whiting (Micromesistius poutassou); functional and bioactive properties. *Journal of Food Science, 76*(1), C14-20.

Ghelichi, S., Sørensen, A.-D. M., García-Moreno, P. J., Hajfathalian, M., & Jacobsen, C. (2017). Physical and oxidative stability of fish oil-in-water emulsions fortified with enzymatic hydrolysates from common carp (Cyprinus carpio) roe. *Food Chemistry*, *237*, 1048-1057.

Grimble, G. K., & Silk, D. B. A. (1986). The Optimum Form of Dietary Nitrogen in Gastrointestinal Disease: Proteins, Peptides or Amino Acids? In K. Miehlke (Ed.), *Verhandlungen der Deutschen Gesellschaft für Innere Medizin: Kongreß Gehalten zu Wiesbaden vom 6.* (pp. 674-685). Munich: J.F. Bergmann-Verlag.

Gringer, N., Osman, A., Nielsen, H. H., Undeland, I., & Baron, C. P. (2014). Chemical Characterization, Antioxidant and Enzymatic Activity of Brines from Scandinavian Marinated Herring Products. *Journal of Food Processing Technology 5*(346).

Halim, N. R. A., Yusof, H. M., & Sarbon, N. M. (2016). Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology, 51*, 24-33.

Hogan, S., & O'Callaghan, D. (2013). Moisture sorption and stickiness behaviour of hydrolysed whey protein/lactose powders. *Dairy Science & Technology, 93*(4-5), 505-521.

Imm, J., & Lee, C. (1999). Production of seafood flavor from red hake (Urophycis chuss) by enzymatic hydrolysis. *Journal of Agriculture and Food Chemistry, 47*(6), 2360-2366.

ACCEPTED MANUSCRIPT

Intarasirisawat, R., Benjakul, S., Visessanguan, W., & Wu, J. (2014). Effects of skipjack roe protein hydrolysate on properties and oxidative stability of fish emulsion sausage. *LWT - Food Science and Technology, 58*(1), 280-286.

Jemil, I., Jridi, M., Nasri, R., Ktari, N., Ben Slama-Ben Salem, R., Mehiri, M., Hajji, M., Nasri, M. (2014). Functional, antioxidant and antibacterial properties of protein hydrolysates prepared from fish meat fermented by *Bacillus subtilis* A26. *Process Biochemistry, 49*(6), 963-972.

Jónsdóttir, R., Geirsdóttir, M., Hamaguchi, P. Y., Jamnik, P., Kristinsson, H. G., & Undeland, I. (2016). The ability of in vitro antioxidant assays to predict the efficiency of a cod protein hydrolysate and brown seaweed extract to prevent oxidation in marine food model systems. *Journal of the Science of Food and Agriculture, 96*(6), 2125-2135.

Karnjanapratum, S., & Benjakul, S. (2017). Antioxidative and sensory properties of instant coffee fortified with galactose-fish skin gelatin hydrolysate maillard reaction products. *Carpathian Journal of Food Science & Technology, 9*(1).

Kimball, S. R., & Jefferson, L. S. (2006). Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. *The Journal of Nutrition, 136*(1), 227S-231S.

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (Selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry, 102*(4), 1317-1327.

Kristinsson, H. G., & Rasco, B. A. (2000a). Fish protein hydrolysates: production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition, 40*(1), 43-81.

Kristinsson, H. G., & Rasco, B. A. (2000b). Biochemical and Functional Properties of Atlantic Salmon (Salmo salar) Muscle Proteins Hydrolyzed with Various Alkaline Proteases. *Journal of Agriculture and Food Chemistry, 48*, 657-666.

Kristinsson, H. G., & Rasco, B. A. (2000c). Kinetics of the hydrolysis of Atlantic salmon (Salmo salar) muscle proteins by alkaline proteases and a visceral serine protease mixture. *Process Biochemistry, 36*, 131-139.

Lee, S.-H., Qian, Z.-J., & Kim, S.-K. (2010). A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry, 118*(1), 96-102.

Lindsay, R. C. (2007). Flavors. In S. Damodaran, K. L. Parkin & O. R. Fennema (Eds.), *Fennema's Food Chemistry* (4th Ed.). Boca Raton, FL: CRC press.

Maehashi, K., Matsuzaki, M., Yamamoto, Y., & Udaka, S. (1999). Isolation of peptides from an enzymatic hydrolysate of food proteins and characterization of their taste properties. *Bioscience, Biotechnology, and Biochemistry, 63*(3), 555-559.

McDermott, A., Visentin, G., De Marchi, M., Berry, D. P., Fenelon, M. A., O'Connor, P. M., Kenny, O. A., McParland, S. (2016). Prediction of individual milk proteins including free amino acids in bovine milk using mid-infrared spectroscopy and their correlations with milk processing characteristics. *Journal of Dairy Science, 99*(4), 3171-3182.

Mendis, E., Rajapakse, N., & Kim, S. K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of Agriculture and Food Chemistry, 53*(3), 581-587.

Mohamed, G., Sulieman, A., Soliman, N., & Bassiuny, S. (2014). Fortification of biscuits with fish protein concentrate. *World Journal of Dairy & Food Sciences, 9*(2), 242-249.

Nilsang, S., Lertsiri, S., Suphantharika, M., & Assavanig, A. (2005). Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. *Journal of Food Engineering, 70*(4), 571-578.

Phanturat, P., Benjakul, S., Visessanguan, W., & Roytrakul, S. (2010). Use of pyloric caeca extract from bigeye snapper (Priacanthus macracanthus) for the production of gelatin hydrolysate with antioxidative activity. *LWT-Food Science and Technology, 43*(1), 86-97.

Picot, L., Bordenave, S., Didelot, S., Fruitier-Arnaudin, I., Sannier, F., Thorkelsson, G., Bergéc, J.P., Guérard, F., Chabeaud, A. Piot, J. M. (2006). Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. *Process Biochemistry, 41*(5), 1217-1222.

ACCEPTED MANUSCRIPT

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnical aspects of microbial proteases. *Microbiology and Molecular Biology Reviews, 62*(3), 597-635.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: meat and fish. *Nutrients, 3*(9), 765-791.

Shen, Q., Guo, R., Dai, Z., & Zhang, Y. (2012). Investigation of enzymatic hydrolysis conditions on the properties of protein hydrolysate from fish muscle (Collichthys niveatus) and evaluation of its functional properties. *Journal of Agricultural and Food Chemistry, 60*(20), 5192-5198.

Sila, A., Sayari, N., Balti, R., Martinez-Alvarez, O., Nedjar-Arroume, N., Moncef, N., & Bougatef, A. (2014). Biochemical and antioxidant properties of peptidic fraction of carotenoproteins generated from shrimp by-products by enzymatic hydrolysis. *Food Chemistry, 148*, 445-452.

Smedes, F. (1999). Determination of total lipid using non-chlorinated solvents. *Analyst, 124*(11), 1711-1718.

Song, R., Wei, R., Zhang, B., & Wang, D. (2011). Optimization of the Antibacterial Activity of Half-Fin Anchovy (Setipinna taty) Hydrolysates. *Food and Bioprocess Technology, 5*(5), 1979-1989.

Spellman, D., McEvoy, E., O'Cuinn, G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal, 13*(6), 447-453.

Tanuja, S., Viji, P., Zynudheen, A., & Joshy, C. (2012). Composition, functional properties and antioxidative activity of hydrolysates prepared from the frame meat of Striped catfish (Pangasianodon hypophthalmus). *Egyptian Journal of Biology, 14*(1), 27-35.

Thiansilakul, Y., Benjakul, S., & Shahidi, F. (2007). Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (Decapterus maruadsi). *Food Chemistry, 103*(4), 1385-1394.

Thorkelsson, G., & Kristinsson, H. G. (2009). Bioactive peptides from marine sources. *State of art. Report to the NORA fund* (pp. 1-19): Matis.

Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chemistry, 116*(1), 240-248.

Wu, H.-C., Chen, H.-M., & Shiau, C.-Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austriasicus). *Food Research International, 36*(9), 949-957.

Yang, S., Liu, X., Zhang, M., Lin, S., & Chen, F. (2017). Moisture-Absorption and Water Dynamics in the Powder of Egg Albumen Peptide, Met-Pro-Asp-Ala-His-Leu. *Journal of Food Science, 82*(1), 53-60.

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Figure Captions

Figure 1. a) Degree of hydrolysis of blue whiting with different commercial proteases (A = Alcalase 2.4L, P = Protamex, S = Savinase 16L, N = Neutrase 0.8L and F = Flavourzyme 500L at optimum pH and temperature and, b) proximate composition of whole blue whiting (BW) and the five BWSPH (A = Alcalase, P = Protamex, S = Savinase, N = Neutrase and F = Flavourzyme).

Figure 2. Protein molecular weight distribution profiles of blue whiting prior to enzymatic hydrolysis (blue whiting) and the four BWSPH (A = Alcalase, P = Protamex, S = Savinase, F = Flavourzyme).

Figure 3. Nitrogen solubility (%) of the four BWSPH in; a) water at different pH levels (2, 4, 6, 8, 10) and, b) a vitamin-tea beverage.

Figure 4. Antioxidant effect of the four BWSPH (Alcalase, Protamex, Savinase and Flavourzyme) assessed using three anti-oxidant assays; a) DPPH free radical scavenging, b) reducing power, and c) ferrous chelating.

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Tables

Table 1. Total and free amino acids (g/100g protein) of the four BWSPH (A = Alcalase, P = Protamex, S = Savinase, F = Flavourzyme) and recommended amino acid requirements of adults (WHO, 2002). Branched chain amino acids are highlighted in bold font. WHO = World Health Organisation.

	A	P	S	F	A	P	S	F	WHO
	Free amino acids				Total amino acids				
His	2.86	2.89	4.30	1.48	3.76	3.93	5.14	1.65	1.5
Ile	**0.03**	**0.07**	**0.11**	**2.44**	**4.41**	**3.80**	**4.39**	**2.94**	**3.0**
Leu	**0.10**	**0.14**	**0.13**	**3.70**	**7.59**	**6.73**	**7.51**	**4.48**	**5.9**
Lys	0.00	0.14	0.33	0.22	8.68	8.18	8.57	5.13	4.5
Met	0.22	0.18	0.31	1.98	3.33	2.91	3.41	1.79	1.6
Cys	0.22	0.21	0.19	0.15	1.29	1.10	1.38	0.78	0.6
Met + cys	0.44	0.40	0.50	2.13	4.62	4.01	4.79	2.57	2.2
Phe	1.16	1.11	3.97	2.30	4.06	3.47	4.20	2.24	
Phe + tyr	1.98	2.55	5.42	3.66	5.65	4.42	4.31	3.90	3.0
Thr	0.11	0.13	0.10	3.31	4.41	4.02	4.57	2.75	2.3
Trp	0	0	0	0.91	N/A	N/A	N/A	N/A	0.6
Val	**0.38**	**0.36**	**0.38**	**2.81**	**5.18**	**4.57**	**5.29**	**3.39**	**3.9**
Ala	0.26	0.29	0.29	3.17	6.92	6.50	6.53	4.21	
Arg	0.16	0.19	0.16	2.55	6.44	6.23	6.57	4.07	
Asp	0.14	0.19	0.13	2.13	10.79	10.14	10.47	6.39	
Glu	0.35	0.33	0.27	3.41	16.34	15.61	15.31	9.59	
Gly	0.20	0.17	0.18	1.83	6.56	6.94	6.55	4.36	
Pro	0.10	0.13	0.10	0.92	4.34	4.28	4.25	2.81	
Tau	0.95	1.00	0.96	0.76	0.85	0.85	0.70	0.45	
Tyr	0.82	1.45	1.45	1.36	1.59	0.95	0.11	1.66	
Ser	0.15	0.17	0.16	2.40	4.82	4.52	4.62	2.99	

Table 2. Emulsifying activity index (EAI, m^2g^{-1}) and emulsion stability index (ESI, min) at different w/v (0.5%, 1%, 2%), and oil binding capacity (OBC, g/g) of the BWSPH.

	EAI (m^2g^{-1})			ESI (min)			OBC (g/g)
BWSPH conc.	0.5%	1%	2%	0.5%	1%	2%	
Alcalase	37.81 ±0.68	17.41 ±0.81	18.44 ±1.69	1.95 ±0.76	3.57 ±0.51	4.83 ±0.73	1.45 ± 0.01
Protamex	57.73 ±0.35	21.37 ±2.13	18.86 ± 0.71	2.97 ±0.56	7.82 ±0.17	7.57 ±0.47	1.44 ± 0.03
Savinase	58.55 ±0.70	34.73 ±1.09	24.29 ±2.43	3.51 ±0.43	5.42 ±0.15	4.89 ±0.23	1.56 ±0.01
Flavourzyme	70.25 ± 1.76	22.69 ±0.52	19.53 ±0.56	5.65 ±0.32	5.10 ±0.82	7.01 ±0.11	1.36 ±0.01

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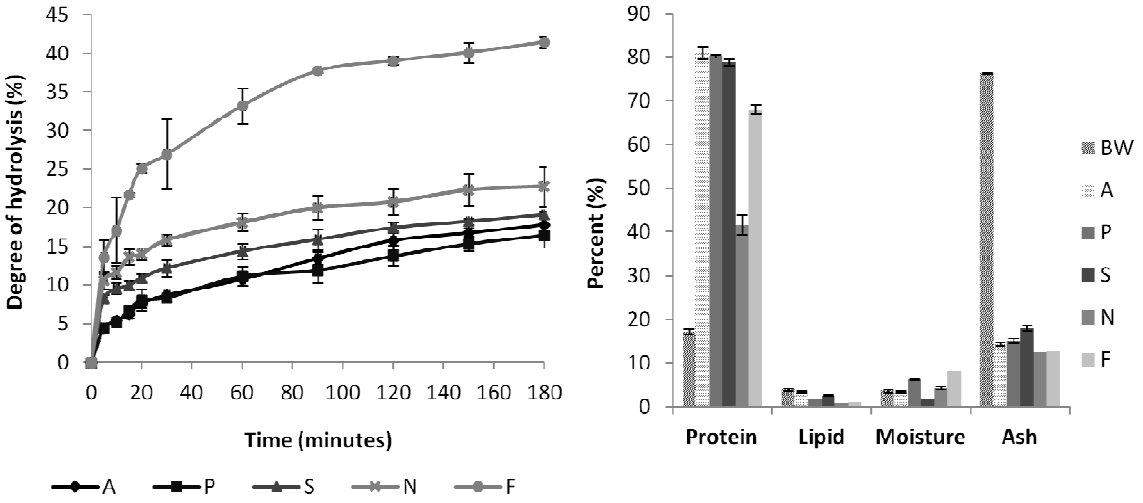
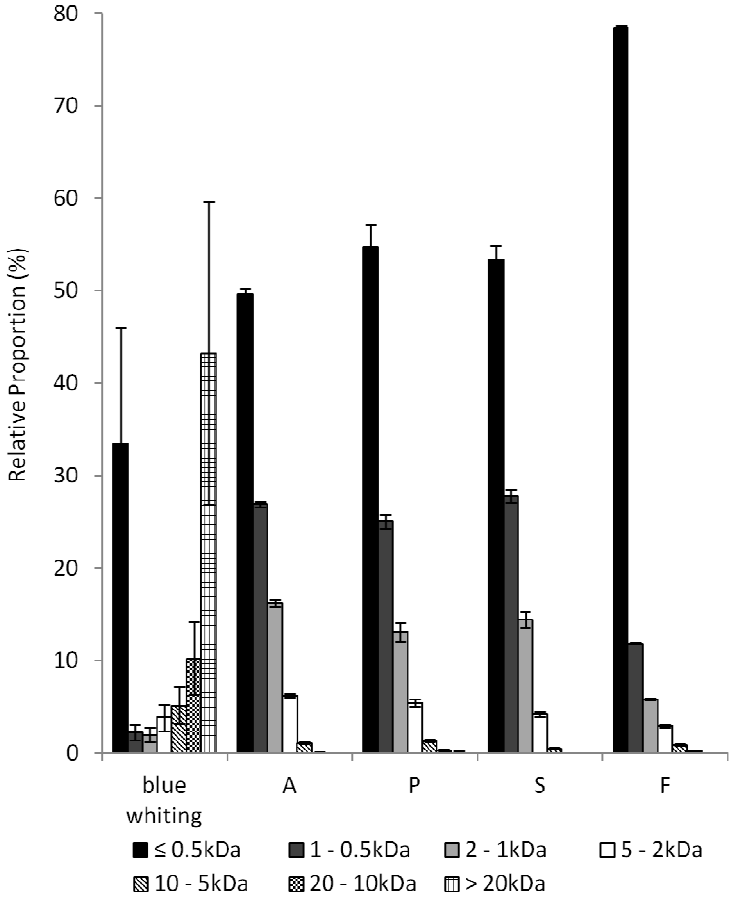


Figure 1. Degree of hydrolysis and proximate composition

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Figure 2. Molecular weight distribution profile



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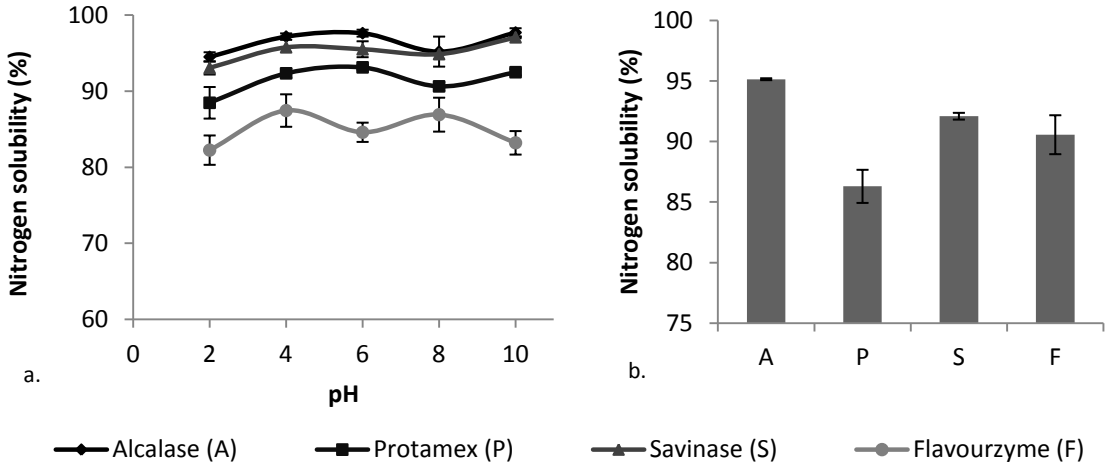
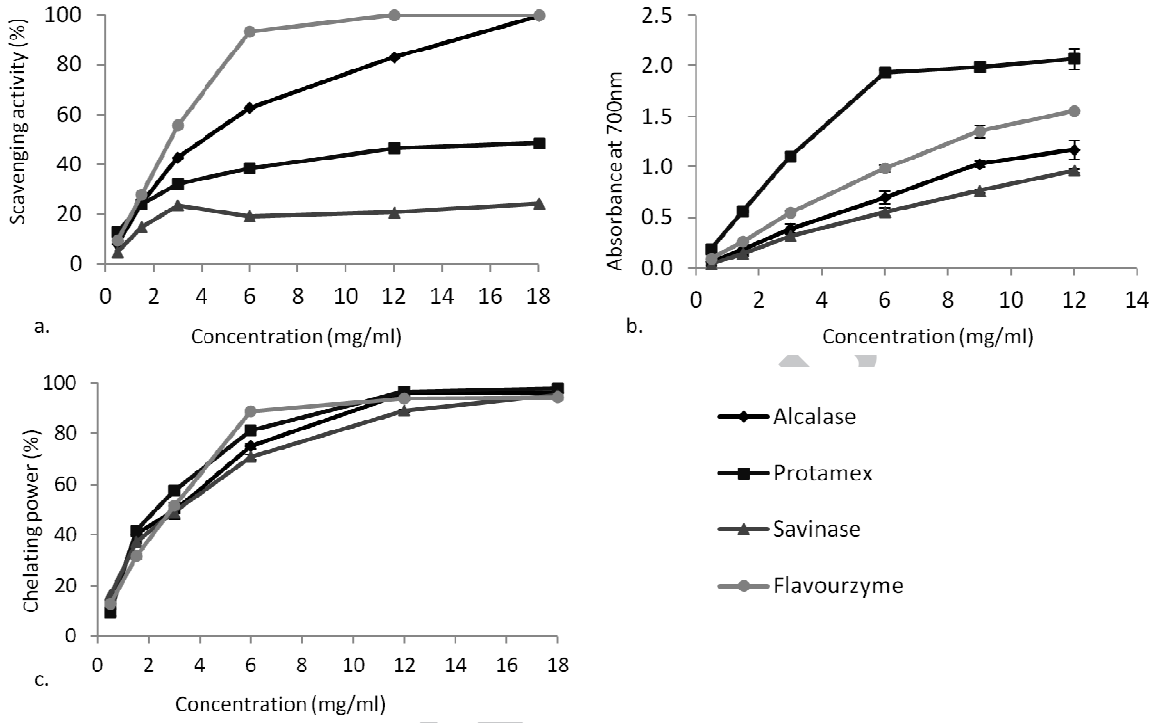


Figure 3. Nitrogen solubility

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Figure 4. Anitoxidant assays



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Highlights

- The degree of hydrolysis of the powders produced ranged from 16.43% to 41.47%.
- Small proteins (< 1 kDa) increased from 35.68% up to 90.18% of protein in the powders.
- Flavourzyme 500L and Protamex produced powders with the most antioxidant properties.
- The powder made with Flavouzyme 500L had an amino acid profile close to that recommended by the WHO.

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