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Functional protein rich extracts from bovine and porcine hearts using acid or alkali solubilisation and isoelectric precipitation.

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Running title: Acid/alkaline bovine and porcine extracts

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Abstract

Alkali solubilisation (ALS) was compared with acid solubilisation (ACS) for preparation of protein rich extracts from bovine and porcine hearts. ACS and ALS recovered 51.53 - 55.74% of the total protein from bovine and porcine hearts. All extracts were rich in myofibrillar proteins with both treatments resulting in reductions in fat, collagen and cholesterol contents compared with starting materials. At 0% NaCl, ACS and ALS extracts had good gelling properties with the ALS gels having lower % cook loss. While treatments did not affect gel hardness, ACS extracts formed gel networks with higher storage modulus after heating and cooling. At 2% NaCl gel hardness, % cook loss and storage modulus values increased, with greater increases occurring for ACS extracts. The results show that ALS and ACS based processes have potential to produce functional ingredients for processed meat products.

Introduction

Slaughterhouses generate a significant volume of meat by-products, many of which have low economic value. The increasing importance of sustainability along with financial pressures has led to increasing interest in adding or recovering value from all processing streams in the meat industry (Toldra *et al*., 2016, Lynch *et al*., 2018). During recent decades, quantities of meat by-products produced from slaughterhouses and meat processors, have significantly increased, while their consumption has gradually declined. Meat by-products are perceived as unattractive due to their unaesthetic appearance and to consumer concerns about food related health risks (Lynch *et al*. 2018, Mullen *et al*. 2017).

However, many meat by-products are rich in proteins and if processed correctly can be used for the production of extracts with desirable functional properties (Chernukha *et al*., 2015, Lynch *et al*. 2018). It is well known that myofibrillar proteins are mainly responsible for the gelling properties of meat products (Chen *et al*. 2015). The production of protein rich extracts from meat by-products represents a good valorization opportunity for the meat industry, and at the same time can improve consumer acceptance.

Various processes have been used to prepare protein rich extracts from offal meat by-products. These mainly include surimi type processes and pH shift technology (James and Mireles DeWitt, 2004). pH shift technology involves acid (ACS) or alkaline (ALS) solubilisation of proteins, with subsequent precipitation of proteins at their isoelectric point (Omana *et al*., 2012).

In previous studies, researchers have extracted proteins from a variety of meat related raw materials, including spent hen and duck meat, mechanically separated turkey and chicken meat, pork and beef lungs, using either ACS or ALS (Khiari *et al*., 2014, Wang *et al*. 2013, Nurkhoeriyati *et al*. 2011, Hrynets *et al*. 2010, Selmane *et al*. 2010).

In addition, Mireles DeWitt *et al*. (2002) used an ACS process to produce protein rich extracts from bovine heart which had excellent heat induced gelation properties. Use of ALS

processes has been focused on poultry by-products and little information is available on the use of this type of pH shift technology in red meat by-products. Therefore, this study will investigate the potential of ALS as a process to prepare protein rich extracts from bovine and porcine hearts. Yield and functional properties of ALS extracts will be compared with ACS to determine if the former offers some advantages.

Materials and methods

Preparation of bovine and porcine hearts

Fresh bovine (from 18-20 months old mixed cross breed steers) and porcine (from 89-108 kg Landrace pigs) hearts were obtained from local abbatoirs 24 hours *post mortem*. Hearts were trimmed to remove valves, caps and adipose tissue, cut into cubes and minced through a 5 mm plate. Minced hearts were vacuum packed in polyethylene bags and kept at -20 °C until use.

Protein extractability

Protein extractability of bovine and porcine hearts at different pH values was determined using the method of Mireles DeWitt *et al*. (2002) with minor modifications. Minced bovine and porcine hearts (100 g) were thawed overnight at 4 °C. Minced heart was mixed with dH_2O (at 4 °C) using a 1:4 w/w ratio and homogenized at high speed, using a Waring blender for 30 s × 4. In between each homogenization the slurry was placed into an ice bath for 10 min. The slurry was further diluted to a final minced heart: water ratio of 1:9 (w/w) by addition of dH_2O (at 4 °C). pH was adjusted from 2.0 to 11.0, in 0.5 pH increments, with 2N HCl or 2N NaOH. At each pH point, aliquots were taken and centrifuged at 4000 × g for 20 min at 4°C, for determination of soluble protein.

Preparation of protein rich extracts using ACS or ALS

Proteins were extracted at pH 2.0 or 11.0 using the procedure described above. The acidified (pH 2.0) or alkali treated (pH 11.0) slurry was centrifuged at 4000 × g for 20 min at 4 °C, and

99 the supernatant was poured through a layer of cheese-cloth prior to isoelectric precipitation of
100 proteins by adjusting the pH to 5.5. Precipitated protein was collected by centrifuging at 4000
101 × g for 20 min at 4 °C and excess water in the pellet was removed by centrifuging at 10000 ×
102 g for 15 min at 4 °C. The pH was adjusted to 7.0 with 5% $NaHCO_3$ before protein
103 determination. Protein yield was calculated as follows:

104 % yield = [(weight of protein in supernatant after 1st centrifugation – weight of protein in
105 supernatant after 2nd centrifugation) / (initial weight protein in homogenate)] × 100

106 Proximate composition analysis

107 Protein (N × 6.25), moisture, fat and ash content was determined using the AOAC methods
108 (AOAC 2012). Cholesterol in hearts and protein extracts was determined using an enzymatic
109 colorimetric method (Boehringer Mannheim / R-Biopharm). Collagen in heart and protein
110 extracts was determined by a colorimetric method for hydroxyproline in meat (Kolar, 1990).

111 Color properties of protein isolates

112 Objective color values (L^*, α^*, b^*) were obtained with a standardized Minolta colorimeter
113 (CR-400, Tokyo, Japan). A white standard plate was used to calibrate the colorimeter. The L^*
114 indicated degree of lightness, α^* indicated redness and b^* indicated degree of yellowness.

115 SDS-PAGE

116 Sample preparation was conducted as described from Mireles DeWitt *et al*. (2002). Protein
117 extracts were 1:10 (w/v) diluted with 5% SDS and homogenised with an Ultra Turrax at high
118 speed. Samples were placed in a water bath at 80 °C for 60 min. Insoluble material were
119 removed by centrifugation at 8000 × g for 20 min at 4 °C and protein in the supernatant was
120 determined with the biuret method. SDS diluted samples were added to sample buffer [0.25M
121 Tris-HCl at pH 6.8, 10% glycerol, 7.12 mM 2-mercaptoethanol, 2.5% bromophenol blue
122 (0.05% w/v)] to a final protein concentration of 3 mg/ml. 5 µl of samples was loaded on a 4–
123 20% ready gel (Bio-Rad Laboratories Inc.). The electrophoretic analysis was performed on a

PowerPack Basic electrophoresis apparatus (Bio-Rad Laboratories Inc.) at constant 160 V. The gel was stained in 0.125% Coomasie blue R-250 and destained in 50:40:10 of water:methanol:acetic acid solution. A high-molecular weight standard was used for the estimation of apparent molecular weight retention of protein bands (Bio-Rad Laboratories Inc.).

Heat induced gelation of protein extracts

Protein content of freshly made protein extracts was standardised at 8% by adding dH_2O or NaCl solution to final salt concentration of 2% (w/w). The protein pastes were allowed to equilibrate overnight at 4 °C, stuffed into gelation tubes (diameter: 1.8 cm, length: 7.9 cm), which were coated with a siliconising agent (Sigmacote, Sigma-Aldrich). Before cooking stuffed tubes were centrifuged at 1500 × g, at 4 °C for 30 s, to eliminate any air bubbles. Then they were heated to 80 °C, at a rate of 1 °C/min, and held at 80 °C for 5 min before cooling in an ice bath for 60 min. All samples were stored at 4 °C overnight prior to analysis.

Gel hardness

The gelled samples were allowed to equilibrate at room temperature for one hour. Gel hardness was evaluated using a texture analyser (TA-XT2i, Stable Micro Systems Ltd.). Gelled samples were cut to a height of 10 mm and then compressed at 30% of their original height, with a constant crosshead speed of 1 mm/s by a 2.5 cm acrylic cylinder. Gel hardness was determined by the peak force during the compression.

Cooking loss

Cooking loss was determined by weighing the samples before and after cooking following removal of free water and it was expressed as follows:

Cook loss % = [(weight before cooking – weight after cooking) / weight before cooking] × 100

148 Dynamic rheological measurements

149 Changes in the storage modulus (G') of the protein extracts (8% (w/w) protein with or
150 without added NaCl) during heating and cooling were monitored using a control stress

151 rheometer (HR-2, TA instruments, USA) in oscillatory mode using a hatched plate-plate
152 geometry of 2.5 cm diameter. Small amplitude deformation (0.5%) strain was applied at an
153 oscillation frequency of 1 Hz and a 2000 μm gap was used. Samples were heated from 4 °C to
154 80 °C at a rate of 1 °C/min and then cooled down from 80 °C to 4 °C at the same rate.

155 Statistical analysis

156 Analysis of variance (ANOVA) was carried out using Minitab® 18 (Minitab Ltd, Coventry,
157 UK) statistical analysis package. The Tukey method was used to obtain grouping information
158 on the treatment means. Significances in differences were defined at $p < 0.05$. Each
159 experiment was repeated at least three times.

160 Results and discussion

161 Proximate composition of raw bovine and porcine hearts

162 As can be seen in Table 1, there was no significant differences ($p > 0.05$) between bovine and
163 porcine hearts in terms of % moisture, % fat, % ash and collagen content. Bovine hearts had
164 significantly ($p < 0.05$) more protein and less cholesterol than porcine hearts.

165 Protein extractability

166 Bovine and porcine hearts proteins exhibited similar extractability behavior over the pH range
167 of pH 2.0 to 11.0 (Figure 1), with low protein extractability at pH 5 to 8, and highest protein
168 extractability at pH 2.0 and 11.0. This is in agreement with Mireles DeWitt *et al*. (2002), who
169 reported that protein extractability from bovine hearts was highest at pH 2.0 and lowest at pH
170 5.0 to 6.0 in the pH range of 2.0 to 6.0. Nurkhoeriyati *et al*. (2011) investigated the effect of
171 pH on protein extractability of spent duck meat and found a highest extractability at pH 2.0
172 and 11.0, as observed in this study but the pH range for minimum solubility was much

narrower, with minimum solubility occurring at pH 5.0 to 6.0. These differences may be due to species and tissue differences, i.e. skeletal muscle vs cardiac muscle.

Based on the protein extractability profile (Figure 1) it was decided to carry out acid and alkali solubilisation at pH 2.0 and 11.0, respectively, followed by isoelectric precipitation at pH 5.5.

Protein recovery and composition of extracts

Proximate composition, yield and color characteristics of protein rich extracts from bovine and porcine hearts are shown in Table 2. For both bovine and porcine hearts there was no significant difference ($p > 0.05$) in % protein recovered between the ACS and ALS process. This is in contrast with the results of Nurkhoeriyati *et al*. (2011), who reported that an ACS process recovered more protein from spent duck meat than an ALS process. These authors speculated that the ACS process induced more changes to protein structure resulting in great protein insolubility on adjustment of the pH at the isoelectric point. These differences may reflect differences in the starting material used.

Extracts prepared from bovine hearts had significantly higher protein content ($p < 0.05$) than those prepared from porcine heart and within a species there was no significant difference ($p > 0.05$) in % protein of extracts between the ACS and ALS process. All of the extracts produced had significantly ($p < 0.05$) lower lipid, cholesterol and collagen content than the starting materials. The initial centrifugation step following acid and alkaline solubilisation sediments membrane lipids and also removes insoluble collagen (Mireles DeWitt *et al*. 2002).

There were no significant differences ($p > 0.05$) in L^* and b^* values for any of the extracts. The a^* values indicate that irrespective of species ALS extracts were redder than ACS extracts. Using spent duck meat as the starting material Nurkhoeriyati *et al*. (2011) also reported that an ALS extracts showed greater retention of myoglobin than ACS extracts which was attributed to greater co-precipitation of myoglobin during isoelectric precipitation.

198 The ALS extracts were visually redder than ACS extracts which may render them more
199 suitable for use as an ingredient in a sausage type product.

200 SDS PAGE

201 SDS electrophoresis indicates that irrespective of the solubilisation process or species the
202 protein profile is dominated by the major myofibrillar proteins myosin and actin (Figure 2).
203 Similar protein profiles were obtained by Mireles DeWitt *et al.* (2002) for acid solubilised
204 proteins from bovine hearts. The major differences observed in protein profile between ACS
205 and ALS process the higher intensity of the protein band of 100 kDa in the ALS extracts and
206 the presence of a protein band with approximate molecular weight 70 kDa in the ACS
207 extracts. Further studies will be required to determine the identity of these bands.

208 Heat induced gel hardness and cook loss

209 Heat induced gels were prepared from the extracts after standardising the protein content at
210 8% (Table 3). Addition of NaCl is a key requirement for good gelling properties for
211 myofibrillar protein rich extracts prepared from bovine hearts using a surimi like processes
212 (James and Mireles DeWitt, 2004). In this study at 0% added NaCl all extracts formed self-
213 supporting gels on heating. There was no significant difference ($p > 0.05$) in hardness values
214 of the gels between process or species. This suggests that ACS and ALS treatment result in
215 modification of the protein structure which facilitates gel formation in the absence of added
216 NaCl. The results indicate that these extracts have potential applications as gelling ingredients
217 in low salt comminuted meat products. When 2% NaCl was added to the extracts gel hardness
218 increased with a greater increase occurring for the ACS extracts.

219 ALS extracts had significantly ($p < 0.05$) lower % cook loss than ACS extracts (Table 3),
220 with and without the addition of NaCl, which highlights the potential of ALS extracts for use
221 as a water binding ingredient in processed meat products. Addition of 2% NaCl increased the
222 % cook loss for ACS and ALS extracts irrespective of species. This suggests that the presence

of NaCl may promote protein-protein interactions, as seen in the increased gel hardness values, but had an adverse effect on protein-water interactions.

Dynamic rheological measurements

The storage modulus of ACS and ALS extracts (protein concentration was standardized at 8%) was determined upon heating and cooling. At 0% added NaCl a sigmoidal pattern of storage modulus during heating was observed for both ACS and ALS treated samples (Figure 3a and 3b). Storage modulus was stable up to 50 °C, where it started decreasing until it reached a minimum at 56 °C, then it rose steadily up to 80 °C. This pattern implies the denaturation of myosin molecules, their subsequent aggregation and network formation (Sun and Holley, 2011). Upon the addition of 2% NaCl storage modulus showed a small decline up to 60 °C where it started increasing. During cooling storage modulus increased steadily until the end of the cycle due to the formation of hydrogen bonds (Hrynets *et al*., 2010), resulting in a firm gel structure (Ingadottir and Kristinsson, 2010) for all the samples.

Storage modulus at 4 °C (after heating and cooling) showed significant variations between treatments for both species (Table 4). Regardless NaCl addition, ACS treated samples produced stronger gels than ALS treated, which is directly related to the extent of protein cross-linking. ACS treated proteins is believed to have more exposed hydrophobic groups on their surface than ALS treated (Nolsøe and Undeland, 2009) and therefore protein-protein interactions and cross-linking are promoted. In all samples, addition of 2% NaCl had a positive effect on storage modulus, with ACS treated samples to exhibit a greater increase than ALS treated. This comes to accordance with the gel hardness results of this study. Similar results were obtained by Hrynets *et al*. (2010), who reported that at 2.5% NaCl ACS treated mechanically separated turkey, after heating at 80 °C and cooling at 5°C, showed higher storage modulus value than ALS treated samples.

Conclusions

The ALS process produced protein rich extracts from porcine and bovine hearts with excellent heat gelling properties and low % fat and cholesterol content. The ALS process has some advantages over ACS, as the extracts had a redder colour which would be desirable in many sausage type products. In addition, ALS extracts had lower % cook loss which is of economic benefit. The ability of ACS and ALS extracts to form gels in the absence of added NaCl suggests that these wet protein extracts have potential as ingredients in low salt meat products.

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Tables

Table 1. Composition of trimmed and minced raw bovine and porcine hearts. Data are presented as mean (n=5) ± standard deviation. Means within same row with different superscript are significant different ($p < 0.05$).

	Bovine Heart	**Porcine Heart**
Moisture (%)	76.71 ± 0.14^{a}	76.37 ± 0.96^{a}
Protein (%)	19.81 ± 0.48^{a}	18.28 ± 0.40^{b}
Fat (%)	1.05 ± 0.38^{a}	1.54 ± 0.39^{a}
Ash(%)	1.24 ± 0.20^{a}	1.52 ± 0.25^{a}
Cholesterol (mg/100 g)	122.85 ± 3.29^{a}	130.46 ± 1.15^{b}
Collagen (mg/100 g)	2.12 ± 0.0^{a}	1.74 ± 0.28^{a}

Table 2. Yield, composition and color properties of protein rich extracts from bovine and porcine hearts using acid or alkali solubilisation followed by isoelectric precipitation. Data are presented as mean (n=3) ± standard deviation. Means within same row with different superscript are significant different ($p < 0.05$).

		Bovine Heart		**Porcine Heart**	
		pH 2.0	**pH 11.0**	**pH 2.0**	**pH 11.0**
Yield (%)		53.30 ± 4.51^{a}	51.53 ± 4.68^{a}	55.74 ± 3.48^{a}	52.01 ± 2.48^{a}
Protein (%)		$12.27 \pm 0.48^{a,b}$	14.33 ± 0.89^{a}	10.68 ± 0.76^{b}	11.31 ± 0.73^{b}
Moisture (%)		81.60 ± 2.43^{b}	81.92 ± 0.69^{b}	87.17 ± 0.75^{a}	85.82 ± 0.05^{a}
Fat (%)		0.28 ± 0.042^{b}	0.29 ± 0.04^{b}	0.36 ± 0.02^{a}	0.37 ± 0.06^{b}
Ash (%)		0.39 ± 0.039^{b}	0.65 ± 0.09^{a}	0.39 ± 0.07^{b}	$0.55 \pm 0.13^{a,b}$
Cholesterol (mg/100 g)		52.15 ± 1.6^{c}	73.98 ± 6.56^{b}	$82.06 \pm 3.95^{a,b}$	92.14 ± 10.50^{a}
Collagen (mg/100 g)		0.45 ± 0.06^{a}	0.42 ± 0.04^{a}	0.38 ± 0.03^{a}	0.20 ± 0.01^{b}
Color properties	***L****	38.99 ± 2.40^{a}	38.93 ± 1.13^{a}	34.74 ± 1.56^{a}	35.97 ± 2.77^{a}
	α*	-0.13 ± 0.09^{b}	2.75 ± 0.38^{a}	2.40 ± 0.59^{a}	2.54 ± 0.16^{a}
	b*	7.49 ± 0.76^{a}	7.25 ± 1.41^{a}	7.17 ± 0.46^{a}	7.55 ± 0.48^{a}

Table 3. Hardness (g) and cook loss (%) of protein rich extracts from bovine and porcine hearts using acid or alkaline solubilisation isoelectric precipitation, after heating (4 °C to 80 °C) and cooling (4 °C). Samples protein content was adjusted to 8 % (w/w) and 0% or 2% NaCl was added. Data are presented as mean (n=4) ± standard deviation. Means within same row with different superscript are significant different ($p < 0.05$).

	ACS Bovine		ALS Bovine		ACS Porcine		ALS Porcine	
	0% Salt	**2% Salt**	**0% Salt**	**2% Salt**	**0% Salt**	**2% Salt**	**0% Salt**	**2% Salt**
Hardness (g)	123.3±0.75^{d}	184.8±2.42^{a}	119.54±0.73^{d}	138.7±4.28^{c}	127.7±0.75^{c,d}	184.5±2.48^{a}	118.47±4.02^{d}	148.6±4.47^{b}
% Cook loss	25.9±0.18^{d}	31.17±0.27^{a}	20.8±0.4^{e}	27.4±1.98^{c,d}	29.6±0.11^{b,c}	33.6±0.19^{a}	17.8±1.1^{f}	27.17±0.58^{d}

Table 4. Storage modulus of protein rich extracts from bovine and porcine hearts, using acid or alkali solubilisation followed by isoelectric precipitation, at 4 °C after heating from 4 °C to 80 °C followed by cooling from 80 °C to 4 °C at 1 °C/min. Samples protein content was adjusted to 8 % (w/w) and 0% or 2% NaCl was added. Data are presented as mean (n=3) ± standard deviation. Means within same row with different superscript are significant different ($p < 0.05$).

	ACS Bovine	**ALS Bovine**	**ACS Porcine**	**ALS Porcine**
0% NaCl	26671.1 ± 305.5^{a}	13542.3 ± 266.6^{b}	25063.5 ± 785.6^{a}	17134.4 ± 493.0^{c}
2% NaCl	32499.9 ± 335.7^{a}	17712.9 ± 403.2^{b}	30675.3 ± 791.8^{a}	26100.2 ± 916.1^{c}

Figures legends

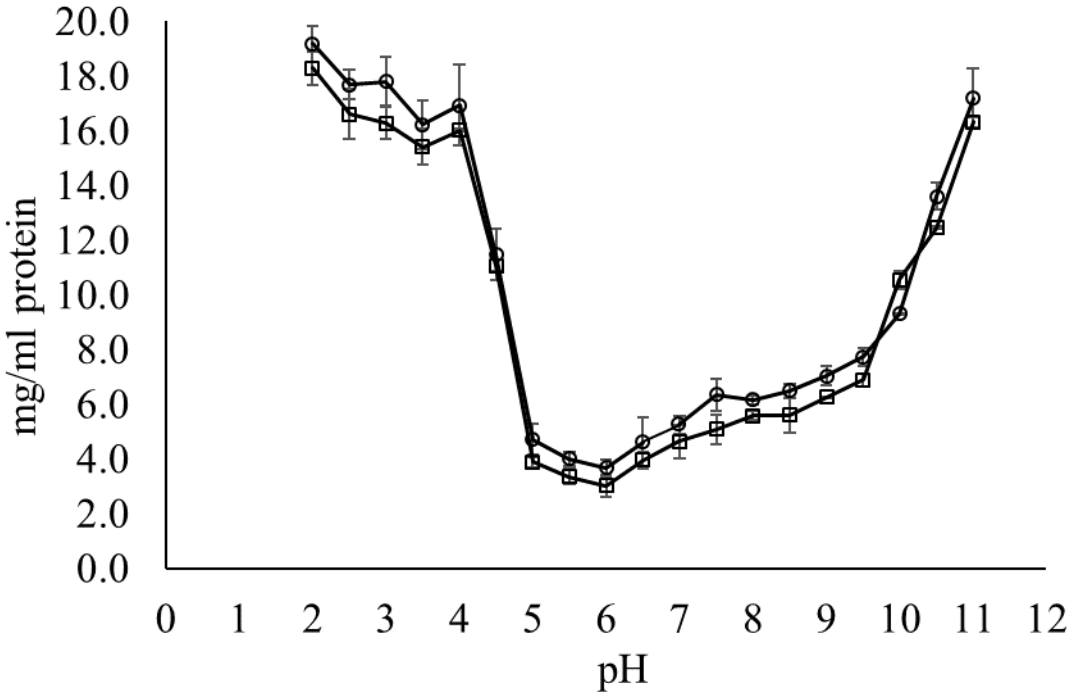
Figure 1. The extractability (mg/ml) profile of proteins recovered from bovine (○) and porcine (□) hearts at the pH range from 2.0 to 11.0.

Figure 2. SDS-PAGE profile of protein rich extracts from bovine and porcine hearts using acid or alkali solubilisation followed by isoelectric precipitation. (Lane 1: high-molecular-weight standard, lane 2: ALS extract from porcine heart, lane3: ACS extract from porcine heart, lane 4: ALS extract from bovine heart, lane 5: ACS extract from bovine heart).

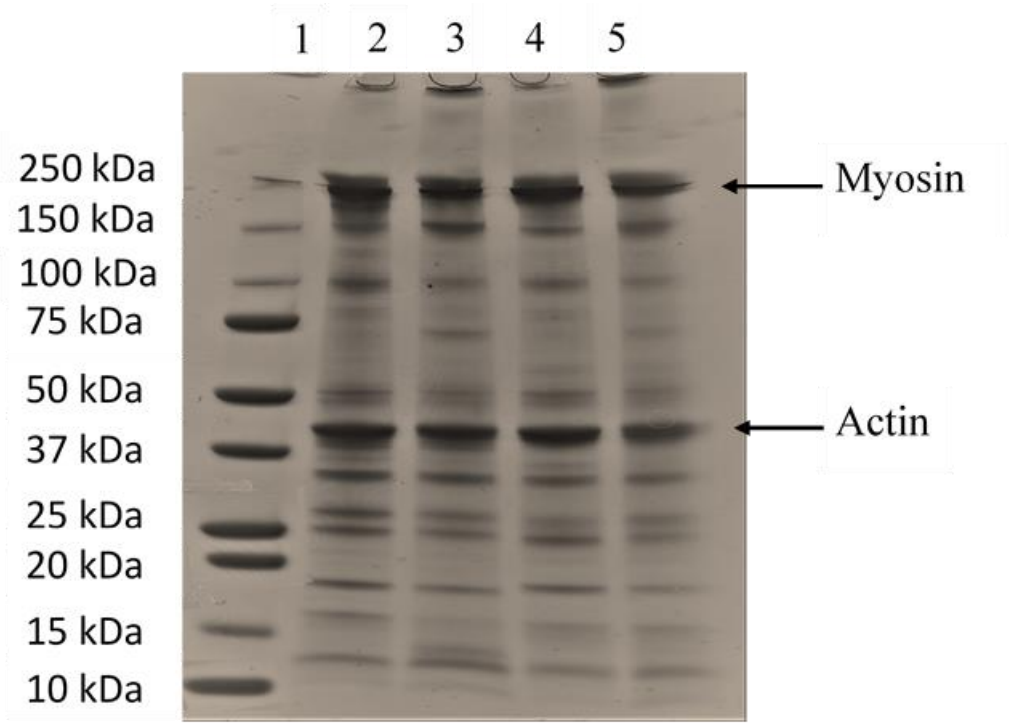
Figure 3. Rheograms of protein rich pellets from bovine (a) and porcine (b) hearts using acid or alkaline solubilisation, isoelectric precipitation with 0% or 2% NaCl addition. The rheograms show storage modulus during heating from 4 °C to 80 °C followed by cooling from 80 °C to 4 °C at 1 °C/min. Samples protein content was adjusted to 8 % (w/w) and 0% or 2% NaCl was added.

Figures

Fig. 1



391 Fig. 2



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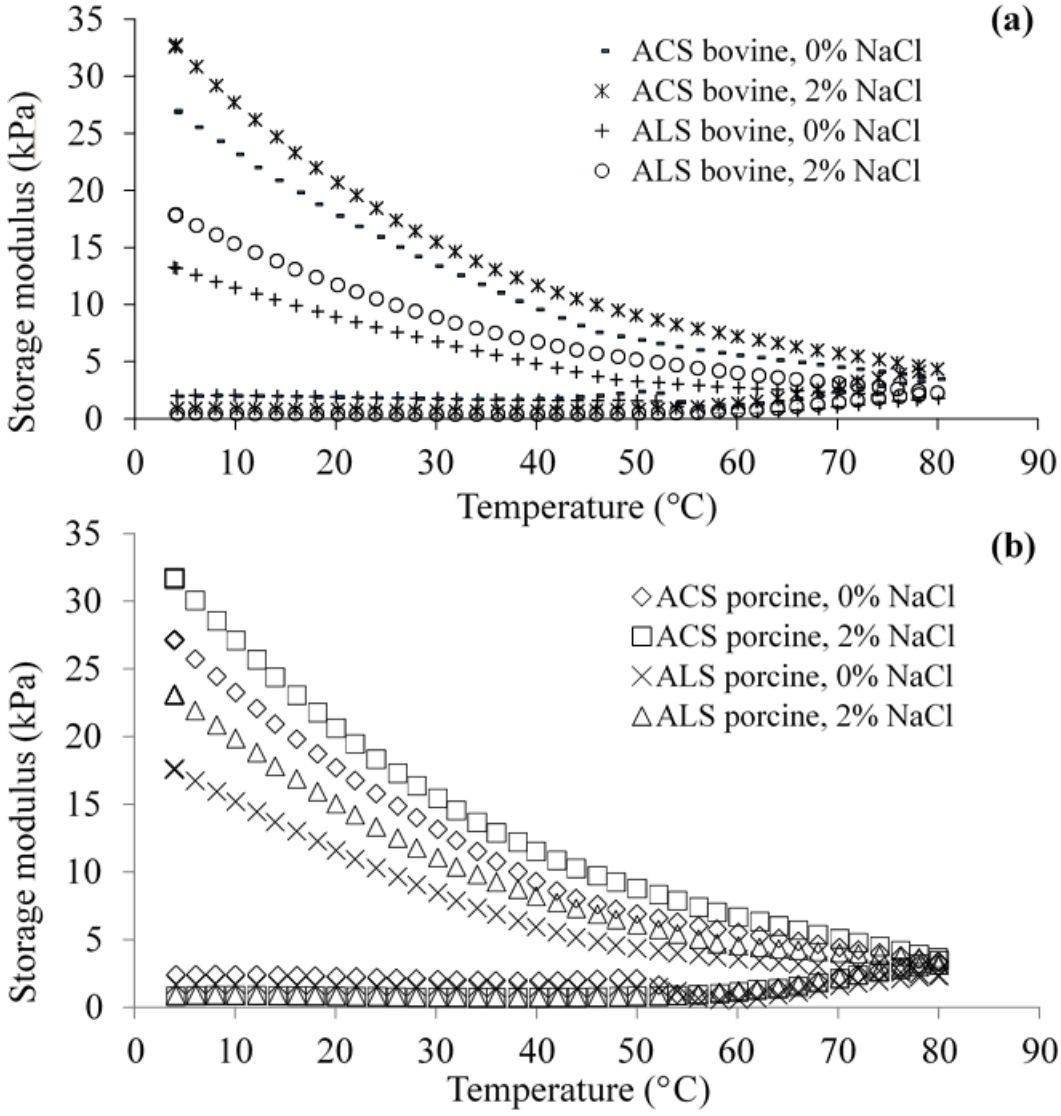
404 Fig. 3


(a)
ACS bovine, 0% NaCl
ACS bovine, 2% NaCl
ALS bovine, 0% NaCl
ALS bovine, 2% NaCl
Storage modulus (kPa)
Temperature (°C)
(b)
ACS porcine, 0% NaCl
ACS porcine, 2% NaCl
ALS porcine, 0% NaCl
ALS porcine, 2% NaCl
Storage modulus (kPa)
Temperature (°C)

405