International Food Research Journal 18(3): 1111-1115 (2011)

Utilisation of gelatine from NZ hoki *(Macruronus novaezelandiae)* fish skins

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Abstract: Response Surface Methodology (RSM) was adopted to optimize the extraction conditions of hoki (*Macruronus novaezelandiae*) gelatine and some of the physico-chemical properties were characterised. The optimum conditions obtained for fish gelatine extraction were 0.75 M concentration of NaCl for 9 min of pre-treatment time and hot water extraction at 49.3^{0}C for 60 min. The predicted yield achieved by RSM (17.4%) intently matched the experimental yield of 17.6%. Even though gel strength of hoki gelatine was significantly higher than those from other cold water fish species as reported in previous studies, it was lower (197 ± 5 g) than those from porcine (307 ± 8.4 g) or bovine (273 ± 16.1 g) gelatine. Therefore, enzymatic modification was applied to improve the properties (especially gel strength) of hoki gelatine, thereby enhance their effective use as foods and food ingredients. The addition of transglutaminase (TGase) enzyme at the concentration of 3.0 mg/g in gelatine increased the gel strength from 197 g to 279 g, which could therefore lead to the formation of products with enhanced rheological as well as sensory properties.

Keywords: Fish gelatine, *Macruronus novaezelandiae*, optimization, transglutaminase, gel strength

Introduction

Gelatine is not a naturally occurring protein, but a high molecular weight polypeptide obtained by hydrolysis of a water insoluble fibrous protein, collagen, which is the primary protein component of mammalian and fish skins, bones and connective tissues. The collagen unit structure is a triple helix of three polypeptide chains coiled around a common axis to form a rigid tropocollagen molecule. Collagen contains at least 18 of the 20 amino acids generally found in proteins, and is characterized by its high content of glycine, proline and hydroxyproline (Bailey and Light, 1989). In contrast, fish collagen has relatively lower concentration of proline and hydroxyproline. Conversion of collagen to gelatine is the most essential transformation in gelatine manufacture. It involves the denaturation of the triple helical structure by heat treatment or hydrolysis for gelatines that gel in water.

Gelatine is one of the most popular biopolymer which is having a widespread application especially in the food industry. The demand for gelatine has been increasing at a steady rate of approximately 2% per annum thus resulting in high prices for gelatine. Gelatine is mainly derived from mammalian sources especially porcine skins and bovine bones. Gelatine from these animal sources has superior gel strength, melting point and viscosity compared to marine sources (Cho *et al*., 2005). However, the rising concern of consumers about bovine spongiform encephalopathy (BSE) outbreaks in commercial mammalian gelatine products has drawn extensive interest among researches to replace gelatine from land animals to marine sources. In addition to that, there is a strong awareness among Muslim/Jews consumers on the requirements for Halal/Kosher ingredient for food product development.

Fish skins are abundant and could be a valuable source of gelatine. It also offers an alternative for those applications where ethical or religious reasons exclude the use of mammalian gelatine products. Fish skins are a major by-product of the fisheries and aquaculture industries. The amount of fish by-products contributes almost 36% of the total weight of the fish (MSC, 2009). Fish processing waste that is currently underutilized in the fishery industry and gelatine from fish skins would be a good way to add value to this waste product.

Hoki is New Zealand's most valuable commercial fish species and lives in cold deep water habitats from depths of 300 metres to over 900 metres. Hoki is a fast growing fish living up to 20- 25 years and can grow to over a metre in length. Total Allowable Catch (TAC) of hoki in New Zealand is 100,000 tonnes per annum (MSC, 2009) and total exports in the year ending December 2008 were worth NZ$ 151 million (~US$ 115 million). The large tonnage of hoki fish processed has resulted in a considerable amount of fish waste.

Fish gelatine is different from mammalian

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gelatines in their properties such as melting and gelling temperatures and gel strength. Extraction of fish gelatine has been reported for several cold water fish species such as Alaskan pollock (Chiou *et al*., 2006), cod (Gómez-Guillén *et al*., 2002; Arnesen and Gildberg, 2007) and hake (Gómez-Guillén *et al*., 2002) and they all seem to have lower melting point and gel strengths due to their lower level of proline and hydroxyproline compared to mammalian gelatine.

The properties of gelatines can be enhanced through enzymatic and chemical modification. Transglutaminase (TGase; E.C. 2.3.2.13) is an enzyme that is responsible for acyl-transfer reaction that occurs between γ-carboxyamide groups of glutamine residues as 'acyl donor' and ε-amine groups of lysine residues as 'acyl acceptor' which resulted in the formation of 3-(γ-glutaminyl) lysine cross-linking bonds in the collagen. The ability of TGase to modify the functional properties of food protein has been extensively reviewed (Motoki and Seguro, 1998; Lorenzen, 2000; Kuraishi *et al*., 2001).

The formation of ε-(γ -glutamyl) lysine isopeptide bonds results in the incorporation of inter or intra-molecular covalent cross-links into food proteins, leading to improving the physical and textural properties of many food proteins such as seafood, meat, surimi, dairy, confectionary, sausages, gelatine (Kuraishi *et al*., 2001), scallops, pork (Kuraishi *et al*., 1997) noodles and pasta (Larre *et al*., 1998, 2000).

Up to date, there have been only a few studies conducted on the effect of fish gelatine gels treated with TGase. There is a lack of information on the physical characteristics of cold water fish gelatine modified by TGase induced cross-linking. Therefore, gelatine extracted from hoki skins by previously described process (Mohtar *et al*., 2010) was used to determine the effects of incorporating TGase enzyme on the gel strength of gelatine gels.

Materials and Methods

Raw materials

Skins of hoki fish were supplied by Independent Fisheries Limited (Auckland, New Zealand) and were kept at -20^{0}C freezer. Porcine gelatine (Type A, Bloom 200, sample no. 378-1208) and bovine gelatine (Type B, Bloom 200, sample no. 379-1208) were kindly provided by International Food Agencies Limited (Auckland, New Zealand). Transglutaminase (TGase) enzyme was a commercial product obtained from Ajinomoto, Malaysia (TG-BW-MH). All chemicals and reagents used were analytical grade.

Raw materials preparation

Fish skins preparation

The frozen skins were thawed at 4^{0}C for 24 hr and any remaining flesh and scales were removed. The cleaned skins were minced without water in a laboratory blender (Waring Commercial®, New Hartford, CT, USA) at low speed and time setting of 15 min. The minced skins were filled into 150 x 90 mm snap-lock plastic bags (GLAD, Clorox (NZ) Ltd., Auckland, New Zealand). The bags were sealed and stored at -20^{0}C until used within one to two weeks.

Partial purification of TGase enzyme

Commercial TGase enzyme obtained from Ajinomoto was purified by ammonium sulfate $(NH_4)_2SO_4$ precipitation between 20% and 80% saturation. To achieve 20% $(NH_4)_2SO_4$ saturation, 17.9 g of $(NH_4)_2SO_4$ granules were weighed out and slowly added to 100 mL of the protein solution. The solution was stirred using a magnetic stirrer at 4^{0}C for 12 hr. After which, the solution was centrifuged using Sorvall® RC-28S Centrifuge (Sorvall®, Newton, CT, USA) at 10 000 x g at 4^{0}C for 15 min. The supernatant was collected in a beaker and granular $(NH_4)_2SO_4$ was added to bring the saturation level step-wise by 10% to a final saturation level of 80%. At each 10% increase in the $(NH_4)_2SO_4$ saturation levels, the contents were stirred at 4^{0}C for 12 hr and centrifuged at 10 000 x g for 15 min at 4^{0}C. The pellets obtained at each of the successive $(NH_4)_2SO_4$ saturation levels were separately dissolved in small volume of 10 mM Tris-acetate buffer (pH 7.5) and dialysed against the same buffer in dialysis tubing with molecular weight cut-off of 50,000 Daltons (Medicell International Ltd., London, UK). The dialysis was done at 4^{0}C for 12 hr and the buffer in the beaker was changed 5 times. After the final dialysis, the tubes were rinsed in Milli-Q water and the contents obtained were frozen at -80^{0}C and freeze dried. The enzyme activity of proteins precipitated at different $(NH_4)_2SO_4$ saturation levels was determined by the method described earlier. The $(NH_4)_2SO_4$ precipitated fraction having the highest enzyme activity was selected for further use in gel strength analysis. The protein concentration of the final enzyme used was determined according to Bradford assay (Wrolstad *et al*., 2005).

TGase enzyme assay

TGase activity was determined according to the method of Folk (1970). A reaction mixture (2 ml) containing 200 µl of 2% TGase solution, 1400 µl of 0.1 M Tris-acetate buffer (pH 6), 100 µl of 2 M hydroxylamine, and 300 µl of 0.1 M

N-Carbobenzoxy-glutaminyl-glycine was prepared. After incubation at 37^{0}C for 10 min, the reaction was stopped by the addition of 2 ml of TCA solution prepared from equal portions of 15 g/100 ml TCA, 5 g/100 ml $FeCl_3$ in 0.1 N HCl and 2.5 N HCl. The precipitate was removed by centrifugation at 4000 x g for 15 min using Sorvall® RC- 28S Centrifuge. The absorbance of the supernatant at 525 nm was then obtained using spectrophotometer (UV-VIS Mini 1240, Shimadzu Scientific Instruments, MD, USA). One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 µ mole of peptide-bound γ- glutamyl hydroxamate per minute.

Extraction of fish gelatine

Gelatine extraction was conducted by the optimized method according to Mohtar *et al*. (2010). Frozen minced skins were thawed at room temperature and were rinsed with tap water. The rinsed minced skins were pre-treated in 0.75 M NaCl solutions (1:6 w/v) for 9 min at 4^{0}C followed by rinsing again with tap water. These steps were conducted twice. The minced skins were gently stirred with Milli-Q water (Millipore Corporation, Billerica, MA, USA) in a ratio of 1:6 w/v for 60 min at 49.3^{0}C in a shaking water bath (Ratek Instruments, Boronia, Victoria, Australia). The samples were centrifuged using Sorvall® RC-28S Centrifuge at 10000 x g at 15^{0}C for 30 min. The clear extract obtained was filtered using a Whatman filter paper No. 5 (Whatman International Ltd., Kent, UK) and the filtrate was freeze dried for further analysis.

Preparation of gelatine gels

The method reported earlier for preparation of gelatine gels was used (Mohtar *et al*., 2010). Gelatine gels were prepared by dissolving 6.67% (w/v) of dry gelatine in Milli-Q water and then heated at 45^{0}C in a water bath for 30 min until gelatine was completely dissolved. The gelatine solutions were cooled at room temperature for 30 min before being chilled in a refrigerator at 10^{0}C for 18 h. The effect of TGase on the gel strength of gelatine gel was investigated by adding the enzyme to gelatine solutions at concentrations of 0.5, 1.0, 3.0, 5.0 and 6.0 mg of enzyme per g of gelatine. Gelatine solutions with different enzyme concentrations were incubated at 45 ^{0}C for 30 min and then cooled to room temperature before maturation at 10^{0}C for 18 h prior to gel strength analysis.

Gel strength analysis

Gel strength of gelatine gels were measured as reported in previous method (Mohtar *et al*., 2010). A 6.67% of dry gelatine were dissolved in Milli-Q water and then heated at 45^{0}C in a water bath for 30 min until gelatine was completely dissolved. The gelatine solutions were cooled at room temperature for 30 min before being chilled in a refrigerator at 7^{0}C for 18 hr. The gel strength was measured immediately after being removed from the fridge using a TA.XT2 Texture Analyser (Stable Micro Systems, Surrey, UK) fitted with a 1.27 cm diameter cylindrical probe at a speed of 1 mm/s with a force of 0.1 N. The force (in g) was recorded as gel strength when the probe had penetrated 4 mm into the gelatine gels. The gel strength of gelatine gels with added TGase was also measured in the same way. The analysis was performed in triplicate.

Results and Discussion

Partial purification of TGase enzyme

Figure 1 shows the TGase enzyme activity (U/mg) in the protein precipitated at different saturation levels of $(NH_4)_2SO_4$. The highest enzyme activity of 71.7 U/mg was obtained at 50% of $(NH_4)_2SO_4$ saturation. The $(NH_4)_2SO_4$ precipitation was conducted to remove some unwanted materials in the enzyme such as sodium caseinate, maltodextrin and sodium chloride from the commercial enzyme supplied by Ajinomoto.

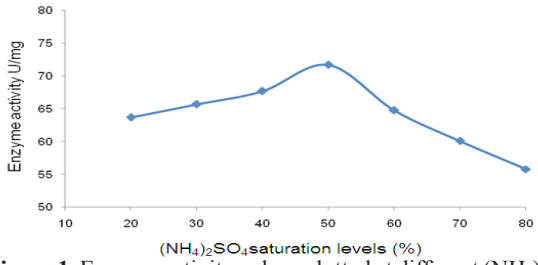

Figure 1. Enzyme activity values plotted at different $(NH_4)_2SO_4$ saturation levels

Optimization of gelatine extraction

The optimized gelatine yield by Response Surface Methodology obtained in our previous studies (Mohtar *et al*., 2010) gave the following equation:

$$Y = 12.8 + 1.00\,X_1 + 1.67\,X_3 + 0.92\,X_1^2 - 5.29\,X_3^2 + 2.25\,X_1X_2 + 1.30\,X_1X_3$$

Where Y is the yield of gelatine (%), X_1 is the concentration of NaCl (M), and X_3 is the temperature of the hot water extraction (^{0}C).

Gel strength analysis

The gel strength of gelatine from hoki skins,

bovine and porcine was achieved as reported in Mohtar *et al*. (2010) (Table 1) where the gel strength of hoki gelatine (197 g) was much higher than those reported in previous literatures for other cold-water fish species (Gómez-Guillén *et al*., 2002; Zhou *et al*., 2006; Arnesen and Gildberg 2007). However, hoki skin gelatine gel strength was significantly lower than those obtained for bovine and porcine gelatine, (gel strengths of 273 g and 307 g were obtained for bovine and porcine gelatine, respectively), at $P<0.05$ (Mohtar *et al*., 2010). The difference in gel strength may possibly be due to some reasons such as lower content of proline and hydroxyproline found in fish gelatine (16-18%) compared to the mammalian gelatines (24%) (Veis, 1964). Karim and Bhat (2009) reported that in cold water species, the ratio of hydroxyproline to proline ratio was 50:100 compared to porcine gelatine, which had a ratio of 62:100. High gelatine gel strengths (250–400 g) are required for a broad range of applications in the food industry, especially in the processing of jellies, canned meat, marshmallows and yoghurts (Holzer, 1996).

Table 1. Rheological properties of gelatines from hoki skin and commercial bovine and porcine

Properties	Gelatine		
	Hoki	Bovine	Porcine
Gel strength (g)	196.72 ± 4.95^{a}	273.32 ± 16.07^{b}	307.30 ± 8.37^{b}
Melting point (^{0}C)	16.6 ± 0.42^{a}	26.9 ± 0.65^{b}	29.1 ± 0.55^{c}
Viscosity (cP)	10.8 ± 0.55^{a}	9.8 ± 0.65^{a}	5.0 ± 0.26^{b}

Values are the mean ± standard deviation of triplicate.
$^{a-c}$ Means with the same superscripts within a row are not significantly different ($P<0.05$).
Adapted from Mohtar *et al*. (2010).

Effect of TGase addition on gel strength

Figure 2 shows the gel strength of gelatine gels added with different concentrations of TGase. Generally, the gel strength of gelatine gel increased with the increment of TGase concentration in which TGase induced the formation of cross-linking via non-disulfide covalent bonds in the gels structure. Gel strength increased significantly ($P<0.05$) at concentration of 3.0 mg/g (279 g) compared to the sample without the addition of TGase (197 g). However, this study found that the increment of TGase concentrations beyond 3.0 mg/g gave a decrease in gel strength which may be due to excessive formation of covalent bonds resulting in the development of intra-molecular covalent bonds (Gómez-Guillén *et al*., 2002). This finding agrees with Fernández-Díaz *et al*. (2001) who found that the gel strength of cod and hake gelatine was decreased at the enzyme concentrations beyond 1.0 mg/g. The excessive addition of TGase will increase the formation of TGase-catalysed covalent bonds which in turn will cause in lowering the aggregation of the triple helix into a collagen fold triple helix, thus decrease the gel network formation (Ledward, 1986). Other than the enzyme concentration, the amino acid variation especially lysine and glutamine could also contribute to the difference in gelatine gel strength (Wangatueai *et al*., 2010).

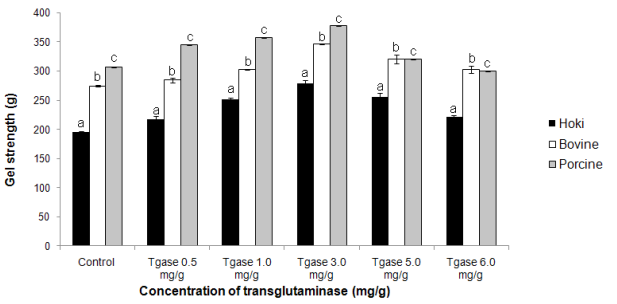

Figure 2. Gel strength of gelatine gels with and without the addition of TGase enzyme
Values are the mean ± standard deviation of triplicate.
Means followed with the same superscripts within a column are not significantly different ($P<0.05$).

Conclusions

Hoki skins gelatine showed lower values of gel strength and melting temperature when compared to commercial mammalian gelatines. However, hoki gelatine had significantly higher gel strength than that of other cold water fish species published in earlier literatures. The addition of TGase enzyme at optimum concentration significantly increased the gel strength of the gelatine gel. This potential property could create significant demand for hoki gelatine which might then be used commonly in food applications to replace available mammalian gelatines. Further study on the effect of enzymatic cross-linking at the microstructure level will be carried out and modification of hoki gelatine through chemical cross-linking will also be conducted.

Acknowledgements

The authors would like to express their gratitude to the University of Auckland for the financial support and to the Ministry of Higher Education, Malaysia for a postgraduate scholarship to the principal author. They would also like to thank Independent Fisheries Limited for the fish skin samples, the International Food Agencies Limited Company for providing the commercial mammalian gelatines and Ajinomoto Malaysia Company for supplying the TGase enzyme.

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