Extraction of Collagen from Catfish (*Clarias gariepnus*) Waste and Determination of its Physico-chemical Properties

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ABSTRACT

Collagen was extracted from catfish (Clarias gariepnus) waste using 0.5M acetic acid and its subsequent precipitation in 2.6M NaCl. The resultant collagen was analysed with respect to its moisture content and physico-chemical properties including yield, pH, protein content, colour, odour and thermal stability. A yield of 16.4% and positive collagen attributes indicate that catfish waste has potential as a collagen source. The snowy white, crystal-like and light textured collagen comprises of 5.97% protein and 0.46% moisture, and exhibits a pH of 4.75. Sensory evaluation indicates that the collagen has a slight fishy odour. Viscosity analysis indicates a steady decrease with increasing temperature over the range considered (20-50°C). The pale colour exhibited and limited odour emitted by the extracted collagen indicate that catfish waste collagen could be applied in the food industry without resulting in any undesirable food products attributes. Differential Scanning Calorimetry (DSC) analysis indicated that the collagen exhibits good thermal stability and denatures at a high temperature in a similar manner to mammalian collagen.

Keywords: *collagen, catfish waste, extraction, Clarias gariepnus*

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Introduction

Collagen is the major fibrous element in connective tissues and the most abundant single protein in the body; constituting approximately 25% of all vertebrate proteins [1]. Collagen is employed in a plethora of industries including the leather and film industries, pharmaceuticals, biomedical and food [2-5]. Collagen has been used as a carrier for antiseptics and enzymes [6] and in the food industry to make sausage casings. In its denatured form (gelatin) it is widely used as a value-added ingredient to improve elasticity, consistency and stability of foodstuffs [2], [7] and [8]. Normally collagen is extracted from the skins of selected vertebrate species, namely porcine and cattle using acid, alkaline or enzymatic processes [4], [9] and [10]. It has also been isolated from other animals, including bird's feet, frog skin, equine tendons, jellyfish and rat tail tendons [11], however the industrially applied collagens generally derive from domestic animals.

Fish collagen denatures at lower temperatures than mammalian collagen, which tends to denature above 30°C whereas fish collagen denatures below 30°C [1]. According to Nagai [8] marine collagen denatures at approximately 10°C less than that of porcine skin collagen, which would indicate that fish collagen is less thermally stable than mammalian derived collagen [1].

The use of cattle and porcine derived collagen may be limited with respect to the inherent health concerns relating to mad cow and foot-and-mouth disease, and religious constraints, respectively [1], [10] and [12]. An alternative safer and permissible collagen source is therefore of significant interest [1] and [3]. Fish collagen exhibits some unique compositional characteristics, including amino acids, thereby presenting the opportunity to develop a non-porcine or cattle based collagen source [13]. The Malaysian fishing industry is burgeoning and consequently so is the quantity of fish waste, which includes fish bones, fins, skin and scales. The consequences of large volumes of fish waste are pollution and unpleasant aromas caused during degradation, however the waste has the potential to be used as an alternative source for collagen. In this study collagen was extracted from catfish (*Clarias gariepnus*) waste, obtained from the fish processing industry, which comprised of fish bones, fins, skin and scales. The resultant extracted collagen was characterized with respect to its inherent physico-chemical properties, thereby enabling comparison between mammalian- and fish-derived collagen.

Materials and Methods

Catfish (*Clarias gariepnus*) waste was obtained from Anyzam Enterprise, a fish processing industry in Pedas, Negeri Sembilan. All the chemicals used in the preparation and extraction of the collagen were purchased from Sigma-Aldrich and of analytical grade.

Extraction of collagen

The collagen was prepared in accordance with the method espoused by Kittiphatannabawon [3] at 4°C with continuous stirring. The fish waste was cut into small pieces and freeze-dried, then mixed with 0.1M NaOH in a sample to alkali solution ratio of 1:10 (w/v) in order to remove non-collagenous proteins. The resultant mixture was stirred for six hours during which time the NaOH solution was changed every two hours. The deproteinised samples were washed with cold water until either a neutral or basic wash water was obtained. The samples were defatted using 10% butyl alcohol in a solid/solvent ratio of 1:10 (w/v) over a period of 18 hours during which time the solvent is changed every six hours. The defatted samples were then washed with cold water and soaked in a 0.5M acetic acid solution in a solid/solvent ratio of 1:30 for 24 hours. The mixtures were subsequently filtered and the residues re-extracted using 0.5M acetic acid. Both filtrates were combined and the collagen precipitated through the addition of NaCl up to a final concentration of 2.6M in 0.05M *tris(hydroxymethyl)aminomethane* (pH 7.0). The resulting precipitate was collected by centrifuge (Model 5420, Kubota Corporation) at a spin rating of 20,000g for 1 hour. The precipitate pellet was dissolved in 0.5M acetic acid, dialysed against 0.1M acetic acid and distilled water, and then freeze-dried.

Collagen Yield

The yield of collagen was calculated according to the formula proposed by Muyonga [14]:

$$\text{Yield of collagen (\%)} = \frac{\text{Weight of powdered collagen} \times 100}{\text{Weight of fresh catfish waste}} \quad (1)$$

Collagen Analysis

The collagen was analysed with respect to pH, moisture content, colour, odour, viscosity and thermal stability. Protein content was determined using the Kjeldahl method according to AOAC [15].

Collagen Solution pH Determination

The pH of the collagen solution was determined in accordance with the method used by Cheow [16]. (2007), whereby 1.0% (w/v) of the collagen solution was prepared by dissolving a given mass of a freeze-dried pellet in distilled water. The pH was determined using a glass electrode [17], which had been standardized using buffers of pH 4.0 and 7.0.

Moisture Content Determination

The moisture content was determined by placing an initial mass of approximately 3 grams of collagen powder into a moisture analyzer (OHAUS Moisture Balance MB45) upon which the sample was heated, to evaporate any inherent water, until a constant mass was attained. The resultant mass difference constitutes the moisture content.

Colour Measurement

Dry collagen powder was evaluated with respect to a chromameter (CR400, model Konica Minolta), which uses three colour coordinates, namely L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness). Samples were placed in a glass sample cup and were assessed in triplicate. Consistency and accuracy was achieved through calibration with respect to white tile and black glass samples.

Collagen Solution Viscosity

Viscosity and DSC measurements are usually used to determine the thermal stability of collagen [8]. Viscosity was measured in triplicate using a 15 Watt LVT Brookfield viscometer (Model 201) operating at 30 rpm in accordance with the method proposed by [3]. A 500ml 0.03% collagen in 0.1M acetic acid (w/v) solution was heated at a constant heating rate of 4°C/min over the range 4 to 50°C and held for 30 minutes at each temperature (10, 20, 30, 40 and 50°C) in order to determine the viscosity.

Differential Scanning Calorimetry

The phase transition temperature for the extracted collagen was determined using a differential scanning calorimeter; Diamond DSC (Perkin-Elmer Instruments, Norwalk, USA), which was calibrated using indium (T_{max}=156.6°C, ΔH_{max}=28.71Jg^{-1}). The freeze-dried collagen samples were rehydrated using deionised water in a solid to solution ratio of 1:40 (w/v). The mixture was allowed to stand for two days at 4°C in order to swell the collagen. A 5 g sample was weighed into a pre-weighed aluminum pan, which was then sealed and analysed using a heating rate of 1°C/min over the range 20 to 50°C.

Statistical Analysis

The data acquired in relation to the extraction process and the physico-chemical properties were analysed using ANOVA and mean comparisons were performed using Duncan's multiple range test. All statistical analysis was performed using Statistical Package for Social Sciences [18].

Results and Discussion

Yield

The average collagen yield extracted from the catfish waste was 16.4% (Table 1), which is in good agreement with that attained by [19] from longbarbel catfish (16.8%). Liu [13] reported 20-38% collagen yields from channel catfish and others have reported yields in the range 18.4-25.64% from grass carp, unicorn leatherjacket and blacktip shark [3], [4] and [20].

Table 1: The Yield, Visual Appearance, Odour, pH, Protein and Moisture-Content of Collagen from Catfish (*Clarias gariepnus*) Waste

Properties	**Collagen from catfish (*Clarias gariepnus*) waste**
Yield (%)	16.40 ± 0.21
Appearance	Light texture, crystal-like and snowy-white
Odour descriptions	Faint fishy odour (dried collagen powder) Moderately detectable fishy odour (collagen solution)
pH	4.75 ± 0.28
Protein content (%)	5.97 ± 0.13
Moisture (%)	0.46 ± 0.04

Values are presented as mean ± standard deviation for triplicate samples.

Collagen yields are influenced by the species or tissues from which the collagen is extracted, the preparation and extraction processes, as well as the feeding period, because if fish are being starved, albumins and globulins degrade and consequently the amount of collagen that can be extracted is reduced [2], [3] and [20]. Pre-treatment and extraction tend to be complex and time consuming, particularly if high collagen yields are desired [20-21]. According to Rodziewicz [2] collagen yields can be increased by 5 and 10% if the extraction time is extended to 48 and 72 hours, respectively.

Extraction with acetic acid is known to affect collagen yield, whereby increased acetic acid concentrations promotes collagen extraction [21]. At low acid concentrations such as 0.5M it is not possible to completely dissolve the raw materials due to lots of cross-linkages within the raw material structures resulting in lower yields [4]. Zalechowska [6] suggested that enzymatic pre-treatment of connective tissues using proteolytic enzymes, which are non-specific and suitable for pepsin, trypsin, pancreatin, bromelain and papain, may increase collagen yields.

In this study, collagen extraction comprises of three important steps, namely non-collagenous protein, fat and soluble matter extraction. Alkali treatment using sodium hydroxide removes the non-collagenous proteins

as a consequence of skin swelling in alkaline solution. Fats are extracted using butyl alcohol and any residual soluble matter is extracted from the fish waste with acetic acid.

Protein Content

The physical and chemical properties of fish collagen differ from that of mammalian collagen [8] and this may be attributed to variations in the types of connective tissues [6]. According to Rodziewicz [2], and Wang [21] the collagenous and non-collagenous protein content in fish determined through extraction is dependent upon the feeding period as well as the fish type, age and the collagen isolation method. The determined protein content in the considered fish waste 5.97% (Table 1), which is much lower than previous studies from silver carp [12] and fish bone [23], which were 14.3% and 15%, respectively. It is of note that the hydroxyproline content is dependent upon the species, environment and body temperature of the fish, which according to [3] and [19] can increase hydroxyproline content in collagen samples and is generally accompanied by an increase in protein content.

Moisture Content

The catfish waste collagen contained very little moisture, 0.46%, (Table 1), which could be attributed to the adoption of a different preparation method than that previously used and the duration of lyophilization. Collagen moisture content reported in previous studies include 7.77% for shark skin, 6.92% for blacktip shark skin and 8.09% for nile perch skin [14] and [22].

pH analysis

Catfish waste collagen exhibits a pH of 4.75 (Table 1), which is less acidic than that reported by [2] who observed pH 3.64 for collagen powder derived from silver carp. The acidic pH is likely a consequence of washing with acetic acid.

Odour Analysis

The extracted dried collagen powder exhibits a faint fishy odour, whereas the collagen solution exhibits a moderately detectable fishy odour (Table 1). According to Morimura [23] collagen produced from fishery waste tends

to retain its characteristic odour and thus it would be necessary to mask the odour by adjusting the amount of material added accordingly. They also reported that collagen of neutral pH exhibited a more acceptable odour, whereas acidic collagen tends to exhibit similar aromatic characteristics to commercial collagen.

Colour analysis

Visual observation indicate that catfish waste collagen exhibits a light textured, snowy white colour, which is crystal-like in appearance (Table 1). This visual analysis is in agreement with the high L^* value of 91.81 (Table 2). The lightness of the collagen obtained in this study implies that it may be suitable for incorporation into food systems without contributing any undesirable colour to the product.

Table 2 : Colour Values for Collagen from Catfish (*Clarias gariepnus*) Waste

Colour values	
L^*	91.81 ± 0.15
a^*	-4.74 ± 0.02
b^*	5.85 ± 0.09

Values are presented as mean ± standard deviation for triplicate samples.

Viscosity Analysis

According to [1] collagen exhibits high viscosity, however it is of note that according to Figure 1 such viscosity decreases with increasing temperature. With respect to the work of Nagai and Suzuki [24] this may be a consequence of collagen structure denaturation caused by heat treatment, whereby the hydrogen bonding in the collagen is gradually broken and the triple-helix structure of collagen converts to a random coil configuration [3], [25] and [26]. Collagen is known to denature above 40°C into a mixture of random-coil single, double and triple strands [3]. Thermal denaturation (T_d) profile of collagen provides useful information on the thermal stability of collagen in relation to the environment and amino acids content [20]. Zhang [12] reported that the T_d for silver carp and grass carp collagen was around 29°C and 28.4°C, respectively, however the T_d for cod skin is only 15°C.

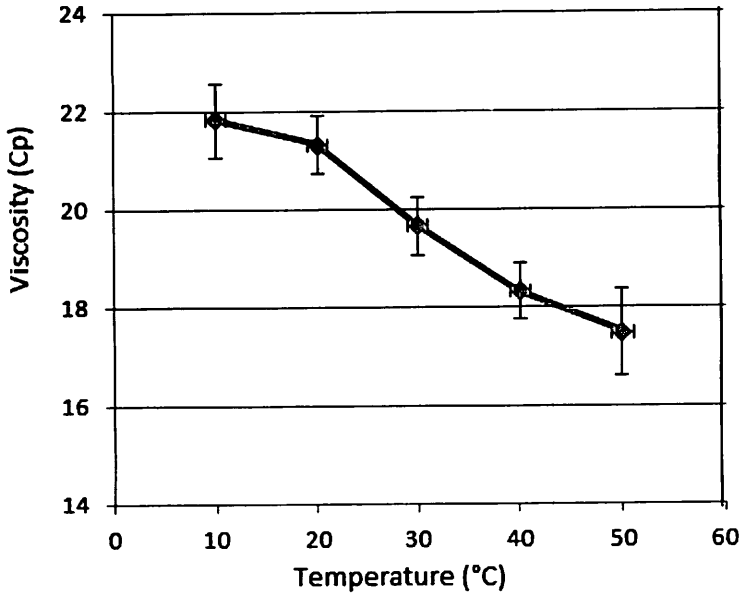

Figure 1: Viscosity of Catfish (*Clarias gariepnus*) Waste Collagen with Temperature

Liu [13] reported that channel catfish collagen denatures at a lower temperature than porcine collagen, 37°C. Senaratne [27] investigated differences in T_d between porcine and brown backed toadfish skin collagen, and determined that brown backed toadfish skin collagen denatures 9°C lower than porcine skin collagen. Senaratne [27] and Pati [28] suggested that the T_d value is related to animal body temperature and the temperature of the environment in which they reside, which may be attributed to the high amino acid content and high quantities of hydroxyproline, which is important in maintaining the stabilization of the trimmers in collagen [13],[19] and proline.

Differential Scanning Calorimetry (DSC) Analysis

The DSC thermogram of collagen from catfish fish waste is presented in Figure 2, in which it is evident that the maximum transition temperature (T_{max}) occurs at 37.36°C. This value is slightly higher than that presented in previous studies, such as collagen extracted from brown banded bamboo

shark, 34.52°C, and blacktip shark, 34.37°C [3]. The cleavage of the telopeptide region by pepsin or the removal of peptides may expedite the denaturation of collagen induced by heat [3].

The T_{max} for catfish waste collagen is slightly lower than that for mammalian collagen, such as cattle skin collagen (40.8°C). However, it is slightly higher than that for porcine skin collagen (37°C) and according to [22] this variation in T_{max} is related to the habitat temperature, and the hydroxyproline and proline composition. Increased amino acid content has been attributed to increases in the denaturation temperature of collagen [8], which is a consequence of amino acid stabilization of the helices resulting in the maintenance of the molecular collagen structure due to restrictions in changes to the secondary structure of the polypeptide chains [28].

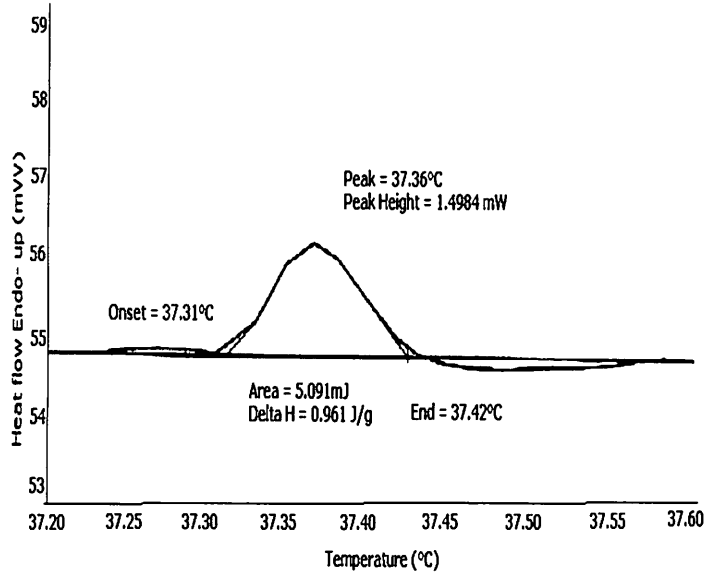

Figure 2: DSC Thermogram of Collagen from Catfish (*Clarias gariepnus*) waste

Conclusion

Collagen has been successfully extracted from catfish waste and preserved by freeze-drying; although the yield is relatively low. The resultant collagen exhibits low moisture content, is light in colour and exhibits comparatively

high thermal stability. As a consequence of the positive characteristics exhibited by the extracted collagen in this study there is potential for use as a valuable collagen alternative in the food industry. Processing conditions such as solvent, time and temperature need to be improved to optimize yields and produce better quality fish waste collagen, and it is in this vein that enzymatic pre-treatment is recommended to enhance future yields.

References

[1] M. Ogawa, R.J. Portier, M.W. Moody, J. Bell, M.A. Schexnayder and J.N. Losso, 2003. Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*), *Food Chemistry*, vol. 88, pp. 495-501.

[2] S. Rodziewicz-Motowidlo, A. Sladewska, E. Mulkiewicz, A. Kolodziejczyk, J. Aleksandrowicz, Miszkiewicz and P. Stepnowski, 2008. Isolation and characterization of a thermally stable collagen preparation from the outer skin of the silver carp (*Hypophthalmichthys molitrix*), *Aquaculture*, vol. 285, pp. 130-134.

[3] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, T. Nagai and M. Tanaka, 2004. Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*), *Food Chemistry*, vol. 89, pp. 363-372.

[4] M. Ahmad, S. Benjakul and S. Nalinanon, 2010. Compositional and physicochemical characteristics of acid solubilized collagen extracted from the skin of unicorn leather jacket (*Aluterus monoceros*), *Food Hydrocolloids*, vol. 24, pp. 588-594.

[5] F. Pati, S. Dhara and B. Adhikari, 2010. Fish collagen: A potential material for biomedical application, Student's Technology Symposium (TechSym), 2010, *Institute of Electrical and Electronics Engineers (IEEE)*, pp. 34-38.

[6] E. Zelechowska, M. Sadowska and M. Turk, 2009. Isolation and properties of collagen from the backbone of Baltic cod (*Gadus morhua*), *Food Hydrocolloids*, vol. 24, pp. 325-329.

[7] S.F. See, P.K. Hong, K.L. Ng, W.M. Wan Aida and A.S. Babji, 2010. Physicochemical properties of gelatins extracted from skins of different freshwater fish species, *International Food Research Journal*, vol. 17, pp. 809-816.

[8] Y. Zhang, W. Liu, G. Li, B. Shi, Y. Miao and X. Wu, 2006. Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodan idella*), *Food Chemistry*, vol. 103, pp. 906-912.

[9] T. Nagai, M. Izumi and M. Ishii, 2003. Fish scale collagen. Preparation and partial characterization. *International Journal of Food Science and Technology*, vol. 39, pp. 239-244.

[10] R. Duan, J.J. Zhang, X. Du, X. Yao and K. Konno, 2009. Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*), *Food Chemistry*, vol. 112, pp. 702-706.

[11] Y.K. Lin and D.C. Liu, 2005. Comparison of physical-chemical properties of type I collagen from different species. *Food Chemistry*, vol. 99, pp. 244-251.

[12] J.J. Zhang, R. Duan, Y. Tian and K. Konno, 2009. Characterisation of acid-soluble collagen from skin of silver carp (*Hypophthalmichthys molitrix*), *Food Chemistry*, vol. 116, pp. 318-322.

[13] H.Y. Liu, D. Li and S. Guo, 2006. Studies on collagen from the skin of channel catfish (*Ictalurus punctaus*), *Food Chemistry*, vol. 101, pp. 621-625.

[14] J.H. Muyonga, C.C.B. Cole and K.G. Duodu, 2003. Characteristic of acid soluble collagen from skins of young and adult Nile perch *(Lates niloticus*), *Food Chemistry,* vol. 85, pp. 81-89.

[15] AOAC, 1995. Official methods of analysis (16th ed.). Washington:Association of Official Analytical Chemists.

[16] C.S. Cheow, M.S. Norizah, Z.Y. Kyaw and N.K. Howell, 2007. Preparation and characterization of gelatins from the skins of sin croaker (*Johnius dussumieri*) and shortfin scad (*Decapterus macrosoma*), *Food Chemistry*, vol. 101, pp. 386-391.

[17] Toledo MPC 227 pH meter, Mettler-Toledo GmbH, Schwerzenbach, Switzerland

[18] SPSS for windows (2006): SPSS Inc., Chicago, IL, USA

[19] M. Zhang, W. Liu and G. Li, 2009. Isolation and characterization of collagens from the skin of largefin longbarbel catfish (*Mystus macropterus*), *Food Chemistry*, vol. 115, pp. 826-831.

[20] C.M. Li, Z.H. Zhong, Q.B. Wan, H.L. Zhao, H.F. Gu and S.B. Xiong, 2008. Preparation and thermal stability of collagen from scales of grass carp (*Ctenopharyngodon idellus*), *Europian Food Research Technology*, vol. 227, pp. 1467-1473.

[21] L. Wang, B. Yang, R. Wang and X. Du, 2008. Extraction of pepsin-soluble collagen from grass carp (*Ctenopharyngodon idella*) skin using an artificial neural network, *Food Chemistry*, vol. 111, pp. 683-695.

[22] P. Kittiphattanabawon, S. Benjakul, W.Visessanguan, H. Kishimura and F. Shahidi, 2010. Isolation and characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*), *Food Chemistry*, vol. 119, pp. 1519-1526.

[23] S. Morimura, H. Nagata, Y. Uemura, A.Fahmi, T. Shigematsu and K. Kida, 2002. Development of an effective process for utilization of collagen from livestock and fishwaste, *Process Biochemistry*, 37, vol. pp. 1403-1412.

[24] T. Nagai and N. Suzuki, 2000. Isolation of collagen from fish waste material – skin, bone, and fins. *Food Chemistry*, vol. 68, pp. 277-281.

[25] L. Wang, Y. Zou, S. Jiang, J. Xu, and Q. Hu, 2011. Chromatographic separation and physicochemical properties collagen species in the skin of deep-sea redfich (*Sebastes entella*), *Food Hydrocolloids*, vol. 25, pp. 1134-1138.

[26] J.W. Woo, S.J. Yu, S.M. Cho, Y.B. Lee and S.B. Kim, 2008. Extarction optimization and properties of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin, *Food Hydrocolloids*, vol. 22, pp. 879-887.

[27] L.S. Senaratne, P.J. Park and S.K. Kim, 2006. Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin, *Bioresource Technology*, vol. 97, pp. 191-197.

[28] F. Pati, B. Adhikari and S. Dhara, 2010. Isolation and characterization of fishscale collagen of higher thermal stability, *Bioresource Technology*, vol.101, pp. 3737-3742.

[29] M. Yan, B. Li, X. Zhoa, G. Ren, Y. Zhuang, H. Hou, X. Zhang, L. Chen and Y. Fan, 2008. Characterization of acid-soluble collagen from the skin of walleye Pollock (*Theragra chalcogramma*), *Food Chemistry*, vol. 107, pp. 1581-1586.