Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.69422

CC BY

Characteristics comparison of collagens from squid skin by different extraction methods

Shaoqian CAO[1], Jinxiu CAI[1], Shunli YING[1], Tianyi CHEN[1], Liang LIU[2], Hua YANG[1], Jiawen MA[1], Luyao HE[1], Xiangyang QI[1]*

Abstract

To provide a scientific basis for the comprehensive utilization of squid processing by-products, squid skin collagen was extracted by acid, enzyme and hot water, respectively, and the physicochemical characteristics of the collagens were compared. The results showed that the yield of acid soluble collagen (ASC), pepsin soluble collagen (PSC) and heat soluble collagen (HSC) was 7.41 ± 0.55%, 32.86 ± 0.67% and 42.23 ± 0.82% (dry weight basis), respectively. FTIR, UV and SDS-PAGE analysis suggested that ASC, PSC and HSC had the typical characteristics of type I collagen and retained the complete triple helix structure. The thermal stability of ASC was higher than that of PSC and HSC. ASC had a more uniform and compact porous network structure than PSC, while HSC was an irregular sheet structure. The solubility of HSC was obviously higher than that of ASC and PSC, and the effect of pH and NaCl on the solubility of HSC was inconspicuous in the experiment conditions. The extraction methods had significant effects on the structure and physicochemical properties of collagens. The solubility of HSC suggested that it was more suitable for liquid products. Moreover, the structural characteristics and high thermal stability of ASC made it suitable for biomedical materials and thermal processing products.

Keywords: squid skin; collagen; extraction; physicochemical characterization.

Practical Application: This study provides a scientific basis for the comprehensive utilization of squid processing by-products and the development of high-value products. HSC is more suitable for liquid products, while ASC is not only suitable for biomedical materials, but also for thermal processing products.

1 Introduction

Collagen is the major component of animal connective tissue, which can maintain the elasticity and firmness of the skin and provide support for the tissue. It has a unique helical structure composed of three polypeptide chains, and the repeated Gly-tripeptide sequences (Gly-X-Y) exist in each chain, where X and Y are proline and hydroxyproline residues commonly (Yu et al., 2014; Tamilmozhi et al., 2013). Collagen has been used in whitening and moisturizing cosmetics, biomedical materials, food additives and imaging industry (Jafari et al., 2020; Sionkowska et al., 2020; Shori et al., 2021; Rama et al., 2021). The skin and bones of pigs, cattle and other terrestrial mammals were the main sources of collagen. Meanwhile, marine collagens are considered as an attractive alternative source. Compared with terrestrial animal sources, collagen derived from aquatic source has low risk of spreading infectious diseases, no religious restrictions, low cost and a wide range of raw materials. In recent years, aquatic collagen has been favored by researchers because of its advantages of beneficial absorption, hypo allergenicity and antigenicity (Jafari et al., 2020; Sionkowska et al., 2020).

Squid is a very popular aquatic product with good taste and high nutritional value. It is rich in a variety of essential amino acids and taurine. The by-products with low market value of squid processing include skins, internal organs and ink sacs, of which skins accounts for about 3-5% of the total weight (Hamzeh et al., 2018). These by-products are usually directly buried or processed into low value-added products. Therefore, innovative methods are needed to process the by-products in order to achieve novel utilization. Squid skin is rich in collagen, and collagen accounts for 80% of the dry weight of squid skin (Gómez-Guillén et al., 2002; Veeruraj et al., 2015). It is a high-quality raw material for extracting collagen and has broad development prospects.

At present, the extraction methods of collagen mainly include acid extraction, enzyme extraction, alkali extraction, hot water extraction and comprehensive extraction, among which acid method, enzyme method and hot water method are widely used. There are some differences in the structure and chemical properties of collagen obtained from different extraction methods and species (Gómez-Guillén et al., 2002; Nagarajan et al., 2012; Song et al., 2021). However, the differences among the extraction methods on the characteristics of collagens from the same species are still ambiguous. In order to make better utilization of squid processing by-products, this investigation extracted collagen from squid skin by citric acid, pepsin and hot water, respectively, and discussed the effects of extraction methods on

Received 27 May, 2022
Accepted 15 July, 2022
[1]*College of Biological and Environmental Sciences, Zhejiang Wanli University, Ningbo, China*
[2]*College of Food Sciences, Zhejiang Pharmaceutical University, Ningbo, China*
*Corresponding author: qixiangyang85@sina.com

the structure and characteristics of collagen, so as to provide a theoretical basis for the practical production and application of squid skin collagen.

2 Materials and methods

2.1 Material and chemicals

Skin of squid (*Dosidicus gigas*) was obtained from Xiangshan Honghai Aquatic and Food Co., Ltd. (Zhejiang, China). The skins were rinsed with running water and cut into small pieces (0.5 × 0.5 cm) with surgical scissors. Then, the pieces were frozen at -20 °C until use. The pepsin from pig stomach and protein molecular weight markers were purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China).

2.2 Pretreatment of squid skin

To eliminate non-collagenous proteins and fat, the skin tissues was soaked with 0.1 M sodium hydroxide solution (solid/solvent ratio of 1:10, w/v) for 6h. After filtration, the skin tissues were rinsed to neutral with distilled water, and then immersed with 15% isopropanol solution (solid/solvent ratio of 1:15, w/v) for 18h. The isopropanol solution was refreshed every 6 h. Thereafter, the tissues were rinsed with distilled water to remove isopropanol solution. The above operations were carried out at 4 °C with gentle stirring. The pretreated squid skins were stored at -20 °C and then used to extract the ASC, PSC and HSC.

2.3 Extraction of collagen

Extraction of ASC

The pretreated squid skins were extracted by 1.5 M citric acid solution (solid/solvent ratio of 1:10, w/v) at 4 °C for 42 h. The mixture was centrifuged (8000 r/min, 8 min, 4 °C) and NaCl (powder) was added to the supernatant to the final concentration of 0.9 M. The precipitate was collected by centrifuging at 10000 r/min for 20 min at 4 °C after standing for 24h. Thereafter, the precipitate was dissolved by 0.5 M acetic acid, and then dialyzed with 0.02 M disodium hydrogen phosphate for 24h, followed by 0.1 M acetic acid for 24h and deionized water for 48h. The dialysis solution was refreshed every 12 h. The dialysate was freeze-dried to obtain ASC.

Extraction of PSC

The pretreated squid skins were extracted by phosphate buffer solution containing 4% pepsin (1200U/g, w/v) with pH 2.2 (solid/solvent ratio of 1:15, w/v) at 4 °C for 60 h. The mixture was centrifuged at 8000r/min for 20 min at 4 °C. The supernatant was treated by the method described above for ASC.

Extraction of HSC

The pretreated squid skins were extracted by citric acid buffer solution with pH 4.0 (solid/solvent ratio of 1:10, w/v) at 60 °C for 8 h. Then, the mixture was cooled and centrifuged at 8000 r/min for 20 min at 4 °C. The subsequent operation was the same as that of ASC.

2.4 UV spectral analysis

The UV spectra of ASC, PSC and HSC were measured by the spectrophotometer (UV-1900PCS, Shanghai spectroscopic instrument Co., Ltd, China). The samples were dissolved in 0.5 M acetic acid to a final concentration of 1 mg/mL, and then scanned in the wavelength range of 190-400 nm with 0.5M acetic acid as blank.

2.5 FTIR spectral analysis

The FTIR spectra of ASC, PSC and HSC were obtained by the Fourier transform infrared spectrometer (VERTEX70, Bruker, Germany). 0.05 mg sample was mixed with 0.01g KBr, and then the mixture was pressed into disc. The discs were analyzed at a spectral range of 500-4000 cm^{-1}.

2.6 SDS-PAGE analysis

Electrophoretic analysis of collagen was determined by the method of Wang et al. (2013) with some modifications. The ASC, PSC and HSC was dissolved in 0.1 M acetic acid at 5 mg/mL, respectively, and then mixed with loading buffer at a ratio of 1:6 (v/v). The mixture was boiled for 10 min, and centrifuged for 10 min at 4000 r/min after cooling down. Electrophoresis was performed with 5% concentrating gel and 7.5% separating gel, and carried out at 80 V followed by 120 V, respectively. After electrophoresis, the gels were dyed with the staining solution containing 0.1% Coomassie brilliant blue R-250, 45% methanol and 10% acetic acid for 30 min, and then decolored with a decolorizing solution containing 75% acetic acid and 50% methanol overnight.

2.7 Amino acid analysis

The amino acid composition of ASC, PSC and HSC were analyzed by the amino acid analyzer (L8800, Hitachi Ltd., Japan) followed the method of Su et al. (2009) with some modifications. 20 mg sample were hydrolyzed under vacuum state with 5 mL of 6 M HCl at 110 °C for 24 h. The hydrolysates were neutralized with NaOH to pH 2.2, and diluted with 0.02 M HCl to 50 mL. A 0.5 mL sample was submitted to the amino acid analyzer.

2.8 Collagen ultrastructure

Morphological properties of the ASC, PSC and HSC were obtained by a scanning electron microscope (S-4800; Hitachi Ltd., Japan). The samples were frozen in liquid nitrogen and cut into thin pieces, then processed in an ion coater for gold coating. The ultrastructure of collagen was observed at 1000 × magnification and at an accelerating voltage of 20 kV.

2.9 Thermal stability analysis

The shrinkage temperature (T_s) was tested by differential scanning calorimetry (DSC 131 Evo, SATARAM, France). The instrument was calibrated taking indium as standards. The ASC, PSC and HSC were weighed accurately (5 mg) into aluminum crucibles and sealed, respectively. Taking an empty

aluminum crucible as the reference, the samples were heated from 20 °C to 150 °C at a rate of 5 °C/min. Then, the Ts was recorded.

Thermal denaturation temperature (T_d) was measured according to the method of Muyonga et al. (2004) with some modifications. The viscosity of ASC, PSC and HSC solutions was measured at a concentration of 1 mg/ mL in the range of 15 °C to 50 °C, and the temperature intervals was 5 °C. The solution was maintained at each temperature for 30 min. The viscosity curves of ASC, PSC and HSC were obtained by plotting the fractional viscosities against temperature. The T_d was considered to be the temperature when the fractional viscosity was 0.5.

2.10 Solubility analysis

The solubility tests were carried out at different pH and NaCl concentrations. The ASC, PSC and HSC was dissolved in 0.5 M acetic acid, respectively, and the final concentration of the solution was 3 mg/mL. For the effect of pH on the solubility, the pH of sample solution was adjusted from 1 to 10 with 6 M HCl or NaOH, respectively. For the effect of NaCl on the solubility, the solution of ASC, PSC and HSC was mixed with NaCl-acetic acid solution with different NaCl concentrations in equal volume to obtain the final NaCl concentrations of 1-6 g/ mL. Thereafter, the sample solution was stirred for 1h at 4 °C, and then centrifuged for 20 min (10000 r/min, 4 °C). Protein contents of the supernatants were determined by Enhanced BCA Protein Assay Kit using bovine serum albumin as a standard. The relative solubility was the ratio of the protein content at each pH or NaCl to the maximal protein content in supernatant.

2.11 Statistical analyses

Graphpad Prism software was used for drawing, and SPSS Statistics software was used for Duncan analysis of variance. The significance level was $P < 0.05$, and the data were expressed as mean ± standard deviation (mean ± SD).

3 Results and discussion

3.1 Yield of ASC, PSC and HSC

The yield of ASC from the squid skin was 7.41 ± 0.55% (dry weight basis), which was higher than those of ASC from skin of sailfish (5.76%, dry weight basis) (Tamilmozhi et al., 2013), swim bladders of yellowfin tuna (1.07%, dry weight basis) (Kaewdang et al., 2014), skulls and spines of skipjack tuna (2.47-3.57%, dry weight basis) (Yu et al., 2014). However, the yield of PSC from the squid skin was 32.86 ± 0.67% (dry weight basis), which was lower than those of PSC from grass carp skin (46.6%, dry weight basis) (Zhang et al., 2007), skins and swim bladders of bighead carp (60.3% and 59.0%, respectively, dry weight basis) (Liu et al., 2012), whereas significantly higher than that of ASC from the squid skin. The low yield of ASC might be explained by the fact that the collagen molecules were likely to form covalent cross-linking through aldehyde condensation in the telopeptide region, resulting in the decrease of the solubility in acidic solution. Nevertheless, limited pepsin digestion could effectively cleave the intermolecular cross-linking at the telopeptide region without damaging the integrity of the triple helix, leading to the higher dissolubility (Zhang et al., 2007; Veeruraj et al., 2013).

Comparatively, the yield of HSC from the squid skin was 42.23 ± 0.82% (dry weight basis), suggesting that the hot water extraction was the most efficient among the three different extraction methods. This was probably attributed to the increasing temperature could provide more energy to partially disrupt the cross-linking of collagen molecules (Nagarajan et al., 2012).

3.2 UV spectra

The UV spectra (190-400 nm) of ASC, PSC and HSC were showed in Figure 1a. ASC, PSC and HSC had maximum absorption peaks at 228, 226 and 228nm respectively, and there is no characteristic absorption peak at 250-280nm. This result indicated that ASC, PSC and HSC were typical collagen and had low content of miscellaneous protein. Most proteins contain aromatic amino acids such as tyrosine, phenylalanine and tryptophan, which contain conjugated double bonds and have strong absorption near 257, 275 and 280 nm respectively (Wang et al., 2018). Previous researchers had shown that collagen contained only a small amount of tyrosine and phenylalanine and almost no tryptophan, and its characteristic triple helix structure had large absorption at about 230nm (Pal et al., 2015). The maximum absorbance near 210-240 nm might be related to

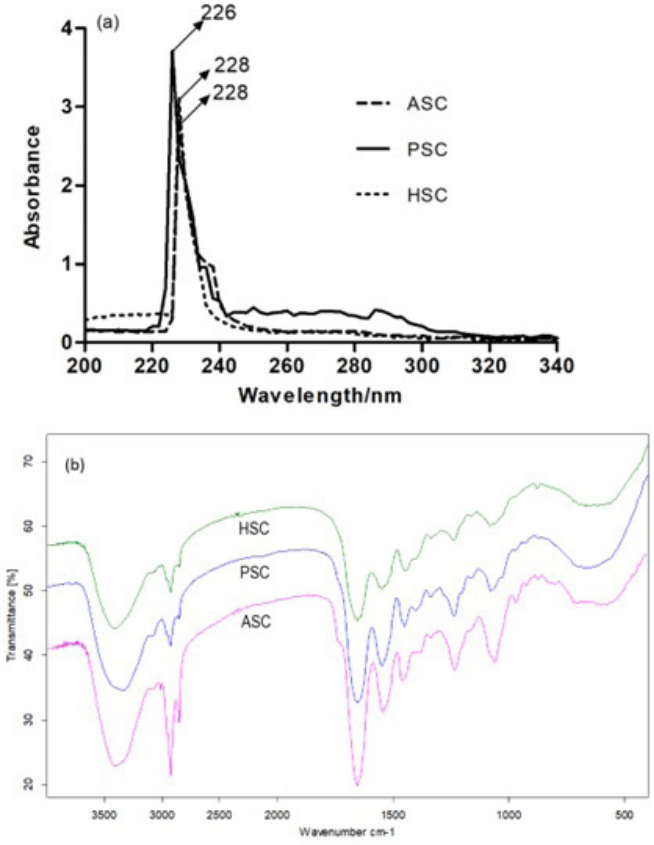

Figure 1. Spectral analysis of ASC, PSC and HSC from squid skin (a: ultraviolet spectra; b: FTIR spectra).

the groups such as C=O, -COOH, $CONH_2$ in polypeptides chains of collagen (Veeruraj et al., 2015; Pal et al., 2015; Song et al., 2021).

3.3 FTIR spectra

The FTIR spectra of ASC, PSC and HSC were showed in Figure 1b. The major characteristic absorption peaks including amide A, amide B, amide I, amide II, and amide III were observed, which were similar to previous report about collagen from squid skin (Veeruraj et al., 2015). It was known that N-H stretching vibration ranges from 3400 to 3450 cm^{-1}, yet the absorption peak decreases by about 100 cm^{-1} when the NH group is involved in a hydrogen bond. The results showed that the amide A bands of ASC, PSC and HSC related to N-H stretching vibration were observed at 3312, 3335 and 3336 cm^{-1} respectively, indicating that the NH groups of the three formed the hydrogen bonds with C=O groups and more NH groups of ASC were involved with hydrogen bonds than those of PSC and HSC. It was speculated that ASC has higher structural stability, which was related to more hydrogen bonds. The amide B bands of ASC, PSC and HSC related to the asymmetrical stretching of CH_2 group were observed at about 2925 cm^{-1}. CH_2 was the characteristic group of tertiary structure, indicating that the tertiary structure of ASC, PSC and HSC had not been destroyed.

Amide I, II and III were related to the skeleton structure of peptide chain. The amide I bands of ASC, PSC and HSC caused by C=O stretching vibration along the peptide backbone were observed at about 1657, 1656 and 1656 cm^{-1} respectively, which was a sensitive indicator of secondary structure of the protein (Surewicz & Mantsch, 1988; Woo et al., 2008; Tekle et al., 2022). This confirmed that ASC, PSC and HSC had the typical triple helix structure of collagen, which was formed via the hydrogen bonds between N-H and C=O (Wang et al., 2018; Ahmed et al., 2018). The amide II bands of ASC, PSC and HSC related to the N-H bending vibrations coupled with C-N stretching vibration were observed at 1549, 1545 and 1546 cm^{-1} respectively. Amide I and II are closely related to the degree of molecular order, and the shift of absorption peak to lower wavenumber is related to the reduction of the molecular order (Muyonga et al., 2004; Yu et al., 2014). ASC showed a slightly higher frequency than PSC and HSC, indicating that there were more molecular crosslinks in ASC which had a higher degree of molecular order. This result suggested that pepsin or high temperature might lead to the loss of amino acids for molecular crosslinks at the telopeptide region during the extraction of PSC and HSC. The amide III bands of ASC, PSC and HSC related to N-H bending vibrations were observed at about 1238 cm^{-1}, which was involved with the triple helix structure. To sum up, the FTIR spectra revealed the extracts of the three methods were typical collagens and maintained helical arrangements.

3.4 SDS-PAGE analysis

SDS-PAGE patterns of ASC, PSC and HSC were showed in Figure 2. They all consisted of two α chains (α_1 and α_2), β chains and γ chains, and the latter two with higher molecular weight were dimers and trimers formed by molecular crosslinking. Their patterns were similar to that of squid skin collagen in the previous reports (Gómez-Guillén et al., 2002; Veeruraj et al., 2015), whose results suggested that the collagen from squid skin were most probable to be classified as type I collagen because the band intensity ratio of α_1 and α_2 chains was found at 2:1 approximately.

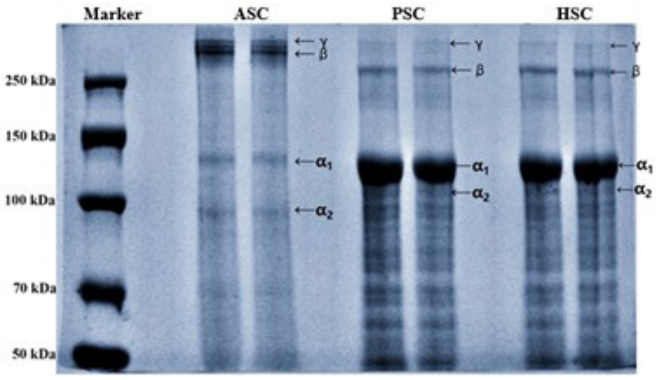

Figure 2. SDS-PAGE image of different collagens from squid skin.

The pattern of ASC was different from those of PSC and HSC. In ASC, the band intensity of β and γ chains was significantly higher than that in PSC and HSC, while the band intensity of α chains were markedly lower. The results indicated that there were more crosslinks in ASC, yet pepsin and high temperature partially damaged the crosslinks and the β and γ chains were broken down into α chains. In addition, ASC had no obvious band below 70 kDa, which indicated that ASC had relatively complete structure. PSC and HSC had a significant increase in the presence of degraded fragments with higher electrophoretic mobility. This appearance might arise from the pepsin or higher temperature used for extraction of PSC or HSC, which might induce fragmentation of α chains or solubilize additional low molecular weight proteins. This result was consistent with the previous literature which reported the molecular weight of PSC was lower than ASC from the same source (Li et al., 2013; Yu et al., 2014).

3.5 Amino acid analysis

The amino acid composition of ASC, PSC and HSC were showed in Table 1, and their profiles were similar and accord with the amino acid characteristics of collagen, indicating that the extraction method had little effect on the amino acid composition of collagen. Among them, the glycine content was the highest, with 315, 310 and 323 residues/1000 residues in ASC, PSC and HSC respectively. This was agreement with the previous literatures which reported that glycine was the dominant amino acid in collagens (Su et al., 2009; Ahmad & Benjakul, 2010; Wang et al., 2018; Ahmed et al., 2019). In addition, ASC, PSC and HSC were also abundant in alanine, proline, hydroxyproline, glutamic acid, aspartic acid and arginine. Among them, proline and hydroxyproline are termed imino acid, which play an important role in preserving the structural stability of triple helix governed by the pyrrolidine rings of imino acid, and the increase of imino acid content resulted in the improvement of thermal stability of collagen (Ahmad & Benjakul, 2011; Matmaroh et al., 2011; Wang et al., 2018). The content of imino acid in ASC, PSC and

HSC was 197, 150 and 156 residues/1000 residues, respectively. It could be seen that its content in ASC was higher than that in PSC and HSC, which suggested ASC might have better thermal stability.

However, the contents of tyrosine, phenylalanine, lysine and cysteine were deficient, especially tryptophan (no found), which were similar to that of gelatin from squid skin by hot water (Nagarajan et al., 2012). Moreover, this results also consistent with the results of UV spectral analysis.

3.6 Thermal stability analysis

DSC profiles of ASC, PSC and HSC were shown in Figure 3a, and the T_s values of ASC, PSC and HSC were 70.5, 64.5 and 68.5 °C, respectively. It showed that the thermal stability of ASC was higher than that of PSC and HSC, which was consistent with the analysis of amino acid composition. In addition, this result might be due to the higher molecular weight of ASC, it was reported that the thermal stability of collagen was related to the molecular weight (Duan et al., 2009).

The changes of fractional viscosity with increasing temperature were depicted in Figure 3b. The fractional viscosity of ASC, PSC and HSC decreased sharply between 20 °C and 30 °C, which indicated that the collagens began to undergo thermal denaturation at about 20 °C. The T_d values of ASC, PSC and HSC were 25.58, 24.86 and 25.57 °C, respectively. The thermal stability of collagen is related to living environment temperature of the source. The squid commonly lives in warm tropical seawater, so T_d values of the collagens from squid skin were similar to that of temperate and tropical fish, such as eel (29.3 °C), saury (23.0 °C), bullhead shark (25 °C), seabass (26.5 °C), chub mackerel (25.6 °C), but much higher than that of cold water fish, such as hake (10.0 °C), deep-sea redfish (16.1 °C), and lower than that of mammalian, such as pig and calf (37.0 °C) (Nagai et al., 2001; Ciarlo et al., 1997; Wang et al., 2007).

Table 1. Amino acid composition of collagens from squid skin extracted using different methods (expressed as residues/1000 residues).

Amino acids	ASC	PSC	HSC
Asp	52	64	63
Thr	24	25	31
Ser	30	33	33
Glu	83	94	97
Gly	315	310	323
Ala	98	107	113
Cys	9	13	7
Val	22	26	23
Met	17	17	16
Ile	19	15	18
Leu	25	34	28
Tyr	10	12	3
Phe	12	16	11
Lys	13	14	11
His	21	17	4
Arg	53	53	63
Pro	126	88	98
Hyp	71	62	58
Trp	—	—	—
Total	1000	1000	1000
Imino acids	197	150	156

—, undetectable.

3.7 SEM analysis

Figure 4 showed the microstructures of ASC, PSC and HSC under magnifications via ×1000. ASC was a uniform and compact porous matrix with good interconnectivity, while PSC was a macroporous network structure with irregularity and filaments, which was similar to the microstructure of collagens from the skin of Nile tilapia (Song et al., 2021). HSC showed a multilayered aggregated and irregular sheet structure without filaments and network, which indicated that high temperature extraction denatured collagen, giving it the characteristic structure of gelatin. The results showed three were differences in the microstructure of the three collagens, and the ASC structure was relatively more porous and looser, indicating that pepsin and high temperature have a great influence on the microstructure.

3.8 Solubility analysis

The effects of pH and NaCl on the solubility of ASC, PSC and HSC were depicted in Figure 5. The solubility of collagen

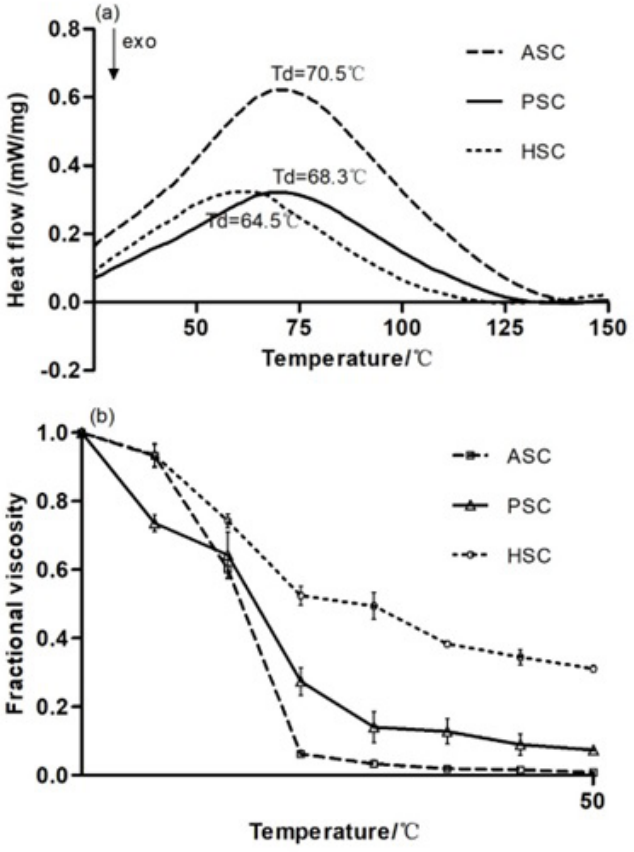

Figure 3. Thermal stability of ASC, PSC and HSC from squid skin (a: DSC spectrum; b: Change curve of viscosity).

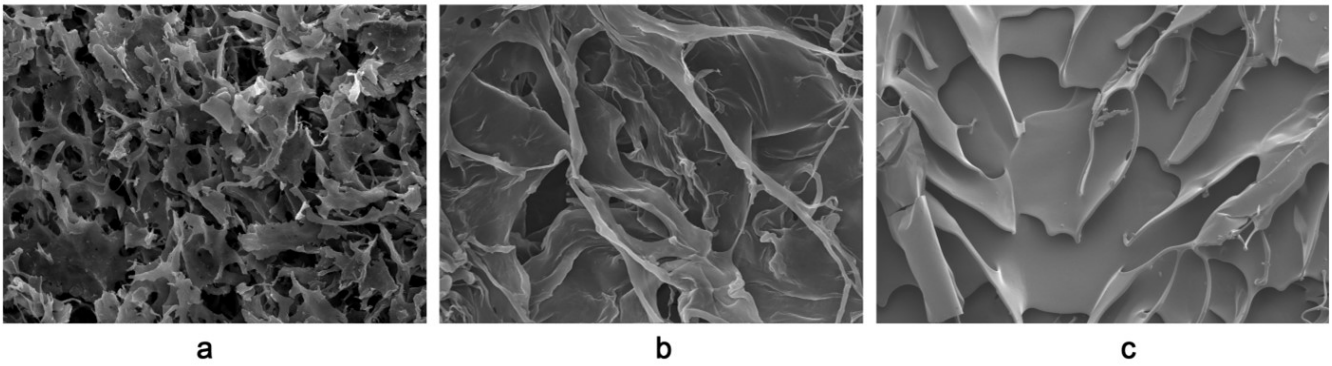

Figure 4. Scanning electron micrographs of ASC, PSC and HSC from squid skin (a: ASC; b: PSC; c: HSC).

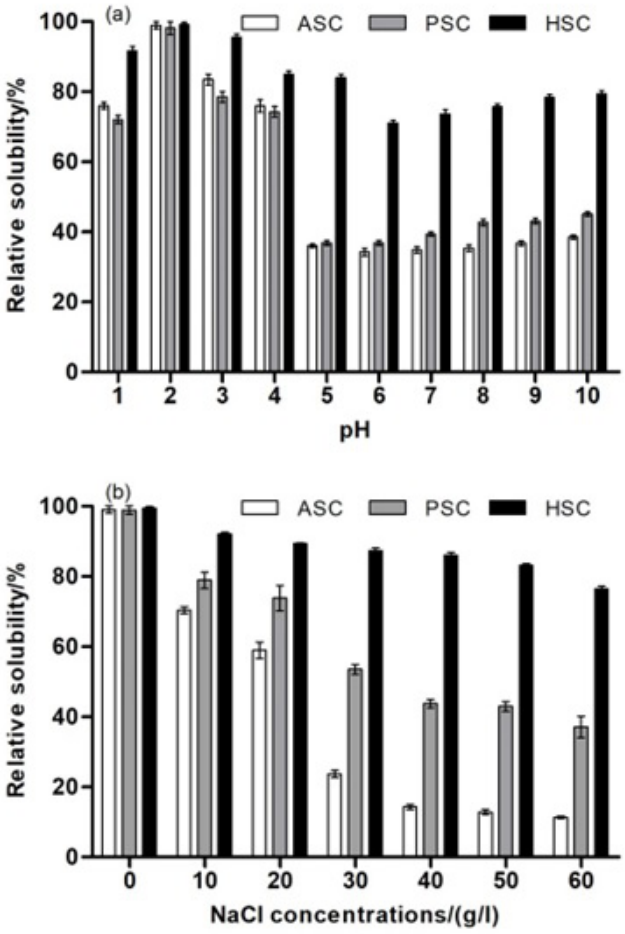

Figure 5. The effects of pH and NaCl concentration on the solubility of collagen from squid skin (a: pH; b: NaCl concentration).

is related to its pI. According to the change trend of the relative solubility with pH, it could be speculated that the pI of ASC, PSC and HSC might be near pH 5-6. The maximum solubility was noticed at pH 2. When pH was above 4, the solubility of ASC and PSC decreases significantly, while that of HSC changes slightly, which was consistent with the collagen of goatfish scale (Matmaroh et al., 2011). Moreover, the solubility of ASC, PSC and HSC increased slightly at pH 6-10, which was due to the increase of net negative charge of molecules resulting in enhanced intermolecular repulsion. In addition, the relative solubility of HSC was higher than that of ASC and PSC, indicating that high temperature extraction had a promoting effect on collagen solubility.

The solubility of ASC, PSC and HSC decreased with the increase of NaCl concentration, but the solubility change of HSC was relatively slight. Compared with ASC and PSC, the solubility of HSC was higher. In general, the solubility of collagens sharply decreased when the NaCl concentration was higher than 3% due to the salting out effect (Matmaroh et al., 2011; Liu et al., 2012; Li et al., 2013). However, when the concentration of NaCl reached 6%, the solubility of HSC was still high, around 76.43%, while that of PSC and ASC was 37.1% and 11.36%, respectively. This result suggested that the high temperature extraction could denature collagen and reduce the degree of molecular crosslinks, resulting in the enhancement of its solubility in salt solution. In addition, it could be seen from the Figure 5 that under the same NaCl concentration, the relative solubility of PSC was higher compared with ASC. This was probably due to the fact that pepsin could cut the terminal peptide region of collagen and reduce the intermolecular crosslinking, leading to the improvement of solubility. Moreover, the difference of solubility was related to the amino acid composition and structure of collagen.

4 Conclusion

The yield of ASC was the lowest and that of HSC was the highest. The results of UV, FTIR and SDS-PAGE showed that ASC, PSC and HSC had the typical characteristics of type I collagen and maintained the triple helix structure. Thermal stability analysis showed that ASC was more stable than PSC and HSC, which was consistent with its greater imino acids content and molecular weight. SEM results showed that there were significant differences in the microstructure of three collagens, and pepsin or high temperature had a great influence on the microstructure. ASC had a uniform and compact porous matrix with good interconnectivity, while HSC showed obvious gelatin properties. HSC had higher solubility compared with ASC and PSC, and the effect of pH and NaCl concentration on the solubility of HSC was inconspicuous.

In conclusion, hot water extraction was more efficient, economical and time-saving than the other two methods, while acid extraction was not only low yield, but also unfriendly to the environment, and enzyme extraction was relatively uneconomical. The collagens obtained by the three methods were partially different in structure and physicochemical properties, and the extraction method could be selected according to different applications. For example, the structural characteristics and high thermal stability of ASC made it not only suitable for biomedical materials, but also for thermal processing products. The solubility of HSC was better than that of ASC and PSC, suggesting it was more suitable for liquid products. It is necessary to further study the functional characteristics and specific application of the collagens. This study provided a scientific basis for the comprehensive utilization of squid processing by-products and the development of high-value products.

Acknowledgements

This work was financially supported by the basic and public welfare research program of Zhejiang Province in China (No. GN22C206180) and the key research and development plan program of Zhejiang Province in China (No. 2019C02071).

References

Ahmad, M., & Benjakul, S. (2010). Extraction and characterisation of pepsin-solubilised collagen from the skin of unicorn leatherjacket (*Aluterus monocerous*). *Food Chemistry*, 120(3), 817-824. http://dx.doi.org/10.1016/j.foodchem.2009.11.019.

Ahmad, M., & Benjakul, S. (2011). Characteristics of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as influenced by acid pretreatment and extraction time. *Food Hydrocolloids*, 25(3), 381-388. http://dx.doi.org/10.1016/j.foodhyd.2010.07.004.

Ahmed, R., Getachew, A. T., Cho, Y. J., & Chun, B. S. (2018). Application of bacterial collagenolytic proteases for the extraction of type I collagen from the skin of bigeye tuna (*Thunnus obesus*). *Lebensmittel-Wissenschaft + Technologie*, 89, 44-51. http://dx.doi.org/10.1016/j.lwt.2017.10.024.

Ahmed, R., Haq, M., & Chun, B. S. (2019). Characterization of marine derived collagen extracted from the by-products of bigeye tuna (*Thunnus obesus*). *International Journal of Biological Macromolecules*, 135, 668-676. http://dx.doi.org/10.1016/j.ijbiomac.2019.05.213. PMid:31154039.

Ciarlo, A. S., Paredi, M. E., & Fraga, A. N. (1997). Isolation of soluble collagen from hake skin (*Merluccius hubbsi*). *Journal of Aquatic Food Product Technology*, 6(1), 65-77. http://dx.doi.org/10.1300/J030v06n01_06.

Duan, R., Zhang, J., Du, X., Yao, X., & Konno, K. (2009). Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). *Food Chemistry*, 112(3), 702-706. http://dx.doi.org/10.1016/j.foodchem.2008.06.020.

Gómez-Guillén, M. C., Turnay, J., Fernández-Díaz, M. D., Ulmo, N., Lizarbe, M. A., & Montero, P. (2002). Structural and physical properties of gelatin extracted from different marine species: a comparative study. *Food Hydrocolloids*, 16(1), 25-34. http://dx.doi.org/10.1016/S0268-005X(01)00035-2.

Hamzeh, A., Benjakul, S., Sae-leaw, T., & Sinthusamran, S. (2018). Effect of drying methods on gelatin from splendid squid (*Loligo formosana*) skins. *Food Bioscience*, 26, 96-103. http://dx.doi.org/10.1016/j.fbio.2018.10.001.

Jafari, H., Lista, A., Siekapen, M. M., Ghaffari-Bohlouli, P., Nie, L., Alimoradi, H., & Shavandi, A. (2020). Fish collagen: extraction, characterization, and applications for biomaterials engineering. *Polymers*, 12(10), 1-37. http://dx.doi.org/10.3390/polym12102230. PMid:32998331.

Kaewdang, O., Benjakul, S., Kaewmanee, T., & Kishimura, H. (2014). Characteristics of collagens from the swim bladders of yellowfin tuna (*Thunnus albacares*). *Food Chemistry*, 155, 264-270. http://dx.doi.org/10.1016/j.foodchem.2014.01.076. PMid:24594184.

Li, Z. R., Wang, B., Chi, C. F., Zhang, Q. H., Gong, Y. D., Tang, J. J., Luo, H. Y., & Ding, G. F. (2013). Isolation and characterization of acid soluble collagens and pepsin soluble collagens from the skin and bone of Spanish mackerel (*Scomberomorous niphonius*). *Food Hydrocolloids*, 31(1), 103-113. http://dx.doi.org/10.1016/j.foodhyd.2012.10.001.

Liu, D. S., Liang, L., Regenstein, J. M., & Zhou, P. (2012). Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp (*Hypophthalmichthys nobilis*). *Food Chemistry*, 133(4), 1441-1448. http://dx.doi.org/10.1016/j.foodchem.2012.02.032.

Matmaroh, K., Benjakul, S., Prodpran, T., Encarnacion, A. B., & Kishimura, H. (2011). Characteristics of acid soluble collagen and pepsin soluble collagen from scale of spotted golden goatfish (*Parupeneus heptacanthus*). *Food Chemistry*, 129(3), 1179-1186. http://dx.doi.org/10.1016/j.foodchem.2011.05.099. PMid:25212354.

Muyonga, J. H., Cole, C. G. B., & Duodu, K. G. (2004). Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). *Food Chemistry*, 85(6), 81-89. http://dx.doi.org/10.1016/j.foodchem.2003.06.006.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N., & Suzuki, N. (2001). Isolation and characterisation of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). *Food Chemistry*, 72(4), 425-429. http://dx.doi.org/10.1016/S0308-8146(00)00249-1.

Nagarajan, M., Benjakul, S., Prodpran, T., Songtipya, P., & Kishimura, H. (2012). Characteristics and functional properties of gelatin from splendid squid (*Loligo formosana*) skin as affected by extraction temperatures. *Food Hydrocolloids*, 29(2), 389-397. http://dx.doi.org/10.1016/j.foodhyd.2012.04.001.

Pal, G. K., Nidheesh, T., & Suresh, P. V. (2015). Comparative study on characteristics and in vitro fibril formation ability of acid and pepsin soluble collagen from the skin of catla (*Catla catla*) and rohu (*Labeo rohita*). *Food Research International*, 76(Pt 3), 804-812. http://dx.doi.org/10.1016/j.foodres.2015.07.018. PMid:28455066.

Rama, G. R., Dullius, D., Agnol, W. D., Esquerdo, V. M., Lehn, D. N., & Souza, C. F. V. D. (2021). Ricotta whey supplemented with gelatin and collagen for the encapsulation of probiotic lactic acid bacteria. *Food Science and Technology*, 41(3), 576-586. http://dx.doi.org/10.1590/fst.19720.

Shori, A. B., Yong, Y. S., & Baba, A. S. (2021). Effects of herbal yogurt with fish collagen on bioactive peptides with angiotensin-I converting enzyme inhibitory activity. *Food Science and Technology*, 41(4), 902-907. http://dx.doi.org/10.1590/fst.24020.

Sionkowska, A., Adamiak, K., & Gadomska, M. (2020). Collagen based materials in cosmetic applications: a review. *Materials*, 13(19), 4217. http://dx.doi.org/10.3390/ma13194217. PMid:32977407.

Song, Z., Liu, H., Chen, L., Chen, L., Zhou, C., Hong, P., & Deng, C. (2021). Characterization and comparison of collagen extracted from the skin of the Nile tilapia by fermentation and chemical

pretreatment. *Food Chemistry*, 340, 128139. http://dx.doi.org/10.1016/j.foodchem.2020.128139. PMid:33010648.

Su, X. R., Sun, B., Li, Y. Y., & Hu, Q. H. (2009). Characterization of acid-soluble collagen from the coelomic wall of Sipunculida. *Food Hydrocolloids*, 23(8), 2190-2194. http://dx.doi.org/10.1016/j.foodhyd.2009.05.013.

Surewicz, W. K., & Mantsch, H. H. (1988). New insight into protein secondary structure from resolution-enhanced infrared spectra. *Biochimica et Biophysica Acta*, 952(2), 115-130. http://dx.doi.org/10.1016/0167-4838(88)90107-0. PMid:3276352.

Tamilmozhi, S., Veeruraj, A., & Arumugam, M. (2013). Isolation and characterization of acid and pepsin-solubilized collagen from the skin of sailfish (*Istiophorus platypterus*). *Food Research International*, 54(2), 1499-1505. http://dx.doi.org/10.1016/j.foodres.2013.10.002.

Tekle, S., Bozkurt, F., Akman, P. K., & Sagdic, O. (2022). Bioactive and Functional properties of gelatin peptide fractions obtained from sea bass (*Dicentrarchus labrax*) skin. *Food Science and Technology*, 42, e60221. http://dx.doi.org/10.1590/fst.60221.

Veeruraj, A., Arumugam, M., & Balasubramanian, T. (2013). Isolation and characterization of thermostable collagen from the marine eelfish (*Evenchelys macrura*). *Process Biochemistry*, 48(10), 1592-1602. http://dx.doi.org/10.1016/j.procbio.2013.07.011.

Veeruraj, A., Arumugam, M., Ajithkumar, T., & Balasubramanian, T. (2015). Isolation and characterization of collagen from the outer skin of squid (*Doryteuthis singhalensis*). *Food Hydrocolloids*, 43, 708-716. http://dx.doi.org/10.1016/j.foodhyd.2014.07.025.

Wang, L., An, X., Xin, Z., Zhao, L., & Hu, Q. (2007). Isolation and characterization of collagen from the skin of deep-sea redfish (*Sebastes mentella*). *Journal of Food Science*, 72(8), E450-E455. http://dx.doi.org/10.1111/j.1750-3841.2007.00478.x. PMid:17995604.

Wang, J., Pei, X., Liu, H., & Zhou, D. (2018). Extraction and characterization of acid-soluble and pepsin-soluble collagen from skin of loach (*Misgurnus anguillicaudatus*). *International Journal of Biological Macromolecules*, 106, 544-550. http://dx.doi.org/10.1016/j.ijbiomac.2017.08.046. PMid:28811205.

Wang, S., Hou, H., Hou, J., Tao, Y., Lu, Y., Yang, X., & Li, B. (2013). Characterization of acid-soluble collagen from bone of pacific cod (*Gadus macrocephalus*). *Journal of Aquatic Food Product Technology*, 22(4), 407-420. http://dx.doi.org/10.1080/10498850.2011.654382.

Woo, J. W., Yu, S. J., Cho, S. M., Lee, Y. B., & Kim, S. B. (2008). Extraction optimization and properties of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin. *Food Hydrocolloids*, 22(5), 879-887. http://dx.doi.org/10.1016/j.foodhyd.2007.04.015.

Yu, D., Chi, C. F., Wang, B., Ding, G. F., & Li, Z. R. (2014). Characterization of acid- and pepsin-soluble collagens from spines and skulls of skipjack tuna (*Katsuwonus pelamis*). *Chinese Journal of Natural Medicines*, 12(9), 712-720. http://dx.doi.org/10.1016/S1875-5364(14)60110-2. PMid:25263986.

Zhang, Y., Liu, W. T., Li, G. Y., Shi, B., Miao, Y. Q., & Wu, X. H. (2007). Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). *Food Chemistry*, 103(3), 906-912. http://dx.doi.org/10.1016/j.foodchem.2006.09.053.