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Partial Characterization of Collagen from Pharaoh Cuttlefish (*Sepia Pharaonis*) Skin

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Abstract. Pepsin solubilized collagen (PSC) from the skin of pharaoh cuttlefish was isolated, partially purified by salt precipitation and dialysis prior to characterization. The yield of PSC was 43.7% (dry weight). PSC had good purity which showed the distinct UV absorption peak at 232 nm and high hydroxyproline content. Total sugar content of PSC was 3.40% (dry weight), which was higher than that of collagen from calf skin (CC) (1.45% dry weight) ($P<0.05$). Based on protein pattern, PSC was considered to contain mixed type collagens, in which consisted of α- and β-chains as major components. PSC was rich in glycine and had high content of imino acids (196 residues/1000 residues). The maximum transition temperature (T_m) of PSC was 34 °C which was about 7 °C lower than that of CC. The skin waste material from cuttlefish processing is one of a promising new source for collagen production.

Keywords: Characterization, Collagen, Extraction, Cuttlefish, *Sepia pharaonis*

1. Introduction

Collagen is a major structural protein in the connective tissue of animal skin and bone. The structural unit of collagen is tropocollagen, a rod-shaped protein consisting of three polypeptides unit (called α-chains) intertwined to form a triple-helical structure [1]. Each polypeptide chain forms a left-handed helix and consists of repeating triplets, $(Gly\text{-}X\text{-}Y)_n$, where X and Y are, with a high possibility, proline or hydroxyproline [2]. Generally, collagen has a wide range of applications in cosmetic, biomedical, pharmaceutical, leather and film industries [3, 4].

Currently, the increasing attention of alternative sources for replacement of mammalian collagen has been paid, especially from seafood processing by-products. Pharaoh cuttlefish skin, a by-product from cuttlefish processing, is one of the alternative sources for collagen preparation since mammalian collagens are associated with several problems such as the outbreak of mad cow disease and the constraint for some religions, mainly Islam and Judaism [5]. Generally, typical processing, especially acid solubilization, renders a low yield of collagen. To tackle the problem, pepsin has been applied since it is able to cleave peptides specifically in telopeptide region of collagen, leading to increased extraction efficiency [4, 5]. Therefore, the objectives of this study were to extract collagen from the skin of pharaoh cuttlefish and to characterize the resultant collagen.

2. Materials and Methods

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2.1. Preparation of Skin Collagen

Collagen from the skin of pharaoh cuttlefish was prepared according to the method of Nalinanon et al. [4] with some modifications. All procedures were performed at 4 °C. The skin was cut into small pieces. After non-collagenous protein and fat were removed, the skin sample was lyophilized and kept at -20 °C until used. The lyophilized skin was extracted with 0.5 M acetic acid in presence of pepsin (10g/100g dry sample) for 72 h prior to filtration through 2 layers of cheesecloth. The filtrate was precipitated by the addition of NaCl to a final concentration of 2.6 M in 0.05 M Tris–HCl (pH 7.5). The mixture was allowed to stand for 1 h for pepsin inactivation. The resultant precipitate was collected by centrifuging at 20,000×*g* for 1 h. The pellet was dissolved in 0.5 M acetic acid prior to dialysis against 0.1 M acetic acid and distilled water, respectively. The dialysate was lyophilized and referred to as "pepsin solubilized collagen" (PSC). PSC was subjected to the determination of hydroxyproline content [4]. The yield of collagen extraction was calculated based on weight of lyophilized collagen in comparison with that of lyophilized skin.

2.2. Characterization of Skin Collagen

2.2.1. SDS–polyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli [6]. Samples (15 μg protein) were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis using a Mini-PROTEAN II unit. After electrophoresis, gel was stained by 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 3 h prior to destain to remove the dark backgound. High-molecular-weight protein markers were used to estimate the molecular weight of proteins.

2.2.2. Amino Acid Analysis

Collagen was hydrolysed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2(2-aminoethyl)indole at 115 °C for 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 mL was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

2.2.3. Hydroxyproline Content

Collagen was dehydrated with acetone and then hydrolysed in 6 M HCl at 110 °C for 24 h prior to determination of hydroxyproline content using the colorimetric method as described by Nalinanon et al. [4]. The hydroxyproline content was calculated and expressed as mg/g of dried collagen sample.

2.2.4. Total Sugar Content

Total carbohydrate content of collagen was determined according to the phenol-sulfuric method as described by Fournier [7] with a slight modification. To calculate the concentration of sugar presented in the sample, calibration curve was performed using D-glucose as a standard. Total sugar content was calculated and expressed as % dry weight of collagen sample.

2.2.5. UV Absorption Measurement

UV absorption spectrum of collagen dissolved in 0.5 M acetic acid (1 mg/mL) was measured using a spectrophotometer according to the method of Nalinanon et al. [8]. Prior to measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 190–350 nm with a scan speed of 50 nm/min at room temperature.

2.2.6. Differential Scanning Calorimetry (DSC)

DSC analysis of collagen samples was carried out following the methods of Nalinanon et al. [4] with a slight modification. The samples were rehydrated by adding 0.05 M acetic acid to dried samples at a solid/solution ratio of 1:40 (w/v) for 2 days at 4 °C. DSC analysis was performed using a differential scanning calorimeter (Model DSC 7, Norwalk, CT, USA). The collagen solutions (5–10 mg) were accurately weighed into aluminium pans and sealed. The samples were scanned at 1 °C/min over the range of 20–50 °C using iced water as the cooling medium. An empty pan was used as the reference. The maximum transition temperature (T_m) and total denaturation enthalpy (ΔH) were estimated from the DSC thermogram.

3. Results and Discussion

3.1. Yield and Some Characteristics of Skin Collagen

The yield and some characteristics of PSC isolated from the skin of pharaoh cuttlefish is shown in Table 1. As per our preliminary study, the yield of acid-solubilized collagen (ASC) was negligible when 0.5 M acetic acid was only used as extracting solution (data not shown). Pepsin was further used as the aid for collagen extraction. The yield of resultant collagen (PSC) was 43.7% (dry weight). Hydroxyproline content of PSC and CC was 113 and 104 mg/g dry sample, respectively. From UV absorption spectra of both PSC and CC, an absorbance at 232 nm with the highest intensity was observed with no absorption peak at 280 nm (data not shown). The results indicated high efficacy of non-collagenous protein removal in PSC preparation. PSC had higher total sugar content than CC ($P<0.05$). The hydroxylysine-linked carbohydrates may have an impact on the structure of the fibrils in the invertebrate collagen [9].

Table 1: Yield, hydroxyproline content, total sugar content and UV absorption peak of pepsin-solubilzed collagen (PSC) from pharaoh cuttlefish skin and type I collagen from calf skin (CC).†

	PSC	CC
Yield (% dry weight)	43.7 ± 0.90	-
Hydroxyproline content (mg/g dry sample)	113 ± 6.18b‡	104 ± 2.45a
Total sugar content (% dry weight)	3.40 ± 0.17b	1.45 ± 0.20a
UV absorption peak (nm)	232 ± 0.05a	232 ± 0.13a

† Mean ± SD from triplicate determinations.

‡ Different letters in the same row indicate the significant difference ($P<0.05$).

3.2. Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophorsis (SDS-PAGE)

Protein patterns of collagens under reducing condition are shown in Fig. 1. Differences in protein patterns of different collagens were observed. The result revealed that PSC comprised β and α chains as the major constituents. With respect to PSC, It possibly contained both $(\alpha1)_3$ homotrimer and $(\alpha1)_2\alpha2$ or $\alpha1\alpha2\alpha3$ heterotrimer.

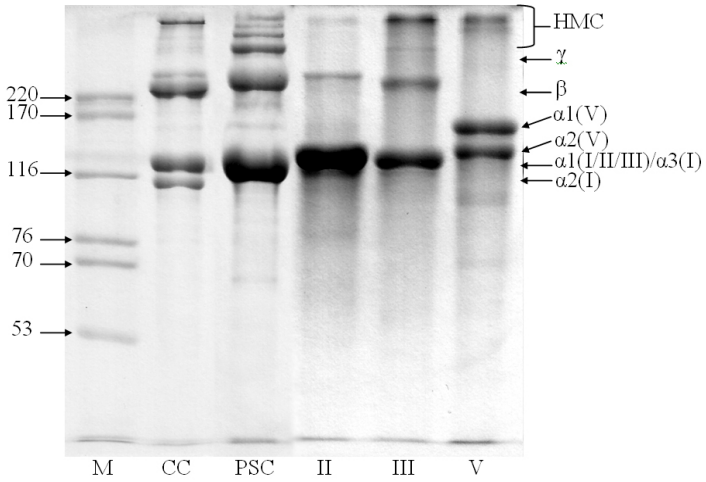

Fig. 1: SDS-PAGE pattern of pepsin solubilized collagen (PSC) from pharaoh cuttlefish skin under reducing condition. HMC, M, CC, II, III and V denote high-MW cross-linked components, MW protein markers, collagen type I, type II, type III and type V, respectively.

3.3. Amino Acid Composition

The amino acid composition, expressed as residues per 1000 total residues, is shown in Table 2. This shows that glycine was the most abundant amino acid in both collagens. PSC had relatively high contents of proline, hydroxyproline and alanine, decreasing in that order. The value of imino acids (proline and hydroxyproline) found in PSC and CC was 196 and 215 residues, respectively.

3.4. Thermal Stability

DSC thermogram of PSC and CC rehydrated in 0.05 M acetic acid are shown in Fig. 2. The maximum transition temperature (T_m) and total denaturation enthalpy (ΔH) of PSC was 34.0 °C and 1.164 J/g,

respectively, which were lower than that of CC (T_m = 40.7 °C; ΔH = 1.204 J/g). This might be owing to the lower content of imino acids (hydroxyproline and proline) of collagen from the skin of pharaoh cuttlefish. The differences in denaturation temperature of collagen from different sources might be determined by different contents of imino acids (proline and hydroxyproline) [2, 3]. Imino acid content shows a direct positive correlation with the thermal stability of protein via the formation of hydrogen bonds, through the hydroxyl group of hydroxyproline [3, 10, 11].

Table 2: Amino acid composition of pepsin solubilized collagen (PSC) from pharaoh cuttlefish skin and type I collagen from calf skin (CC).

Amino acid	PSC	CC
Alanine	83	119
Arginine	60	51
Aspartic acid/asparagine	59	45
Cysteine	0	0
Glutamic acid/glutamine	82	75
Glycine	331	330
Histidine	7	5
Isoleucine	20	11
Leucine	26	23
Lysine	12	26
Hydroxylysine	14	7
Methionine	15	6
Phenylalanine	10	3
Hydroxyproline	96	94
Proline	100	121
Serine	40	39
Threonine	23	18
Tyrosine	4	3
Tryptophan	1	3
Valine	18	21
Total	1000	1000
Imino acids[a]	196	215

[a] Imino acids include proline and hydroxyproline

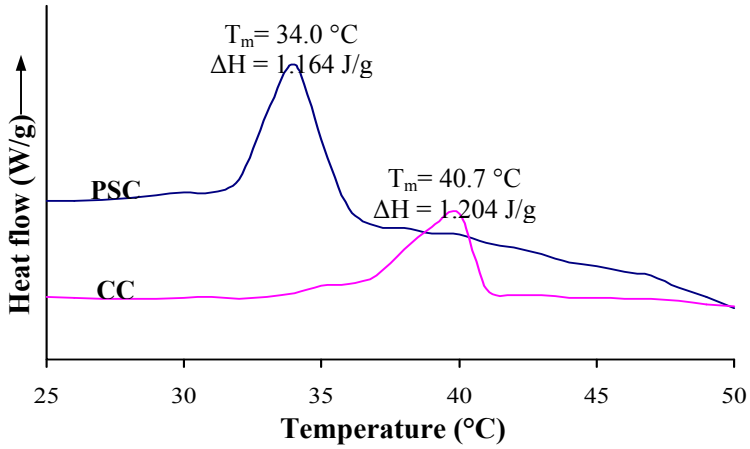

Fig. 2: DSC thermograms of pepsin solubilized collagen (PSC) from pharaoh cuttlefish skin and type I collagen from calf skin (CC) rehydrated in 0.05 M acetic acid.

4. Conclusion

Extraction of collagen from the skin of pharaoh cuttlefish could be achieved by pepsin solubilization under acidic condition. PSC from pharaoh cuttlefish was characterized biochemically and physicochemically.

It contained mixed type collagens that α- and β-chains were the major components. PSC presented high imino acid content (196 residues/1000 residues) and transition temperature (34 °C).

5. Acknowledgements

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6. References

[1] S. Nalinanon, S. Benjakul, H. Kishimura, and K. Osako. Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*): Characteristics and effect of pepsin hydrolysis. *Food Chem.* 2011, **125** (2): 500-507.

[2] M. Ogawa, M.W. Moody, R.J. Portier, J. Bell, M.A. Schexnayder, and J.N. Losso. Biochemical properties of black drum and sheepshead seabream skin collagen. *J. Agric. Food Chem.* 2003, **51** (27): 8088-8092.

[3] A. Jongjareonrak, S. Benjakul, W. Visessanguan, T. Nagai, and M. Tanaka. Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brownstripe red snapper (*Lutjanus vitta*). *Food Chem.* 2005, **93** (3): 475-484.

[4] S. Nalinanon, S. Benjakul, W. Visessanguan, and H. Kishimura. Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). *Food Chem.* 2007, **104** (2): 593-601.

[5] S. Nalinanon, S. Benjakul, W. Visessanguan, and H. Kishimura. Tuna Pepsin: Characteristics and its use for collagen extraction from the skin of threadfin bream (*Nemipterus* spp.). *J. Food Sci.* 2008, **73** (5): C413-C419.

[6] U.K. Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, **227** (5259): 680-685.

[7] E. Fournier. (2005). Basic protocol: Determination of reducing and nonreducing sugars using the phenol-sulfuric acid assay. In: R.E. Wrolstad et al. (eds.), *Handbook of food analytical chemistry*. New Jersey: John Wiley & Sons, Inc. 2005, pp. 653-655.

[8] S. Nalinanon, S. Benjakul, and H. Kishimura. Collagens from the skin of arabesque greenling (*Pleurogrammus azonus*) solubilized with the aid of acetic acid and pepsin from albacore tuna (*Thunnus alalunga*) stomach. *J. Sci. Food Agric.* 2010, **90** (9): 1492-1500.

[9] Z.E. Sikorski, A. KolaKoska, and B.S. Pan. The nutritive composition of the major groups of marine food organisms. In: Z.E. Sikorski (ed.). *Seafood : resources, nutritional composition and preservation*. Florida: CRC Press.1990, pp. 29-54.

[10] Z.E. Sikorski, D.N. Scott, and D.H. Buisson. The role of collagen in the quality and processing of fish. *Crit. Rev. Food Sci. Nutr*. 1984, **20** (4): 301-343.

[11] Y. Zhang, W. Liu, G. Li, B. Shi, Y. Miao, and X. Wu. Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). *Food Chem.* 2007, **103** (3): 906-912.