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Characteristics of Collagen from Nile Tilapia *(Oreochromis niloticus)* Skin Isolated by Two Different Methods

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ABSTRACT

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) isolated from Nile tilapia skin using two different methods were compared for biochemical properties and thermal stability. Method 1 (the Noitup method) was performed at 4-6°C for the whole process with a centrifuge speed of 30,000 g, while Method 2 (the Ogawa method) was performed at a higher temperature of 22-23°C, with a centrifuge speed of 10,000 g. The dry weight yields of ASC from Methods 1 and 2 were 38.84 and 20.70%, while the dry weight yields of PSC were 48.21 and 38.27%, respectively. The denaturation temperatures of ASC from Methods 1 and 2 were 34.29 and 34.43°C, while those of PSC were 34.32 and 34.61°C, respectively. From these amino acid and molecular weight profiles and SDS-PAGE results, ASC and PSC obtained by both methods were characterized as type I collagen with no disulfide bond, which were composed of α-1 (2 chains) α-2 (1 chain), β, and γ subunits. Amino acid profiles of ASC and PSC obtained from both methods were similar. Except for the lower yield obtained from Method 2, there were no differences in the molecular weight, amino acid composition and denaturation temperature between ASC and PSC obtained from both methods.

Key words: collagen, denaturation temperature, fish skin, fish by-product, Nile tilapia

INTRODUCTION

Nile tilapia, a freshwater fish, is widely cultivated and consumed in Thailand. In addition, it is also exported as a whole fish or as fillets. Thus, an abundance of waste from the head, blood, scale, bone and skin is discarded from processing plants and sold at a low value to feed mills or fertilizer plants.

Collagen is the most abundant protein in vertebrates making up approximately 30% of total protein. Collagen is a major component of connective tissue, muscle, teeth, bone and skin. There are 19 types of collagen, labeled I-XIX. Collagen is composed of three similarly sized triple helix polypeptide chains. Each chain contains about 1000 amino acid residues in size and has an average length of 300 nm and a diameter of 1.4 nm. Collagen has a repetitive primary sequence of which every third residue is glycine. The sequence of the polypeptide chain can be described as Gly-X-Y, in which X and Y are often found to be proline and hydroxyproline forming a left-hand super helix with the other two chains (Whitford, 2005).

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Collagen has been used in the biomedical, pharmaceutical (Lee *et al.*, 2001), food and cosmetic industries (Kim and Mendis, 2006; Senaratne *et al.*, 2006).

Most commercial collagens come from bovine skin, pig skin or chicken waste. These land animal sources are unsuitable for many religious and ethnic groups, face regulatory and quality control difficulties and can contain biological contaminants and poisons, such as BSE (Mad Cow Disease), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD). Thus, it is of interest to search for new sources of collagen originating from fish and other seafood (Zhang *et al.*, 2007).

The three major methods of collagen extraction produce neutral salt-solubilized collagen, acid-solubilized collagen and pepsin-solubilized collagen (Ward and Courts, 1977). Many researchers have studied the pepsin-solubilized collagen (PSC) extraction method from different sources, such as from the skin of brownstripe red snapper (Jongjareonrak *et al.*, 2004), fish waste material (Nagai and Suzuki , 2000), albacore tuna and silver-line grunt skin (Noitup *et al.*, 2005), bone and scale of black drum and sheepshead seabream (Ogawa *et al.*, 2004), and black drum and sheepshead seabream skin (Ogawa *et al.*, 2003). Some of the methods are performed at 4°C using a high-speed centrifuge. However, some are performed at a higher temperature and use lower speed centrifugation.

Noitup *et al.* (2005) extracted a high yield of collagen (90%) from silver-line grunt and albacore tuna. This method (hereafter referred to as the Noitup method) was performed at 4-6°C for the whole process with a centrifuge speed of 30 000 g, while Ogawa *et al.* (2003) used a method (hereafter referred to as the Ogawa method) involving a higher temperature of 22-23°C, with a centrifugation speed of 10,000 g. Thus, the Ogawa method seemed to be more convenient for a pilot scale. However, the biochemical properties and thermal stability of collagen extracted by these two methods might be different. Therefore, the objective of this study was to compare the biochemical and physical properties of collagen extracted by these two methods in order to select the appropriate method for extraction of collagen from Nile tilapia skin to be used as a moisturizing cream ingredient.

MATERIALS AND METHODS

Preparation of acid and pepsin-solubilized collagen by the Noitup method

Skin of Nile tilapia was used as the raw material. The remaining meat and scales were removed and the skin was cut into small pieces (0.5 × 0.5 cm) with scissors. The cleaned skin was suspended in 20 volumes of 0.1 N NaOH (pH 12) and stirred for 4 h to remove non-collagenous proteins. The skin was washed thoroughly with cold distilled water (temperature not greater than 10°C) until a neutral pH was obtained and then it was lyophilized according to the method of Nagai and Suzuki (2000) and stored at low temperature (4-6°C) until use.

To produce acid-solubilized collagen, the prepared sample was extracted with 70 volumes of 0.5 M acetic acid for 24 h at 4-6°C and centrifuged at 30,000 × g for 60 min. The supernatant was collected, while the precipitate was re-extracted two times. Then, it was salted out by adding NaCl to 0.9 M and centrifuged at 30,000 × g for 60 m. The precipitate was dissolved in 20 volumes of 0.5 M acetic acid, then dialyzed against 0.1M acetic acid and deionized water in a dialysis membrane (spectra/ por mwco 12-14,000 RC membrane, Spectrum Laboratories, Rancho Dominguez, CA, USA.) and lyophilized. The dialyzate was referred to as ASC.

To produce pepsin-solubilized collagen, the prepared sample was extracted with 70 volumes of 0.5 M acetic acid for 24 h at 4-6°C and centrifuged at 30,000 × g for 60 min. The supernatant was collected and 0.1% pepsin added with 6 h incubation to remove telopeptides. Then, it was salted out by adding NaCl to 0.9 M and centrifuged at 30,000 × g for 60 min. The precipitate was dissolved in 20 volumes of 0.5 M acetic acid, then dialyzed against 0.1 M acetic acid and deionized water in a dialysis membrane. The dialyzate was referred as PSC. The yield of lyophilized collagen, calculated from the lyophilized extracted collagen was compared with the lyophilized fish skin sample.

Preparation of acid and pepsin-solubilized collagen by the Ogawa method

All procedures to prepare the acid and pepsin-solubilized collagen were carried out at 22-23°C except for centrifuging that was performed at 4°C. The skin was de-scaled and cut into small pieces (3 cm) with scissors. The pieces were suspended in 10 volumes of 0.1 M NaOH and the suspension was stirred overnight with a magnetic stirrer. After decanting, the skin pieces were re-suspended in 20 volumes of 0.1 M. NaOH solution with stirring for 24 h. The alkaline-insoluble components were stained through a cheesecloth and rinsed with distilled water repeatedly until a neutral pH was obtained.

To produce acid-solubilized collagen, the insoluble components were extracted with 10 volumes of 0.5 M acetic acid for three days. The resulting viscous solution was centrifuged at 10,000 g for 20 min at 4°C. The residue was re-extracted with 10 volumes of 0.5 M acetic acid for three days and the extract was centrifuged again. The supernatants of the two extracts were combined and were salted out by adding NaCl to give a final concentration of 0.9 M. After standing overnight, the precipitate was collected by centrifuging at 10,000 g for 20 min. Then, it was dissolved in 10 volumes of 0.5 M acetic acid. The salting out and solubilization procedures were repeated three times. The resultant solution was dialyzed against 0.1 M acetic acid in a dialysis membrane (spectra/ por mwco 12-14,000 RC membrane, Spectrum Laboratories, Rancho Dominguez, CA, USA.) and lyophilized. The dialyzate was referred to as ASC.

To produce pepsin-solubilized collagen, the insoluble components after neutralization were solubilized with 10 volumes of 0.5 M acetic acid containing 0.1% (w/v) pepsin for three days and subsequent steps duplicated the previous ASC extraction. The dialyzate was referred to as PSC. The yield of lyophilized collagen calculated from the lyophilized extracted collagen was compared with the lyophilized fish skin sample.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE technique was performed on the collagen samples to compare protein patterns according to the method of Laemmli (1970). Samples were solubilized with 5% SDS to a concentration of 5 μg/ μl. The amount of protein applied on the gel was 25 μg. SDS-PAGE was performed on 6-7.5% separating gel with a 4% stacking gel, using an electrophoresis buffer of tris-glycine containing 0.1% SDS (pH 8.3). After electrophoresis, protein bands were stained with Coomassie brilliant blue R-250 (Bio-Rad Richmond, CA., USA). The molecular weights of the protein bands were determined by comparing their mobility with molecular weight markers ranging from 36-205 kDa.

Amino acid composition

The collagen samples were analyzed for amino acid composition by the AOAC method (AOAC, 1995).

Thermal analysis

The thermal analysis was determined by a differential scanning calorimeter (DSC) (Netzsch DSC 200 PC, Germany). Lyophilized collagen samples were dissolved in 0.05 M acetic acid to give a 3% solution according to the method of Rose and Mandal (1996) and sealed in a DSC cell. The cell was held at 4-6°C for 16-20 h to obtain equilibrium, then DSC was carried out under a heating rate of 1°C/min. The characteristic temperatures of the onset (To), the peak (Tp) and the recovery (Tr) in each DSC curve were recorded.

RESULTS AND DISCUSSION

Acid-solubilized collagens (ASC) extracted from Nile tilapia by the Noitup and Ogawa methods had dry weight yields of 38.84 and 20.70%. These yields were higher than those extracted from: ocellate puffer, 10.70% (Nagai *et al*., 2002); black drum, 2.30% and sheepshead sea bream, 2.60% (Ogawa *et al*., 2003); and grass carp, 8.00% (Zhang *et al*., 2007). However they were lower than from: Japanese sea bass, 51.40%; chub mackerel, 49.80%; bull head shark, 50.10% (Nagai and Suzuki, 2000); and adult nile perch, 58.70% (Muyonga *et al*., 2004).

The dry weight yield of pepsin-solubilized collagens (PSC) extracted from Nile tilapia by the Noitup and Ogawa methods was 48.21 and 38.27%, respectively. PSC extracted from either method was lower than from: albacore tuna, 67.00%; silver-line grunt, 90.00% (Noitup *et al*., 2005); brown backed toadfish, 54.30% (Senaratne *et al*., 2006); bigeye snapper, 53.68% (Nalinanon *et al*., 2007), and grass carp, 46.60% (Zhang *et al*., 2007). However, they were higher than from: black drum, 15.80%; sheepshead seabream, 29.30% (Ogawa *et al*., 2003); and skate, 25.60% (Hwang *et al*., 2007) as shown in Table 1.

The differences in yields between the Ogawa and Noitup methods could be attributed to the fact that in the samples prepared by the Noitup method, the fish skins were lyophilized to remove water and obtain a higher protein concentration. The ratio of skin to 0.5 M acetic acid in the Noitoup method was 1:70, while in the Ogawa method it was 1:40. For PSC extraction, 0.1% pepsin was added to the supernatant obtained after ASC extraction, while 0.1% pepsin was added at the first step of the Ogawa method. Subsequently, the pepsin acted with a higher efficiency when digested in the supernatant without any precipitate matter. Furthermore, the Noitup method was performed with a high centrifuge speed (30,000 g, 60 min), whereas the sample preparation in the Ogawa method used fresh fish skin and was performed at a lower centrifuge speed (10,000 g, 20 min). Therefore, the dry weight yields of ASC and PSC obtained by the Noitup method were higher than the yields of the Ogawa method. The differences in the extraction steps are shown in Table 2.

Table 1 Amount of pepsin-solubilized collagen.

PSC	Dry weight (%)	Method
Blackdrum	15.80	Ogawa *et al*. (2003)
Sheephead seabream	29.30	Ogawa *et al*. (2003)
Albacore tuna	67.00	Noitup *et al*. (2005)
Silver line grunt	90.00	Noitup *et al*. (2005)
Brown backed toadfish	54.30	Senaratne *et al*. (2006)
Skate	25.60	Hwang *et al*. (2007)
Big eye snapper	53.68	Nalinanon *et al*. (2007)
Grass carp	46.60	Zhang *et al*. (2007)
Nile tilapia by Ogawa method	38.27	
Nile tilapia by Noitup method	48.21	

Table 2 Differences between the extraction methods used by the Noitup and Ogawa methods.

Method	Raw material	Extraction			Centrifugation	
		Temperature	Time	Number	Speed	Time
Noitup *et al*. (2005)	Lyophilized skin	4-6°C	30 h	3	30,000 g	60 min
Ogawa *et al*. (2003)	Fresh skin	22-23°C	72 h	2	10,000 g	20 min

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The SDA-PAGE pattern of extracted collagens is shown in Figure 1.

Nile tilapia ASC extracted by the Noitup and Ogawa methods had at least two different α chains: α-1 and α-2. The molecular weight of the α-1 chain from the two methods was 117.13 and 116.45 kDa, while the α-2 chain values were 105.51, and 106.90 kDa, respectively. The PSC extracted by both methods also had at least two different α chains: α-1 and α-2. The α-1 chain molecular weight from the two methods was 114.50 and 113.50 kDa, respectively, with the molecular weight for the α-2 chain being 103.75, and 104.62 kDa, respectively. The calf-skin type I collagen had two different α chains (α-1 and α-2) with a molecular weight of 116.45 and 109.38 kDa, respectively.

These results indicated that ASC and PSC extracted by these methods could be characterized as type I collagen which contained α-1 2 chains and α-2 1 chains with reference to the standard acid-soluble type I calf-skin collagen. These results were similar to the studies of Nagai and Suzuki (2000), Yata *et al*. (2001), Ogawa *et al*. (2003), Muyonga *et al*. (2004), Noitup *et al*. (2005), Senaratne *et al*. (2006), Hwang *et al*.

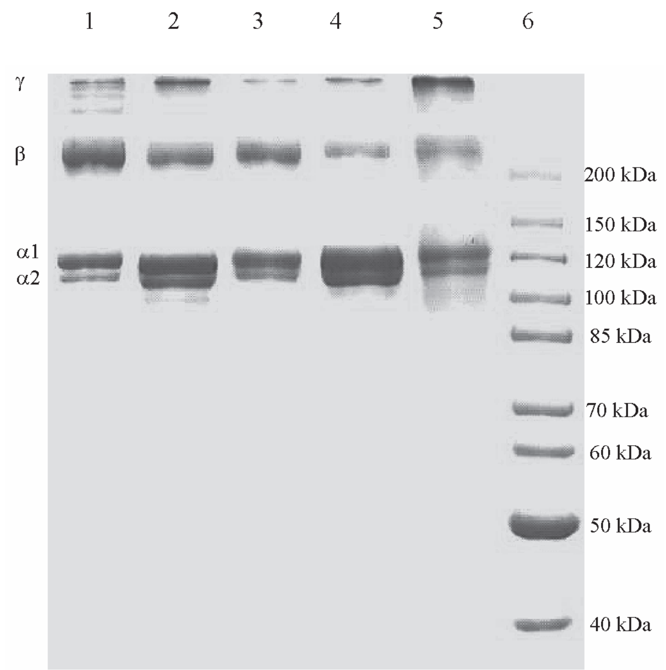

Figure 1 SDS-PAGE patterns of collagens
(1) ASC/Noitup; (2) PSC/Noitup; (3) ASC/Ogawa; (4) PSC/Ogawa; (5) standard acid-soluble type I calf-skin collagen (Sigma); (6) molecular weight protein marker.

(2007) and Zhang *et al*. (2007).

The band intensity ratio of cross-linked chains (β or γ) to total non-cross-linked monomer chains (α1+α2) on the SDS-PAGE is shown in Table 3.

From Table 3, the band intensity ratio of the cross-linking chain (β+γ) to total non-cross-linking monomer chains (α1+α2) of ASC and PSC based on the Noitup method were 0.64 and 0.39, and γ/(α1+α2) ratios were 0.17 and 0.14.

ASC and PSC based on the Ogawa method had a β/(α1+α2) ratio of 0.48 and 0.11, with the γ/(α1+α2) ratio being 0.18 and 0.10, respectively.

The β and γ, intra – and/or inter molecular cross-linking of ASC from both methods were higher than for PSC. Cross-linking containing telopeptides was removed by the pepsin and the β chain was converted to non cross-linking monomer chains (α-1 + α-2) (Ogawa *et al*., 2003). Thus, these cross-linking molecules generally caused a decrease in solubility of the collagen (Jonjareonrak, 2006). Therefore, the smaller band intensity ratio indicated that the purity of PSC was higher than for ASC. These band intensity ratios were in accordance with the studies of Ogawa *et al*. (2003) and Noitup *et al*. (2005).

Amino acid composition

Table 4 shows the amino acid composition of the extracted collagens.

Table 4 shows that the highest yielding amino acid in ASC from the Noitup and Ogawa methods was glycine at 25.15 and 25.32%, followed by proline at 11.00 and 11.36%. Cysteine was not found in any sample. As with ASC, the most abundant amino acid in PSC from both methods was glycine at 25.66 and 25.36 % and the second was proline at 11.14 and 11.31 %. Again, cysteine was not found. The amino acid profiles expressed were consistent with others reported in Ogawa *et al*. (2003); Jongjareonrak *et al*. (2004); Noitup *et al*. (2005); Senaratne *et al*. (2006); and Zhang *et al*. (2007).

The tyrosine and histidine contents in PSC from both methods were less than their contents in ASC, which was explained by the fact that PSC extraction was a modified process of ASC using pepsin to hydrolyze the telopeptide bond in ASC. When the telopeptide bond was degraded, tyrosine and histidine located near the telopeptide zone were also degraded leading to a decline of both amino acids levels in PSC (Montero *et al*., 1990). Other supporting evidence was the specificity of pepsin to break down the tyrosine-histidine bond (Belitz *et al*., 2004). These results were consistent with those of other investigators (Jongjareonrak *et al*., 2004; Noitup *et al*., 2005).

The amino acids, proline and hydroxyproline, from ASC extracted by the Noitup and Ogawa methods had yields of 194.00 and 198.00 g/ 1000 g, respectively. With PSC from both methods, the corresponding yields were 196.80 and 198.40 g/ 1000 g, respectively. The amino acid content was correlated in the same direction as the denaturation temperature; if the extractives were rich in amino acids, the denaturation temperature was high too. The amino acids have a tropocollagen triple-helix structure and can form a strong hydrogen bond with surrounding molecules leading to increased collagen heat stability and a higher denaturation temperature (Belitz *et al*., 2004; Zhang *et al*., 2007).

Table 3 Band intensity ratio.

	ASC Noitup	PSC Noitup	ASC Ogawa	PSC Ogawa
β/(a1+a2)	0.64	0.39	0.48	0.48
γ/(a1+a2)	0.17	0.14	0.18	0.10

Denaturation temperature

The denaturation temperature of calf skin and extracted collagens is shown in Table 5.

As shown in Table 5, the Tp denaturation temperature of ASC extracted by the Ogawa and Noitup and methods was 34.43 and 34.29°C, respectively, while the Tp of PSC from both methods was 34.61 and 34.32°C, respectively. In the PSC process, pepsin hydrolyzed the telopeptide bond with intermolecular crosslinks but had no

Table 4 Amino acid composition of collagens from Nile tilapia skin isolated by the Ogawa and Noitup methods (g / 1000 g protein).

Amino acid	Ogawa		Noitup		Calfskin
	ASC	PSC	ASC	PSC	
Hydroxyproline	84.40	85.30	84.00	85.40	87.80
Aspartic acid	58.20	58.60	59.70	60.10	46.40
Serine	32.80	31.00	31.10	25.50	31.30
Glutamic acid	93.60	99.10	96.10	96.80	74.10
Glycine	253.20	253.60	251.50	256.60	334.80
Histidine	10.10	7.30	7.80	5.80	4.10
Arginine	83.60	85.20	82.30	95.90	57.40
Threonine	28.00	26.20	25.80	28.30	17.60
Alanine	103.50	108.50	104.40	104.41	112.70
Proline	113.60	113.10	110.00	111.40	118.80
Tyrosine	3.80	2.50	5.30	2.00	4.10
Valine	18.30	16.50	18.90	17.20	22.30
Hydroxylysine	19.40	17.20	16.20	14.60	4.60
Isoleucine	10.00	10.60	13.40	9.40	12.00
Leucine	26.50	22.60	27.30	23.10	25.10
Lysine	30.00	35.10	34.90	37.00	27.00
Phenylalanine	18.50	15.20	17.50	12.30	13.60
Methionine	12.50	12.10	13.80	14.50	6.40
Cysteine	0.00	0.00	0.00	0.00	0.00
Total	1,000.00	1,000.00	1,000.00	1,000.00	1,000.00

ASC= acid-soluble collagen; PSC= pepsin-soluble collagen; Calfskin= standard acid soluble type I calf-skin collagen.

Table 5 Thermal denaturation of collagens from Nile tilapia skin isolated by the Ogawa and Noitup methods.

Collagen	Denaturation temperature (°C)			
	To	Tp	Tr	H (J/g)
Calf-skin collagen	34.70	40.80	42.80	1.50
ASC-N	33.43	34.29	40.96	0.50
PSC-N	32.27	34.32	41.09	0.35
ASC-O	33.53	34.43	38.13	1.37
PSC-O	33.40	34.61	40.86	0.37

ASC-N= acid-soluble collagen isolated by the Noitup method; PSC-N= pepsin-soluble collagen isolated by the Noitup method; ASC-O= acid-soluble collagen isolated by the Ogawa method; PSC-O= pepsin-soluble collagen isolated by the Ogawa method; To= onset temperature; Tp= peak temperature; Tr= recovery temperature; H= enthalpy of melting.

effect on the triple-helix, a basic structure of both collagens. Hence, both ASC and PSC had similar denaturation temperatures, which were accordingly correlated with the amino acid profile (Hickman *et al*., 2000).

The amino acid contents of the fish skin were less than those of bovine collagen (206.60 g /1000g), which was correlated to the lower denaturation temperature of fish collagen compared to bovine collagen. Li *et al*. (2004) reported that collagen from land animals had a higher denaturation temperature than that from fish because of its higher amino acid content.

The ASC and PSC from Nile tilapia had a higher denaturation temperature compared with the other fish varieties reported (Ciarlo *et al*., 1997, Nagai and Suzuki, 2000, Nagai *et al*., 2002, Sadowska *et al*., 2003, Senaratne *et al*., 2006, Zhang *et al*., 2007).

In addition, for aquatic animals, habitat and body temperature affected the denaturation temperature of collagen (Belitz *et al*., 2004; Zhang *et al*., 2007).

Jongjareonrak (2006) reported that three factors affected the collagen functional properties: aging and the living period of fish samples; the processing steps for raw fish; and the pH and NaCl concentrations during the collagen extraction step. In the current study, both methods used Nile tilapia fish skin, and had controlled pH and NaCl concentrations. Kolodziejska *et al*. (2008) reported that temperature correlated with the collagen extraction yield. The solubilization of collagen increased according to increasing temperature and completely solubilized at 45°C. Moreover, the extraction temperature of the Ogawa and Noitup methods was 22-23 and 4°C, respectively, which was lower than the denaturation temperature shown in Table 5.

CONCLUSION

ASC and PSC from Nile tilapia skin isolated by the method of Ogawa *et al*. (2003) and Noitup *et al*. (2005) were characterized as type I collagen. Their molecular weights, amino acid profiles and denaturation temperatures indicated there was no difference between them. It was thus concluded that both methods provided the same quality of collagen.

Finally, the Ogawa method was considered a more convenient procedure than the Noitup method. In the Ogawa method, fresh fish skin was used and the temperature was controlled (≤4°C) only in the centrifugation step with a lower centrifuge speed (10,000g). Contrarily, the Noitup method recommended lyophilized fish skin and high-speed centrifugation (30,000g), as well as a controlled temperature (4-6°C) during the whole process. Despite its lower yield, the Ogawa method was selected for collagen extraction as an ingredient in moisturizing cream ingredient for a subsequent experiment.

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