

Isolation and Characterization of Collagen from Skin of Bullfrog, *Rana catesbeiana* Shaw

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In order to utilize skin of bullfrog (*Rana catesbeiana* Shaw) as an alternative source of collagen, we investigated and compared biochemical and physical properties of collagens isolated from bullfrog skin. Two kinds of collagen (BSASC; bullfrog skin acid-soluble collagen and BSPSC; bullfrog skin pepsin-solubilized collagen) were isolated by subsequent treatments with acetic acid and pepsin. The amounts of skin collagen isolated in the subsequent treatments were 7.3% BSASC and 18.2% BSPSC on the basis of lyophilized bullfrog skin weight, respectively. According to the electrophoretic pattern and CM-cellulose column chromatogram, the BSASC has the chain composition of $\alpha 1\alpha 2\alpha 3$ heterotrimer, and the BSPSC consists of two α chains of $\alpha 1\alpha 2$. In addition, the denaturation temperatures of all collagens tested were ranged from 30°C to 38°C. This study suggests that there is a possibility to use bullfrog skin collagen as an alternative source of collagen for industrial purposes, and subsequently it may increase the economical value of the bullfrog.

Key words: Collagen, Bullfrog skin, Isolation, Characterization

Introduction

Collagen exists in nearly all organs of vertebrates, and is the major structural element of skin, bone, tendon, cartilage, blood vessels and teeth. It is the most abundant and ubiquitous protein in the body of vertebrates. The feature of a typical collagen molecule includes a long, stiff, triplestranded helix, in which three collagen polypeptide chains are wound around one another in form of a ropelike superhelix. Each polypeptide chain forms a lefthanded helix and consists of repeating triplet, (Gly-X-Y)_n, where X and Y have a high possibility to be proline or hydroxylproline. In addition, collagen is unique in possessing different levels of structural order: primary, secondary, tertiary, and quaternary. Nearly 15 types of collagen have been reported up to date. Type I collagen has a helical structure which is composed of three polypeptide chains; two identical chains, termed $\alpha 1$ (I) and another chain, termed $\alpha 2$ (I).

Each of the three chains consists of 1,000 amino acid residues with an approximate molecular weight of 100,000 Da. It is a natural material with good biological compatibility and well characterized for low antigenicity. It can also be incorporated into aqueous bases for enhanced cellular penetration and wound repair. Therefore, collagen has attracted great interest as a biomaterial for medical uses such as drug delivery, tissue engineering (Keiji et al., 1998; Shanmugasundaram et al 2001; Wolfgang, 1998; Willoughby et al., 2002) as well as for the production of cosmetics due to its good moisturizing property. Moreover, in food industry, collagen has been utilized to produce edible casings, which are needed in the meat packing for sausages, salami, snack sticks, etc. and as dietary fibers in nutraceutical industry (Bailey and Light, 1989; Cavallaro et al., 1994; Hood, 1987; Slade and Levine, 1987; Stainsby, 1987). However, highly infectious and contagious diseases like bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) in landbased animals have limited the use of collagen derived from

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cattle and pigs for industrial applications as there is a possibility to be transmitted those diseases from animals to human being via animal tissues (Helcke, 2000). In addition, the collagen extracted from pigs can not be used due to religious barriers in several nations. Therefore, many researches have been focused on alternative sources of collagen derived from cattle and pigs.

Bullfrogs of the genus *Rana* (Ranidae), an extensive group of approximately 250 extant species of amphibians, have proved to be particularly rich sources of collagen so that bullfrog skin has a potential as an important source of collagen without the threat of BSE and TSE. In the present study, we isolated and characterized two kinds of collagen extracted from skin of bullfrog, *Rana catesbeiana* Shaw. Furthermore, we compared the characterization of two collagens from skin of bullfrog to that of cattle or fish collagens by investigating biochemical and physical properties of bullfrog collagen in order to use bullfrog skin collagen as an alternative source of collagen for industrial purposes and subsequently it may increase the economical value of the bullfrog.

Materials and Methods

Materials

Bullfrog (*Rana catesbeiana* Shaw) was collected from ponds in Yangsan, Korea. The skin was rapidly separated from bullfrog and rinsed with deionized water to eliminate contaminants under -4°C , and then stored at -20°C until use. Pepsin (EC 3.4.23.1, 3085 U/mg protein), p -dimethylaminobenzaldehyde, collagen type I (from porcine) and Achromopeptidase from *Achromobacter llyticus* (EC 3.4.21.50; 4.5 amide activity/mg protein) were purchased from Wako Pure Chemicals industries Tokyo, Japan). All other reagents used in this study were reagent grade chemicals, and all procedures to isolate collagen were carried out at 4°C .

Isolation of the collagen from muscle

Bullfrog skin was cut into small pieces with scissors. The muscle was suspended in 10 volumes (w/v) of 0.1 M NaOH, and the suspension was stirred overnight. Each solution was re-suspended in 20 volumes of 0.1 M NaOH solution with stirring for 24 h. The alkaline insoluble components were strained through cheesecloth, and rinsed with distilled water repeatedly until a neutral pH was obtained. The insoluble matter was extracted with 10 volumes of 0.5 M acetic acid for 3 days, and the resulting viscous solution was centrifuged at $12,000\times g$ for 1 h. The residue was re-extracted with 10 volumes of 0.5 M

acetic acid for 3 days, and the extracts were centrifuged at the same speed again. The supernatants were salted out by adding NaCl at the final concentration of 0.7 M. After standing overnight, the resulting precipitate was collected by centrifuging at $12,000\times g$ for 1 h. The precipitate was dissolved in 10 volumes of 0.5 M acetic acid. Salting out and solubilization procedures were repeated 3 times. The resultant solution was dialyzed against 0.1 M acetic acid, and lyophilized for further experiments. The lyophilized collagen was named as BSASC, which is bullfrog skin acidsoluble collagen. In addition, insoluble residues from bullfrog muscle were also lyophilized for preparing pepsinsolubilized collagen. The lyophilizates were suspended in 0.5 M acetic acid, and digested with 10% (w/v) pepsin for 48 h. The pepsinsolubilized collagens were centrifuged at $12,000\times g$ for 1 h, and the supernatants were dialyzed against 0.02 M Na_2HPO_4 (pH 7.2) for 3 days. After dialysis, the resultant was dissolved in 0.5 M acetic acid, and was salted out by adding NaCl to a final concentration of 1.0 M. The precipitate was dissolved in 0.5 M acetic acid and, dialyzed against 0.1 M acetic acid, and then lyophilized. The lyophilizate was named as BSPSC, which is bullfrog skin pepsinsolubilized collagen.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). The collagen samples were dissolved in 0.02 M phosphate buffer (pH 7.2) containing 1% SDS and 3.5 M urea. Electrophoresis was performed on 6% gels in 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS. Each gel was visualized with Coomassie Brilliant Blue R250 dissolved in water, methanol and trichloroacetic acid (5:4:1, v/v/v) and destained using a solution containing methanol, distilled water and acetic acid (5:4:1, v/v/v).

CM-cellulose column chromatography

To investigate each subunit composition of each collagen sample, the samples were applied to a CM-cellulose column chromatography. Collagen samples were dissolved in 20 mM sodium acetate buffer (pH: 4.8) containing 6 M urea at 4°C , and then incubated at 45°C for 30 min. The solution was centrifuged at $20,000\times g$ for 1 h and the supernatant was applied to a CMcellulose column (1.5 \times 15 cm) previously equilibrated with the same buffer. Each subunit was eluted with a linear gradient of 0-0.1 M NaCl in the same buffer at a flow rate of 0.4 ml/min. The subunit component was detected by absorbance at 230 nm,

and the fractions indicated by the numbers were examined by SDS-PAGE.

Peptide mapping

Each collagen sample (0.2 mg) was dissolved in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5% SDS, and heated at 100°C for 5 min. The digestion was carried out at 37°C for 30 min by adding 5 μ L of lysyl endopeptidase from *Achromobacter lyticus* (EC 3.4.21.50; 4.5 amidase activity/mg protein; Wako, Osaka, Japan). After adding SDS to a final concentration of 2%, the proteolysis was stopped by boiling for 5 min. SDS-PAGE was performed by the method of Laemmli (1970) using 12.5% gel.

Determination of denaturation temperature

The denaturation temperature (Td) was determined by the method of Kimura et al (1981). Five milliliters of 0.03% collagen solution in 0.1 M acetic acid was used for the viscosity measurement. The flow rates used as an index for calculation of reduced viscosities were average of five observations. The relation is described as follows: relative viscosity (η_{rel}) = flow time of sample/flow time of control (0.1 M acetic acid); specific viscosity (η_{sp}) = η_{rel} - 1. The denaturation temperature was taken as the mid point of the linear portion of the sigmoidal curve obtained by plotting η_{sp} at t°C against various temperatures.

UV-Vis spectra

The UV-Vis adsorption spectra of collagens from bullfrog skin were recorded by a Cary 1-C UV-Visible spectrophotometer (Varian Inc., Australia). Data collection and plotting were accomplished by the UVPC program supplied by the manufacturer.

Determination of amino acid composition

Amino acids in isolated collagens were determined by hydrolyzing collagen with 6 N HCl at 110°C for 24 h using amino acid auto-analyzer (Biochrom 20; Pharmacia Biotech Ltd, Cambridge, UK). Amino acid composition was determined by derivatization with ninhydrin and measuring absorbance at 570 nm except for proline and hydroxyproline, for which absorbance at 440 nm was measured. The amino acid content was expressed by the number of residues per 1,000 residues.

Results and Discussion

Isolation of collagen from bullfrog skin

The bullfrog skin was hardly solubilized with the treatment of 0.5 M acetic acid. The yield of acid-soluble collagen isolated from bullfrog skin (BSASC)

was about 7.3%, on the basis of lyophilized dry weight. That value was similar to the results, 5.2% of collagen on the basis of dry weight that was isolated from nautilus outer skin (Pitchumani et al., 2000), and 3.6% and 2% collagens that were produced from wing muscle of skate (Yamashita et al., 1975) and cuttlefish skin (Nagai et al., 2001), respectively. After the acid treatment, the insoluble residue in 0.5 M acetic acid was subsequently treated with 10% (w/v) pepsin. The yield of pepsinsolubilized collagen from bullfrog muscle (BSPSC) was 18.2% (on the dry weight basis). This result was lower compared to those from edible jellyfish exumbrella (46.4%) (Nagai et al., 1999), rhizostomous jellyfish mesogloea (35.2%) (Nagai et al., 2000), cuttlefish outer skin (35.0%) (Nagai and Suzuki, 2002) and brown backed toadfish skin (Senaratne et al., 2006), respectively. As shown in Fig. 1, two collagens (BSASC, and BSPSC) isolated from the bullfrog skin were mainly composed of two different α chains, α_1 and α_2 . Moreover, a small amount of the β chain was also contained in each collagen. Nagai and Suzuki (2002) reported the isolation and characterization of acidsoluble collagen and pepsinsoluble collagen from paper nautilus (*Argonauta argo*, *Linnaeus*) outer skin with two chains. Kimura et al. (1988) have prepared collagen from carp scale and have reported its properties. Carp scale collagen had three different α_1 , α_2 and α_3 , giving a heterotrimer with a chain composition of $\alpha_1\alpha_2\alpha_3$.

Subunit composition of bullfrog skin collagen

After chemical and thermal treatments with dissolution in 20 mM sodium acetate buffer, pH: 4.8,

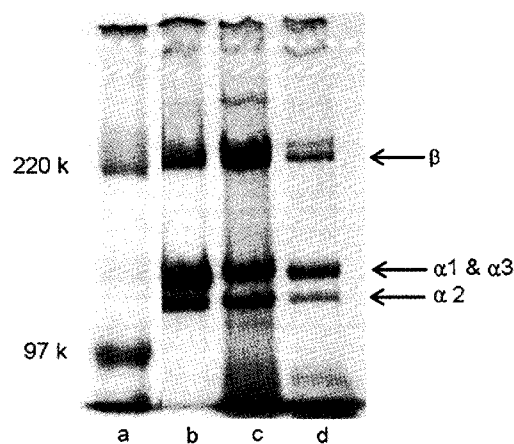


Fig. 1. SDS-Polyacrylamide gel electrophoresis patterns of collagens isolated from bullfrog skin. Lane a, molecular weight markers, Lane b, porcine skin collagen; Lane c, BSASC; Lane d, BSPSC

containing 6 M urea at 4°C followed by incubation at 45°C for 30 min, the denatured BSASC was applied to a carboxymethyl cellulose (CM-cellulose) column chromatography, and it was resolved into two main peaks corresponding to α subunits disentangled from the collagen fiber (Fig. 2A). To visualize α subunits, the several chromatographic fractions, as indicated by the numbers were analyzed by SDS-PAGE. These results suggested that BSASC consists of three α chains. The $\alpha 1$ and $\alpha 3$ chains were found in number 1, a $\alpha 2$ chain was found in numbers 2 and 3. This result suggests that this collagen is a heterotrimer with a chain composition of $\alpha 1\alpha 2\alpha 3$. Nagai and Suzuki (2002) reported that the acidsolubilized collagen from paper nautilus (*Argonauta argo*, Linnaeus) outer skin had a chain composition of $\alpha 1\alpha 2\alpha 3$ heterotrimer. The BSPSC was also applied to CM-cellulose column chromatography, and it was separated into three peaks, two having large peaks and a small peak containing a α chain as a major component (Fig. 3B). This result suggests that the collagen consists of two α chains, alpha 1 and alpha 2. Kimura (1988) reported that the $\alpha 3$ chain was widely distributed in fish skin collagen. In addition, many researcher have reported that the $\alpha 3$ chain was detected in 14 fish species of 17 teleosts (Kimura et al., 1985, 1987, 1987, 1988; Piez, 1965). Therefore, it seems that the $\alpha 3$ chain is widely distributed not only in the fish skin, but also in the skin of amphibians.

Peptide mapping

To compare the patterns of peptide fragments with BSASC and BSPSC, the collagen hydrolysates digested by lysyl endopeptidase were migrated on 12.5% SDS-polyacrylamide gel. The electrophoretic patterns of BSASC (Fig. 3) were similar to those of BSPSC and porcine skin collagen (data not shown).

Denaturation temperature

The thermal denaturation curves are shown in Fig 4. The Td of BSASC and BSPSC were about 33.8°C and 38.0°C, respectively. These results indicate that the Td of BSASC was lower than that of BSPSC. In addition, the Td of bullfrog muscle collagens was similar to that of porcine skin collagen (37°C) (Kimura et al., 1981), but higher than collagens originated from other marine organisms. For examples, the Td of collagen obtained from paper nautilus outer skin was 27.0°C (Nagai and Suzuki, 2002), and that of collagens investigated in fish species such as sardine, red sea beam, Japanese sea bass, and cod skin were 28.5, 28.0, 28.0 and 25.0°C respectively (Nagai et al., 2004; Kim et al., 1993). These results can be attri-

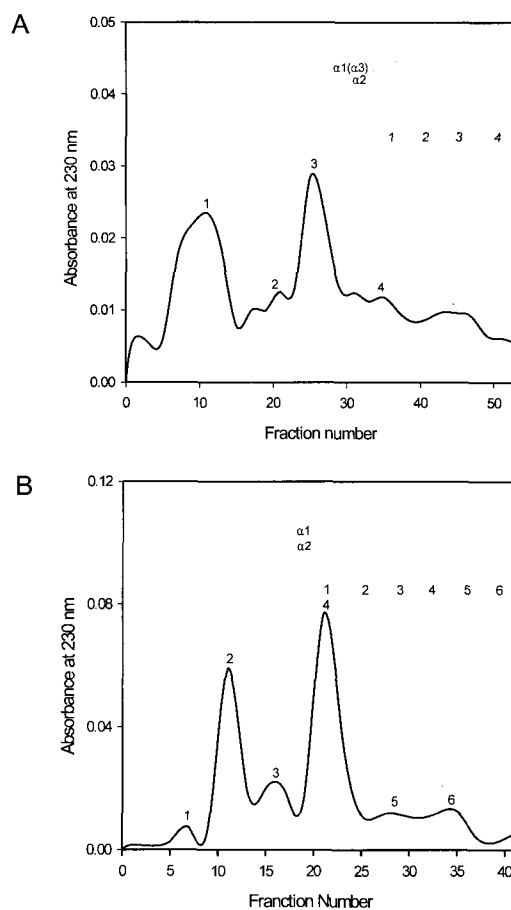


Fig. 2. CM-Cellulose column chromatography of denatured collagen samples. A, BSASC; B, BSPSC. A 1.0×15 cm column of CM-cellulose was equilibrated with 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea, and maintained at 4°C. The collagen sample (15 mg) was dissolved in 5 ml of the same buffer, denatured at 60°C for 15 min, and then eluted from the column with a linear gradient of 0-0.15 M NaCl at a flow rate of 0.4 ml/min. The fractions indicated were examined by SDS-PAGE as shown in the inset.

buted to the physiological and biochemical nature of the amphibian. The Td is proportional to the content of hydroxylproline (Table 1), which might play a singular role in the stabilization of the triplestranded collagen helix due to the ability of formation of hydrogen bonds through hydroxyl group (Nagai et al., 2004).

UV-Vis spectra

Fig. 5 shows UV-Vis spectra of collagens isolated from skin of bullfrog. It is generally known that tyrosine and phenylalanine are sensitive chromophores, which absorb light below 300 nm. As a result,



Fig. 3. Peptide mapping of lysyl endopeptidase digests from collagen samples. A, BSASC; B, BSPSC.

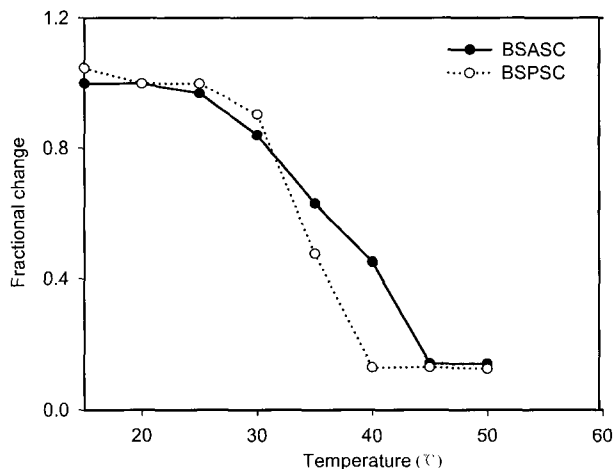


Fig. 4. Thermal denaturation curve of collagens isolated from bullfrog skin. BSASC, acid-soluble collagen; BSPSC, pepsin-solubilized collagen.

both BSASC and BSPSC only contain one peak at 224 nm (Fig. 5A) and 226 nm (Fig. 5B), respectively. Porcine skin collagens, however, contain two peaks at 229 nm and 276 nm, respectively (data not shown), because the tyrosine and phenylalanine from BSASC and BSPSC showed lower level than those from the porcine skin collagen.

Amino acid composition

Amino acid composition is expressed as residues per 1,000 total amino acid residues as shown in Table

Table 1. Amino acid composition of collagens isolated from bullfrog skin (Residues/ 1,000 residues)

Amino acid	Skin	
	BSASC	BSPSC
Hydroxyproline	93	90
Aspartic acid	48	45
Threonine	6	6
Serine	3	2
Glutamic acid	73	71
Proline	89	86
Glycine	428	435
Alanine	90	89
Cystine	1	1
Valine	21	22
Methionine	15	15
Isoleucine	16	17
Leucine	25	28
Tyrosine	1	1
Phenylalanine	10	10
Histidine	15	16
Lysine	32	32
Arginine	34	34
Total	1000	1000

1. Amino acid profiles of acidsoluble collagens (BSASC) and pepsinsolubilized collagens (BSPSC) were almost similar to each other. The two collagens composed of glycine as the major amino acid and that was about 42.8% and 43.5% of total amino acids, respectively. Because glycine represents approximately one third of the total residues of amino acids and could be found as the every third residue in collagen molecules except for the first 10 amino acid residues from the N-terminus and the first 10 amino acid residues from the C-terminus of the collagen molecules (Senaratne et al., 2006; Foegeding et al., 1996). In addition, two collagens isolated from bullfrog skin were rich in proline, glycine, hydroxylproline, alanine, but poor in threonine, serine, cysteine, and tyrosine. The dehydroxylation degrees of hydroxylproline in BSASC and BSPSC were 51.0%, and 51.1%, respectively. In previous studies (Nagai et al., 2001; Marie-Madeleine et al., 2000; Senaratne et al., 2006; Mizuts et al 2002), it was reported that the degrees of hydroxylation of proline were measured as follows: ocellate puffer fish (39.4%), purple sea urchin test (48.0%), brown backed toadfish skin (45.3%) and muscle layer of the skate (51.3%). The results elucidated that the degree of Hydroxyl lation in collagens originated from fish species is greatly lower than 78% of calf skin collagen (Madeleine et al., 2000), which suggests that the cross linking and stability of bullfrog skin collagens are similar. Moreover, hydroxylation of

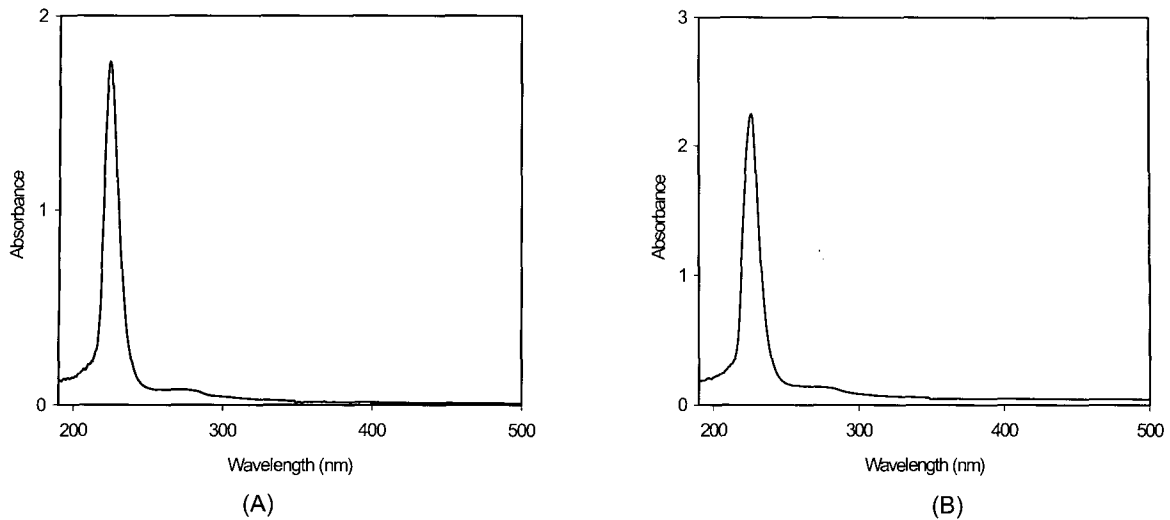


Fig. 5. UV-Vis spectra of collagens isolated from bullfrog skin. A, BSASC; B, BSPSC.

proline and lysine are important for the thermal stability of collagen since hydroxyproline stabilizes the triple helix of collagen (Ramachandran, 1988). Bullfrog skin collagens have cystine residues, while calf skin collagen (Madeleine et al., 2000), ocellate puffer fish skin collagen (Nagai et al., 2002) and wing muscle collagen (Mizuts et al., 2002) have none. Also the amino acid composition indicates that bullfrog skin collagens, BSASC and BSPSC have 48 and 45 aspartic acid residues.

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