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Studies on bullfrog skin collagen

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Abstract

Pepsin-soluble collagens were prepared from bullfrog skin and partially characterized. This study revealed interesting differences in the frog skin collagen when compared with known vertebrate collagens. This may be attributed to the position of the amphibians in the vertebrate hierarchy. Therefore, detailed investigations on various physicochemical properties, such as molecular weight, amino acid composition, denaturation temperature, UV–Vis and IR spectra of bullfrog skin collagen were carried out. The study confirms the structural relationship of collagen to habitat and function. Moreover bullfrog skin has potential as an alternative source of collagen to calf skin and bone for use in various fields.

Keywords: Bullfrog skin; Collagen; Amphibian; Denaturation temperature

1. Introduction

Collagen is the most abundant and ubiquitous protein in the body of vertebrates. The term 'collagen' is generally applied to a series of related, yet chemically distinct, macromolecular species.

Collagen is a natural material with good biological compatibility and well characterized low antigenicity. It can be degraded into physiologically well tolerated compounds. Also, it can also be processed in aqueous base for enhanced cellular penetration and wound repair. Therefore, collagen has attracted great interest as a biomaterial for medical use, such as drug delivery, and tissue engineering (Keiji, Miho, Takami, & Akihiko, 1998; Shanmugasundaram, Ravichandran, Neelakanta Reddy, Nalini, Subrata, & Panduranga Rao, 2001; Wolfgang, 1998; Willoughby et al., 2002).

Collagen is the predominant protein in the living body. The main sources of industrial collagen are limited to those from calf skin and bone. There is active discussion of its role in BSE (bovine spongiform encephalopathy) or TSE (transmissible spongiform encephalopathy). The risk of contamination has to be evaluated on a case by case basis. So, an alternative safe collagen should be sought. In this paper, bullfrog skin was used to extract collagen. Bullfrog is an amphibian, it poses no threat of BSE and can be taken as a safe collagen source. The skin of bullfrog contains a large quantity of collagen so that bullfrog skin has potential as an important source of collagen. The preparation and characterization of collagen from bullfrog skin are therefore described.

2. Methods and materials

2.1. Extraction of bullfrog skin collagen

Collagen from bullfrog skin was prepared according to the method of Epstein (1974). In brief, 40 g fresh bullfrog skin sample were denuded and the subcutaneous tissue and some fat were eliminated. After rinsing with water, it was cut into parts and triturated. Then it was rinsed with 5% NaCl and acetone, respectively, to remove some soluble substances and fat. After rinsing with water, it was put into 250 ml 0.5 M acetic acid, and 0.1 g pepsin was added. The mixture solution was stirred intermittently for 8 h at a temperature below 4 °C. The ropy collagen solute was filtered by gauze. The pH value of filtrate was

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adjusted to 7–8. Collagen was collected by centrifugation. Then collagen sediment was dissolved in 0.5 M acetic acid. The above dissolution–precipitation procedure was repeated twice and pure collagen sediment was preserved in 0.5 M acetic acid. The yield of bullfrog skin collagen was determined based on the dry weight.

2.2. SDS–polyacylamide gel electrophoresis (SDS–PAGE)

Protein samples were analyzed by SDS–PAGE according to Laemmli (1970) using 5% resolving gels in an electrophoresis instrument (Eastern Teli Science and Trade Centre) at 25 mA/gel. Protein bands were stained with Coomassie brilliant Blue R250.

2.3. Amino acid analysis

Pepsin-soluble and denatured collagen samples extracted from bullfrog skin were hydrolysed in 6 N hydrochloric acid at 110 °C for 22 h with the usual protection for cysteine, methionine and tyrosine. The hydrolysates were analysed on a Hitachi 835-50 amino acid analyser.

2.4. Determination of denaturation temperature

The method of Pitchumani, Lonchin, and Gowri (2001) was used. The denaturation temperature and intrinsic viscosity of bullfrog skin collagen were determined by the measurement of reduced viscosity of collagen solutions in 0.5 M acetic acid at concentration of 0.1%, at various temperatures. The flow rates, used as an index for calculation of reduced viscosity, were the average of three observations. The relationship is described as follows: relative viscosity $= \eta_{rel} =$ flow time of sample/flow time of control (0.5 M acetic acid); specific viscosity $= \eta_{sp} = \eta_{rel} - 1$.

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 temperatures.

2.5. UV–Vis spectra

The UV–Vis adsorption spectra of bullfrog skin collagen were recorded by a Hitachi spectrophotometer (model U-2010). Data collection and plotting were accomplished by the UVPC programme supplied by the manufacture.

2.6. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) of the pepsin-soluble collagen was recorded using FTIR (Nicolet 200SXV) spectroscopy.

3. Results and discussion

3.1. Collagen

The bullfrog skin was not completely solubilized with 0.5 M acetic acid. The collagen of bullfrog skin was easily solubilized by limited pepsin proteolysis. This was similar to those of edible jellyfish (Nagai et al., 1999). The yield of pepsin-soluble collagen was 12.6% on a dry weight basis. It appears that a large amount of collagen can be obtained from bullfrog.

3.2. Electrophoresis

The pepsin-soluble bullfrog skin collagen was analysed by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 1). The electrophoretic profiles of bullfrog skin collagen were significantly different from those of pig species (Lane 2), showing that high molecular weight components were scarce. Pepsin-soluble bullfrog skin collagen(lane 1) showed only a single Á band, $Á_1$. It may be that during the digestion of pepsin, the high molecular weight components were degraded. It also means that bullfrog skin collagen is more easily degraded by pepsin than other known collagens. A great amount of the Á chain was obtained in pepsin-soluble bullfrog skin collagen.

3.3. Amino acid composition

The amino acid composition of pepsin-soluble collagen of bullfrog skin, compared with calf skin collagen

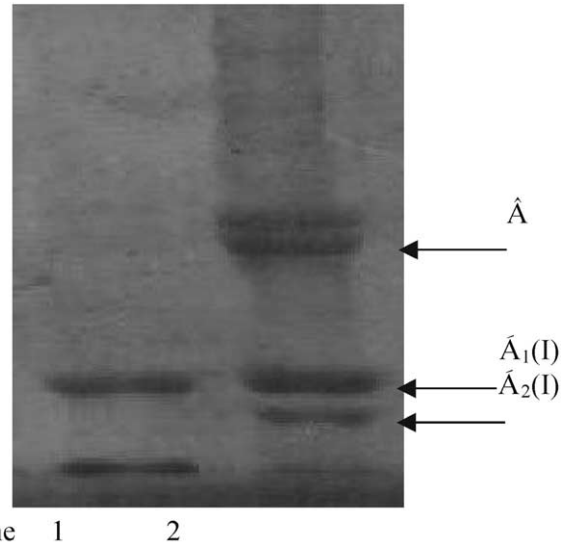

Fig. 1. Electrophoretic analysis of the bullfrog collagen and denatured collagen (gelatin) preparation. Lane 1, pepsin-soluble bullfrog skin collagen; Lane 2, pig skin collagen. Pig skin collagen was used as mobility markers for ↑-chains and ↓-chains.

Table 1
Amino acid compositions of the pepsin-soluble of bullfrog skin, compared with calf skin collagen (results are expressed as residues/1000 residues)

Amino acid	Bullfrog skin collagen	Calf skin collagen
Hydroxyproline	54	94
Glycine	300	330
Hydroxylysine	11	7
Aspartic acid	53	45
Leucine	27	23
Threonine	23	18
Tyrosine	5	3
Serine	53	39
Phenylalanine	18	3
Glutamic acid	80	75
Lysine	33	26
Alanine	119	119
Histidine	6	5
Cystine	8	–
Valine	25	21
Arginine	56	50
Methionine	5	6
Proline	113	121
Isoleucine	11	11

(Marie-Madeleine, Laurence, Christine, Patrick, & Daniel, 2000), is presented in Table 1. Glycine was the most abundant amino acid in pepsin-soluble bullfrog skin collagen. Glycine accounted for about 30% of all amino acids in this collagen with approximately 300 residues, which is lower than that of calf skin collagen. As shown in the Table, bullfrog skin collagen has cystine residues, while calf skin collagen has none. The amino acid composition also indicates that the number of phenylalanine residues (18) of bullfrog skin collagen is more than that (3) of calf skin collagen. Hydroxyproline is derived from proline by post-translational hydroxylation mediated by prolylhydroxylase. The proline to hydroxyproline ratio (0.48) of bullfrog skin collagen was far below that (0.78) of calf skin collagen, which suggests that the crosslinking and stability of bullfrog collagen are lower. The degree of hydroxylation of lysine residues in the bullfrog skin collagen was a little higher than that of calf collagen, reflecting a slightly higher amount of carbohydrate than that of the former.

3.4. Denaturation temperature

Thermal denaturation profile of bullfrog has provided useful clues to the thermal stability of collagen in relation to enviroment and amino acid content. The denaturation temperature (T_d) of pepsin-soluble bullfrog skin collagen was calculated from the thermal denaturation curve. It was calculated that T_d of pepsin-soluble bullfrog skin collagen was 30.3 °C (Fig. 2). This

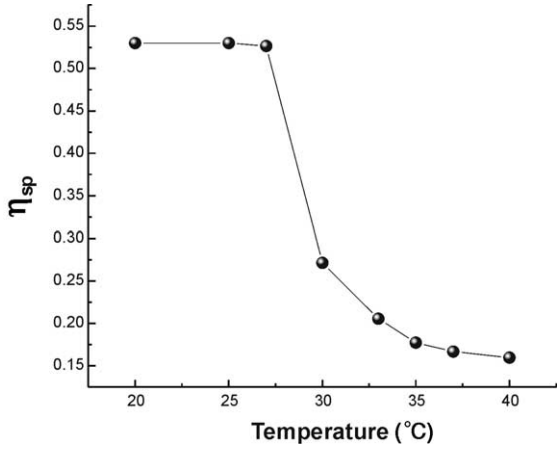

Fig. 2. Thermal denaturation curve of pepsin-soluble bullfrog skin collagen solution, measured by viscosity in 0.5 M acetic acid. The incubation time at each temperature was 30 min. Collagen concentration: 0.1%.

was about 6 °C lower than that of pig skin collagen (Takeshi, Yoko, & Nobutaka, 2002), which can be attributed to the nature of the amphibian. The denaturation temperature is proportional to the hydroxyproline content (Doty & Nishihara, 1957). The denaturation temperature is known to increase with an increase of amino acid residues. Hydroxyproline is believed to play a singular role in the stabilization of the triple-stranded collagen helix due to its hydrogen bonding ability through its –OH group (Burjandze, 1979). According to the amino acid analysis of bullfrog skin collagen, the content of hydroxyproline is 5.36%, far below that of pig skin collagen (~10%).

3.5. UV–Vis spectra

It is generally known that tyrosine and phenylalanine are sensitive chromophores, which absorb light below

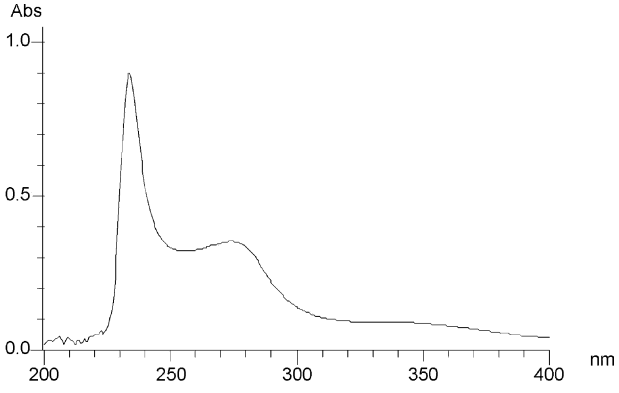

Fig. 3. UV–Vis spectra of pepsin-soluble collagen.

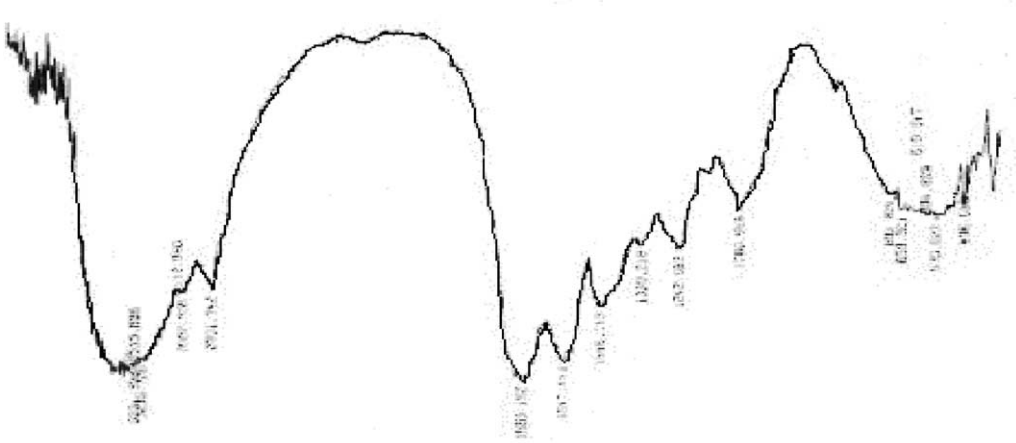

Fig. 4. Fourier transform infrared spectrum of pepsin-soluble bullfrog skin collagen. (The unit of abscissa is cm^{-1}).

300 nm. As can be seen from the UV–Vis spectra, pepsin-soluble collagen has adsorption near 236 nm and 280 nm (Fig. 3).

3.6. Fourier transform infrared spectroscopy

Fig. 4. shows the FTIR spectra of pepsin-soluble bullfrog skin collagen. The amide A band is associated with the N–H stretching frequency. A free N–H stretching vibration occurs in the range of 3400–3440 cm^{-1}, and when the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequency, usually 3300 cm^{-1}. The amide A band of bullfrog skin collagen was found at 3335 cm^{-1}, suggesting its involvement in hydrogen bonding.

The amide I band position of bullfrog skin collagen was found at 1653 cm^{-1}, fitting well the range 1650–1655 cm^{-1} for other collagens.

The helical structure of the collagen was confirmed from the IR absorption ratio between the 1235 cm^{-1} (amide III) and 1450 cm^{-1} bands, which was approximately equal to one for all preparations (Plepis, Goissis, & Das Gupta, 1996).

4. Conclusion

In conclusion, a great quantity of collagen could be prepared from bullfrog skin by a pepsin treatment process. The bullfrog is one of the most delicious of foods so that the amount of bullfrog consumed is great. However, the bullfrog skin has always been regarded as waste without any utilization, resulting in environment pollution. Moreover bullfrog lives in water that it poses no threat of BSE. For these reasons, bullfrog skin has potential as an alternative source of collagen to calf and pig skin and bone. This study is only a preliminary report of work for exploiting collagen from underutilized resources.

Acknowledgements

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References

Burjandze, T. V. (1979). Hydroxyproline content and location in relation to collagen thermal stability. *Biopolymers*, *18*, 931–936.

Doty, P., & Nishihara, T.. In G. Stainsby (Ed.), *Recent advances in gelatin and glue research* (pp. 72). London: Pergamon Press.

Epstein, E. H. (1974). [$\alpha I(III)_3$ human skin collagen. Release by pepsin digestion and preponderance in fetal life. *J. Biol. Chem.*, *249*, 3225–3231.

Keiji, F., Miho, M., Takami, H., & Akihiko, S. (1998). Protein release from collagen matrices. *Advance Drug Delivery Reviews*, *31*, 247–266.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.

Marie-Madeleine, G. G., Laurence, B., Christine, C., Patrick, D., & Daniel, H. (2000). Structural aspects of fish skin collagen which forms ordered arrays via liquid crystalline states. *Biomaterials*, *21*, 899–906.

Nagai, T., Ogawa, T., Nakamura, T., Ito, T., Nakagawa, H., Fujiki, K., Nakao, M., & Yano, T. (1999). Collagen of edible jellyfish exumbrella. *Journal of the Science of Food & Agriculture*, *79*, 855–858.

Pitchumani, S., Lonchin, S., & Gowri, C. (2000). Molecular species of collagen in the intramuscular connective tissues of the marine crab, Scylla serrata. *Comparative Biochemistry & Physiology Part B*, *125*, 555–562.

Plepis, A. M. D. G., Goissis, G., & Das Gupta, D. K. (1996). Dielectric and pyroelectric characterization of anionic and native collagen. *Polymer Eng. Sci.*, *36*, 2932–2938.

Shanmugasundaram, N., Ravichandran, P., Neelakanta Reddy, P., Nalini, R., Subrata, P., & Panduranga Rao, K. (2001). Collagen-chistan polymeric scaffolds for the in vitro culture of human epidermoid carcinoma cells. *Biomaterials*, *22*, 1943–1951.

Takeshi, N., Yoko, A., & Nobutaka, S. (2002). Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chemistry*, *78*, 173–177.

Willoughby, C. E., Batterbury, M., & Kaye, S. B. (2002). Collagen Corneal Shields. *Survey of Ophthalmology*, *47*, 174–182.

Wolfgang, F. (1998). Collagen—biomaterial for drug delivery. *European J. Pharm. Biopharm*, *45*, 113–136.