

*Full Length Research Paper*

# Type I collagen from bullfrog (*Rana catesbeiana*) fallopian tube

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**Pepsin-soluble collagen (PSC) was extracted from the fallopian tube of bullfrog (*Rana catesbeiana*) with a yield of 16.4%, on a dry weight basis. Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) showed that the PSC contained two alpha components ( $\alpha 1$  and  $\alpha 2$ ) and was classified as type I collagen. PSC was found to contain 19.5% of amino acids. PSC exhibited a maximum absorbance at 230 nm, but little absorbance near to 280 nm. The denaturation temperature for PSC was determined to be 29.6°C. These results suggest that bullfrog fallopian tube has potential as a supplement to the sources of vertebrate collagens.**

**Key words:** Bullfrog (*Rana catesbeiana*), pepsin-soluble collagen, type I collagen.

## INTRODUCTION

Collagen is the most abundant protein of animal origin. It has been utilized in food, cosmetics and biomedical materials (Kittiphattanabawon et al., 2005). More than 21 types of collagen have so far been identified in various tissues and their roles have been investigated (Gelse et al., 2003) and type I collagen is the major protein, comprising approximately 90% of total collagen (Nakamura et al., 2003). The main sources of industrial collagen are

limited to mammals (for example, bovine and pig) and birds (for example, chicken).

Bullfrog, *Rana catesbeiana*, is one of the commercially important frog species in the world. Bullfrog is also an important commercial meat in Taiwan, especially in Pingtung County. Therefore, a larger amount of bullfrog fallopian tube is dumped as processing waste; on the other hand, bullfrog possesses no threat of bovine spongiform encephalopathy (Yan et al., 2008; Muyonga et al., 2004) and foot-and-mouth disease and can be taken as a safe collagen source.

The bullfrog skin collagen was characterized (Li et al., 2004); however, little is known about the collagen of bullfrog fallopian tube. For making effective usage of the dumped bullfrog fallopian tube and avoiding environmental pollution, the aim of this study was to investigate the extraction and characterization of collagen from the fallopian tube of bullfrogs.

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**Abbreviations:** PSC, Pepsin-soluble collagen; SDS-PAGE, sodium dodecyl sulphate polyacrylamide-gel electrophoresis; CBB, coomassie brilliant blue R-250; FTIR, fourier transform infrared spectroscopy.

## MATERIALS AND METHODS

### Sample preparation

Bullfrog fallopian tube was provided by the Jia-Young Seafoods Co., a commercial bullfrog breeding and processing industry at Pingtung (Taiwan). After gutting, it was immediately stored at  $-18^{\circ}\text{C}$  and transported to the laboratory. The frozen fallopian tubes were thawed under  $4^{\circ}\text{C}$  for 1 day for use. All other reagents used were of analytical grade.

### Extraction of collagen

All procedures were performed at  $4^{\circ}\text{C}$ , as previously described (Jongjareonrak et al., 2005) with some modification. In brief, the minced fallopian tubes were soaked in 0.1 M NaOH for 1 day with gentle stirring to remove non-collagenous materials effectively and centrifuged at  $20,000 \times g$  for 10 min. The alkali-treated fallopian tubes were then thoroughly rinsed with distilled water until a neutral pH was reached. Fat was removed in 10% butyl alcohol for 1 day with gentle stirring. Defatted fallopian tubes were centrifuged at  $20,000 \times g$  for 10 min, thoroughly rinsed with distilled water and then, lyophilized. The freeze-dried sample was soaked in 0.5 M acetic acid containing 10% pepsin for 2 day. Supernatants were collected after centrifuging at  $20,000 \times g$  for 1 h and was then salted out by adding NaCl to a final concentration of 0.7 M and centrifuged with the same condition again. The resultant pellet was re-dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid for 1 day (1:15, v/v, changed every 4 h), distilled water for 2 day (1:15, v/v, changed every 4 h) and then lyophilized. The yield of pepsin-soluble collagen (PSC) from bullfrog fallopian was calculated based on the dry weight.

### Ultraviolet-visible spectroscopy (UV-vis) spectra

PSC sample was dissolved in 0.5 M acetic acid to obtain a concentration of 2 g/l. The UV-vis adsorption spectra of bullfrog fallopian tube collagen were recorded by a Hitachi spectrophotometer UV-2010 (Hitachi, Tokyo, Japan) from 200 to 400 nm and a scanning speed of 200 nm/min.

### Amino acid composition

The hydrolysis of collagen was carried out according to the method of Simpson et al. (1976). In brief, 0.2 mg of collagen was hydrolyzed in vacuum at  $115^{\circ}\text{C}$  for 22 h with 1.0 ml of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole. Following hydrolysis, the hydrolysates were partially neutralized with 1.0 ml of 3.5 N NaOH and filtered through 0.45  $\mu\text{m}$  disposable syringe filters for amino acid analysis. High performance liquid chromatography was performed using an Agilent 1100 liquid chromatograph equipped with G1312A binary pump and G1315A diode array detector. The analytical column was a reverse phase Zorbax eclipse-AAA,  $150 \times 4.6$  mm with a packing material of 5  $\mu\text{m}$  particle size. Mobile phase A was 40 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.8); mobile phase B was acetonitrile, methanol and pure water in a ratio of 45:45:10 and at a flow rate of 2 ml/min. The gradients of mobile A and B were according to the suggestion of Agilent 1100 (No. 5980-1193E, Agilent, Andover, MA, USA). Retention time and spectrum was compared with that of the standard (Agilent, PN 5061-3330).

### Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE)

Protein sample was performed by SDS-PAGE according to

Laemmli (1970), using a 7.5% resolving gel and 4.5% stacking gel. Protein samples was applied and subjected to electrophoresis. A calf skin acid-soluble collagen (Sigma C-9791, Sigma Chemical Co., St Louis, MO, USA) regard as a marker to contrast with the sample. Electrophoresis was conducted at a content of 25 mV for 4 h. Protein bands were stained using 2.5% Coomassie brilliant blue R-250 (CBB) (2.5 g CBB was dissolved in 400 ml of methanol, 100 ml of glacial acetic acid and then made up to 1000 ml with distilled water) and distained using a solution containing methanol and glacial acetic acid in a ratio of 5:1.

### Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) spectra were obtained by an infrared spectrophotometer (model IR300, Thermo Mattson, USA). The spectra were recorded from  $4000$  to  $700$   $\text{cm}^{-1}$  at a data acquisition rate of  $4$   $\text{cm}^{-1}$  per point.

### Determination of denaturation temperature

The denaturation temperature was measured from the viscosity changes according to the method of Kittiphattanabawon et al. (2005) with a slight modification. Five hundred milliliters of 0.03% collagen solution in 0.1 M acetic acid were subjected to viscosity measurements using a Brookfield synchroelectric viscometer (model DV II+, Brookfield Eng Labs Inc., Stoughton, MA, USA.) with spindle no. 3 and speed of 300 rpm. The thermal determination curve was obtained by measuring solution viscosity at several temperatures from 4 to  $50^{\circ}\text{C}$  with a heating rate of  $4^{\circ}\text{C}/\text{min}$ . At the designated temperature, the solution was maintained for 30 min prior to viscosity determination. Measurement was carried out in triplicate. The relative viscosity was calculated in comparison to that obtained at  $4^{\circ}\text{C}$ .

## RESULTS

### Collagen

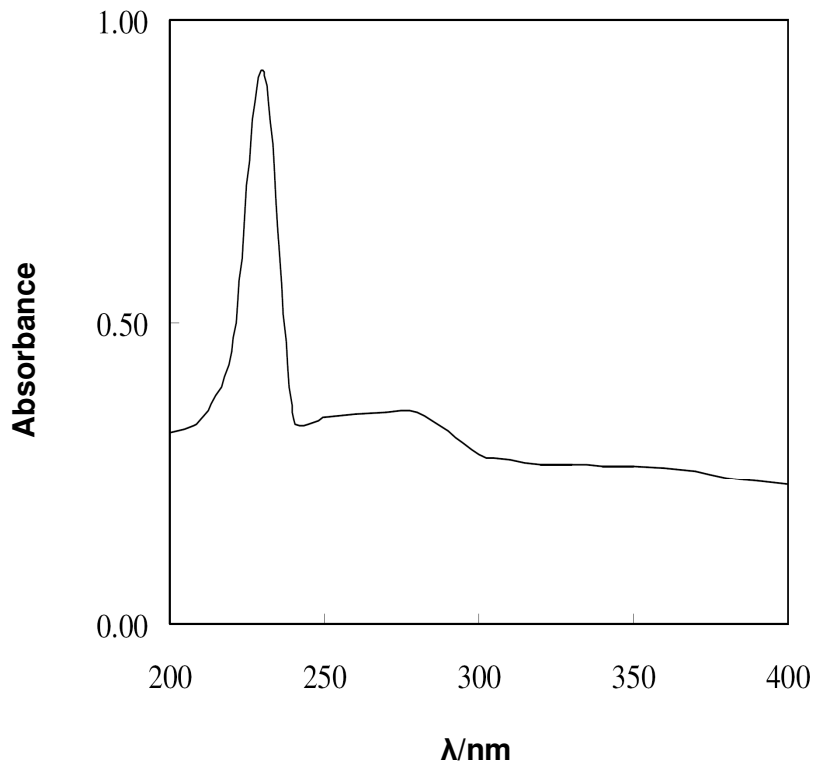
The PSC from bullfrog fallopian tube was easily solubilized by limited pepsin proteolysis. The yield of PSC was 16.4% on a dry weight basis.

### UV-vis spectra

As can be seen from the UV-Vis spectra (Figure 1), the distinct absorbance of the collagen was obtained near 230 nm. There is absorbance at 220 to 230 nm, with little or no absorbance near 280 nm.

### Amino acid composition

The amino acid composition is shown in Table 1. This shows that glycine was the most abundant amino acid in bullfrog fallopian collagen and that there were relatively high contents of proline, alanine and glutamic acid, decreasing in that order. Glycine accounted for more than 30% of all amino acids in this collagen.



**Figure 1.** UV-vis spectra of pepsin-soluble collagen from bullfrog fallopian tube.

**Table 1.** Amino acid composition and content of pepsin-soluble collagen from bullfrog fallopian tube.

Amino acid	Content (g/100 g protein)
Hydroxyproline	7.2
Aspartic acid	5.1
Threonine	2.4
Serine	4.8
Glutamic acid	7.3
Proline	12.3
Glycine	31.2
Alanine	10.5
Valine	2.4
Methionine	0.6
Isoleucine	1.1
Leucine	2.4
Tyrosine	0.3
Phenylalanine	1.5
Hydroxylysine	0.8
Lysine	3.4
Histidine	0.7
Arginine	6.0

## SDS-PAGE

The acid-soluble collagen from bullfrog fallopian tube was

examined by SDS-PAGE (Figure 2). This collagen consisted of  $\alpha$  chains ( $\alpha 1$  about 138 kDa and  $\alpha 2$  chain about 115 kDa), which showed two distinct species varying in their mobility and their dimer ( $\beta$  chain) and small amounts of  $\gamma$  components were also found.

## FTIR

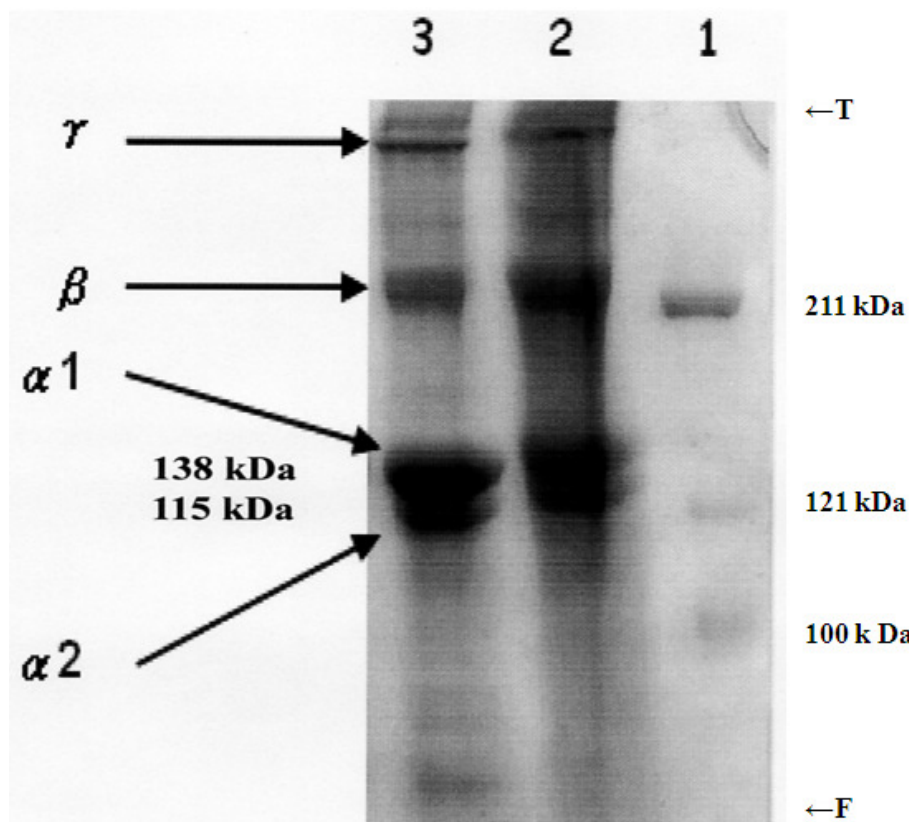
Figure 3 shows the FTIR spectra of the PSC from bullfrog fallopian tube. The amide I band position was observed at  $1654\text{ cm}^{-1}$ , which is the absorption band of C=O stretching which is associated with the secondary structure of the protein. The absorption between the  $1233\text{ cm}^{-1}$  (amide III) and  $1462\text{ cm}^{-1}$  band was observed.

## Thermal stability

Figure 4 shows the thermal denaturation curve of PSC from bullfrog fallopian tube. The denaturation temperature ( $T_d$ ) of bullfrog fallopian tube collagen was calculated from the thermal denaturation curve. It was calculated that  $T_d$  of bullfrog fallopian tube collagen was about  $29.6^\circ\text{C}$ .

## DISCUSSION

The PSC yield of bullfrog fallopian tube was lower than



**Figure 2.** SDS-PAGE patterns of collagen from bullfrog fallopian tube. Lane 1, Protein markers; Lane 2, type I collagen; Lane 3, Collagen of bullfrog fallopian tubes; T, gel top and F, buffer front.

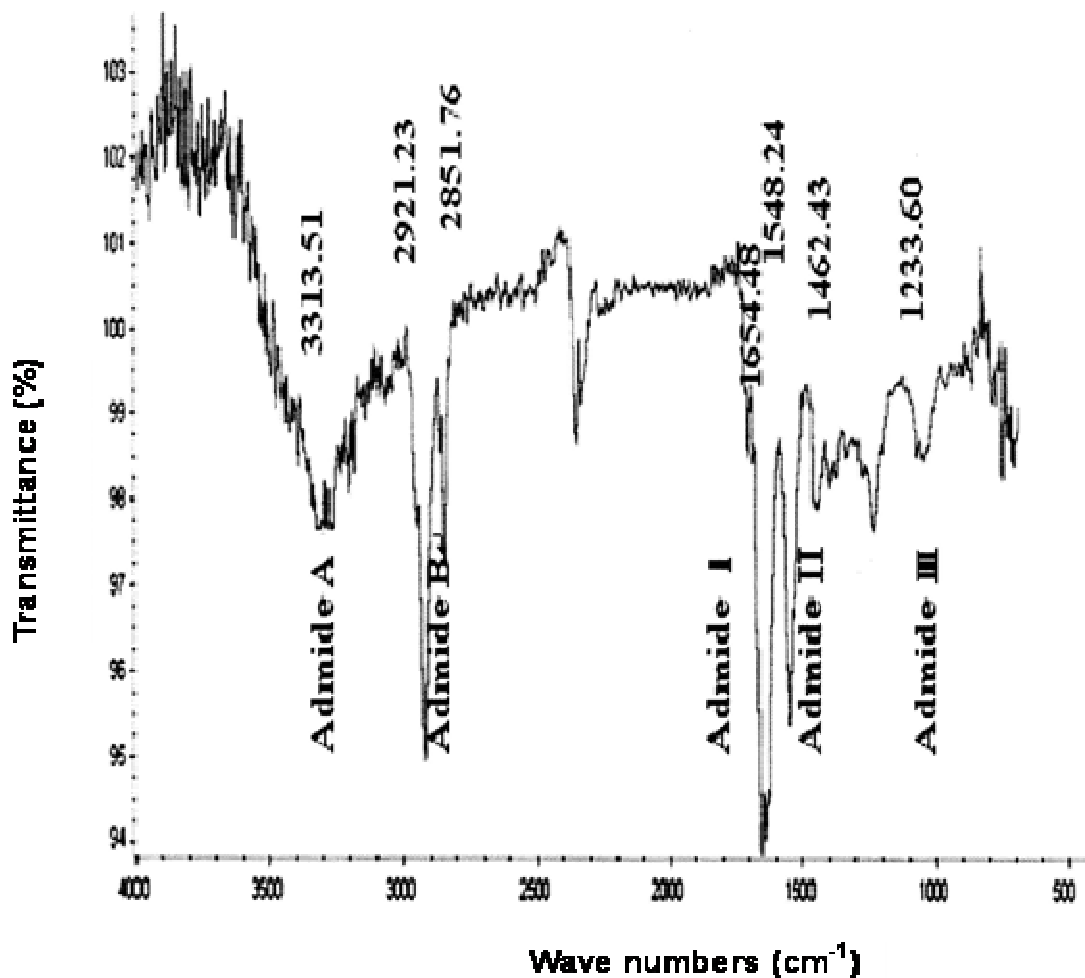
43.5% of horse mackerel bone (Kimura et al., 1988) and 38.4% of channel catfish skin (Liu et al., 2007), but higher than 12.6% of bullfrog skin (Li et al., 2004) and 4.7% of brownstripe red snapper (Jongjareonrak et al., 2005). It appears that a large amount of collagen can be obtained from bullfrog.

Generally, tyrosine and phenylalanine are sensitive chromophores and absorb UV light at 283 and 251 nm (Lin and Liu, 2006), where PSC has no evident absorbance. Therefore, PSC from bullfrog fallopian tube well supports the property of collagen that there is absorbance at 220 to 230 nm, with little or no absorbance near 280 nm. The results indicated high efficacy of non-collagenous protein removal (Kittiphattanabawon et al., 2010).

The degree of hydroxylation of proline was calculated to be 37.5%, similar to the 39% of ocellate puffer fish (Nagai et al., 2002), but lower than 51.7% of octopus arm (Nagai et al., 2001a), 42.8% of channel catfish skin (Liu et al., 2007) and 40.1% of Nile perch skin (Muyonga et al., 2004). Among them, it was found that bullfrog fallopian tube collagen was unstable against temperature. Foegeding et al. (1996) concluded that, fish collagens have lower amino acid contents than mammalian collagens. The amino acid (proline and hydroxyproline)

percentage of the PSC from bullfrog fallopian tube was 19.5%, which was similar to Nile perch skin (20.0%, Muyonga et al., 2004), bigeye snapper skin (19.3%, Kittiphattanabawon et al., 2005) and cuttlefish skin (18.8%, Nagai et al., 2001b), but was lower than pig skin (22%, Giraud-Guille et al., 2000) and calf skin (21.5%, Ikoma et al., 2003). Yan et al. (2008) indicated that the thermal stability is influenced by the amino acid content. Zhang et al. (2007) also demonstrated that, the higher the amino acid content, the more stable are the helices. On the other hand, Gustavson (1955) reported that, molecular structure of collagen is maintained mainly by the pyrrolidine rings of proline and hydroxyproline and also maintained partially through the hydrogen bond ability of hydroxyproline (Piez and Gross, 1960). Therefore, the collagen helices of bullfrog fallopian tube might be less stable than those of mammalian skins, due to the lower amino acid content.

The literature indicated that, bullfrog skin collagen was more easily degraded by pepsin (Li et al., 2004). Type I collagen is the most abundant and best studied collagen and type I triple helix is usually formed as a heterotrimer by two identical  $\alpha 1(I)$ -chains and one  $\alpha 2(I)$ -chain (Gelse et al., 2003). Cui et al. (2007) reported that a  $1\alpha$  chain resembled  $\alpha 1$  chain of type I collagen of vertebrate.



**Figure 3.** Fourier transform infrared spectroscopy of pepsin-soluble collagen from bullfrog fallopian tube.

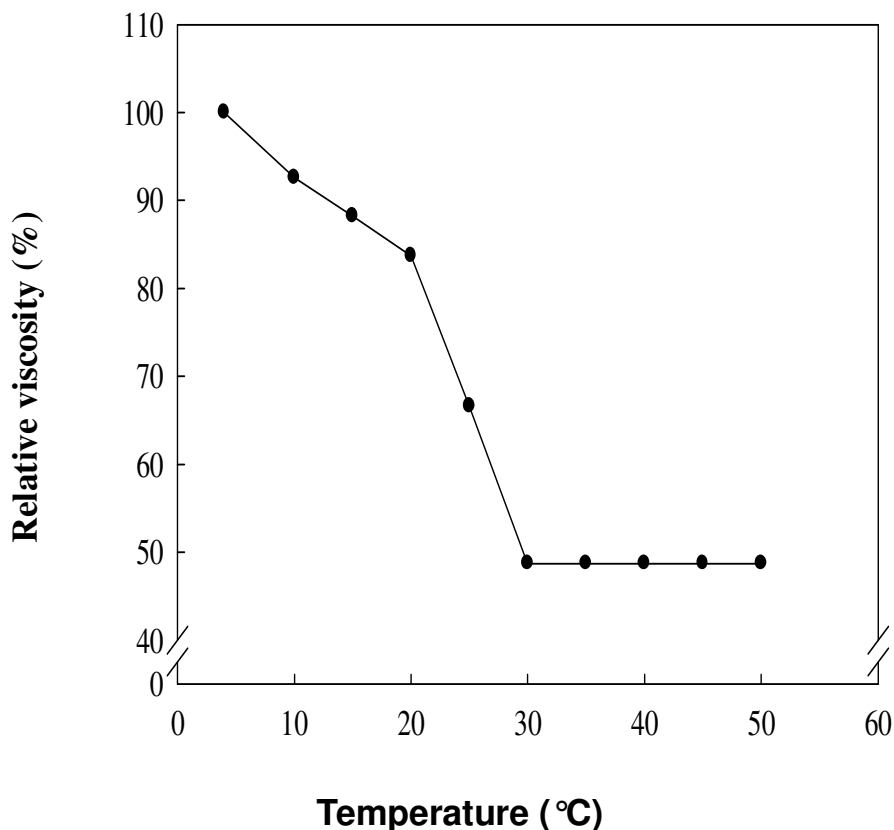
Therefore, the existence of at least two different subunits implicating that a major collagen from bullfrog fallopian tube might be classified as type I collagen.

The FTIR spectrum of the PSC from bullfrog fallopian tube was similar to that exhibited by other collagens (Muyonga et al., 2004; Liu et al., 2007). The amide A band is associated with the N–H stretching frequency. A free N–H stretching vibration occurs in the range of 3400 to 3440  $\text{cm}^{-1}$  and when the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequency, usually near 3300  $\text{cm}^{-1}$  (Li et al., 2004). The amide A band of walleye pollock skin collagen was found at 3313  $\text{cm}^{-1}$ , which showed that there were NH groups involved in hydrogen bonds. Li et al. (2004) also indicated that, the amide B band of bullfrog skin collagen was found at 2921  $\text{cm}^{-1}$ , which is related to asymmetrical stretch of  $\text{CH}_2$  (Muyonga et al., 2004). The absorption between the 1233  $\text{cm}^{-1}$  (amide III) and 1462  $\text{cm}^{-1}$  bands demonstrated the existence of helical structure (Liu et al., 2007). Therefore, the FTIR investigation indicates the existence of helical arrangements of bullfrog fallopian tube collagen.

Thermal stability of collagen is usually described by the temperature ( $T_d$ ) at which the triple helix structure of collagen in solution is disintegrated into random coils (Hao and Li, 1999). This value is similar to other marine organisms: Bone of Japanese sea bass (30.0°C), skip-jack tuna and ayu (29.7°C), yellow sea bream and horse mackerel (29.5°C) and Japanese sea bass fin (29.1°C) (Nagai and Suzuki, 2000), but lower than Nile perch skin (36°C, Muyonga et al., 2004) and channel catfish skin (32.5°C, Liu et al., 2007); however, higher than grass carp (28.4°C, Zhang et al., 2007), ocellate puffer fish (28.0°C, Nagai et al., 2002) and walleye pollock (24.6°C, Yan et al., 2008).

## Conclusions

A great quantity of collagen could be prepared from bullfrog fallopian tube by a pepsin treatment process. PSC extracted from bullfrog fallopian tube was classified as type I collagen. FTIR investigation shows the existence of helical arrangements of PSC. Meanwhile, from



**Figure 4.** Thermal denaturation curve of pepsin-soluble collagen from bullfrog fallopian tube.

the perspective of waste utilization and standpoint of avoidance of environmental pollution, fallopian tube has potential as an alternative source of collagen to calf and pig skin and bone.

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