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Effects of pepsin digestion at different temperatures and times on properties of telopeptide-poor collagen from bird feet

Yung Kai Lin, Deng Cheng Liu *

Department of Animal Science, National Chung-Hsing University, 250 Kao Kuang Road, Taichung 40227, Taiwan, ROC

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Abstract

This study was conducted to evaluate the effect of pepsin digestion at different temperatures (4, 12, 18 and 24 °C) and times (24, 36, 48 and 72 h) on properties of telopeptide-poor collagen from bird feet (TPCBF). The yield, SDS-PAGE, in vitro fibrillogenesis and denaturation temperature of the TPCBF from different treatments were determined. The results showed that the TPCBF yield increased as temperature and time increased. The yield of lots treated at 4 °C were significantly lower than those of the treatments at 12, 18 and 24 °C. In the SDS-PAGE electrophoretogram, the low molecular fragments of samples treated at 12, 18 and 24 °C were higher than those at 4 °C. The fibrillogenesis rate of lots at different temperatures decreased as treated time increased. The denaturation temperatures of the samples treated at 24 °C for 48 and 72 h were significantly lower than those of the treatments at 4, 12 and 18 °C, for all times. These changes could be related to telopeptide removal and lost molecular integrity of collagen by pepsin digestion at a high temperature and long time of treatment. However, the optimum conditions of pepsin treatment should be controlled at 12 °C for 24 h due to the best fibril-forming capacity, high denaturation temperature and an acceptable yield shown in this study.
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Keywords: Bird feet; Telopeptide-poor collagen

1. Introduction

Collagen is a major component in the body of vertebrates (Bannister & Burns, 1972). It is widely used in clinical and cosmetic applications, eg. as a wound dressing, hemostat, a carrier of drugs, cell culture system and skin mask (Lee, Singla, & Lee, 2001). Inherent difference in phyicochemical properties of collagen arise directly from diversification by chemical or physical modification depending on the species collagen used (Angele et al., 2004). Bird feet are usually an edible poultry by-product in Asia. However, bird feet contain abundant collagen, and may be a good source to replace mammalian collagen (Liu, Lin, & Chen, 2001).

Collagen has a unique structure, size and amino acid sequence. The molecule consists of three polypeptide chains intertwined and resembles a three-stranded rope. Non-helical telopeptides are attached to both ends of the molecule and serve as the major source of antigenecity (Werkmeister & Ramshaw, 2000; Wu et al., 1999). Telopeptide-poor collagen, which is produced by elimination of the telopeptide by non-specific enzymes and pepsin, is reported as a good example formed under acid conditions (Bannister & Burns, 1972; Wu et al., 1999). The pepsin activity can be increased at low pH and body temperature. Severe conditions will damage the integrity of collagen; therefore, most studies of collagen extraction have been limited to low temperature and short time exposure (Nomura, Sakai, Ishii, & Shirai, 1996).

* Corresponding author. Tel.: +886 4 22872622; fax: +886 4 22860265.
E-mail address: dcliu@dragon.nchu.edu.tw (D.C. Liu).

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Limited pepsin digestion has been performed as a method of solubilizing collagen which is otherwise highly insoluble (Moller & Rhode, 1982; Nagai et al., 2000; Nomura et al., 1996). However, there has been little information available on structural studies in relation to change of collagen by pepsin digestion, (Bannister & Burns, 1972; Purna & Babu, 2001). This study was conducted to evaluate the effects of pepsin digestion at different temperatures (4, 12, 18 and 24 °C) and time (24, 36, 48 and 72 h), on properties of telopeptide-poor collagen from bird feet (TPCBF), to determine the optimum treatment conditions.

2. Materials and methods

2.1. Sample

Frozen broiler feet (storage less than 1 month) of 6-week-old birds (Arboracres) were obtained from local poultry plant and thawed overnight at 4 °C. Fingertips of bird feet were excised, then the feet were cut into 2 cm pieces, ground by a meat grinder and frozen at −20 °C for TPCBF preparation.

2.2. Preparation of TPCBF

The preparation was according to the modified method of Nagai et al. (2000). All the preparations were performed at 4 °C. Removal of fat and pigments was carried out by stirring in 20% ethanol for 24 h, centrifugation at 10,000*g* for 20 min and discarding the supernatant, this procedure was repeated twice. Removal of non-collagenous proteins was accomplished by stirring with 0.2 N NaOH for 24 h, centrifugation at 10,000*g* for 20 min and discarding the supernatant. 5% (w/w) pepsin (EC 3.4.23.1; P-7000, Sigma, USA) and 10 volumes of 0.5 M acetic acid was later added to the precipitate. The mixture was subjected to different temperature and time treatments. TPCBF solution was centrifuged at 20,000*g* for 40 min, supernatant adjusted to pH 7.0 by 1 N NaOH and maintained for 24 h to inhibit pepsin activity, then adjusted to pH 3.5 with 0.5 M acetic acid. Finally, crystalline NaCl was slowly added to a final concentration of 0.9 M and the mixture centrifuged at 20,000*g* for 40 min; the resultant precipitate was dissolved in 0.5 M acetic acid, dialyzed against 0.05 M acetic acid, and then dry material was obtained by lyophilization.

2.3. Yield of TPCBF

The yield of TPCBF was calculated using the following formula: the dry matter weight of TPCBF (g)/the weight of ground bird feet (kg). The final dry product contained 5% moisture.

2.4. SDS-PAGE analysis

SDS-PAGE of TPCCF was performed by the method of Laemmli (1970), using 7.5% separating gels and 5% stacking gels. The gels were stained with Coomassie Blue R250 (Sigma, B7920) and destained in methanol:acetic acid (2:1). The separated protein bands were identified by comparison with those of standard molecular mixture marker (Sigma, SDS-6H), which included myosin (205 kDa), β-galacctosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa) which were purchased from Sigma Chemical (St. Louis, MO, USA).

2.5. Determination of denaturation temperature

Denaturation temperature (T_d) was determined by the method of Kimura, Miura, and Park (1983); TPCBF was dissolved in volumes of 0.5 M acetic acid and then determined by a digital viscometer (Brookefield, USA) with 10 rpm test speed. The thermal transition curve of TPCBF solution was obtained by measuring its viscosity from 18 to 45 °C (temperature rise rate 0.3 °C/min). T_d was indicated as the temperature at which the change in viscosity was half completed.

2.6. In vitro fibrillogenesis test

The in vitro fibrillogenesis test was carried out by the method of Sivakumar and Chandrakasan (1998). The TPCBF was dissolved in physiological buffers (0.13 M phosphate, 0.02 M NaCl, pH 7.0) maintained at 37 °C by a temperature controller (Hitachi U-2001 UV, Japan) and examined by recording the increase in absorbance at 313 nm, at various time intervals using a visible spectrophotometer. The highest absorbance indicated more reconstituted collagen fibre.

2.7. Statistical analysis

All data of this experiment were analyzed by a GLM programme and Duncan's new multiple range test contained in the SAS system (2000).

3. Results and discussion

3.1. General properties of TPCBF

The telopeptide-poor collagen of bird feet was easily dissolved after treating by limited pepsin digestion. All wet samples exhibited slime with white turbidity and were highly viscous solutions. These TPCBF lyophilized products visually had a white cotton-like appearance and were easily soluble in 0.1 M acetic acid.

3.2. Yield of TPCBF

The pepsin treated conditions were closely related to the ultimate TPCBF properties. The yield of TPCBF with different treatments ranged from 16.79 to 66.55 g (Table 1) and increased as the temperature and time were increased up to the 18 °C and 72 h evaluated in this work. The TPCBF yield with treatment at 4 °C (ranged from 16.79 to 39.14 g/kg, dry TPCBF/wet raw material) was significantly lower than with other temperature and time treatments ($p < 0.05$). The TPCBF yield with treatment at 4 °C for 24 h had the lowest value (16.79 g/kg, dry TPCBF/wet raw material) when compared to other treatments. Moreover, TPCBF yield with treatment at 12 and 18 °C for 48 and 72 h had the highest values when compared to other treatments. Nomura et al. (1996) reported that optimum yield of fish scale collagen, was highest when produced at 15–20 °C for 72 h (70–80% soluble). In this study, the yield of TPCBF reached maximum values at 12 and 18 °C for 72 h, respectively, and this agrees with previous research (Nomura et al., 1996). The TPCBF yield at 24 °C was slightly lower than the TPCBF yields at 18 °C for 36, 48 and 72 h. Those results were probably caused by pepsin digestion at a high temperature and long time and resulted in formation of lower molecular weight peptide fragments which cannot be precipitated by NaCl. This phenomenon can be seen in the analysis of the SDS-PAGE electrophoretogram.

3.3. SDS-PAGE analysis

The secondary structure of all TPCBF samples contained two different main α-chains, namely α-1 and α-2, and these fragments can be observed in the SDS-PAGE electrophoretogram (Fig. 1). Therefore, our results also indicated that type I collagen is a major component of chicken feet collagen. Among subunit fragments of the SDS-PAGE electrophoretogram, low molecular weight fragments could be observed when treatment temperature was 12 °C on above, for all time treatments. This means that the high molecular weight fragments significantly lost their integrity during high temperature (18 and 24 °C) treatments. Nomura et al. (1996) reported that enzyme reaction, above 15 °C, produced the highest yield, but the major problem was the production of lower molecular weight fragments of α chain from collagen. Lee et al. (2001) also pointed out that collagen should maintain a 3D structure for biomaterial usage. In our findings, similar results were observed with treatments which were above 12 °C and resulted in a higher amount of lower molecular weight fragments which can be observed in the SDS-PAGE

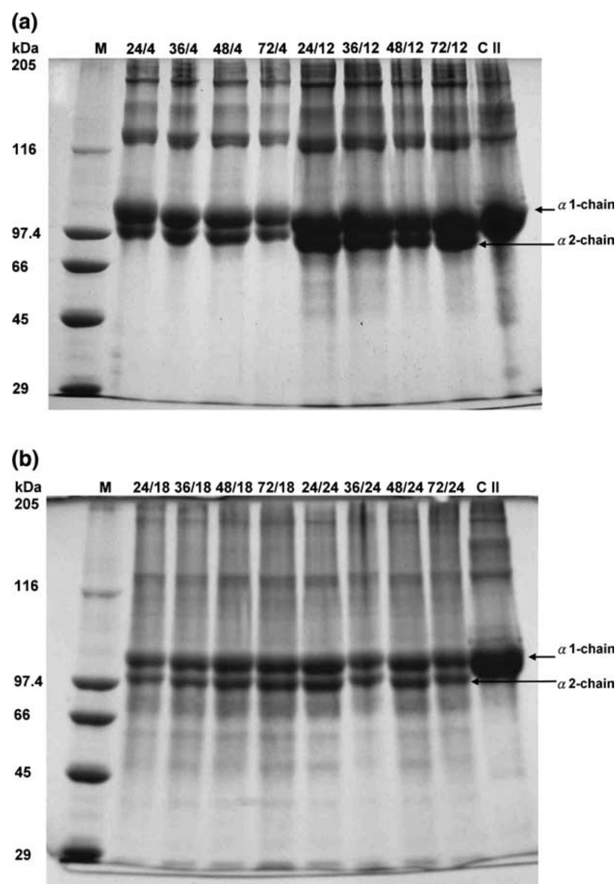

Fig. 1. Electrophoretogram (SDS-PAGE) of TPCBF with pepsin digestion at 4, 12 °C (showed as (a)) and 18, 24 °C (showed as (b)) for 24, 36, 48 and 72 h, respectively. M: Sigma high molecular marker; treatments indicated as digestion time/digestion temperature; C II: Sigma chicken type II collagen.

Table 1
Effect of pepsin digestion at different temperatures and times on TPCBF yield

Temperature (°C)	Time (h)			
	24	36	48	72
	TPCBF yield (dry matter, g/kg)*			
4	16.79 ± 4.79^{bx}	16.84 ± 2.94^{by}	24.68 ± 5.06^{by}	39.18 ± 6.44^{by}
12	36.39 ± 10.28^{by}	59.43 ± 8.39^{az}	63.03 ± 1.94^{az}	64.48 ± 11.13^{az}
18	31.56 ± 5.02^{by}	60.67 ± 6.39^{az}	61.32 ± 3.18^{az}	66.55 ± 2.12^{az}
24	53.12 ± 1.20^{az}	53.12 ± 1.95^{az}	58.94 ± 0.18^{az}	58.95 ± 3.29^{az}

a,b: Means within the same row without the same superscript are significantly different ($p < 0.05$).
x–z: Means within the same column without the same superscript are significantly different ($p < 0.05$).
* Means ± standard deviation ($n = 6$ replicates/treatment).

electrophoretograms (Fig. 1). Thus, to obtain the best quality of TPCBF it should be treated at below 12 °C for further biomedical application.

3.4. In vitro fibrillogenesis test of TPCBF

Collagen solution can re-form fibril that closely resembles native fibril under physiological conditions and this was detected by optical density at 313 nm (Bannister & Burns, 1972). In vitro fibrillogenesis curves of collagens after having undergone pepsin digestion in different treatments are shown in Fig. 2. The fibrillogenesis rate of treatments at different temperatures decreased as digestion time increased. Observably, the slope of the fibrillogenesis rates of collagens at 24 °C were more flattened than those of other treatments. The treatment at 24 °C for 24 h only maintained a small fibril-forming capacity, while the other treatments at 24 °C for 36, 48 and 72 h almost caused loss of the fibril-forming capacity. The 'telopeptides' are necessary for end-to-end aggregation of tropocollagen molecules and this is also an important step of fibrillogenesis for collagens (Bannister & Burns, 1972; Purna & Babu, 2001). These results indicated that a higher temperature and longer time of pepsin digestion resulted in larger amounts of telopeptide of tropocollagen and with less fibril forming capacity in TPCBF. Moreover, this phenomenon also clearly indicates that TPCBF with serious pepsin digestion (i. e. at 24 °C for 36, 48 and 72 h) almost lost all of their fibril-forming capacity in this study. The fibril-forming capacity is an important index of collagen molecular integrity and the denaturation of collagen caused a reduction in fibril-forming capacity (Bannister & Burns, 1972; Meredith & Kezdy, 1981). However, the best fibril forming capacity is in the TPCBF with pepsin digestion at 12 °C for 24 h when compared to the results under other conditions of this study. This result can be explained and supported by SDS-PAGE analyses (Fig. 1). The collagens from pepsin digestion at 4 and 12 °C had fewer subunit fragments and more molecular integrity (collagen α-1 and α-2) when compared to the samples treated at 24, 36 and 48 °C.

3.5. Denaturation temperature of TPCBF

The denaturation temperature of TPCBF was determined by viscosity measurement (Kimura et al., 1983). The T_d values of TPCBF, with pepsin digestion at differ-

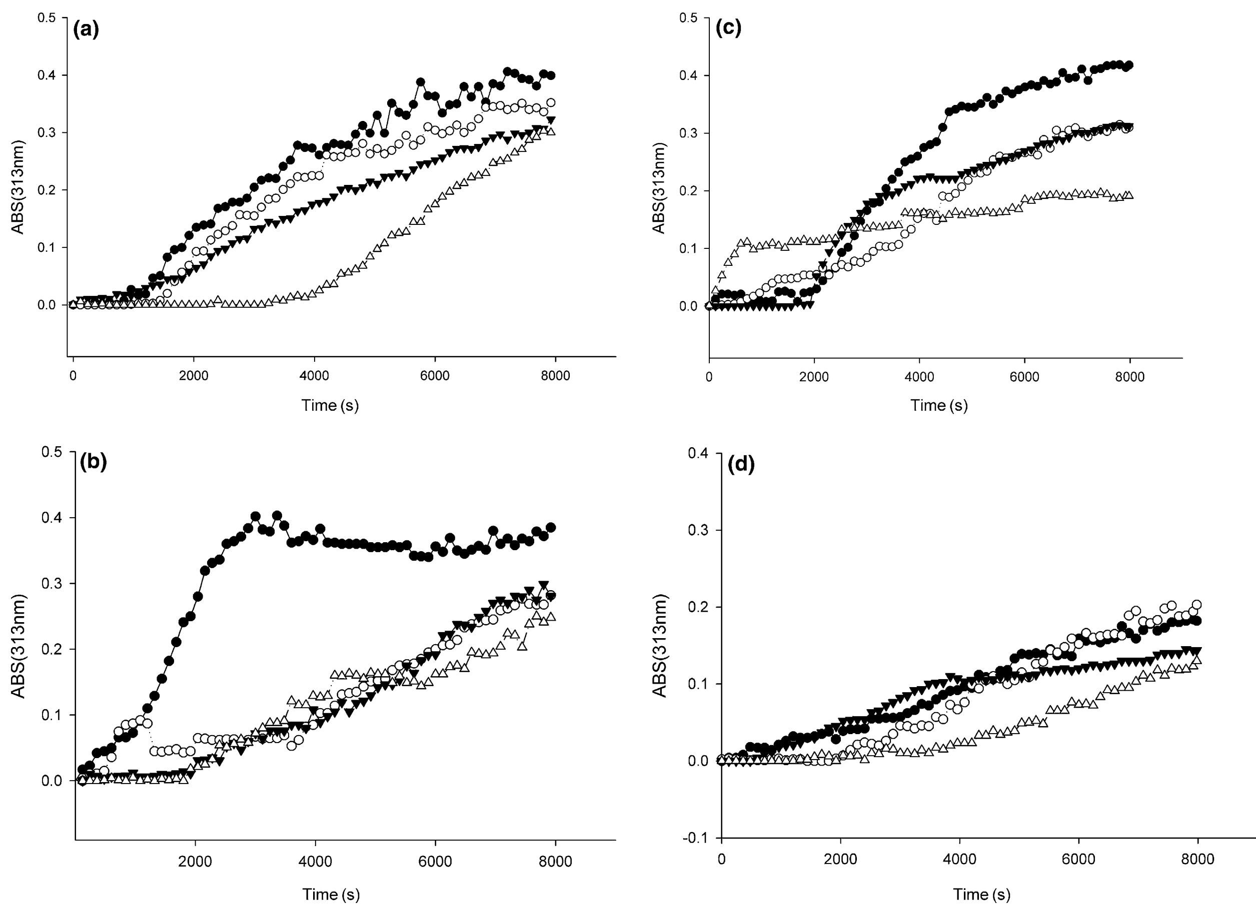

Fig. 2. In vitro, fibrillogenesis rate of TPCBF with pepsin digestion at 4 °C (a), 12 °C (b), 18 °C (c) and 24 °C (d) for 24, 36, 48 and 72 h (closed circle 24 h, open circle 36 h, closed triangle 48 h, open triangle 72 h), respectively.

Table 2
Effect of pepsin digestion at different temperatures and times on TPCBF denaturation temperature

Temperature (°C)	Time (h)			
	24	36	48	72
	T_d (°C)*			
4	37.75 ± 0.63^{ab}	37.95 ± 0.63^{az}	36.83 ± 0.32^{by}	36.57 ± 0.28^{cy}
12	37.73 ± 0.11^{ab}	38.02 ± 0.27^{az}	37.33 ± 0.28^{abyz}	37.10 ± 0.96^{byz}
18	37.43 ± 0.11^{b}	38.30 ± 0.28^{az}	37.90 ± 0.43^{abz}	37.70 ± 0.17^{abz}
24	37.56 ± 0.55^{a}	36.70 ± 0.60^{by}	35.93 ± 0.83^{bx}	36.00 ± 0.42^{by}

a–c: Means within the same row without the same superscript are significantly different ($p < 0.05$).
x–z: Means within the same column without the same superscript are significantly different ($p < 0.05$).
* Means ± standard deviation ($n = 6$ replicates/treatment).

ent temperatures and times are shown in Table 2. The T_d values of the samples at 24 °C for 48 and 72 h (35.93 and 36.00 °C) were significantly lower than those after the other treatments and TPCBF with pepsin digestion at 12–18 °C had a higher denaturation temperature than TPCBF after to other treatments (ranged from 37.10 to 38.30 °C). A higher T_d value of collagen indicates an important element for the preservation of collagen structure (Meredith & Kezdy, 1981). Therefore, heat stability is an important property of collagen when used for biomedical applications (Lee et al., 2001). Our results also indicated that TPCBF with pepsin digestion at 12–18 °C had higher denaturation temperature than TPCBF after other treatments (Table 2). Nagai et al. (2000) reported the denaturation temperatures of jellyfish and porcine collagen were 28.8 and 38.8 °C, respectively. In this study, we have also demonstrated that the denaturation temperature of TPCBF ranged from 35.93 to 38.30 °C (with various conditions) which was higher than jellyfish and similar to porcine collagen. This is important information if TPCBF is to replace porcine collagen in biomedical use.

4. Conclusion

In conclusion, it is suggested that biomedical use of telopeptide-poor collagen from bird feet should be instigated. The optimum conditions for pepsin treatment in the production of telopeptide-poor collagen from bird feet are 12 °C for 24 h. Moreover, for tissue engineering purposes, the TPCBF has also shown higher thermal stability and lower risk of transmissible disease and may be a good substitute for bovine or porcine collagen in biomedical usage.

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