



IgE reactivity to type I collagen and its subunits from tilapia (*Tilapia zillii*)

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ABSTRACT

Acid-soluble collagen (ASC) was isolated from the skin of tilapia (*Tilapia zillii*) via acetic acid (HAc) extraction and NaCl precipitation. ASC from tilapia consists of α chains ($\alpha 1$ and $\alpha 2$), β chains and γ chains and is classified as type I collagen. A comparison of the properties of tilapia collagen and silver carp parvalbumin showed that tilapia collagen was less stable under heat treatment and more resistant to pepsin digestion. Both tilapia collagen and silver carp parvalbumin were degraded at pH 2.0 but stable at pH 3.0–11.0. Subunits $\alpha 1$ and $\alpha 2$ were further purified from tilapia collagen by carboxymethyl (CM) cellulose column chromatography with linear gradient elution and stepwise elution, respectively. Enzyme-linked immunosorbent assay (ELISA) and immunoblotting results demonstrated the specific IgE activity of different fish-allergic patients' sera towards the $\alpha 1$ and $\alpha 2$ chains of tilapia collagen. It can be inferred that tilapia collagen and its subunits are all potential allergens.

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1. Introduction

Anaphylactic reactions to measles, mumps, and rubella vaccines stabilised by bovine gelatin have been reported repeatedly. Kelso reported that a child who developed anaphylaxis after receiving a combined measles, mumps, and rubella vaccine had IgE antibodies to gelatin (Kelso, Jones, & Yunginger, 1993). Thus, bovine gelatin included in live vaccines is considered to be an allergen (Nakayama, Aizawa, & Kuno-Sakai, 1999). Type I collagen, which is the main component of gelatin, was confirmed as the major allergen of bovine gelatin allergy (Sakaguchi et al., 1999).

Fish is one of the most commonly allergenic foods (Aas, 1966). It has been reported that many fish cause food allergies. Yuki Hamada reported that fish collagen may possibly be a new allergen (Hamada, Nagashima, & Shiomi, 2001; Hamada et al., 2003), in addition to the major allergen of fish, parvalbumin (Elsayed & Bennich, 1975). The skin and meat of fish, but especially the skin, contains abundant type I collagen. A previous study by Japanese researchers has preliminarily investigated the IgE reactivity of human sera against marine fish collagen. Sakaguchi et al. (2000) compared discrepancies between the IgE reactivity towards fish collagen in patients with fish allergy and bovine allergy. They concluded that fish gelatin (type I collagen) may be a potential allergen in subjects with fish allergy.

Currently, collagen is widely used in the film industry and tissue engineering, as well as in sausage casings, food additives, and cosmetics (Zhang, Liu, & Li, 2009). A number of collagen samples from

the skin and bones of fish have been isolated and characterised. Recently, collagen from the skin and scales of several freshwater fish has also been isolated and characterised (Duan, Zhang, Du, Yao, & Konno, 2009; Pati, Adhikari, & Dhara, 2010; Singh, Benjakul, Maqsood, & Kishimura, 2011). Freshwater fish is one of the most popular foods in China, where tilapia is widely distributed. The skin and meat of tilapia are known to contain a large quantity of collagen. Nowadays, tilapia is also a major source of collagen for industrial uses (Zhang, Duan, Tian, & Konno, 2009; Zhang, Liu, et al., 2009).

Whether freshwater fish collagen also has the potential ability to cause an anaphylactic reaction in consumers and affect the healthy, especially in light of the extensive applications of tilapia collagen, is currently an important issue. A previous study confirmed an allergenic response can be invoked by the whole collagen molecule. However, little is known about the subunits of fish collagen and the relationships between them. We do know that the structure of type I collagen is a right-handed triple super-helical rod consisting of three polypeptide chains. Although one report indicated that it is the $\alpha 2$ subunit of bovine gelatin that mainly causes an anaphylactic reaction (Sakaguchi et al., 1999), there has not been sufficient research on fish collagen and its subunits to identify the specific source of its allergenicity. On this basis, it is meaningful to study the allergenicity of tilapia collagen and its subunits.

2. Materials and methods

2.1. Materials and pretreatment

Live tilapia (about 1000 g) were purchased from a local market in Jimei, and brought to the laboratory alive in a bucket of water.

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Tilapia skin was prepared manually and immediately washed with chilled distilled water to remove the adhering tissues. All materials were stored at -70°C until use.

2.2. Human sera

Sera from patients were obtained from the Hospital of Jimei University and the Second Hospital of Xiamen. Sera from 8 tested patients, who had been found to be allergic to fish, were used for ELISA and immunoblotting, and sera from non-allergic individuals were pooled and used as a negative control. All sera samples were stored at -30°C until use.

2.3. Isolation of acid-soluble tilapia collagen

The methods described by Zhang, Duan, et al. (2009) and Liu, Zhong, et al. (2010), with some slight modifications, were used to isolate and purify tilapia collagen.

All procedures were performed at 4°C with gentle stirring. Tilapia skin was cut into small pieces using scissors and homogenised with 0.1 M NaOH at a solid/solvent ratio of 1:10 and extracted for 24 h; the solution was changed twice during that time to remove non-collagenous proteins. The residue from the alkaline extraction was then rinsed with cold distilled water repeatedly until the pH became stable or nearly neutral. It was then soaked in 10% *n*-butyl alcohol with a solid/solvent ratio of 1:10 for 24 h, with a change of solution each 12 h, to remove fat. The residue was washed several times and extracted in 0.5 M acetic acid with stirring for 3 days. The supernatant was salted out by adding NaCl to a final concentration of 0.9 M, and the precipitate was collected by centrifugation at 20,000g for 30 min. The precipitated collagen was re-dissolved in 0.5 M acetic acid and dialysed against 0.1 M acetic acid for 3 days; the sample was then lyophilised.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970), with slight modification, using the discontinuous Tris-HCl/glycine system with 7.5% resolving gel and 4% stacking gel. Tilapia collagen was mixed with loading buffer and heated at 95°C for 3 min. A high molecular weight marker containing bands from 10 to 250 kDa was used as a reference. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250.

2.5. Comparison of the properties of tilapia collagen and silver carp parvalbumin

The properties were compared with simulated gastric fluid (SGF) digestion, heat treatment, and different pH buffer treatment, respectively.

In the SGF digestion, silver carp parvalbumin isolated by our laboratory was used for comparison. Briefly, the silver carp muscle was homogenised with 20 mM Tris-HCl (pH 7.5) containing 5 mM EDTA, the supernatant was collected and fractionated with ammonium sulphate from 60% to 100% saturation. The precipitate was dissolved and fractionated into DEAE-Sephacel; the proteins were eluted by NaCl and applied to Superdex 75, then the column was eluted and fractions were collected for SDS-PAGE (Liu, Wang, et al., 2010). SGF was prepared as described in the *US Pharmacopoeia* (1995) and consisted of porcine pepsin in 25 mM NaCl at pH 1.2. The contents of collagen and parvalbumin were adjusted to be equal and dissolved in SGF. The total volume of the reaction solution was 1 mL. For each sample, SGF was preheated at 37°C for 15 min before adding the test protein. Digestion was performed at 37°C with continuous rocking. At each scheduled time point (0, 1, 2, 5, 10, 15, 30, and 60 min), a 100- μL aliquot of the reaction

solution was removed, and the reaction was immediately terminated by adding 30 μL 200 mM Na_2CO_3 . A protein sample dissolved in SGF with no added pepsin was the control. SDS-PAGE was performed using a linear gradient gel ranging from 6% to 20%.

In the study on thermal stability, tilapia collagen and silver carp parvalbumin were treated for 30 min at temperatures of 0, 20, 30, 40, 50, 70, and 100°C . After heat treatment, the samples were then mixed with the loading buffer and reacted at 95°C for 3 min before SDS-PAGE.

To investigate pH stability, the proteins were mixed with buffer at pH 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 11.0. The sample and the buffer were mixed at a ratio of 1:10 and maintained at ambient temperature for 1 h. The samples were then mixed with the loading buffer and reacted at 95°C for 3 min before SDS-PAGE.

2.6. Isolation of the subunits of tilapia collagen

CM (5 mL) column chromatography was used to isolate the $\alpha 1$ and $\alpha 2$ subunits, according to the method of Piez, Eigner, and Lewis (1963) with slight modification. The tilapia collagen sample was dissolved in 10 mL of sodium acetate buffer (20 mM, pH 4.8) at a concentration of 1 mg/mL. Prior to fractionation on the column, the sample was heated at 45°C for 40 min. To isolate the $\alpha 1$ subunits, elution was achieved using a starting buffer with a linear gradient of 0–1 M NaCl, at a flow rate of 60 mL/h. Fractions were collected automatically every 3 min. To isolate the $\alpha 2$ subunits, a starting buffer containing 0.13 M NaCl, followed by a starting buffer containing 0.17 M NaCl, was used. The absorbance was monitored at 223 nm. The fractions indicated by numbers were examined by SDS-PAGE (7.5% gels).

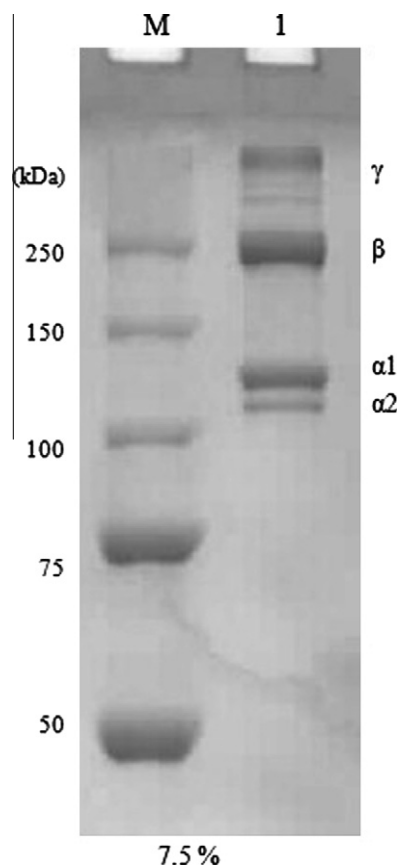


Fig. 1. SDS-PAGE analysis of the purified collagen from tilapia skin using 7.5% gel. M: High molecular protein marker ranging from 10 to 250 kDa. 1: Tilapia skin collagen.

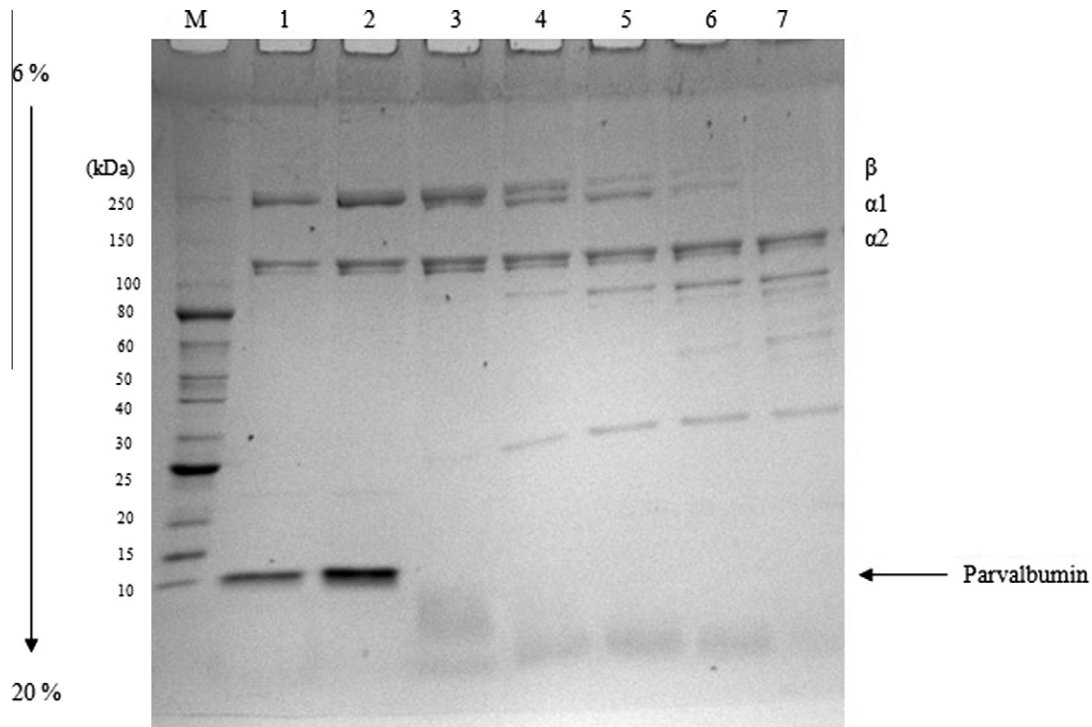


Fig. 2. Comparison of the stability of tilapia collagen and silver carp parvalbumin to SGF digestion using 6–20% gels. M: High molecular weight protein marker. 1: Control, SGF digestion without pepsin for 60 min. 2–7, Sample digestion at 0, 1, 5, 15, 30, and 60 min, respectively.

2.7. Measurement of specific IgE activity towards tilapia collagen and its subunits

The specific IgE activity towards tilapia collagen was determined by ELISA and immunoblotting. A flat-bottomed polystyrene plate with 96 wells was used for ELISA. In brief, the sample (0.2 µg) was diluted in a coating buffer (containing 0.16% (w/w) Na₂CO₃ and 0.29% (w/w) NaOH, pH 9.6). The plate was coated with 100 µL diluted sample and blocked at 4 °C overnight with 250 µL 5% skim milk in PBS. After blocking, samples of patient sera diluted (1:5) with 1% skim milk in PBS were used as primary antibody and incubated at 37 °C for 2.5 h, followed by HRP-conjugated goat anti-human IgE (diluted 1:2000) as secondary antibody incubated at 37 °C for 1.5 h. For the enzyme reaction, 100 µL 3,3',5,5'-tetramethylbenzidine (TMB) were added to each well followed by incubation at 37 °C for 20 min, and the reaction was terminated by adding 50 µL 2 M sulphuric acid. The absorbance was measured at 450 nm.

Western blotting was achieved by the method of Motoyama et al. (2008), with some modification. Collagen (0.3 µg) was carried on SDS-PAGE, then transferred to the membrane. The membrane was then blocked with 5% skim milk and successively treated with primary antibody and secondary antibody. For the detection of IgE-binding bands, human serum (1:5 diluted) was used as the primary antibody, incubated at 37 °C for 3 h; goat anti-human IgE (diluted 1:2000) was used as the secondary antibody, incubated at room temperature for 1.5 h. Then the antigen–antibody reaction was visualised using 3,3'-diaminobenzidine tetrahydrochloride (DAB).

3. Results and discussion

3.1. Isolation of tilapia skin collagen

Acid-soluble collagen from tilapia skin was analysed by SDS-PAGE (7.5% resolving gel and 3.9% stacking gel). The SDS-PAGE

pattern showed that tilapia collagen consists of an $\alpha 1$ subunit (about 120 kDa), an $\alpha 2$ subunit (about 115 kDa), high molecular weight components including a β subunit (about 250 kDa), and small amounts of γ subunit (Fig. 1).

The SDS-PAGE pattern of tilapia collagen was similar to that of other fish, such as Nile perch (Muyongaa, Cole, & Duodu, 2004), silver carp (Zhang, Duan, et al., 2009), channel catfish (Liu, Li, & Guo, 2007), and brown-backed toadfish (Senaratne, Park, & Kim, 2006). The α components ($\alpha 1$ and $\alpha 2$), although they have approximately the same molecular weight, can be separated by SDS-PAGE, as the $\alpha 2$ subunit has a greater affinity for SDS and consequently exhibits higher mobility in the gels (Muyongaa et al., 2004). The γ component has a high molecular weight and low density. The tilapia collagen obtained was found to be type I collagen (Bailey & Light, 1989).

3.2. Comparison of the properties of tilapia collagen and silver carp parvalbumin

Fig. 2 shows the results of SGF digestion by porcine pepsin. The control indicated that the protein did not decompose when incubated at 37 °C for 1 h without pepsin. After SGF digestion for 1 min, collagen presented little change (the β subunit appeared slightly degraded), but the parvalbumin was markedly decomposed into small fragments. After digestion for 5 min, the silver carp parvalbumin was mostly decomposed, whereas tilapia collagen displayed little degradation. However, there was an obvious degradation of the β subunit of tilapia collagen after digestion for 30 min, and fragments ranging from 40 to 100 kDa were observed. The results showed tilapia collagen appears more stable to SGF digestion than silver carp parvalbumin.

Fig. 3A and B shows the changes in the proteins when treated at different temperatures. Tilapia collagen remained intact at temperatures under 50 °C, but when the temperature reached 70 °C, tilapia collagen began to decompose (the α and β components were

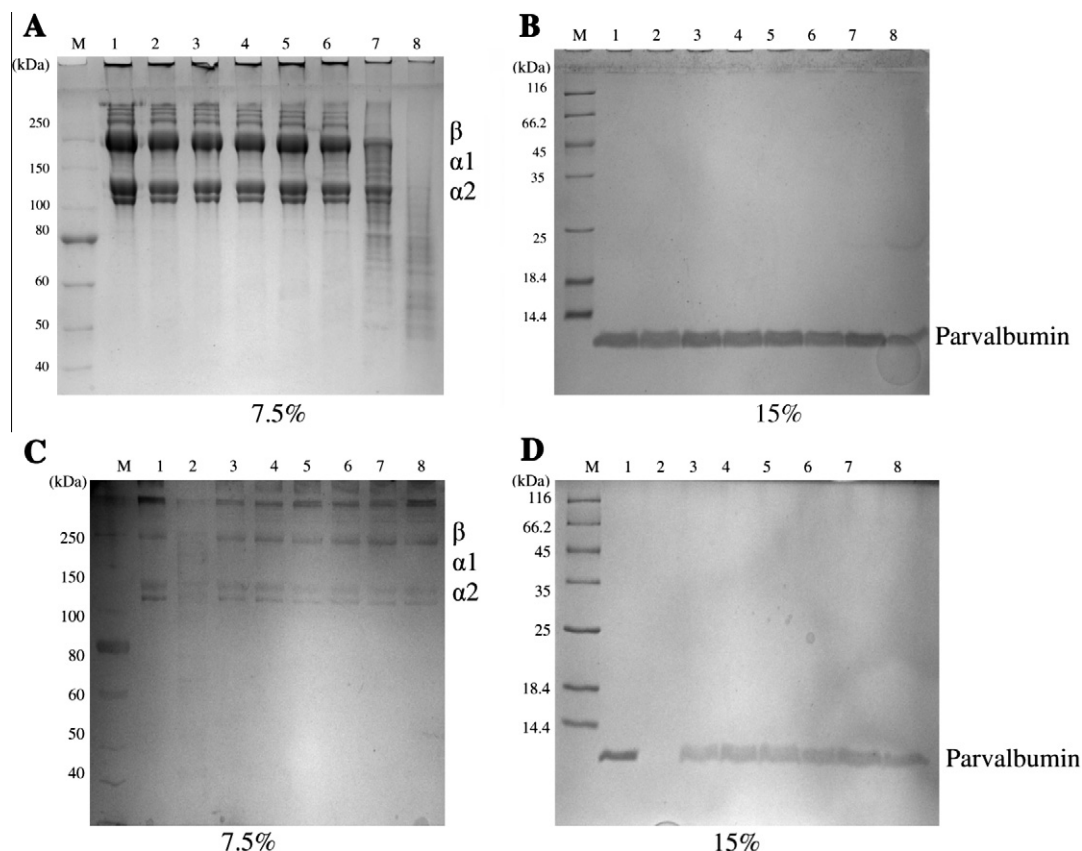


Fig. 3. Comparison of the properties of tilapia collagen and silver carp parvalbumin when heated at different temperatures and treated in buffers of different pH values. A: SDS-PAGE analysis of tilapia collagen heated at different temperatures using 7.5% gel. M: Marker. 1: Control, protein without treatment. 2–8: Protein treated for 30 min at 0, 20, 30, 40, 50, 70, and 100 °C, respectively. B: SDS-PAGE analysis of silver carp parvalbumin heated at different temperatures using 15% gel. M: Marker. 1: Control, protein without treatment. 2–8: Protein treated for 30 min at 0, 20, 30, 40, 50, 70, and 100 °C, respectively. C: SDS-PAGE analysis of tilapia collagen treated at different pH values using 7.5% gel. M: Protein marker. 1: Control, protein without treatment. 2–8: Protein treated for 1 h at pH 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 11.0, respectively. D: SDS-PAGE analysis of silver carp parvalbumin treated at different pH values using 15% gel. M: Protein marker. 1: Control, protein without treatment. 2–8: Protein treated for 1 h at pH 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 11.0, respectively.

transformed into fragments ranging from 20 to 100 kDa). However, silver carp parvalbumin showed no degradation even at a temperature of 100 °C, although a small quantity of parvalbumin aggregated to high molecular weight polymers.

Fig. 3C and D shows a comparison of the pH stabilities. When mixed with buffers at pH values ranging from 3.0 to 11.0, both tilapia collagen and silver carp parvalbumin presented no distinct changes. However, when treated with a buffer at pH 2.0, there was an obvious degradation of the tilapia collagen and the parvalbumin, which were both transformed into small fragments. The bands associated with the $\alpha 1$, $\alpha 2$ and β components of tilapia collagen appeared thinner, and the original band of silver carp parvalbumin had disappeared.

High stability against harsh conditions is one of the characteristics of food allergens, such as tropomyosin or parvalbumin. These allergens cannot be decomposed easily even at high temperatures or in strong acids. Due to their stability, food allergens are not broken down easily by industrial processing or cooking, and always remain intact in the human stomach and cause anaphylaxis. From the above results, tilapia collagen appeared more resistant to gastric digestion than silver carp parvalbumin, although the heat stability of tilapia collagen might not be as good as parvalbumin. Both tilapia collagen and silver carp parvalbumin decomposed at pH 2.0, but stayed stable even at pH 11.0 and in SGF (pH 1.2 containing HCl). This stability comparison offers indirect evidence to indicate that tilapia collagen is a potential allergen.

3.3. Isolation of the subunits of tilapia collagen

Tilapia collagen was fractionated on the CM column under denaturing conditions. After heating at 45 °C for 40 min, the collagen was denatured, and the subunits (including $\alpha 1$, $\alpha 2$, and β) were separated. Different concentrations of NaCl in the starting buffer were used to purify the subunits of tilapia collagen, reflecting the different ionic binding strengths of the distinct chains to the column. Elution was achieved by two different methods.

Purification of subunit $\alpha 1$ was achieved using a starting buffer with a linear gradient of 0–1 M NaCl. As shown in Fig. 4A, tilapia collagen eluted as one major peak. Fig. 4B shows the SDS-PAGE pattern results of linear gradient elution. Subunit $\alpha 1$ was eluted first when the concentration of salt increased gradually, followed by the β subunit and then the cross-linked dimers of $\alpha 1$ and β . Then subunit $\alpha 2$ appeared, but mixed with the $\alpha 1$ and β subunits, as the $\alpha 1$ and β subunits had not completely eluted. From the above we deduced the $\alpha 1$ subunit could be obtained first and the $\alpha 2$ subunit could be purified after the other subunits were completely eluted.

To isolate the $\alpha 2$ subunit, elution was achieved using different concentrations of NaCl (0.13 M and 0.17 M) in the starting buffer. Fig. 2C shows that, when the starting buffer contained 0.13 M NaCl, the $\alpha 1$ subunit eluted along with a small quantity of $\alpha 2$ subunit. After eluting completely with 0.13 M NaCl, the buffer was changed to 0.17 M NaCl and the $\alpha 2$ subunit was purified (Fig. 3).

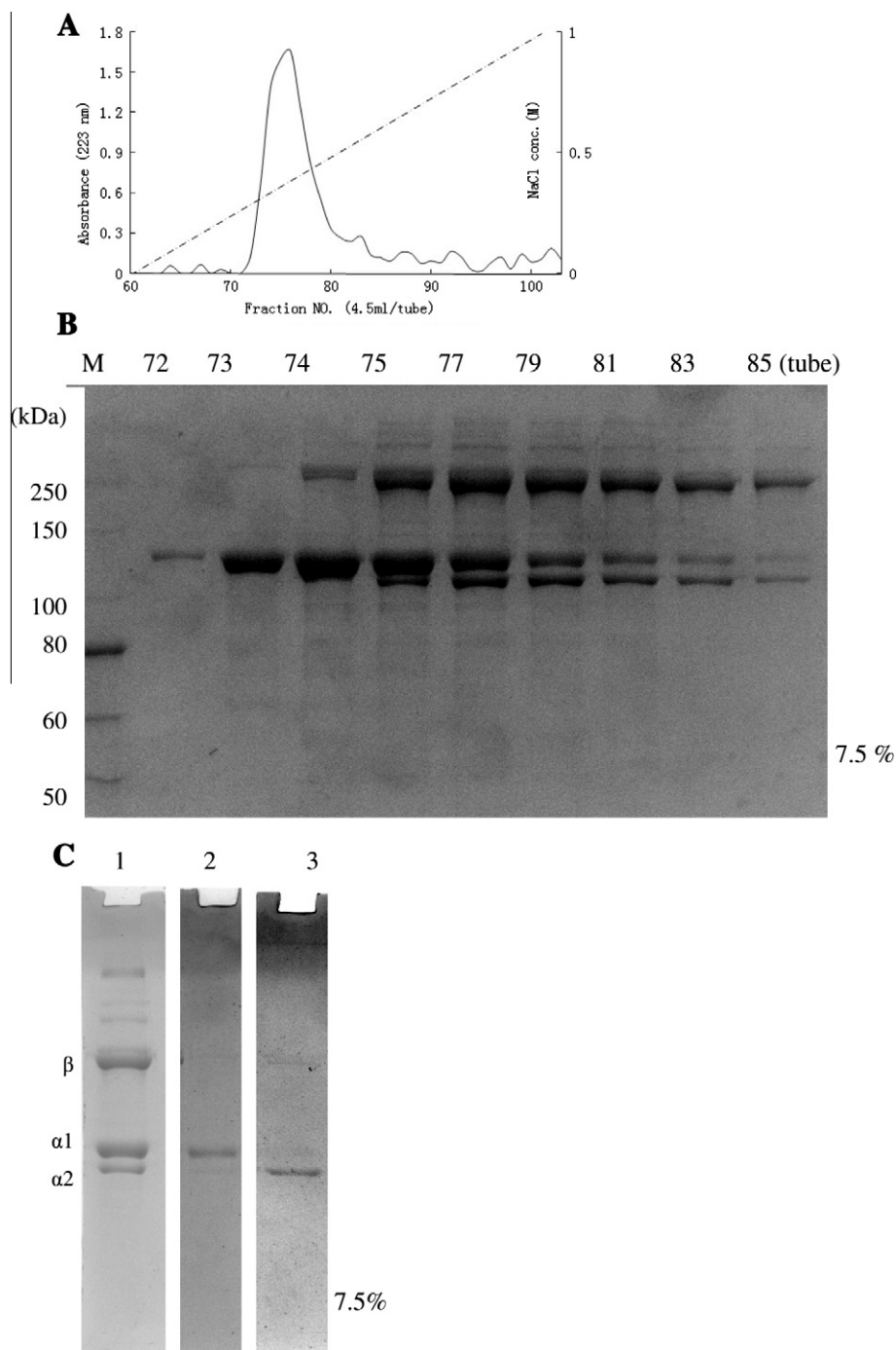


Fig. 4. Purification of the subunits of tilapia collagen with column chromatography. A: CM column chromatography of the $\alpha 1$ subunit using a linear gradient of 0–1 M NaCl. B: SDS–PAGE of the fractions, indicated by numbers. C: CM column chromatography of the $\alpha 2$ subunit. 1, Collagen isolated from tilapia skin. 2, Fractions eluted by 0.13 M NaCl. 3, Fractions eluted by 0.17 M NaCl.

Although there are several complex components in tilapia collagen, such as intramolecular cross-linked $\beta 1$ and $\beta 2$, $\alpha 1$ and $\alpha 2$ were the basic elements constituting the dimers. Sakaguchi et al. (1999) studied the influence of the $\alpha 1$ and $\alpha 2$ subunits on the sensitisation of bovine gelatin and found that the $\alpha 2$ subunit played a more important role in causing food allergy than the $\alpha 1$ subunit. A study on soybean β -conglycinin confirmed that all three of its subunits are potential allergens (Krishnan, Kim, Jang, & Kerley, 2009). As IgE binding might be different for the different components of tilapia collagen, it was important to compare the allergic effects of subunits $\alpha 1$ and $\alpha 2$ and the whole collagen entity, to elucidate the relationship between allergenicity and the different subunits.

3.4. Comparison of the specific IgE reactivity of tilapia collagen and its subunits

The specific IgE reactivity was examined by ELISA and immunoblotting, using sera from 8 patients with fish allergy and sera from non-allergic individuals as a negative control. The ELISA results showed that the sera of patient No. 1 and No. 2 had specific IgE reactivity to all proteins (collagen, $\alpha 1$, and $\alpha 2$). No. 3 reacted strongly to $\alpha 2$, but weakly to collagen and $\alpha 1$. No. 5 reacted to collagen and $\alpha 2$, but not to $\alpha 1$. No. 6 showed IgE reactivity only to $\alpha 1$. No. 4 and No. 8 scarcely reacted to any of the proteins, indicating that these two patients might not be allergic to collagen. The

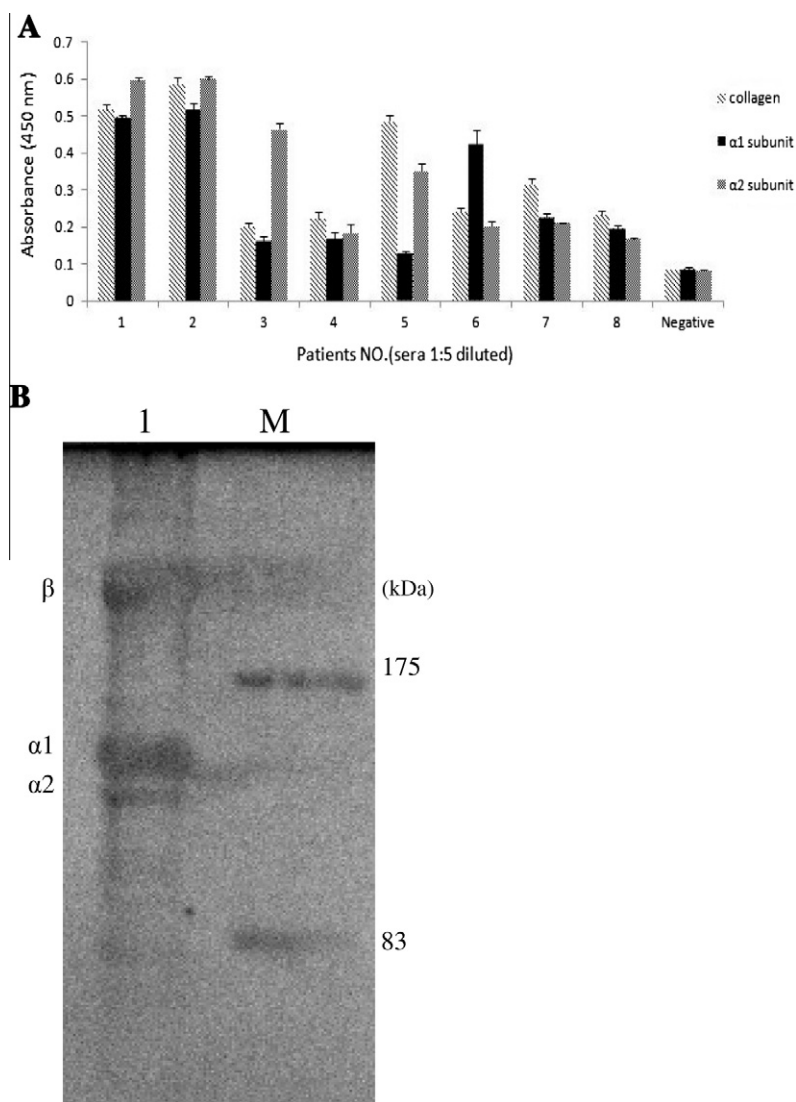


Fig. 5. ELISA (A) and western blotting (B) analysis of the specific IgE activity of tilapia collagen and its subunits. M: Prestained marker. 1: Collagen isolated from tilapia skin.

reaction rates of sera from the patients with fish allergy to collagen, $\alpha 1$, and $\alpha 2$ were 50%, 37.5%, and 50%, respectively.

Immunoblotting results using the sera from the patients with fish allergy (Fig. 5B) demonstrated a specific antigen–antibody reaction between the collagen molecule and IgE of sera from patient No. 1. The specific IgE reaction to tilapia collagen could be confirmed clearly on the nitrocellulose membrane, with subunits $\alpha 1$, $\alpha 2$, and β all having specific IgE reactivity, while the whole tilapia collagen molecule also reacted with patient sera.

In a previous study, fish gelatin was confirmed to have specific IgE reactivity towards human sera and was reported to be a potential allergen in patients with fish allergy (Hamada et al., 2001; Sakaguchi et al., 2000). In this study, the IgE-binding activities of tilapia collagen and the $\alpha 1$ and $\alpha 2$ subunits were quite different and did not present a clear picture. This is different from bovine gelatin, in which the $\alpha 2$ subunit mainly causes the bovine gelatin allergy (Sakaguchi et al., 1999). However, our results are similar to those of soybean β -conglycinin, in which the three subunits are all potential allergens (Krishnan et al., 2009). We inferred that the IgE reactivity of tilapia collagen and the $\alpha 1$ and $\alpha 2$ subunits were distinctly different. Moreover, it is possible that heating at 45 °C for 30 min prior to isolation of the subunits might destroy their three-dimensional structure and affect the specific IgE activ-

ity of the collagen and the subunits. This should be investigated by further study.

The IgE-binding activities of the $\alpha 1$ and $\alpha 2$ subunits of tilapia collagen were detected by ELISA and immunoblotting. The subunits used for ELISA detection were native protein, compared to immunoblotting, in which the proteins were denatured by heating and treatment with SDS. Thus, it was remarkable that the denaturation of tilapia collagen did not cause a decrease in IgE-binding activity, which indicates that tilapia collagen still might cause specific hypersensitivity even after food processing.

4. Conclusions

Acid-soluble collagen extracted from tilapia skin was composed of $\alpha 1$ and $\alpha 2$ subunits, and was classified as type I collagen. A comparison of the stabilities of tilapia collagen and silver carp parvalbumin showed that parvalbumin is more stable at high temperatures but collagen is more resistant to pepsin under SGF conditions, with no detectable difference in pH stability between the two. This property comparison provides indirect evidence that tilapia collagen is a potential allergen.

Subunit $\alpha 1$ was isolated using CM column chromatography via elution with a linear gradient of 0–1 M NaCl. Subunit $\alpha 2$ was

purified by stepwise elution with 0.13 M NaCl and 0.17 M NaCl. ELISA and immunoblotting results revealed the specific IgE activities towards tilapia collagen, $\alpha 1$, and $\alpha 2$ of sera from 8 patients allergic to fish. It can be inferred that tilapia collagen and its subunits are potential allergens.

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