

Purification and Characterization of Protamine, the Allergen from the Milt of Large Yellow Croaker (*Pseudosciaena crocea*), and Its Components

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ABSTRACT: The protamine in fish milt can cause anaphylaxis in humans. To determine the allergen in the milt of large yellow croaker (*Pseudosciaena crocea*), crude extracts were incubated with sera from allergic patients. The results showed that a 12 kDa multicomponent protein was the major allergen in the milt of large yellow croaker. The multicomponent protein was purified, and physicochemical characterization showed that it was a glycoprotein, highly stable in acid–alkali conditions, and weakly retained immunoglobulin E (IgE)-binding activity at high temperatures. Separation and immunoreactivity analysis of the components of the multicomponent protein showed that it had six components, and component 5 had the strongest IgE-binding activity with patient sera. N-terminal sequencing confirmed the multicomponent protein was protamine. Following analysis of protamine from different fish by reversed-phase liquid chromatography and circular dichroism spectra, the protamines from different fish were found to have a similar secondary structure, although their components were different.

KEYWORDS: milt, large yellow croaker (*Pseudosciaena crocea*), allergen, protamine, characterization, components

INTRODUCTION

Large yellow croaker (*Pseudosciaena crocea*), one of the most commercially important marine fish in southeastern China,¹ is geographically distributed from the southern part of the Yellow Sea to the northern part of the Southern Sea of China with a total production of around 127,917 tons in 2014.² It is popular among consumers due to its good taste and high nutritional value. China is the largest fish-producing nation. In recent years, due to the formation of the characteristic processing area for large yellow croaker in Fujian and the development of aquatic products processing, the quantity of processed large yellow croaker has increased, and the development of uses for the milt of large yellow croaker has attracted increased attention. Milt, one of the byproducts of fish processing, mainly consists of nucleoproteins, enzymes, and microelements.³ Drugs containing the nucleoproteins in milt can be used for leucopenia and hepatitis, which may be caused by anticarcinogens or radiotherapy. The milt contains an alkaline protein called protamine, which is a hemostatic agent, and not only can be used in the treatment of pulmonary hemoptysis and bleeding caused by serious hepatitis but also can be used in the pharmaceutical industry in relation to heparin anticoagulant activity.⁴ In addition, some researchers have found that milt protamine has broad-spectrum antibacterial activity and could potentially be used as a natural antimicrobial agent. Protamine has been used to preserve a wide variety of foods ranging from confectionary to fruits and rice. It has been added to foods in combination with a reducing agent.⁵ A number of researchers have also studied protamine hydrolysate, which has antioxidant activity and an antifatigue function.^{6,7} Therefore, protamine products might have a broad application in the food industry in the future.

However, in modern clinical medicine, protamine can lead to an allergic reaction with symptoms of urticaria or heart arrest.^{8,9} It was reported that the injection of protamine causes allergic reactions in China every year. For example, protamine injections have caused allergic reactions in 36 of 619 patients undergoing open-heart surgery in the past 5 years, and the morbidity rate was 5.8%.¹⁰ Allergic reactions to protamine injections have also been observed in other countries.^{11–13} Some researchers have indicated that allergic shock caused by protamine is a type I hypersensitivity reaction.¹⁴ Once protamine enters the body, it can stimulate the immune system to produce immunoglobulin E (IgE), which then combines with mastocytes in vessels or the bronchial wall and basophilic granulocytes in the blood. In addition, internal protamine can specifically combine with IgE to form a protamine–IgE complex and activate mastocytes and basophilic granulocytes to induce degranulation, which releases bioactive compounds such as histamine and plasmakinin. These bioactive compounds can expand capillaries and increase capillary permeability, muscle contractions, and glandular secretion, leading to many allergic symptoms such as shock, bronchospasm, and asthma.¹⁴

Protamine is a polymer cationic peptide consisting mostly of arginine. It is also called nucleoprotamine, which can combine with DNA in the nucleus in mature fish milt, and its isoelectric point (pI) is between 11 and 13.¹⁵ Protamine may be isolated from the spermatid cells of fish, birds, and mammals and is

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commercially recovered from herring (clupeine) and salmon (salmine) milt.¹⁶ Gusse et al.¹⁷ separated and purified the protamine from dog-fish using ion-exchange chromatography, analyzed its amino acid composition, and found that the protamine had four components with high arginine content. Gill et al.¹⁸ achieved quantitative purification of protamine from frozen herring milt using heparin affinity chromatography. Protamine is heat-resistant and able to withstand steam sterilization without loss of antimicrobial activity.¹⁹ Awotwe-Otoo et al.²⁰ characterized and evaluated the differences in protamine sulfate obtained from different sources. The results showed that the five protamine sulfate samples evaluated all had relatively good thermal properties such as moisture content, glass transition, and melting temperature. Furthermore, researchers also studied how to isolate hypoallergenic protamine sulfate. He et al.²¹ obtained a low molecular weight protamine fragment following thermolysin enzymatic digestion of the native protamine and found that it could completely neutralize the anticoagulant function of both heparin and low molecular weight heparin, with reduced antigenicity and cross-reactivity to mouse-derived anti-protamine antibodies. However, there are currently few reports on protamine allergy.

The objectives of the present study were to determine and purify the major allergen in the milt of large yellow croaker, to analyze its physicochemical characteristics, and to isolate and identify the components of the protamine of large yellow croaker (LPt). In addition, as we have previously researched parvalbumin and collagen in carp in our laboratory, the main allergen in carp milt was also investigated in this study.

MATERIALS AND METHODS

Materials. Large yellow croaker (*P. crocea*) and common carp (*Cyprinus carpio* Linnaeus) were purchased from Jimei market, Xiamen, China. Fish milt was collected immediately and used for the experiments or stored in a freezer at -70°C .

Human Sera. Human sera were provided by the Xiamen Second Hospital from patients (20–35 years old) who had been diagnosed with an allergic reaction to fish milt, and the samples were numbered 1–36.

The pooled sera were mixed with sera from 10 patients (numbered 4, 12, 21–25, 29, 32, and 36, respectively). Personal information is shown in Table 1. Serum from a nonallergic individual was used as negative control. All serum samples were stored at -30°C until use. The human ethical approval number of the Xiamen Second Hospital Ethical Council is XSH2012-EAN019.

Isolation of the Major Allergen. A crude extract was prepared from the fish milt using the method of Gill et al.,¹⁸ with slight modifications. The milt was homogenized with a 2-fold volume of ice-cold water containing 0.15 mol/L NaCl, 25 mmol/L ethylenediamine-tetraacetic acid (EDTA), and 0.05 mmol/L phenylmethanesulfonyl fluoride (PMSF). The extract was centrifuged at 8000g for 20 min at 4°C . The precipitate was then suspended in ultrapure water and centrifuged twice under the same conditions. The precipitate was placed in 2.5 volumes of 0.75 mol/L sulfuric acid for 2 h at 40°C . After centrifugation at 10000g for 20 min at 4°C , the supernatant was collected. This procedure was repeated, and all supernatants were combined for distillation and condensation. The concentrate was mixed with a 3-fold volume of 95% glacial acetic acid and stirred at 4°C overnight. The concentrate was then centrifuged at 10000g for 20 min at 4°C , and the precipitate was collected and washed with cold acetone and diethyl ether. After lyophilization, the crude extract of milt was obtained.

The method for purifying the target protein from fish milt was as follows: The crude extract was dissolved in 25 mmol/L acetic acid–sodium acetate (pH 5.4) containing 0.15 mol/L NaCl and subjected to Sephacryl S-200 HR (GE Healthcare, Waukesha, WI, USA) chromatography after centrifugation at 8000g for 15 min at 4°C . To further purify the target protein, the samples were loaded onto quick flow heparin affinity (Iduron, Cheshire, UK) chromatography after dialysis and concentration. The elution conditions used were a linear gradient of 0.15–2.0 mol/L NaCl at a flow rate of 1 mL/min. All steps were performed at 4°C .

Amino Acid Analysis of the Target Protein. The sample powder was hydrolyzed under reduced pressure in 6.0 mol/L HCl containing 0.1% phenol at 110°C for 24 h. After hydrolysis, HCl was removed, and the amino acid composition of an aliquot was analyzed using an amino acid autoanalyzer model L-8900 (Hitachi Co., Tokyo, Japan).

Carbohydrate–Peptide Linkage Characterization. β -Elimination was used to characterize the carbohydrate–peptide linkage of protamine. The protocol was carried out according to the method described by Zhang et al.,²² with some modifications. The target protein

Table 1. Clinical and Laboratory Characterizations of 36 Fish Milt-Allergic Patients^a

serum	patient	sex	age (years)	symptoms	IgE titer	serum	patient	sex	age (years)	symptoms	IgE titer
1	763	F	30	P	2.02	19	720	M	23	N, GIT	3.39
2	642	F	25	E, N	1.84	20	911	F	34	U	3.21
3	626	M	21	GIT	2.67	21	424	F	20	U	5.47
4	614	M	27	U	4.82	22	551	M	28	A, D	5.71
5	595	M	35	Dr	1.94	23	109	M	21	P	2.65
6	737	F	20	GIT	2.10	24	193	F	35	Dr	4.59
7	646	M	23	U	2.29	25	555	F	29	U	4.69
8	178	F	33	A, D	2.98	26	440	M	23	ND	1.85
9	470	F	26	P	1.90	27	067	F	27	E, N	2.64
10	933	M	20	E, N	1.82	28	911	M	32	U	1.73
11	433	M	21	GIT	2.61	29	240	F	30	P	1.98
12	757	F	32	ND	2.82	30	362	F	33	GIT	1.80
13	024	F	22	U	2.14	31	370	M	22	Dr	1.69
14	471	F	25	E, N	2.75	32	670	F	24	A	2.62
15	717	M	27	N	2.02	33	557	M	29	U	1.87
16	314	F	24	Dr	2.08	34	411	M	21	U	1.93
17	499	M	30	P	2.35	35	221	F	30	E, N	2.18
18	307	F	26	ND	1.80	36	203	M	25	E, Dr	4.35

^aF, female; M, male; U, urticaria; P, pruritus; E, emesis; N, nausea; A, asthma; D, dyspnea; GIT, gastrointestinal problems; Dr, diarrhea; ND, not determined/unknown. The titer was defined following the equation $\text{titer} = A_p/A_N$, in which A_p is the absorbance of each positive sera in ELISA test and A_N is the absorbance of negative serum in ELISA test.

solution (90 μ L, 0.2 mg/mL) was mixed with 10 μ L of 0.5 mol/L NaOH and incubated at 37 °C for 16 h. The pH of the reaction system was adjusted to neutral using HCl (1 mol/L) to stop the reaction. The ultraviolet absorption values of the reaction system were then determined in the wavelength range of 210–300 nm using a Lambda 35 spectrometer (PerkinElmer, Waltham, MA, USA).

Thermal and pH Stability. To evaluate the thermal stability of the main allergen, the purified target protein was adjusted to a concentration of 0.2 mg/mL and incubated at different temperatures (4, 30, 40, 50, 60, 70, 80, 90, and 100 °C) for 30 min. In the study of pH stability, the target protein was incubated in buffer solutions at different pH values (1.0, 3.0, 5.0, 6.0, 7.0, 8.0, 9.0, 11.0, and 13.0). The final concentration of the protein was 0.2 mg/mL after being mixed with the buffer solutions at a ratio of 1:5 (v/v) and was maintained at ambient temperature for 1.5 h.

Purification of the Components. Isolation of the components was based on obtaining protamine. After concentration, the samples were loaded onto a quick flow heparin affinity chromatograph, and determination of the protein concentration and electrophoresis analysis were used to analyze the separation and purification of the samples. The elution conditions were NaCl solution, a linear gradient of 0.15–2.0 mol/L, and a flow rate of 0.8 mL/min.

Identification of the Components by N-Terminal Sequencing. N-terminal sequencing was used to analyze the components of the target protein. Following electrophoresis of the purified target protein, the protein was transferred to a PVDF film using an electrotransfer method. The PVDF film was soaked in 200 mL of 10 mmol/L sodium borate buffer and oscillated for 5 min after electrotransfer. The target band was cut after the following processes: Coomassie brilliant blue staining, 60% methanol washing, soaking in distilled water for 5 min, and air-drying. The film containing the target band was then cut up, coupled, cut, extracted, and transformed using an automatic Edman sequence analyzer. High-performance liquid chromatography (HPLC) was then performed to analyze the types of phenylthiohydantoin (PTH)-amino acids to obtain the protamine N-terminal sequence information.

Determination of the Components of Different Protamines by RP-HPLC. Protamines from different types of fish and target proteins were determined using an Agilent 1260 HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) consisting of a quaternary pump, a standard automatic sampler, and a variable-wavelength detector (VWD). The components of protamine and target proteins were separated by an Agilent ZORBAX 300SB-C18 column (4.6 mm i.d. $\times$ 150 mm) with an isocratic elution program consisting of different ratios of acetonitrile/water, a flow rate of 1.0 mL/min, a column temperature of 25 °C, a sample volume of 5 μ L, and a detection wavelength of 220 nm. The control samples were salmine grade X (Sigma, St. Louis, MO, USA) and the protamine sulfate from herring (clupeine) (Shanghai No. 1 Biochemical & Pharmaceutical Co., Ltd., Shanghai, China), respectively.

Analysis of the Structure of Different Protamine by Circular Dichroism (CD) Spectra. The parameters of CD analysis were a scanning wavelength range of 190–250 nm and a scanning speed of 60 nm/min. After the sample, with a concentration exceeding 1 mg/mL, was dissolved in buffers, the spectra were obtained and analyzed on an MOS-500 (Bio-Logic, Claix, France) spectrometer in 1 mm cells.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was performed as previously reported,²³ with some modifications. In brief, the samples (0.2 μ g) were placed in each well of polystyrene 96-well ELISA plates (Nunc Maxisorb, Roskilde, Denmark) and incubated at 37 °C for 2 h. After the washing and blocking processes, the coated plates were incubated with 50 μ L of patient sera (diluted 1:5 with 0.1% skimmed milk in PBS) as the primary antibody at 37 °C for 2.5 h, followed by horseradish peroxidase (HRP)-conjugated goat anti-human IgE (diluted 1:2000) as the secondary antibody incubated at 37 °C for 1.5 h. Then, 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) (Tiangen, Beijing, China) was added to each well and then incubated at 37 °C for 20 min. After the reaction was terminated, the absorbance was measured at 450 nm by an automated ELISA plate reader (Benchmark 96; Bio-Rad Laboratories, Hercules, CA, USA).

Tricine–Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (Tricine-SDS-PAGE) and Western Blotting. In the present study, the proteins were analyzed by Tricine-SDS-PAGE, which

was performed under reducing conditions according to the method of Schagger.²⁴ Western blotting was carried out as described by Shen et al.,²⁵ with minor modifications. In brief, the proteins (1.0 mg/mL purified protein) were transferred from the gel to nitrocellulose (NC) membranes and then blocked with 5% skimmed milk and incubated with human sera (1:4 dilution) for 2 h. Following incubation with HRP-conjugated goat anti-human IgE (1:20000 dilution) (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), the immunoassay was completed using the enhanced chemiluminescent (ECL) technique (Pierce, Rockford, IL, USA).

Dot Blotting. The proteins (1.0 mg/mL purified protein) were directly blotted on NC film and incubated with human sera (1:4 dilution) after blocking. Following incubation with HRP-conjugated goat anti-human IgE (1:20000 dilution), the immunoassay was carried out using ECL.

RESULTS

Confirmation of the Major Allergen Using IgE-Binding Activity in Patient Sera. ELISA was used to analyze the specific IgE-binding activity between the crude extract of large yellow croaker milt and sera from allergic patients. The specific reaction of crude extract incubated with allergic patient sera was positive. The highest value was 0.582, and the average measured value was 2.9 times that of the negative control (Figure 1A). Therefore, it was determined that there were some allergens present in the milt of large yellow croaker. To further determine the allergens and the molecular weight of the crude extract, Western blotting analysis was performed. There were eight clear protein bands in Tricine-SDS-PAGE, and most of them were of small molecular weight, which revealed that a few proteins were present in the milt of large yellow croaker. It was noted that the 12 kDa protein showed the most obvious binding with sera and reacted with 84% of patient sera. In addition, the 10 and 14 kDa proteins had IgE-binding activity with a fraction of the sera (Figure 1B). Therefore, it was initially identified that the 12 kDa protein may be the major allergen in the milt of large yellow croaker. Furthermore, these three proteins were identified as a multi-component protein.

The crude extract of carp milt was also analyzed by ELISA and Western blotting. As shown in Figure 1C, the crude extract incubated with allergic patient sera was also positive. The highest measured value was 0.479, and the average measured value was 3.0 times that of the negative control. The protein components in the carp milt were more complex than those in the milt of large yellow croaker, of which the 15 kDa protein reacted with 67% of patient sera, and the 18, 26, 27, and 34 kDa proteins had IgE-binding activity with a fraction of the sera (Figure 1D). Therefore, the 15 kDa protein was initially identified as the major allergen in the milt of carp.

Isolation of the Target Protein. The washed milt of large yellow croaker was first homogenized with precooled ultrapure water to precipitate deoxyribonucleoprotein (DNP) in the milt. The protamine, which was in the precipitate, dissolved in the sulfuric acid extract, so the weak bond between deoxyribonucleic acid (DNA) and protamine was then broken by sulfuric acid digestion. The crude protamine extract was obtained from the concentrated sample solution by ethanol precipitation. To purify the target protein, the redissolved crude extract was loaded onto a Sephacryl S-200 HR column. Due to the effect of the molecular sieve, the high molecular weight protein was eluted early, and the small molecular protein was eluted later (Figure 2A). It can be seen from Tricine-SDS-PAGE that the 45 kDa impure protein was removed, and the 12 kDa protein and its close protein components were in the sample solution.

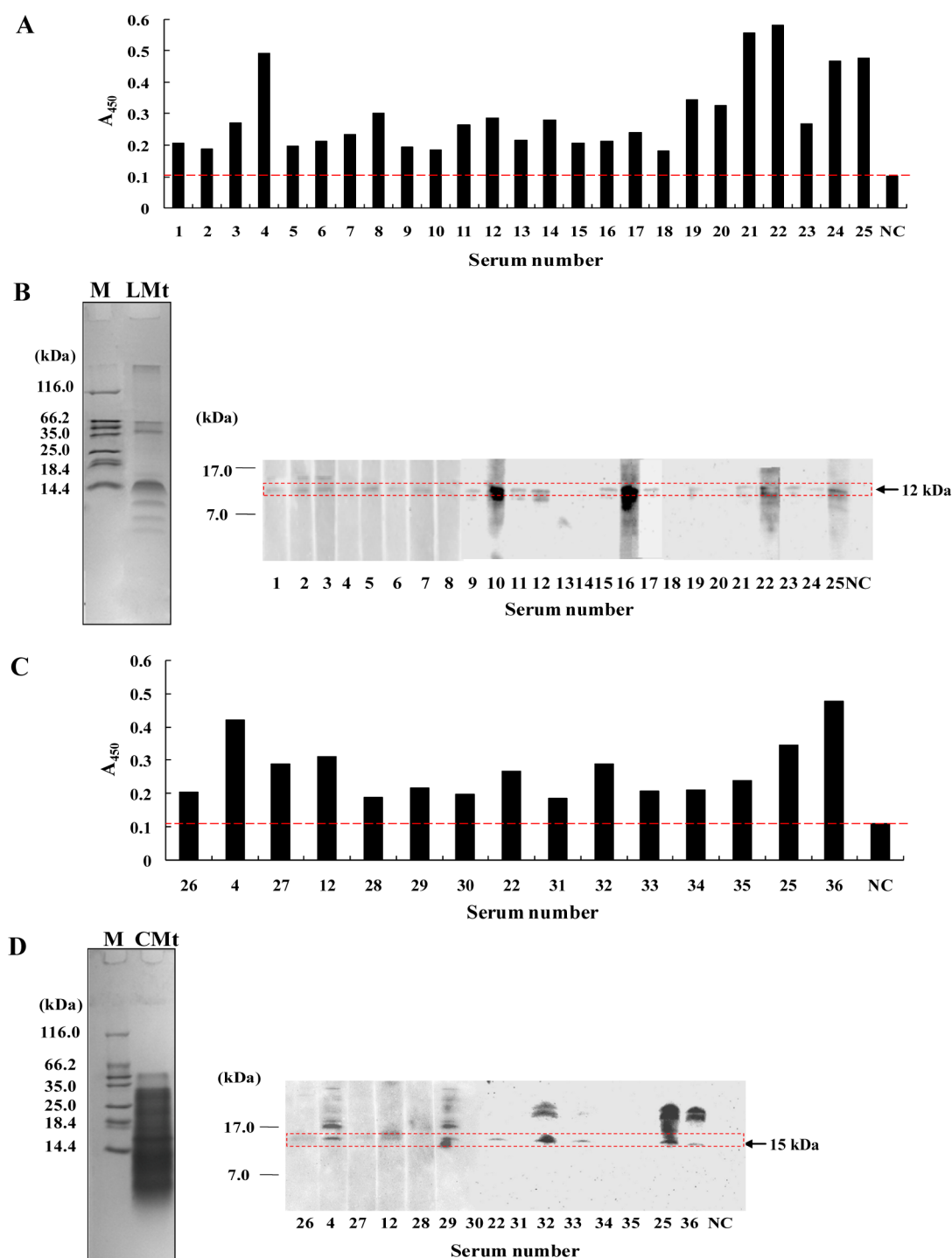


Figure 1. Determination of the major allergen in the milt of large yellow croaker and common carp: (A) investigation of the IgE-binding activity of the milt of large yellow croaker, 1–25, as immunoblotting profiles of IgE-binding activity in patient sera (NC, negative control); (B) for determination of the major allergen, the IgE-binding activity of crude extract was analyzed by Western blotting (M, protein marker; LMt, crude extract of the milt of large yellow croaker); (C) investigation of the IgE-binding activity of the milt of common carp, 26–36, as immunoblotting profiles of IgE-binding activity in patient sera (NC, negative control); (D) for determination of the major allergen, the IgE-binding activity of crude extract was analyzed by Western blotting (M, protein marker; CMt, crude extract of the milt of common carp).

In addition, the purification process of the target protein from carp milt was as follows: the crude extract was obtained after homogenization, separation, sulfuric acid digestion, and ethanol precipitation. The samples were then loaded onto a Sephacryl S-200 HR column. As shown in Figure 2B, the high molecular weight proteins were removed, and the small molecular weight proteins (include the 15 kDa target protein)

were collected, pooled, concentrated, and subsequently loaded onto a quick flow heparin affinity column. The 15 kDa protein was eluted with 0.15–2.0 mol/L NaCl (Figure 2C).

The IgE-binding activity of the two target proteins was confirmed by Western blotting using sera of patients, and serum from a nonallergic individual was used as a negative control. As shown in Figure 2D, three components of the purified

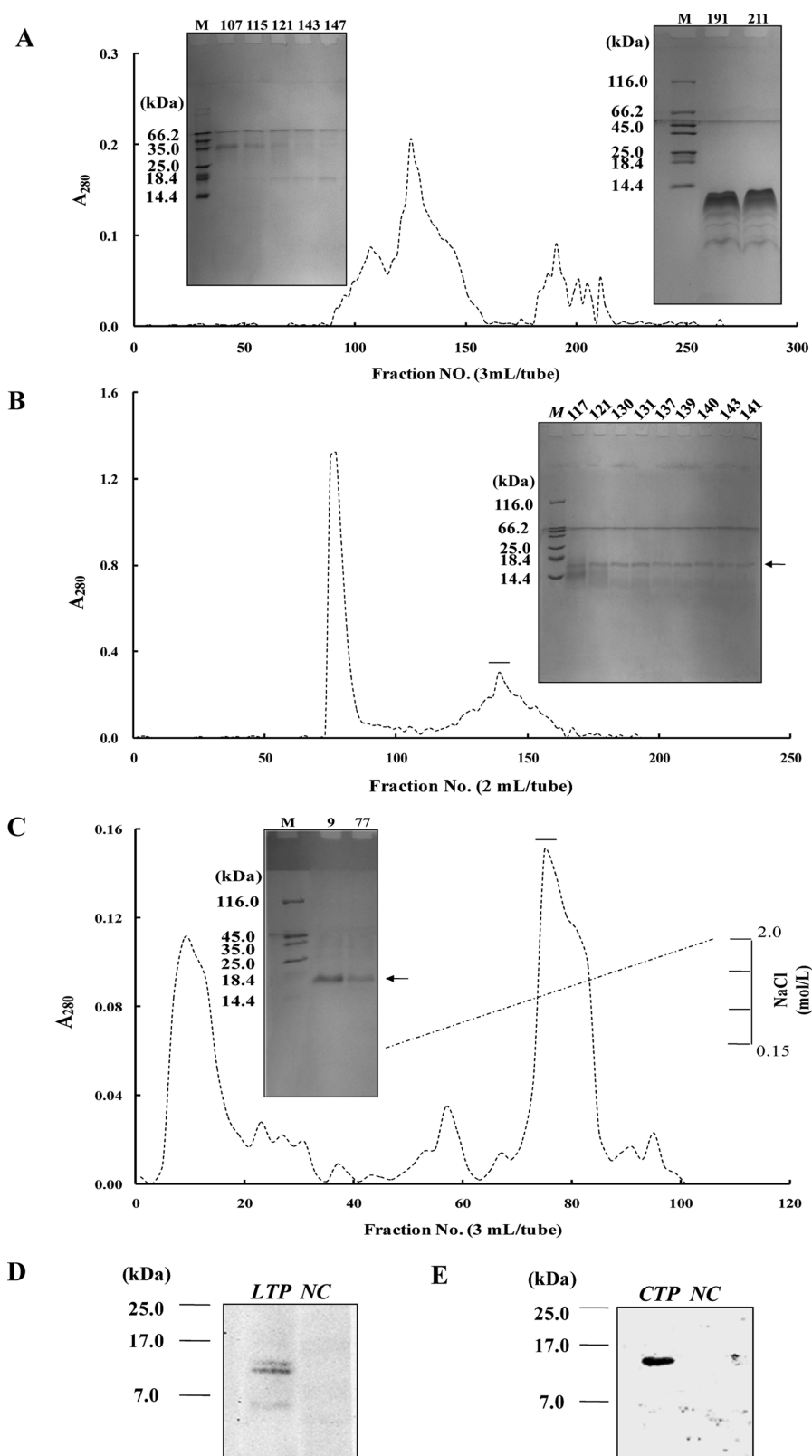


Figure 2. Column chromatography and IgE-binding activity of the target protein in the milt of large yellow croaker and common carp: (A) Sephacryl S-200 HR gel chromatography of the target protein in the milt of large yellow croaker; (B) Sephacryl S-200 HR gel chromatography of the target protein in the milt of common carp; (C) quick flow heparin affinity chromatography of the target protein in the milt of common carp; (D) Western blotting analysis of the purified protein in the milt of large yellow croaker (lane 1, sera of fish milt-allergic patients; lane 2, NC, negative control); (E) Western blotting analysis of the purified protein in the milt of common carp (lane 1, sera of fish milt-allergic patients; lane 2, NC, negative control).

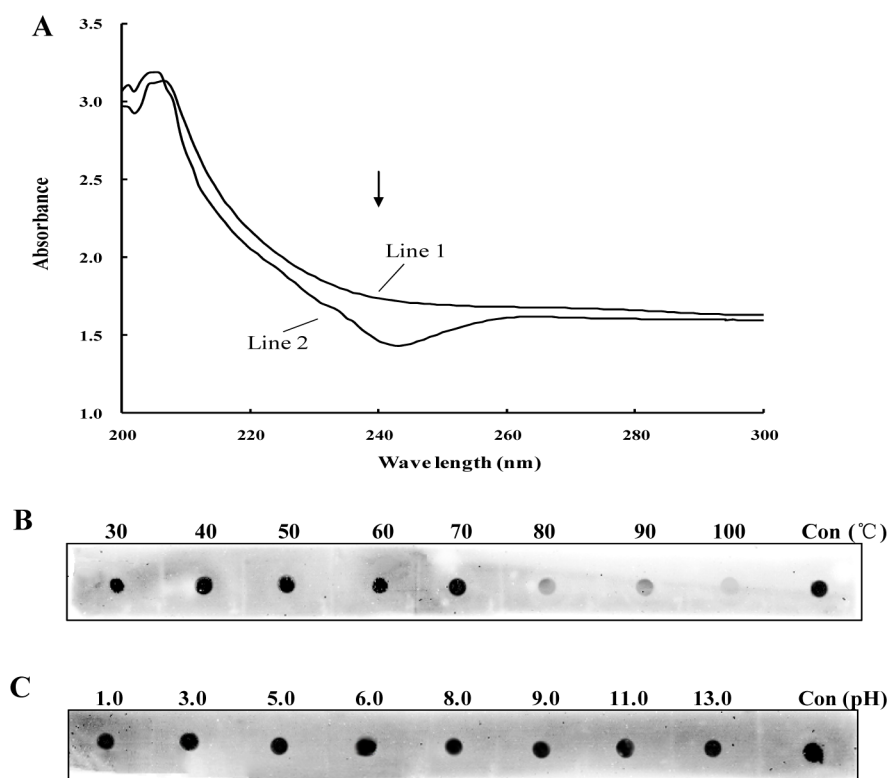


Figure 3. Physicochemical characteristics of the target protein: (A) UV wavelength scanning atlas of the target protein in the milt of large yellow croaker (line 1, target protein treated with low-concentration alkali; line 2, untreated target protein; the change at 240 nm is marked by an arrow); (B) dot blotting analysis of the IgE-binding activity of the target protein of large yellow croaker treated at different temperatures (the target protein treated at 4 °C is the control); (C) dot blotting analysis of the IgE-binding activity of the target protein of large yellow croaker treated with buffers at different pH values (the target protein treated with buffer (pH 7.0) is the control).

multicomponent protein in the milt of large yellow croaker had IgE-binding activity with patient sera. In addition, two components with high molecular weight had stronger IgE-binding activity, whereas the IgE-binding activity of the component with small molecular weight was weak. The purified protein in the carp milt had strong IgE-binding activity following incubation with allergic patient sera and no IgE-binding activity with negative control. Therefore, this proves that two purified target proteins were major allergens in the milt of large yellow croaker and carp, respectively. The result was consistent with the previous prediction.

Analysis of the Amino Acid Composition of the Target Proteins. Because protamine is rich in arginine and basic amino acids, analysis of the amino acid composition of the two purified proteins was performed. The results showed that the purified multicomponent protein in the milt of large yellow croaker had only one basic amino acid, and arginine was 60% of the total amino acid content. The 15 kDa protein isolated from the milt of carp had three basic amino acids, which were histidine, arginine, and lysine, and these amounted to 40% of the total amino acid content.

Glycoprotein Characterization of the Target Protein. Most proteins causing allergies are glycoproteins. The determination of total carbohydrate using the phenol–sulfuric acid method indicated that carbohydrates in the multicomponent protein of the milt of large yellow croaker amounted to 2.5% (w/w) of the whole protein content. β -Elimination was carried out to provide evidence for the existence of O-linked glycan in the target protein's structure. As shown in Figure 3A, compared with the samples that were not treated with alkali, the UV absorbance at

240 nm of the samples treated with alkali increased significantly. These results suggested that O-glycans were present in the structure of the multicomponent protein. Using the same method to determine the 15 kDa protein in the milt of carp, we found that it was a type of glycoprotein with O-glycans.

Thermal and pH Stabilities. The thermal and pH stabilities of the multicomponent protein were analyzed by dot blotting to detect IgE-binding activity. In the thermal stability test, the multicomponent protein had strong IgE-binding activity at low reaction temperatures (≤ 70 °C). However, when the reaction temperature increased to 70 °C, the IgE-binding activity of the multicomponent protein was gradually weakened, and when the reaction temperature increased to 100 °C, the IgE-binding activity was almost undetectable (Figure 3B). High temperature weakened the IgE-binding activity of the multicomponent protein with patient sera. In the pH stability test, the IgE-binding activity of the target protein was stable under different pH values (Figure 3C). This indicated that acid and alkali treatment had no effect on the IgE-binding activity of the target protein with patient sera. Compared with the multicomponent protein in the milt of large yellow croaker, the IgE-binding activity of the 15 kDa protein in carp milt was weaker at high reaction temperatures (≥ 60 °C) and in a strong acid (pH 1.0) or strong alkali (pH 13.0) environment. This indicated that similar to general food allergens, the purified target proteins were relatively stable in thermal and acid/alkali conditions.

Purification of the Components in the Multicomponent Protein. A quick flow heparin affinity gel column was used to purify the components by electrostatic force in a neutral environment. The avidity between different components of

the multicomponent protein and heparin were different, so six components (defined as components 1–6) in it were orderly eluted depending on the binding force with heparin (Figure 4A).

As shown in Tricine-SDS-PAGE (Figure 4B), the molecular weight of the six components was close, and the component with larger molecular weight had stronger avidity with heparin, which

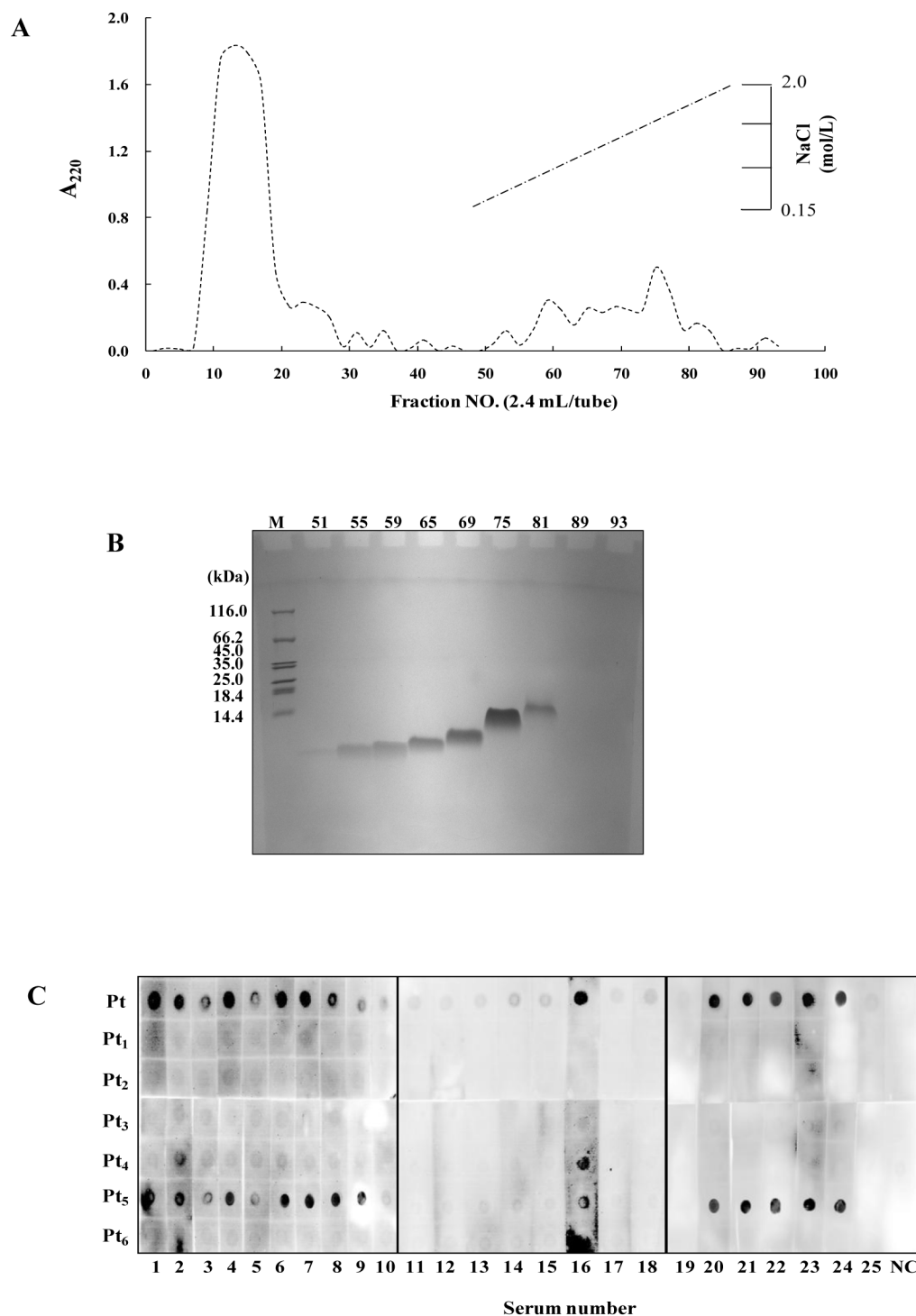


Figure 4. continued

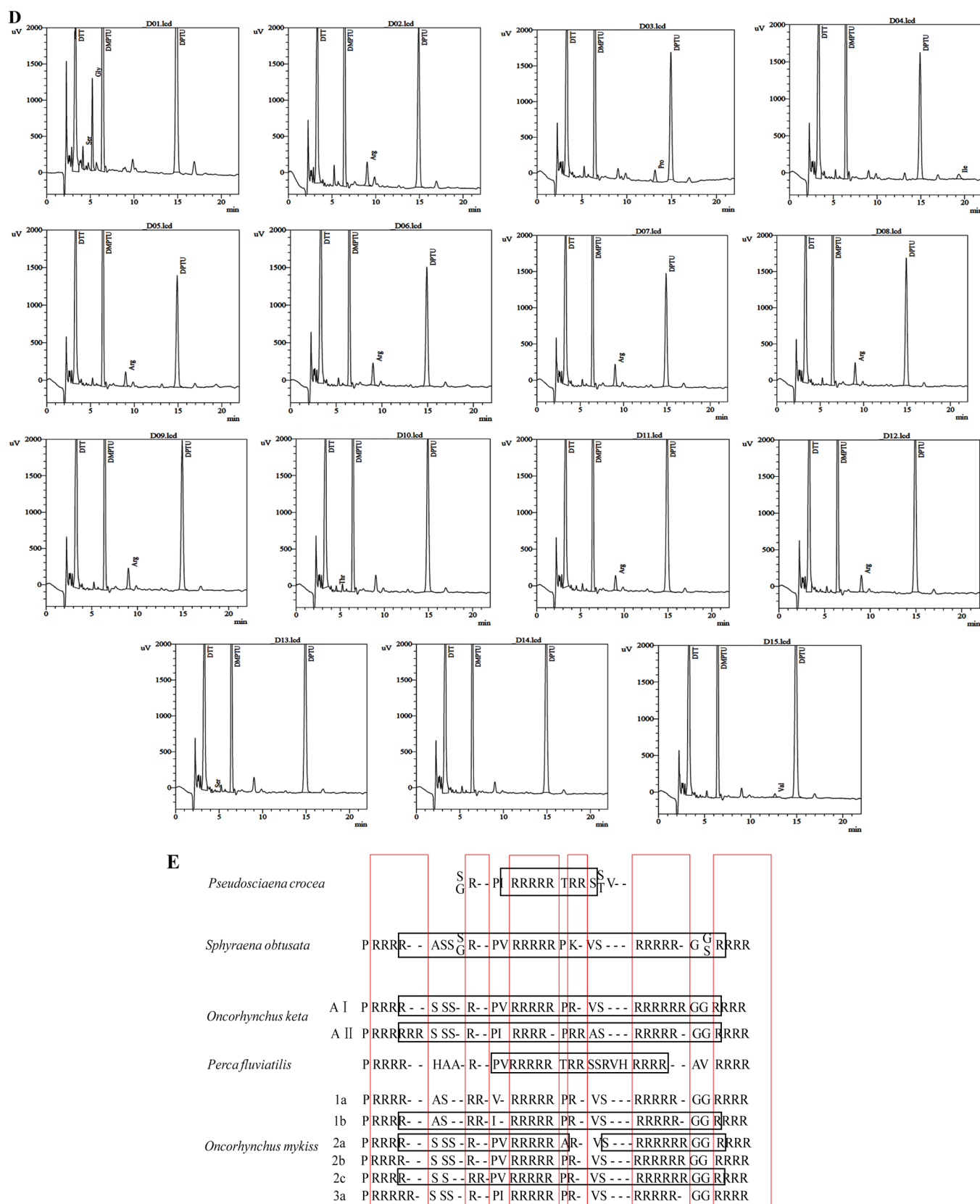


Figure 4. Preparation, IgE-binding activity, and amino acid sequence analysis of the components of the target protein: (A) quick flow heparin affinity chromatography; (B) Tricine-SDS-PAGE analysis of all components of the target protein; (C) dot blotting analysis of the IgE-binding activity of the components in the target protein; (D) HPLC analysis of Edman degradation products; (E) amino acid sequence alignment results of the target protein (marked section in the black box is the predicted epitope).

was hard to wash off. To investigate the immunoreactivity of the different components in the target protein, the six components were incubated with sera of 25 patients, and dot blotting was used to detect the IgE-binding activity of each component. The target protein in the milt of large yellow croaker had IgE-binding activity with more than half of the patient sera; moreover, component 5 with a molecular weight of 12 kDa had the strongest IgE-binding activity (Figure 4C). In addition, of the other five components, components 4 and 6 had IgE-binding activity with the sera of only two patients, but other components did not have IgE-binding activity with patient sera. These results were consistent with those of the serological test, which revealed that the 12 kDa component (component 5) was the major allergen in the milt of large yellow croaker.

Identification of Component 5 by N-Terminal Sequencing. N-terminal sequencing analysis was performed to further investigate component 5 in the milt of large yellow croaker. Following Edman cyclic enzymolysis, the protein was decomposed into 15 amino acids and its amino acid sequence was S(G)RPIRRRRRTRRSS(T)V (Figure 4D). The sequence obtained in this study was compared with the reported amino acid sequence of protamine. It was observed that there was a longer arginine cluster and two shorter arginine clusters in the sequence, and different arginine clusters were separated by Ser, Gly, and Ala. Therefore, component 5, the 12 kDa protein, was one of the components of protamine, and the target protein of large yellow croaker was proved to be protamine. In addition, the linear epitope of the reported protamine and component 5 were analyzed by DNA Star Protean software. The results showed that the value of the prediction range of the hydrophilicity plot, flexible region, antigenic index, and surface probability in protamine were all >1. The predicted linear epitope of protamine had almost a greater mass of amino acids in the amino acid sequence after all of the positive overlap regions were added. Moreover, the predicted linear epitope of component 5 was the 5–14 amino acid IRRRRRTRRS (Figure 4E).

Analysis of the Components of Protamine by RP-HPLC. To analyze and compare the components of different protamines, RP-HPLC was performed to detect the protamine in salmon, herring, and large yellow croaker. Following separation by RP-HPLC, the chromatograms of the different fish protamines were different. Salmine had four peaks with a close peak area, whereas clupeine had three obviously different peaks. LPt had six peaks, of which the first and second peaks appeared early, whereas the retention time from the third to the sixth peak was close. In addition, after analyzing Tricine-SDS-PAGE of three types of protamine, the results showed that salmine had four components with close molecular weight; clupeine had three components and their molecular weights were 14, 12, and 10 kDa, respectively; and the LPt had six components (Figure 5). These results were consistent with the results of RP-HPLC analysis revealing that the components of the different fish protamines were different.

Secondary Structure Identification by CD. The secondary structures of salmine and LPt were analyzed and compared using CD. The CD chromatogram of LPt was similar to that of salmine, and there was a positive peak at 190 nm, a small and wide negative peak at 220–230 nm, and a wide positive peak at 210–220 nm (Figure 6). Thus, an α -helix, β -turn, and random coil were present in the secondary structure. To compare the differences between the protamines from saltwater fish and freshwater fish, the purified protein in the carp milt, which was determined to be a protamine, was analyzed. The CD spectrum

of the carp protamine showed that although there were differences in terms of location and strength of peaks, the second structures of the protamine in carp were speculated to be similar to the salmine or LPt, because there was a negative peak at 208 nm, a positive peak at 205 nm, and a small and wide positive peak at 210–230 nm.

DISCUSSION

In the present study, the major allergen in the milt of large yellow croaker (25 sera from patients) and carp (15 sera from patients, in which 4 sera were the same as that in large yellow croaker) were analyzed by Western blotting, and their crude extracts were incubated with sera. The results showed that the 12 kDa protein reacted with more than half of the patient sera, and the 10 and 14 kDa proteins from the milt of large yellow croaker had IgE-binding activity with a fraction of the patient sera. According to Balhorn's study,²⁶ the protamine evolved from specialized histones through protamine-like proteins to true protamine. As protamine-like proteins have structural features in common with both histones and protamine, and there are potential isomers, protamines do not generally have a single component. Therefore, we supposed that the 12 kDa multicomponent protein was the major allergen in the milt of large yellow croaker, and further studies were carried out on this protein. The Western blotting analysis results of the crude extract of carp milt showed that not only the 15 kDa protein but also the 18, 26, 27, and 34 kDa proteins had IgE-binding activity with a fraction of the patient sera. It was reported that the components and content of protamine are different in different developmental stages of fish milt, and the protein component was complicated in immature or incomplete mature fish milt, which had more specialized histones and protamine-like proteins.³ The carp milt in our study was possibly in the stage of incomplete maturation; thus, the four proteins that had IgE-binding activity with a fraction of the patient sera may be specialized histones or protamine-like proteins. In the ELISA results, most sera reacted with the native protein, but many fewer sera had strong specific binding activity with the denatured protein as shown in the Western blotting results. This indicated that the conformational epitope (native protein) was recognized by more patient sera than the linear epitopes (denatured protein) in the major allergen of the two fish milt. In addition, we found that patient sera 4, 12, 22, and 25 had IgE-binding activity with the allergen in both large yellow croaker milt and carp milt. Patient sera that had specific binding with the crude extracts also had immunologic reactions with the protein in fish muscle and the myofibril or myosinogen of shellfish in serologic tests. In clinical medicine, the patient investigated by Knape et al.²⁷ was found to be allergic to codfish and had high levels of IgG, IgM, and IgE antibodies against protamine sulfate. Furthermore, Collins et al.²⁸ also found that although there was a distinct genetic difference between shellfish and vertebrate fish, an allergic reaction caused by protamine injection could not be ruled out. Therefore, we speculate that there was cross-reactivity between the allergens in different fish milts and other allergens.

The physicochemical characteristics of the target protein were analyzed in this study. The purified multicomponent protein in the large yellow croaker milt had only one basic amino acid, but the purified 15 kDa protein in the carp milt had three basic amino acids. According to the differences in types and numbers of basic amino acids, protamine can be divided into monoprotoamine, diprotoamine, and triprotoamine.²⁹ Xu et al.³⁰ proved that the protamine in carp was a triprotoamine, with a molecular weight of 15 kDa, and contained three basic amino acids, which

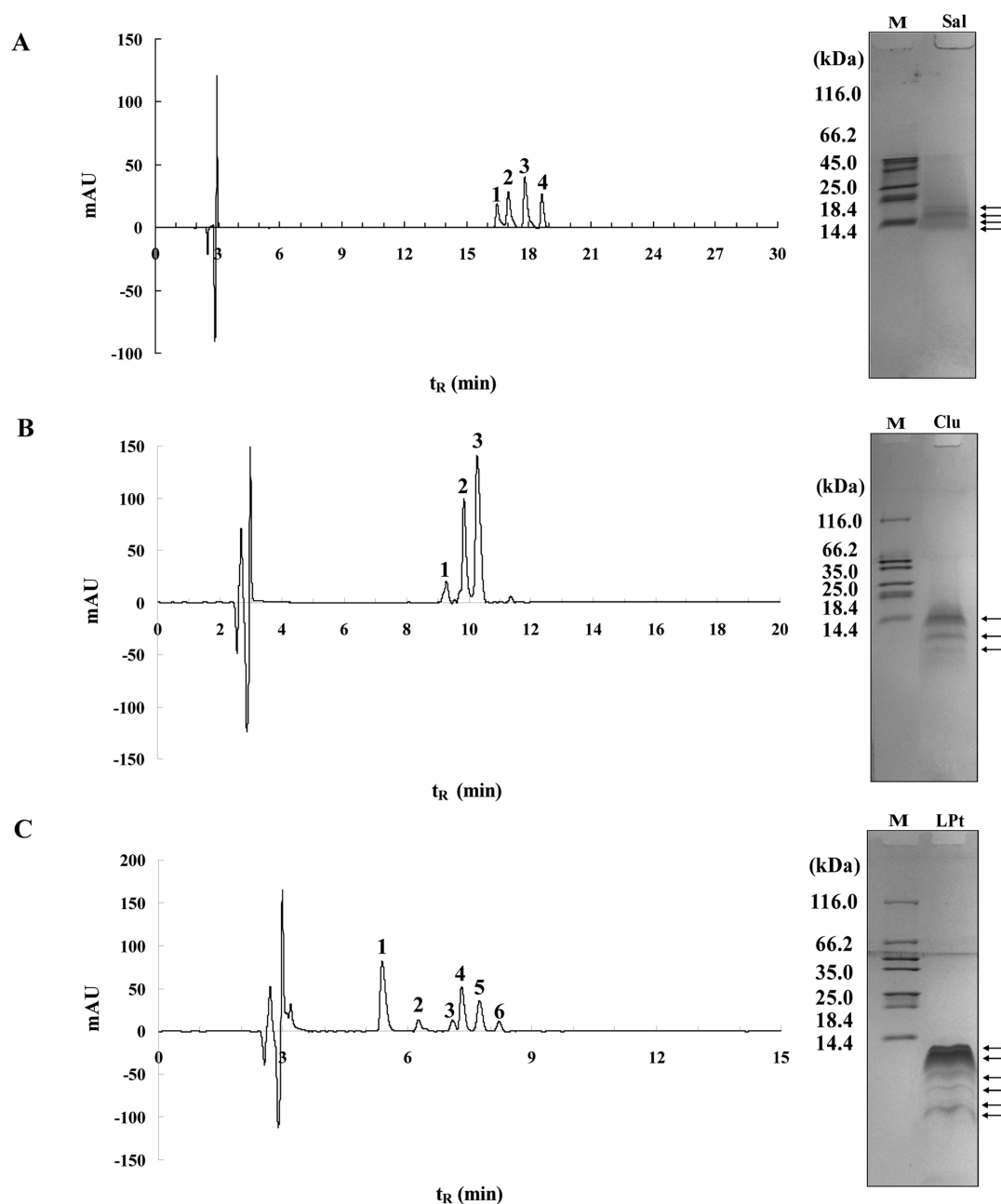


Figure 5. RP-HPLC and Tricine-SDS-PAGE analysis of protamine: (A) salmine; (B) clupeine; (C) LPT. Components of protamine are marked by an arrow.

was consistent with our study. Therefore, it can be speculated that the multicomponent protein in the large yellow croaker milt was a monoprotoamine and that the 15 kDa protein in the carp milt was a triprotoamine. In the thermal stability test, the two target proteins had weak IgE-binding activity at high reaction temperature. This may be due to the unstable β -turn and random coil structure of the target protein, and when the temperature increased above a certain value, its dimensional structure was broken and covered the fractional IgE-binding site. In the pH stability test, the multicomponent protein had stronger IgE-binding activity and the 15 kDa protein had weak IgE-binding activity in a strong acid (pH 1.0) or strong alkali (pH 13.0) environment. This may be because extreme pH conditions can affect the acting force that maintained the protein structure, destroyed its structure, and consequently affected the binding activity. However, the physicochemical properties of the two

proteins were similar, although their protein components and amino acid compositions were different.

The multicomponent protein in the large yellow croaker milt was studied further. The results of separation, purification, and immunologic analysis showed that this multicomponent protein had six components, and component 5 had the strongest IgE-binding activity with patient sera. The antibacterial activity of the components of large yellow croaker milt was also investigated. Component 5 was identified by N-terminal sequencing. The protein's amino acid sequence was S(G)RPPIRRRRTRRSS(T)V, and it was found to be successfully paired with the amino acid sequence of the other fish protamine. Okamoto et al.³¹ found that there were four longer arginine clusters and two shorter arginine clusters in the whole amino acid sequence of fish protamine, and they were separated by characteristic residues such as Thr, Ser, Gly-Gly, Pro-Ile, and Val-Val. Furthermore, two structural

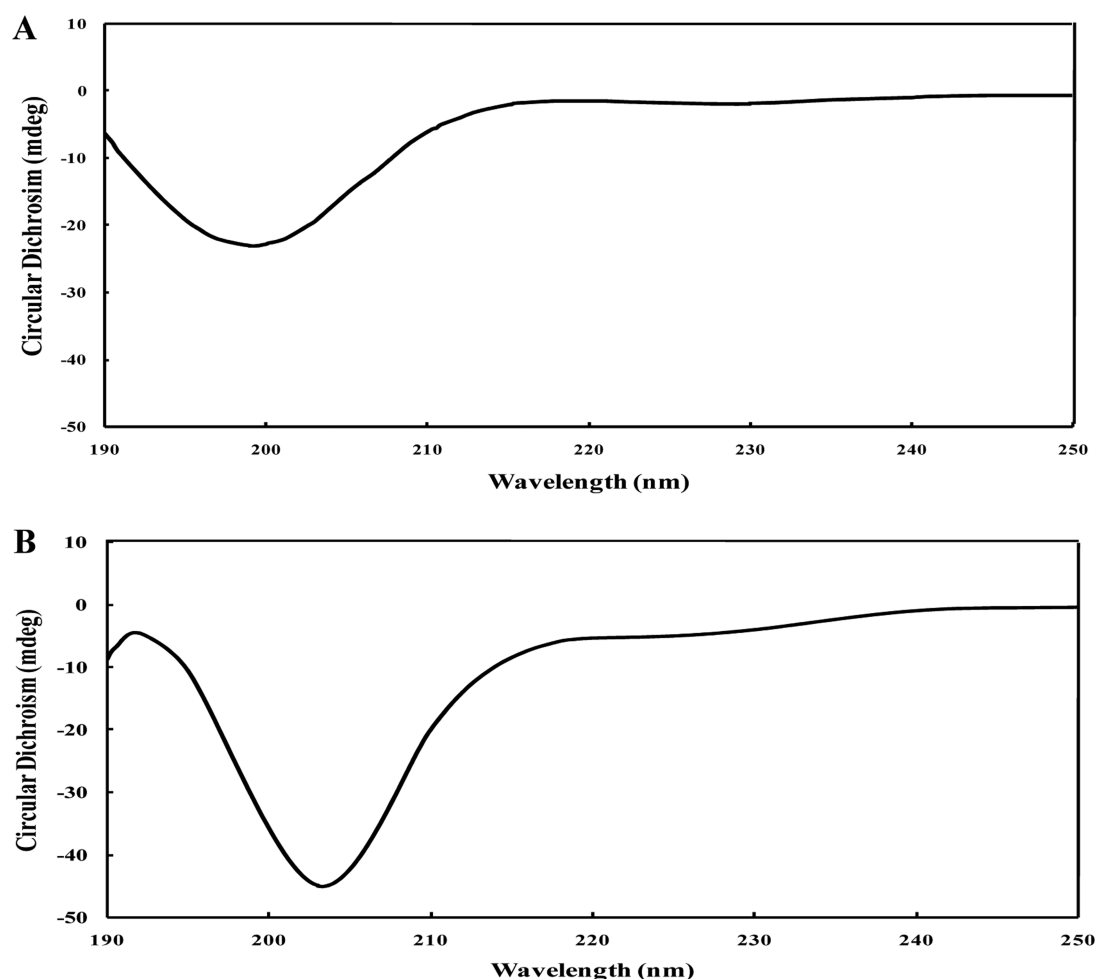


Figure 6. Circular dichroism spectra of protamine: (A) salmine; (B) LPt.

elements have been identified in all vertebrate protamine. One is a series of small “anchoring” domains containing multiple arginine or lysine amino acids that are used to bind the protein to DNA. The second is the presence of multiple serine and threonine residues that can be used as phosphorylation sites.²⁶ Therefore, it was confirmed that the purified multicomponent 5 of protamine and that the purified multicomponent protein was protamine. Furthermore, the obtained sequence in the present study was in the middle of the sequence of protamine and did not start from the N-terminus. This may because the N-terminus was cut off by endonuclease in the protein during the process of protein maturity. During the process of digestion, when circulated to Ser or Thr, trifluoroacetic acid, which plays a splitting role in the previous cycle, reacted with the hydroxy in Ser or Thr and closed its α -amino; hence, Ser or Thr was not detected in the 14th cycle.³² In our study, we also used DNA Star Protean software to analyze the epitope of other reported fish protamine and component 5. The predicted linear epitope of protamine almost had a greater mass of amino acids in the amino acid sequence. This may because protamine is a simple protein containing abundant Arg and a number of stretches of arginine residues usually separated by neutral amino acids, such as Ala, Ser, and Gly. However, earlier studies showed that the amino acids Ala, Ser, Asn, Gly, and, particularly, Lys play a key role in IgE binding to allergenic epitopes.³³ However, Raman spectroscopy has shown that the protamine not combined with DNA is unstructured in solution.³¹ Therefore, we suggest that protamine

may belong to one of the intrinsically unstructured proteins. Intrinsically unstructured proteins are entire proteins or proteins with large segments that lack a fixed or ordered three-dimensional structure. This type of protein is characterized by a low content of bulky hydrophobic amino acids and a high proportion of polar and charged amino acids and often contains repetitive sequences.³⁴ Nevertheless, protamine intrinsically has characteristics of unstructured proteins, and its disordered sequences are often found as flexible linkers that can connect globular domains, but with an insufficient hydrophobic core to fold stable globular proteins.

Using RP-HPLC to analyze and compare different fish protamines, we showed that salmine, clupeine, and LPt had three, four, and six components, respectively. Awotwe-Otoo et al.³⁵ indicated that salmine consisted of four major polypeptides, made up of only aliphatic amino acids, of which approximately 67% of the residues were arginine. Chang et al.³⁶ separated four homogeneous fractions from salmine by RP-HPLC. In addition, a previous paper showed that three similar components were isolated from clupeine by ion-exchange column chromatography.³ This proved that RP-HPLC could accurately separate the components of protamine. Because different fish protamines have different components, the retention time and molecular weight of the components were different. This may due to different fishes having different closely related protamine genes per haploid genome, coding for different proteins.²⁶ The evolution process of protamine, histones, and protamine-like proteins could affect

their formation. In addition, in salmine, for example, amino acid sequence analysis revealed that there were 30–32 amino acids in each of its components, and the molecular weight of a single component was around 4 kDa. However, the electrophoresis results showed that its single molecular weight was around 15 kDa. This may be because protamine is a hydrophilic basic protein. During the denaturation process, its autocoupling SDS was less and its electrophoretic mobility was slow due to its character. The apparent molecular weight was mainly related to the average number of proteins combined with SDS and the shape of the denatured protein; thus, the theoretical molecular weight of protamine was inconsistent with the apparent molecular weight. According to a comparison of the CD spectra of salmine, LPT, and the protamine from carp and other reported methods of obtaining secondary structure,³⁷ we found that there were α -helices, β -turns, and random coils in their secondary structures. A previous study showed the same structural elements in its secondary structure but their contents were different; there were no β -pleated sheets or other polypeptide chain sheets, and the CD chromatogram of salmine was consistent with that reported previously.³⁸ However, as protamine is an intrinsically unstructured protein. Protamines does not have highly ordered structures like other proteins, so this might result in there being no obvious characteristic peak in the CD chromatogram to directly determine the secondary structure. Furthermore, there was difference in the location and strength of peaks in the CD chromatogram of carp protamine compared with the protamine from two other marine fish species. This may be because salmine and LPT are monoproamines, whereas the carp protamine is a triproamine, and there were differences in their amino acid compositions.

In conclusion, the major allergen in the milt of large yellow croaker was protamine, a multicomponent protein that contained six components. LPT is a monoproamine containing 60% Arg and O-glycans, is thermally stable, and is stable in acid and alkaline environments. Furthermore, a comparison of different fish protamines showed similar secondary structures, but different components. On the basis of the above research, follow-up work should focus on analyzing the protein structure and cross-reactivity with other species and identifying a method for reducing the immunoreactivity of the allergen. These findings may be used to aid further research on controlling protamine allergy.

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Notes

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ABBREVIATIONS USED

Ala, alanine; Arg, arginine; Asn, asparagine; CD, circular dichroism; Clu, clupeine; CMt, milt of common carp; ELISA, enzyme-linked immunosorbent assay; Gly, glycine; IgE, immunoglobulin E; Ile, isoleucine; LMt, milt of large yellow croaker; LPT, protamine of large yellow croaker; LTP, target protein

of large yellow croaker; Lys, lysine; Pro, proline; RP-HPLC, reversed phase high-performance liquid chromatography; Sal, salmine; Ser, serine; Thr, threonine; TMB, 3,3',5,5'-tetramethylbenzidine; Tricine-SDS-PAGE, Tricine-SDS-polyacrylamide gel electrophoresis; Val, valine; VWD, variable-wavelength detector

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