



Purification and characterisation of sarcoplasmic calcium-binding protein, a novel allergen of red swamp crayfish (*Procambarus clarkii*)

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ABSTRACT

Crayfish sarcoplasmic calcium-binding protein (SCP) was purified. The physicochemical and polymorphic characterisations were also analysed. SCP was purified by column chromatography to reveal a single band with molecular mass of 22 kDa and further confirmed by mass spectrometry. The results of physicochemical characterisation showed that SCP was stable in the processes of thermal or acid/alkali treatment, and could be digested by simulate gastrointestinal fluid. Importantly, the comparison of SCP polymorphism using sera from crustacean-allergic patients demonstrated SCP-II had a weaker IgE-binding activity. The isoelectric points of SCP subunits a, b and c were 4.6, 4.7, and 4.8, respectively, as determined by two-dimensional electrophoresis and IgE immunoblotting analysis showed that patients' sera reacted to three subunits of SCP. Finally, it can be concluded that SCP is a stable polymorphic allergen in crayfish, and all of its isotypes and subunits have allergenicity.

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1. Introduction

There is a high consumer demand for aquatic products, particularly in the coastal areas of China, because of their delicious taste and nutritional value. These products are, however, also well known as one of the most common causes of food allergy. Fish and crustaceans are two of the eight major sources of allergenic food proposed by the Food and Agriculture Organisation (FAO) of the United Nations and the World Health Organisation (WHO) (FAO/WHO, 2001). The increasing production and consumption of aquatic products has resulted in more hypersensitive reactions, most which are mediated by IgE antibodies and manifest as urticaria, asthma, diarrhea, and even life-threatening anaphylaxis (Lopata, O'Hehir, & Lehrer, 2010). Allergy to aquatic products has become an important public health issue.

Various worldwide studies on the allergens of aquatic products that cause IgE-mediated hypersensitivity have been reported. Parvalbumin, a 12 kDa protein, is the major allergen in fish (Elsayed & Aas, 1971; Guo, Kubota, & Shiomi, 2011). Collagen and its subunits have also been reported as potential allergens that cause allergy reactions to various fish (Hamada, Nagashima, & Shiomi, 2001; Pan et al., 2012). Tropomyosin, a thermally stable myofibrillar protein consisting of two subunits with molecular masses of 36–38 kDa, has been identified as the major allergen in molluscs and crustaceans (Leung et al., 1996; Shanti, Martin, Nagpal, Metcalfe,

& Rao, 1993). Arginine kinase, which is normally involved in cell metabolism in invertebrates, is a 40 kDa water-soluble protein found in myosinogen and has been well-documented as an important cross-reactive allergen in crustaceans (Yu, Lin, Chiang, & Chow, 2003). In recent years, myosin light chain (Ayuso et al., 2008), hemocyanin (Piboonpocanun, Jirapongsananuruk, Tipayanon, Boonchoo, & Goodman, 2011), and sarcoplasmic calcium-binding protein (SCP) have been reported to be the novel allergens of shrimp.

SCP was identified as a shrimp allergen in *Penaeus monodon* (Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008) and its IgE-binding activity was demonstrated at the molecular level in *Litopenaeus vannamei* (Ayuso et al., 2009). The water-soluble SCP protein is found in myosinogen and functions as an invertebrate EF-hand calcium-binding protein. There is also some evidence to indicate that it plays a role in muscle relaxation, similar to that of parvalbumin in vertebrates (Hermann & Cox, 1995). One of the typical properties of parvalbumin and SCP is their polymorphism (Liu et al., 2010; Wnuk & Jauregui-Adell, 1983). It has been asserted that for an allergen to retain its allergenicity, it must be stable enough to survive food processing treatments and *in vivo* digestion after consumption. Although SCP is of particular interest in that it has similar characteristics to arginine kinase, collagen, and parvalbumin, it is nevertheless important to study the physicochemical and polymorphic characteristics of this protein.

Due to its worldwide popularity as a gourmet food, the red swamp crayfish (*Procambarus clarkii*), which is native to south-central United States and Northeastern Mexico, has been artificially

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propagated for a number of years. With increasing consumption of crayfish, cases of hypersensitive reactions after its ingestion have been reported. Crustacean-sensitive patients in the United States had a high prevalence of skin test positivity to crayfish extract (70%) (Daul, Morgan, & Lehrer, 1993). *P. clarkii* is one of the important economic species in China and its total production in 2010 was around 563,281 tons (Anonymous, 2011). A survey of 182 children suffering from urticaria in China indicated that 58.79% of subjects had an allergic reaction to aquatic products containing crayfish, which suggests that crayfish is an important allergenic food (Wang & Yu, 2009). It is still uncertain whether SCP is the allergen in *P. clarkii* that causes IgE-mediated hypersensitivity.

The aim of this study is to purify crayfish SCP, confirm its allergenicity, and further analyse its physicochemical and polymorphic characteristics. Then it will make the contribution to the future research of the desensitisation investigation of crayfish.

2. Materials and methods

2.1. Materials

2.1.1. Animal material

Fresh red swamp crayfish (*P. clarkii*), blue scud (*Decapтерus maruadsi*), crucian carp (*Carassius auratus*), manila clam (*Ruditapes philippinarum*), Pacific white shrimp (*L. vannamei*), and mud crab (*Scylla paramamosain*), were purchased from a local market in Xiamen. Muscle samples were immediately removed and used for immediate experimentation or stored at -80°C for later use.

2.1.2. Chemicals

Chemicals were purchased from various suppliers: Q-Sepharose and Sephacryl S-200 HR from Amersham Biosciences (Uppsala, Sweden); protein standards for SDS-PAGE from Fermentas (Vilnius, Lithuania) or New England BioLabs (Beverly, USA); periodic acid-Schiff (PAS) staining kit from Genmed (Grand Rapids, USA); horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibody from Kirkegaard and Perry Laboratories (Gaithersburg, USA); HRP-conjugated goat anti-rabbit IgG antibody and the enhanced chemiluminescent (ECL) substrate for immunoblotting from Pierce (Rockford, USA); 3,3',5,5'-Tetramethylbenzidine (TMB) from Tiangen (Beijing, China), and porcine pancreatic juice from Sigma-Aldrich (St. Louis, USA). Rabbit anti-crayfish polymorphic SCP polyclonal antibody and porcine pepsin were prepared in our laboratory. All other reagents were of analytical grade.

2.1.3. Human sera

Sera were obtained from 17 crustacean-allergic patients (No. 475, 362, 112, 252, 303, 555, 743, 468, 354, 336, 083, 300, 384, 282, 437, 221, 347) provided by the Hospital of Jimei University. Crustacean allergy was confirmed on the basis of extensive history, physicochemical examination and objective manifestations observed after ingestion of crustacean products. Written informed consent was obtained from each patient. Sera from healthy individuals (No. 307, 776) without a history of crustacean allergy were pooled and used as a negative control. All sera were stored at -80°C until use.

2.2. Myosinogen preparation and immunoassay

Crayfish muscle tissue was minced and homogenized with five volumes of ice-cold 20 mM Tris-HCl buffer (pH 7.0). The homogenate was then centrifuged at 7200 rpm for 20 min at 4°C . The supernatant was the crayfish myosinogen.

SDS-PAGE was performed. Samples were separated on 15% polyacrylamide gels with 5% stacking gels. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 (CBB).

The separated sample was transferred to a nitrocellulose membrane, after which Western-blotting was carried out as described by Huang et al. (2010). Human serum (1:4 dilution) was used as the primary antibody, and HRP-labelled goat anti-human IgE antibody (1:20000 dilution) as the secondary antibody. Blot was visualized using ECL.

2.3. Protein purification and peptide analysis by matrix-assisted laser desorption ionisation mass spectrometry (MALDI MS)

The 22 kDa protein was purified according to the method of Yu et al. (2003) and Shiomi et al. (2008), with some modifications. All procedures were performed on ice or at 4°C . The muscle tissue of crayfish was minced, then subjected to homogenization and centrifugation, and the supernatant fractionated with ammonium sulphate from 70% to 90% saturation. The resulting precipitate was dissolved and dialysed before being subjected to a Q-Sepharose column. The binding proteins were eluted with a linear gradient of 0–0.3 M NaCl and subsequently eluted with buffer (10 mM Tris-HCl, pH 8.0) containing 0.3 M NaCl at a flow rate of 1 ml/min. The fractions with SCP were concentrated and then applied to a Sephacryl S-200 HR gel column. The column was eluted at a flow rate of 0.4 ml/min and fractions with purified SCP were collected and identified. Protein concentration was estimated by measuring the absorbance of the sample solution at 280 nm.

The purified protein was migrated on 15% SDS-PAGE gel and CBB stained for mass spectrometry analysis. The target protein area was selected and sent to Shanghai Applied Protein Technology Co. Ltd. (Shanghai, China) for analysis by MALDI MS. Mass spectrometric conditions were as follows: laser source: ND; wavelength: 355 nm; accelerating voltage: 2 kV; and substrate: α -cyano-4-hydroxycinnamic acid (α -CHCA). The positive ion mode was adopted and the data were obtained automatically. Results were compared with the data base of NCBI.

2.4. Physicochemical characterisation of SCP

2.4.1. Thermal and pH stability and dot-blotting

In the study on thermal stability, purified SCP was incubated at different temperatures (30°C , 40°C , 50°C , 60°C , 70°C , 80°C , 90°C , and 100°C) for 30 min. For pH stability, SCP was incubated in buffers with different pH values (pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0) for 1.5 h at room temperature. After incubation, all the samples were analysed by SDS-PAGE and dot-blotting.

For dot-blotting, the purified SCP, treated at different temperatures and different pH values, was directly blotted on nitrocellulose membrane and incubated with pooled human sera (1:4 dilution) after blocking. Following incubation with HRP-labelled goat anti-human IgE, the immunoassay was carried out using ECL.

2.4.2. Simulated digestion stability and inhibition ELISA

The digestibility of purified SCP in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was examined according to the method of Huang et al. (2010) with some modifications. The total volume of the reaction solution was 1 ml. The ratio of pepsin to purified SCP was 1:1000 (w:w). At different time intervals (0, 1, 2, 5, 10, 15, 30, and 60 min) a 100 μl aliquot of the reaction solution was removed and terminated by adding 30 μl of 200 mM Na_2CO_3 . For intestinal digestion, the ratio of pancreatic juice to purified SCP was 1:1000 (w:w). The reaction interval times were 0, 1, 15, 30, 60, 120, 180, and 240 min and the reactions were immediately terminated by heating at 95°C for 5 min. A protein sample, dissolved in

SGF/SIF without adding the corresponding enzyme, was used as a control. Samples were analysed by SDS–PAGE. All experiments were performed in triplicate.

The specific IgE-binding activity of purified SCP digestion fragments was determined by inhibition ELISA, according to Huang et al. (2010). A 50 µl aliquot of human serum (diluted 1:2 with 0.1% non-fat milk in PBS) was mixed with the same volume of inhibitor (digested SCP, in the concentration range of 0.03–60 µg/ml). A 50 µl aliquot of this mixture was then transferred to the ELISA plate that had been pre-coated with SCP. After incubation with HRP-conjugated goat anti-human IgE antibody (1:2000 dilution), the color development was performed using TMB. Absorbance was measured at 450 nm. The hypersensitivity of the digested SCP fragments was determined by calculating the inhibition rate.

2.4.3. PAS staining and carbohydrate-peptide linkage characterisation

To determine whether SCP was a glycosylated protein, PAS staining was performed according to Liu et al. (2010). β-elimination was used to characterise the carbohydrate-peptide linkage of SCP. The protocol was carried out according to the method described by He (2004), with some modifications. A 90 µl sample of SCP (0.3 mg/ml) was mixed with 10 µl of 0.5 M NaOH and incubated at 37 °C for 16 h, after which the absorbance at 220–280 nm was measured.

2.5. Polymorphic characterisation of SCP

2.5.1. Native-PAGE

Native-PAGE can be regarded as a modification of the SDS–PAGE procedure. In the former method the samples are not heated and no SDS or reducing agent is added to samples, gel or the electrophoretic buffer. Electrophoresis is then carried out at 4 °C, after which the gel is stained with CBB.

2.5.2. Two-dimensional electrophoresis (2-DE)

Purified SCP was applied to a 7 cm IPG gel strip with an immobilized pH gradient (3.9–5.1) and isoelectric focusing was performed in an Ettan IPGphor 3 unit (GE Healthcare). After isoelectric focusing, the strip was equilibrated and subjected to SDS–PAGE on 12% gel, followed by CBB staining.

2.6. Polyclonal antibody preparation and immunoassay

Purified SCP was adjusted to a concentration of 200 µg/ml and then emulsified with an equal volume of complete Freund's adjuvant (St. Louis, USA) as an antigen. A 100 µg quantity of antigen was added to a total volume of 1 ml, which was then subcutane-

ously injected into an adult female rabbit. Further injections, with the same amount of purified SCP mixed with incomplete Freund's adjuvant, were carried out at 2, 4, and 5 weeks later. Five days after the final injection, the blood was collected from the retro-orbital plexus of the rabbit, in order to obtain antiserum.

Polyclonal antibody was used for the immunoassay of crude extracts from aquatic products and processed crayfish (soft-boiled and boiled). The muscle tissue of each sample was minced and homogenized with five volumes of ice-cold 20 mM Tris–HCl buffer (pH 7.5, containing 3% NaCl w/w). Protein concentrations of all samples were adjusted to the same volume, after which western-blotting was carried out. Rabbit anti-crayfish polymorphic SCP polyclonal antibody (1:8000 dilution) was used as the primary antibody. HRP-labelled goat anti-rabbit IgG antibody (1:20000 dilution) was used as the secondary antibody.

3. Results and discussion

3.1. The IgE-binding activity of patients' sera to crayfish myosinogen

Western-blotting analysis of crayfish myosinogen was performed using sera from 17 crustacean-allergic patients, to identify the specific IgE-binding allergens. As shown in Fig. 1, the sera from 15 out of 17 subjects (except No. 303 and 221) indicated IgE-binding activity in relation to the protein at 40 kDa, whereas a 22 kDa protein was recognised in myosinogen with a particular intensity in 4 sera (No. 362, 303, 083, and 300). It should be noted that the 22 kDa protein was recognised with higher intensity than that of the 40 kDa protein, which corresponds to arginine kinase. The control sera did not show any labelling. In recent studies, SCP with a molecular mass of about 20 kDa was confirmed to have a specific IgE-binding activity towards human sera and was considered to be a novel allergen in shrimps (Ayuso et al., 2009; Shiomi et al., 2008). It can thus be inferred that the 22 kDa protein may be the water-soluble protein SCP.

3.2. Purification and identification of the 22 kDa protein

In the present study, the 22 kDa was highly purified from the muscle of crayfish by ammonium sulphate fractionation and sequential column chromatography with Q-Sepharose and Sephacryl S-200 HR. Fractions of Q-Sepharose, eluted at a NaCl concentration of 0.3 M, contained the 22 kDa target protein and a 40 kDa contaminated protein (Fig. 2A). The fractions were then pooled, concentrated, and loaded on the following Sephacryl S-200 HR gel-filtration column, which allowed the removal of proteins with high molecular mass. The target protein and the 40 kDa protein

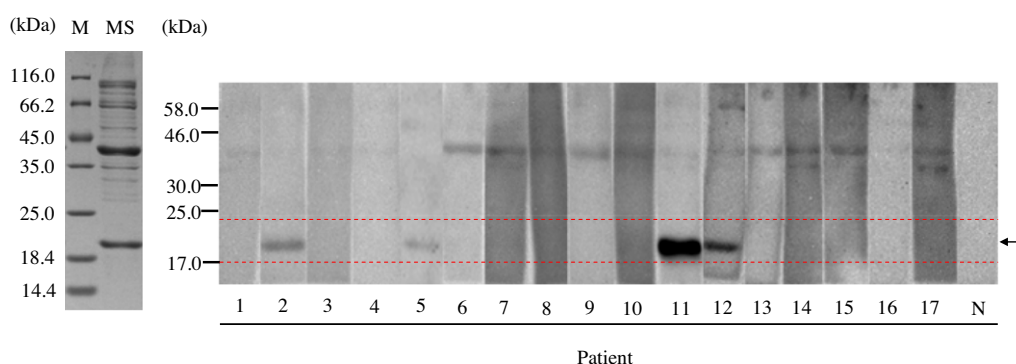


Fig. 1. SDS–PAGE analysis and IgE immunoblotting assay of crayfish myosinogen. M: protein marker; MS: SDS–PAGE analysis of myosinogen; 1–17: immunoblotting profiles of IgE-binding activity of myosinogen to patient sera (1–17 correspond to sera No. 475, 362, 112, 252, 303, 555, 743, 468, 354, 336, 083, 300, 384, 282, 437, 221, 347); N: immunoblotting using pooled normal sera (No. 307, 776).

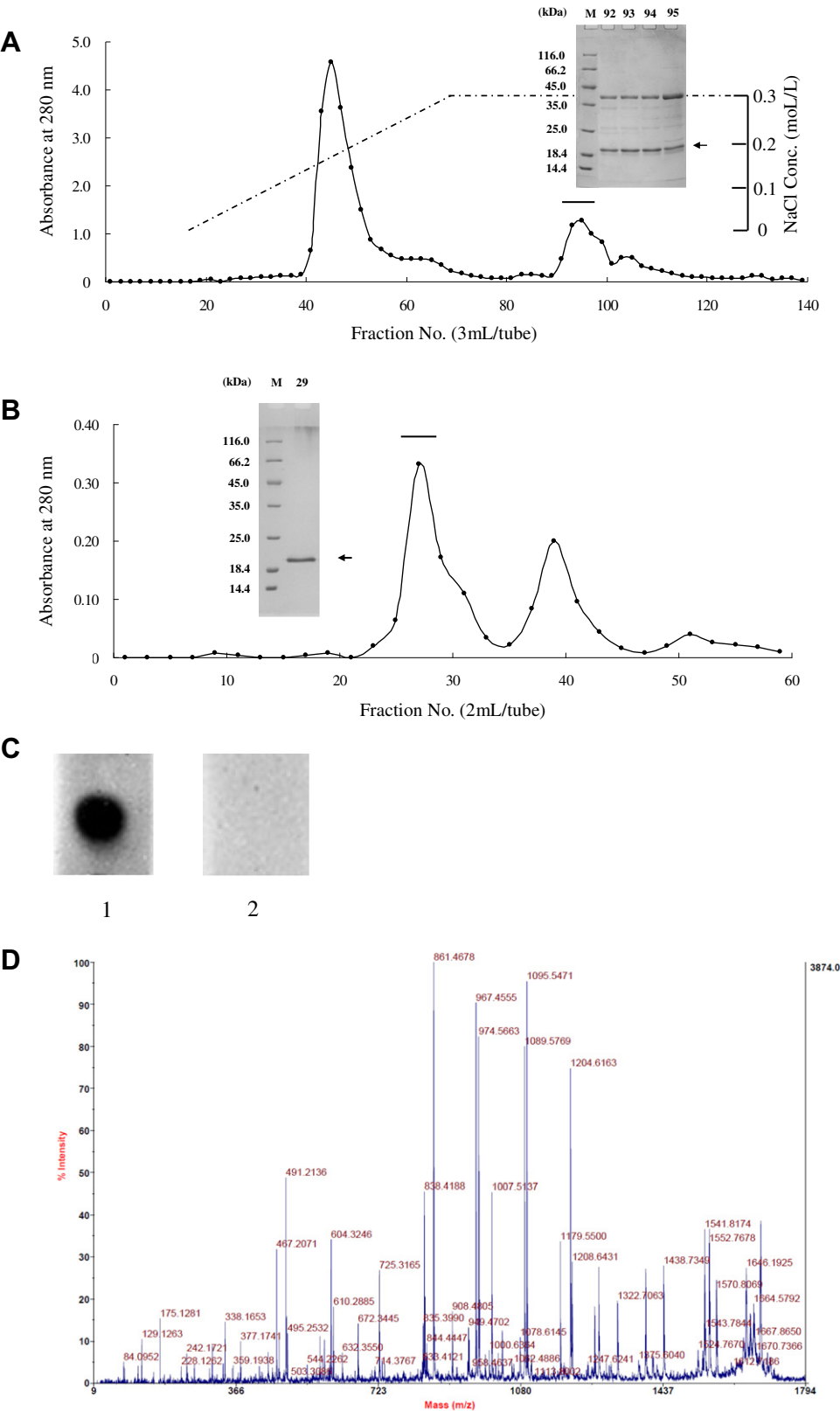


Fig. 2. Chromatographic purification, IgE reactivity identification, and mass spectrometric analysis of the 22 kDa protein in crayfish. (A) Q-Sepharose chromatography. (B) Sephacryl S-200 HR gel chromatography. The numbers on the tops of the lanes correspond to the fraction number. Target protein fractions under the bar were pooled. (C) Dot-blotting analysis of the purified protein. dot 1, sera of crustacean-allergic patients; dot 2, sera of healthy individuals (negative control). (D) The map of mass spectrometry/mass spectrometry (MS/MS). (E) Effective peptides from mass spectrometry (MS) compared with data in the NCBIInr. (F) Protein sequence alignments of SCPs from *Procambarus clarkii*, *Litopenaeus vannamei*, and *Penaeus monodon*. The N-glycosylation site is marked by a dashed box.

E					
	Start-End	Calc. Mass	Obsrv. Mass	$\pm$ da	Peptide Sequence
	15-36	2726.2227	2726.2017	-0.021	YMYDIDNNGFLDKNDFECLALR
	28-36	1137.5358	1137.5546	0.0188	NDFECLALR
	37-43	802.4417	802.4645	0.0228	NTLIEGR
	37-56	2239.0737	2239.1187	0.045	NTLIEGRGEFNEAAYANNQK
	44-56	1455.6499	1455.6484	-0.0015	GEFNEAAYANNQK
	94-103	1042.5356	1042.5466	0.011	AFSAFPGAFK
	104-111	968.52	968.5348	0.0148	TFIANQFK
	112-127	1721.8341	1721.8439	0.0098	TIDVNGDGLVGVDEYR
	112-133	2466.1931	2466.2209	0.0278	TIDVNGDGLVGVDEYRLDCISR
	134-148	1698.8334	1698.8374	0.004	SAFANIKEIDDAYNK
	165-190	2977.4619	2977.4272	-0.0347	YQELYAQFISNPDESANAVYLFGLPK
	165-193	3333.6313	3333.6436	0.0123	YQELYAQFISNPDESANAVYLFGLPK EVQ

F					
	Purified protein	YMYDIDNNGFLDKNDFECLALRNTLIEGRGEFNEAAYANNQK			
	<i>Procambarus clarkia</i>	MAYSWDNRVKYVVRMYDIDNNGFLDKNDFECLALRNTLIEGRGEFNEAAYANNQKIMSN	60		
	<i>Litopenaeus vannamei</i>	MAYSWDNRVKYVVRMYDIDNNGFLDKNDFECLALRNTLIEGRGEFSADAYANNQKIMRN	60		
	<i>Penaeus monodon</i>	MAYSWDNRVKYVVRMYDIDNNGFLDKNDFECLALRNTLIEGRGEFSADAYANNQKIMRN	60		
	Purified protein	AFSAFPGAFKTFIANQFKTIDVNGDGL			
	<i>Procambarus clarkia</i>	LWNEIAELADFNKDGAVTIEQFKQAVQKNCSSGKAFSAFPGAFKTFIANQFKTIDVNGDGL	120		
	<i>Litopenaeus vannamei</i>	LWNEIAELADFNKDGEVTVDEFKQAVQKHCQGGKYGFDPGAFKTFIANQFKTIDVNGDGL	120		
	<i>Penaeus monodon</i>	LWNEIAELADFNKDGAVTIEQFKQAVQKHCQGGKYGFDPGAFKTFIANQFKTIDVNGDGL	120		
	Purified protein	VGVDYRLDCISRSFANIKEIDDAYNK			
	<i>Procambarus clarkia</i>	VGVDYRLDCISRSFANIKEIDDAYNKLATDEDKKAGGISLARYQELYAQFISNPDESA	180		
	<i>Litopenaeus vannamei</i>	VGVDYRLDCISRSFANIKEIDDAYNKLTTEDDRKAGGLTLERYQELYAQFISNPDESC	180		
	<i>Penaeus monodon</i>	VGVDYRLDCISRSFANIKEIDDAYNKLTTEDDRKAGGLTLERYQELYAQFISNPDESC	180		
	Purified protein	NAVYLFGLKEVQ			
	<i>Procambarus clarkia</i>	NAVYLFGLKEVQ	193		
	<i>Litopenaeus vannamei</i>	SACVYLFGLKEVQ	193		
	<i>Penaeus monodon</i>	SACVYLFGLKEVQ	193		

Fig. 2. (continued)

were separated effectively. After Sephacryl S-200 HR chromatography, highly purified protein migrated as a single band with a molecular weight of about 22 kDa on SDS–PAGE (Fig. 2B).

To confirm the IgE-binding activity of the 22 kDa protein, purified protein was used as antigen and investigated by dot-blotting with sera samples from crustacean-allergic patients. As shown in Fig. 2C, dot 1 showed a strong positive result with patients' sera. No IgE-binding activity was detected with negative control sera (dot 2). The 22 kDa protein could be identified as an allergen in crayfish.

Mass spectrometry was used to identify the 22 kDa protein. Peptide fragments, produced upon digestion of the purified protein with trypsin, were analysed by MALDI MS. The mass spectrometry of the purified protein showed multiple peaks ranging from 800 to

4000 Da. The effective peaks that were further analysed by mass spectrometry/mass spectrometry (MS/MS), as shown in Fig. 2D. A total of twelve peptides matched SCP from different crustacean species. The matched peptides had 100%, 84.9%, and 83.3% identity with aligned amino acid sequences of SCP from *P. clarkii* (gi|81171160), *L. vannamei* (gi|223403273) and *P. monodon* (gi|380503696), respectively (Fig. 2E and F). These results thus revealed that the purified protein shared high identities with SCPs from different species of shrimp. Based on these results it can thus be confirmed that the purified protein was the crayfish (*P. clarkii*) SCP, with a molecular mass of 22 kDa, which corresponds to the results from SDS–PAGE. The different molecular masses of crayfish and shrimp may be derived from the familia difference or the differences in amino acid sequences. In addition, according to the

amino acid sequence of *P. clarkii*, there is only one N-glycosylated site, which was marked by a dashed box. The N-linked glycan is usually identified by its conserved sequence -Asn-X-Ser/Thr/-.

3.3. Physicochemical characterisation of SCP

3.3.1. Thermal stability and pH stability

The thermal stability of purified SCP was checked by SDS-PAGE. Results, as illustrated in Fig. 3A, indicate that during the process of thermal treatment, SCP appeared to be intact. Even at temperatures of up to 100 °C, the SCP had not been decomposed. To further detect the change in SCP IgE-binding activity when treated at different temperatures, dot-blotting was performed using patients' sera. The result showed that the IgE-binding activity of SCP increased gradually with an increase in temperature, up to 80 °C. However, when the temperature reached 80 °C and above, the IgE-binding activity began to decrease (Fig. 3B). It can be speculated that the epitopes of SCP that had been inward-oriented, were turned outward and that unfolding increased according to the extent of heating. The possibility that unfolding may have led to the formation of new antigenic structures (50–70 °C) should also be considered. When the temperature exceeds 80 °C there is a loss of the secondary and the tertiary structure of SCP, which causes the protein to adopt a fully unfolded conformation (Davis & Williams, 1998). It is possible that there is a potentially allergenic structure, created not simply by exposing the epitopes but by new interactions and folding that occurs after heat denaturation. Reorganisation of protein structure and/or aggregation of SCP may have a masking effect on the recognition of sequential (or lin-

ear) epitopes (Taheri-Kafrani et al., 2009). Further research is needed to investigate the possibility that the thermal stability of SCP depends on the presence of binding calcium ions, such as parvalbumin (Bugajska-Schretter et al., 2000).

Fig. 3C shows the pH stability of purified SCP. When mixed with buffers at pH values ranging from 1.0 to 11.0, SCP remained stable. It was however, interesting to find that dot-blotting analysis indicated that the IgE-binding activity did not decrease when SCP was exposed to acidic (pH 1.0–5.0) and alkaline (pH 10.0–11.0) conditions (Fig. 3D). There are two possible explanations for this. Firstly, that the IgE-binding activity to SCP increased at low and high pH levels (at pH 1.0–5.0 and at pH 10.0–11.0) in comparison with the control sample. Different environmental conditions could induce conformational changes: some allergens may undergo conformational changes associated with the exposition of different combinations of epitopes recognised by the IgE antibodies of allergic subjects (Bernardi et al., 2010). Thus the results may be due to changes in conformational structures of SCP by acid or alkali treatment, which then give rise to the exposure of epitopes at the surface of SCP. Alternatively, some subjects may have IgE antibodies that more readily recognise the conformational epitopes exposed in acidic/alkalic conditions.

A second possibility is that there was no substantial change in IgE-binding activity to SCP in acidic (pH 1.0–5.0) and alkalic (pH 10.0–11.0) buffers, but the IgE-binding activity was reduced by exposure to pH 6.0–9.0. The control sample was also in a buffer of pH 8.0, since the pH value of the buffer used for purification was 8.0. It can therefore be hypothesized that, as is the case for β -lactoglobulin, SCP exists as a dimer at neutral pH (6.0–9.0), while

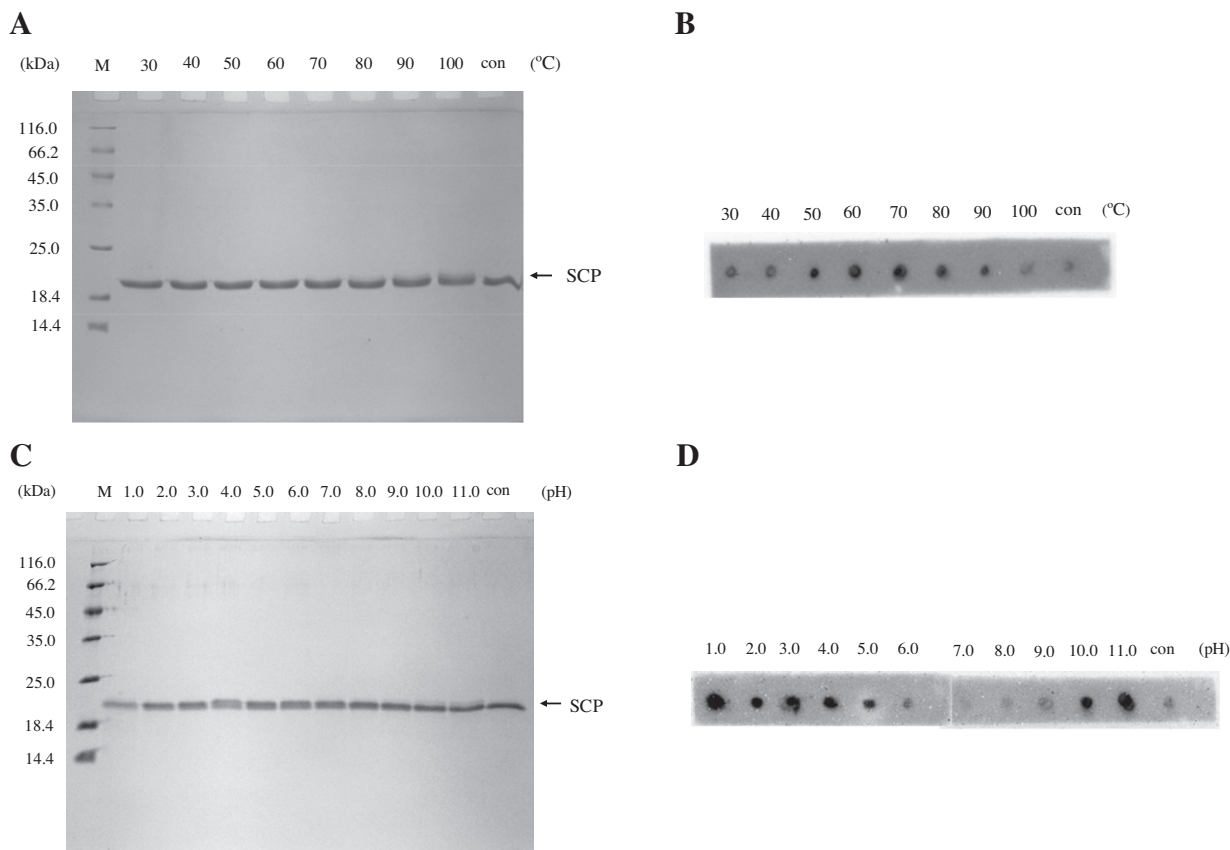


Fig. 3. Physicochemical characterisation of the purified SCP. (A) SDS-PAGE analysis of thermal stability of SCP. (B) Dot-blotting assay of thermal treated SCP. (C) SDS-PAGE analysis of pH stability of SCP. (D) Dot-blotting assay of SCP treated at different pH values. (E) SDS-PAGE analysis of the stability of SCP to SGF digestion. (F) SDS-PAGE analysis of the stability of SCP to SIF digestion. M: protein marker, con: control. (G) Inhibition-ELISA analysis of the digested fragments of SCP. SCP digested by pepsin (◆; 1 h), pancreatic juice (▲; 4 h), and undigested (□).

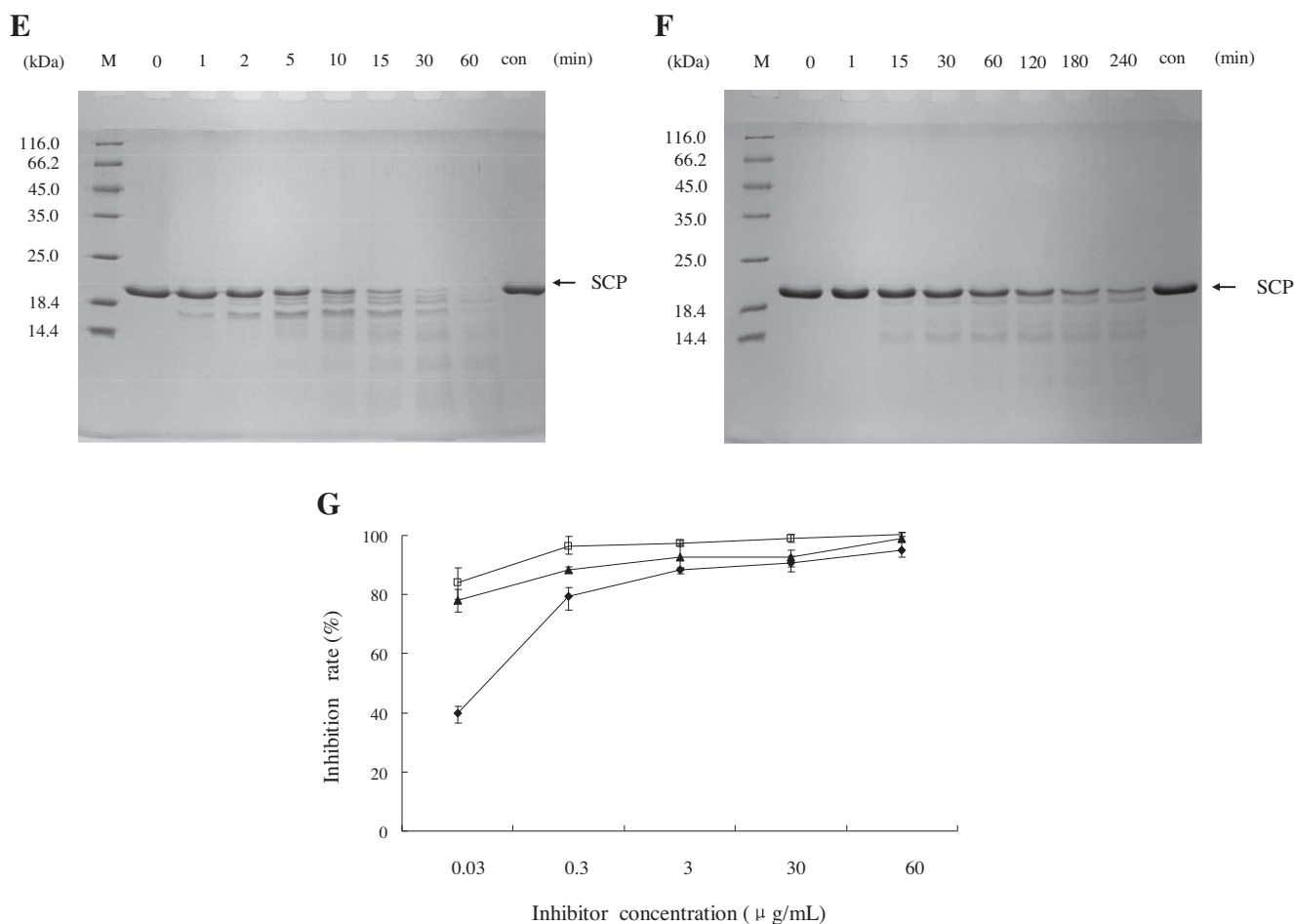


Fig. 3. (continued)

it dissociates to a monomer at acidic pH (1.0–5.0) (Sakurai & Goto, 2002). At pH > 9.0, a Tanford transition occurs, consisting of dimer dissociation (Taheri-Kafrani et al., 2009). The existence of a dimer may possibly decrease the exposition of epitopes. To confirm this explanation it would be necessary to carry out experiments on circular dichroism (CD).

High stability against harsh conditions is one of the characteristics of food allergens, such as tropomyosin or parvalbumin. These allergens are not easily decomposed, even at high temperatures or in strong acidic or alkaline conditions. Crayfish SCP was similarly found to be resistant to high temperature treatment and was highly stable in acidic or alkaline conditions.

3.3.2. Digestion stability

In vitro testing was performed to determine whether SCP was stable to digestion under simulated human gastrointestinal conditions. The digestibility of purified SCP in SGF was analysed by SDS-PAGE. The original band with molecular mass 22 kDa was gradually digested over time. As a result of pepsin digestion, a major fragment of ~16 kDa was produced after 1 min and more proteolytic fragments that were smaller than 14.4 kDa appeared after a prolonged digestion time. After 1 h, the original band was digested completely (Fig. 3E). On the other hand, the digestibility of SCP in SIF was relatively decreased when compared with that in SGF. Pancreatic juice digestion produced observed fragments within the range of 14.4–18.4 kDa, or fragments of smaller relative molecular

weight after 15 min, but SCP was detected to be incompletely degraded at 4 h (Fig. 3F).

To further analyse the IgE-binding capabilities of SCP-derived digestion fragments, inhibition ELISA, which is more sensitive for detecting proteolytic fragments with IgE-binding activity, was conducted. As shown in Fig. 3G, the IgE-binding activity of SCP that had been digested by pepsin for 1 h, was significantly more reduced, in comparison with an undigested sample. However, pancreatic juice digestion over a period of 4 h had little effect on SCP allergenicity.

Food allergens always remain intact in gastrointestinal-like digestion and cause anaphylaxis. From the above results, crayfish SCP appeared more resistant to intestinal digestion than to gastric digestion, and the IgE-binding activity of SCP remained highly potent in intestinal digestion conditions. In addition, pepsin treatment may be an appropriate means of reducing SCP IgE-binding activity.

3.3.3. PAS staining and carbohydrate-peptide linkage characterisation

PAS staining was used to determine whether SCP from crayfish is a glycoprotein. No band could be observed in the negative control BSA (Fig. 4A, lane 1), while a magenta band was observed on IgG heavy chain, which was used as a positive control (Fig. 4A, lane 2). Purified SCP subjected to PAGE was shown to have a positive band after PAS staining (Fig. 4A, lane 3). This suggests that SCP is a glycoprotein, which conforms to the current opinion that most food allergens were identified as glycoproteins

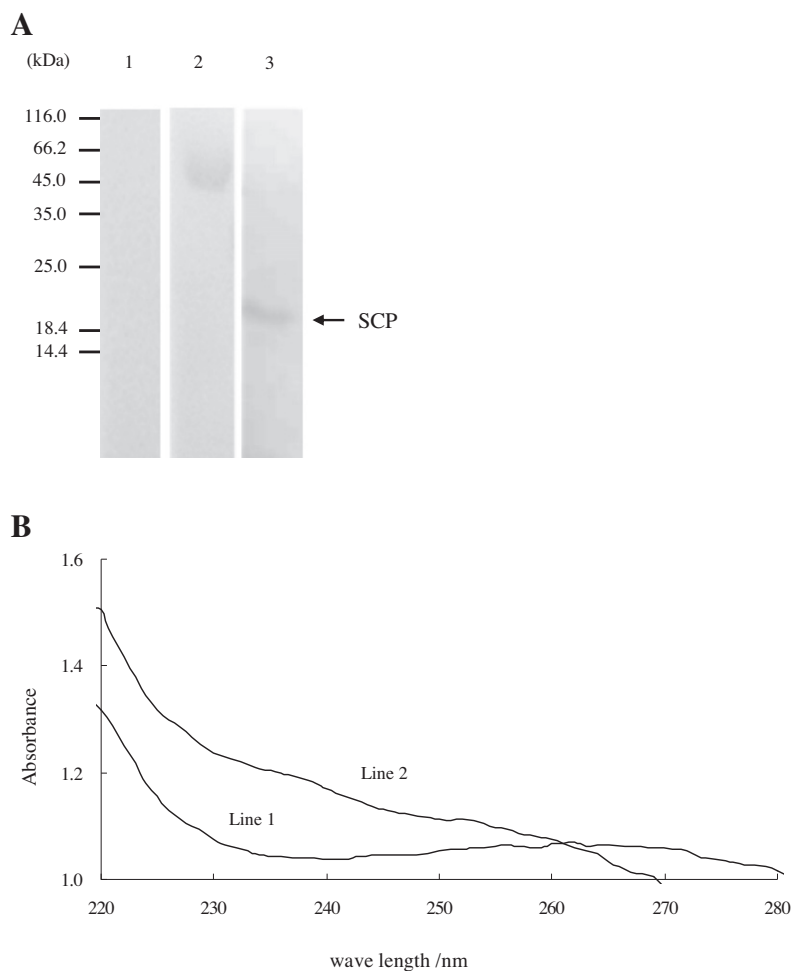


Fig. 4. Glycoprotein properties analysis of the purified SCP. (A) PAS staining of SCP, 1: BSA as negative, 2: IgG heavy chain as positive control; 3: the purified SCP. (B) UV spectra analysis of the purified SCP, Line 1, untreated SCP; Lane 2, SCP treated with low-concentration alkali.

with molecular masses in the range of 10–40 kDa (Radauer, Büblin, Wagner, Mari, & Breiteneder, 2008). A determination of total carbohydrate by the phenol–sulphuric acid method indicated that carbohydrates in SCP were ~4.9% (w/w) of the whole protein.

β -Elimination was applied to provide evidence for the existence of O-linked glycan in the SCP structure. As shown in Fig. 4B, when the SCP was treated with alkali, the value of UV absorbance at 240 nm increased significantly, in comparison with the absorbance levels prior to alkali treatment. This result suggests that one or more O-glycans exist in the structure of SCP (Hang & Bertozzi, 2005). The O-glycan, which is usually attached to Ser or Thr, is unstable in low alkali concentrations at a low temperature. During β -elimination, Ser and Thr are transformed to α -amino acids. These two unsaturated amino acids exhibit a characteristic UV absorption at 240 nm. Since glycosylation is reported to be associated with the IgE-binding capacity, it is clear that the relationship between glycosylation and allergenicity of SCP from crayfish needs further investigation.

Most of food allergens are glycoproteins and share the characteristics that they are stable in the processes of thermal or acid/alkali treatment and may still have immunological activity after digestion. The results of the characteristic investigation indicated that SCP shared the physicochemical characteristics of most food allergens.

3.4. Polymorphic characterisation of SCP

It was reported that crayfish SCP had polymorphism (three isotypes, SCP-I, SCP-II, and SCP-III) and existed as dimers (Wnuk & Jauregui-Adell, 1983). It is unclear whether there are some differences of the IgE-binding activity among SCP isotypes and subunits.

Native-PAGE was performed to confirm the polymorphism of SCP and it was surprising to observe three bands on the PAGE (Fig. 5A). The three bands were then extracted and analysed by MALDI MS and results indicated that they all belonged to SCP. It can be concluded that the purified crayfish SCP consisted of three isotypes and that the motilities on native-PAGE are quite different. According to Wnuk and Jauregui-Adell (1983), SCP-I was the slowest, SCP-III moved to the foremost position, and the pI values of the three isotopes were 5.05, 4.90, and 4.75: the first and third values agreed with those estimated for the SCP-I and SCP-III isotypes respectively. The IgE-binding activity was further examined by western-blotting, using sera from patients with a crustacean allergy (Fig. 5B). The specific IgE reaction to crayfish SCP isotypes could be confirmed clearly on the nitrocellulose membrane. All three isotypes had a strong reaction to the sera. It should be noted that the IgE-binding activity to SCP-II was relatively low compared to the binding associated with SCP-I and SCP-III.

It has also been reported that crayfish SCP is a dimer of 22 kDa subunits. White et al. (2011) identified three SCP variants (subunit

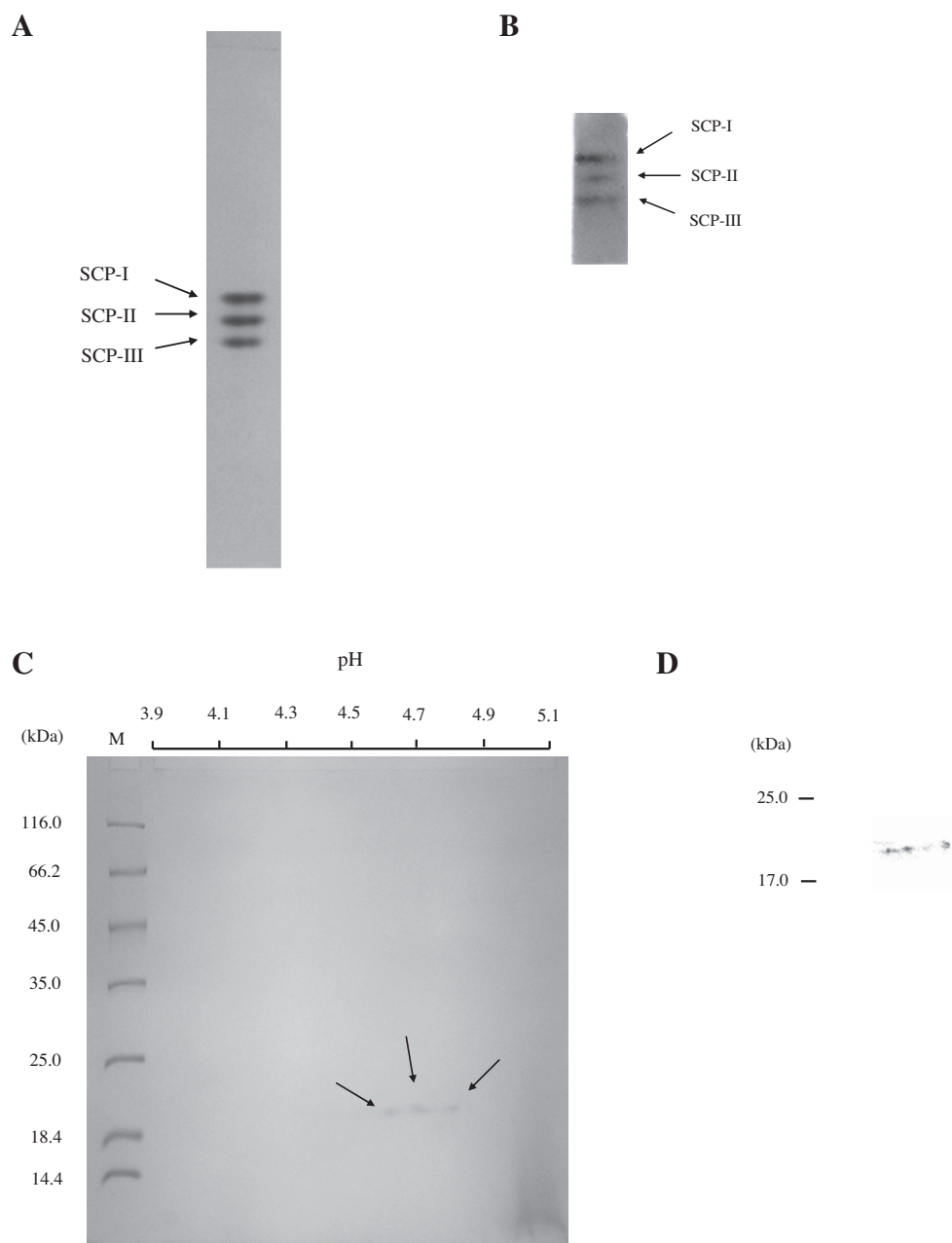


Fig. 5. Polymorphic characterisation of the purified SCP. Native-PAGE (A) analysis of the purified SCP and western-blotting (B) analysis of the specific IgE-binding activity to SCP isotypes. 2-DE (C) and western-blotting (D) analysis of the IgE-binding activity to SCP subunits, M: protein marker.

a, b, and c) in the freshwater crayfish *P. clarkii* and the subunits were the basic elements constituting the dimers. It is unclear whether the subunits, or the form of which the subunits were composed, resulted in the slight differences in the IgE-binding activity in relation to polymorphism. It can be assured that the SCP subunits may have a direct influence on the sensitization of SCP isotypes. Furthermore, the question of which subunit plays a more important role in causing IgE-mediated allergy needs to be further investigated.

To better characterise the subunits of SCP, the purified sample was subjected to 2-DE for separation by isoelectric point in one dimension (the horizontal direction) and by molecular mass in the second dimension (the vertical direction). The 2-DE profile of the purified SCP from crayfish is shown in Fig. 5C and three spots were detectable. The molecular masses of these three spots were

consistent with the result from SDS-PAGE. The pI values of the three subunits were approximately 4.6, 4.7, and 4.8, but the question of which subunits corresponds to a, b, and c is still uncertain. The bioinformatics information on the isoelectric points of SCP subunits constitutes the only information that has so far been reported: the pI values of SCP subunits predicted by bioinformatics method are 4.59 (subunit a: gi|334545623), 4.48 (subunit b: gi|334545642), and 4.53 (subunit c: gi|334545668). A comparison of the allergic effects of subunits, to elucidate the relationship between allergenicity and the different subunits, thus becomes more important. Fig. 5D illustrates a specific antigen–antibody reaction between the SCP subunits and the sera from patients. All three SCP subunits had specific IgE-binding activity, but were immunologically indistinctive. It can be inferred that they were all allergens of crayfish.

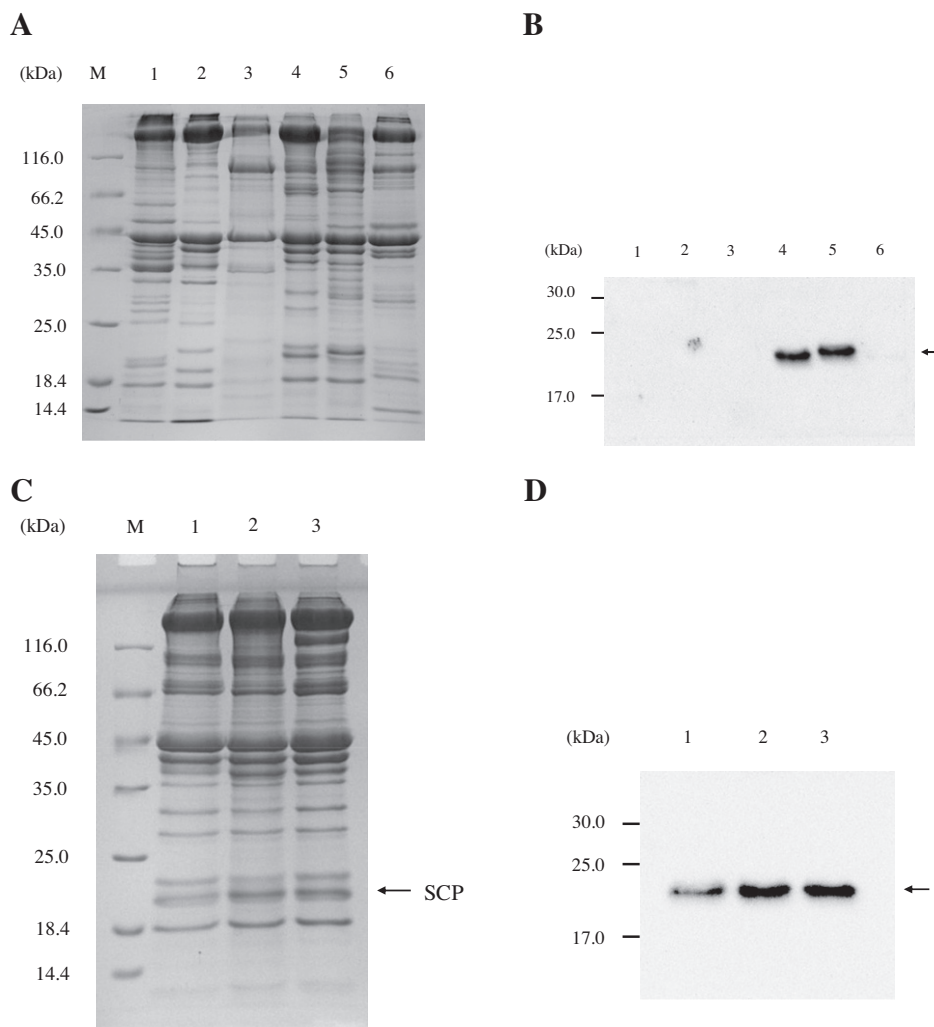


Fig. 6. SDS-PAGE (A) and immunoblotting (B) analysis of the crude extracts from aquatic products using rabbit anti-crayfish polymorphic SCP polyclonal antibody. M: protein marker; 1–6: crude extracts from blue scud, crucian carp, manila clam, red swamp crayfish, Pacific white shrimp, and mud crab, respectively. SDS-PAGE (C) and immunoblotting (D) analysis of the crude extracts from the processed crayfish. M: protein marker; 1–3: the crude extracts from the boiled crayfish, the soft-boiled crayfish, and the raw crayfish, respectively.

The results outlined above demonstrate that the purified crayfish SCP was a mixture of three isotypes (SCP-I, SCP-II, SCP-III) among which there were three different subunits a, b, and c that appeared in the form of three dimers, by the way of two subunits in combination. The IgE-binding activity of SCP isotypes had slight differences, while the IgE-binding activity of SCP subunits was not dissimilar. All of the isotypes and subunits had allergenicity. This is analogous to the observations of Krishnan, Kim, Jang, and Kerley (2009), who found that all three of the soybean β -conglycinin subunits are potential allergens. For future research, isolation of crayfish SCP isotypes is needed, to further investigate the relationship between isotypes and subunits relating to IgE-binding activity.

In summary, SCP was a stable and polymorphic allergen. The differences of characteristics between SCP and other food allergens could provide the reference and instruction for the future desensitization investigation of crayfish.

3.5. Polyclonal antibody preparation and immunoassay

Since the IgE-binding activity displayed no obvious difference among SCP isotypes, the rabbit anti-crayfish SCP polyclonal antibody was prepared. The antibody reacted with SCP even after dilution to 1/50,000, suggesting its higher titer.

Crude extracts from blue scud, crucian carp, manila clam, red swamp crayfish, Pacific white shrimp, and mud crab were subjected to SDS-PAGE (Fig. 6A). Immunoblotting analysis (Fig. 6B), using rabbit anti-crayfish polymorphic SCP polyclonal antibody, indicated that only the SCPs from red swamp crayfish and Pacific white shrimp could be detected, while no bands were detectable for blue scud, crucian carp, manila clam, and mud crab. It can be speculated from these results that SCP does not exist, or that the percentage of SCP is low in the crude extracts of blue scud, crucian carp, manila clam, and mud crab. Alternatively, SCPs from blue scud, crucian carp, manila clam, and mud crab may not have been recognised by the rabbit anti-crayfish polymorphic SCP polyclonal antibody.

Crude extracts of crayfish under different heating conditions were also analysed by SDS-PAGE (Fig. 6C) with the resulting profiles showing multiple protein bands ranging from 10 to 200 kDa. The protein component was more simple in the extract of boiled crayfish (the bands at about 40 kDa and 116 kDa had disappeared or had a lower band density) in comparison with that obtained from raw and soft-boiled crayfish. However, all extracts had a prominent band at approximately 22 kDa, which corresponded with the molecular weight of SCP. The protein presented no distinct changes among the three different extracts. The 22 kDa pro-

teins from raw, soft-boiled and boiled crayfish were recognised by the rabbit anti-crayfish SCP polyclonal antibody in western-blotting studies (Fig. 6D). The 22 kDa proteins in the extracts all reacted with the rabbit anti-crayfish polymorphic SCP polyclonal antibody, although the extract from boiled crayfish was detected at a lower intensity. These results infer that extracts from both soft-boiled crayfish and boiled crayfish retained an IgG-binding protein at 22 kDa.

The prepared polyclonal antibody could be used to specifically detect SCPs from different aquatic products which would contribute to improving the safety control of aquatic products allergens. For the purpose of a future study, it is recommended that anti-crayfish polymorphic SCP monoclonal antibody should be prepared and further used in order to develop an indirect ELISA for semi-quantification of SCPs from the commercially-important species of crayfish and shrimps. Such a detection method would have a high sensitivity and accuracy and could play an important role in developing diagnostic reagents for aquatic-product allergens.

4. Conclusions

The purified SCP with molecular mass of 22 kDa was confirmed through mass spectrometry and was identified as a novel allergen in crayfish by the determination of its IgE-binding activity. The physicochemical characterisation of SCP showed that glycosylated protein SCP was a highly stable allergen; the polymorphic characterisation revealed that the IgE-binding activity was slightly different in the SCP isotypes and the subunits were not dissimilar in terms of IgE-binding activity. The SCP isotypes and their subunits all have allergenicity.

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