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Original article

# Isolation and characterisation of collagen from the ribbon jellyfish (*Chrysaora* sp.)

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**Summary** Pepsin-solubilised collagen from the ribbon jellyfish (*Chrysaora* sp., morphotype 1) umbrella (JPSC) was isolated and characterised. The yield of collagen varied (9–19%, based on ash-free dry weight) depending on the amount of pepsin used. Type II collagen was the major component of extracted collagen. The peptide map of JPSC differed from that of standard collagen type II, which indicates their different primary structures. FTIR spectra of JPSC, however, did not differ significantly from those of type II collagen. The $T_{max}$ of JPSC was 37.38 °C, which is higher than that of other marine collagens. Glycine was the main amino acid in JPSC (320 residues per 1000 residues), followed by glutamic acid, alanine, proline, aspartic acid and hydroxyproline. The isoelectric point of JPSC was 6.64. These results indicate that this jellyfish species has the potential to be a marine source of type II collagen that can be used in place of land-based sources.

**Keywords** *Chrysaora* sp, collagen, ribbon jellyfish.

## Introduction

Collagen is the most abundant protein in animals, accounting for ~30% of total body proteins (Lee *et al.*, 2001; Addad *et al.*, 2011). It is the main structural material of the extracellular matrix of all connective tissues (i.e. skin, bones, ligaments, tendons and cartilage) as well as interstitial tissues of all parenchymal organs (Gelse *et al.*, 2003). This fibrous protein provides the tissues with mechanical strength and physiological functions (Kittiphattanabawon *et al.*, 2010b; Pati *et al.*, 2010). The 26 types of collagen identified to date constitute the collagen superfamily (Gelse *et al.*, 2003). All collagens have a triple helical structure composed of three polypeptide chains (α chains) with a repeated sequence of three amino acids, glycine-X-Y, in which X and Y are mostly proline and hydroxyproline (Gelse *et al.*, 2003). For many years, collagen and its denatured form (gelatin) have been widely used in food, pharmaceutical, biotechnology, biomedical and cosmetics industries (Ogawa *et al.*, 2004).

Land-based animals such as cows and pigs (particularly their skin and bones) are the traditional sources of collagen for industrial use (Jongjareonrak *et al.*, 2005). However, use of porcine and bovine collagen poses the risk of transmitting diseases such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), and foot and mouth disease (FMD) (Ogawa *et al.*, 2004; Song *et al.*, 2006). In addition, Muslims and Hindus do not consume porcine or bovine products, respectively, for religious reasons. These obstacles along with several environmental issues have led scientists to look for new sources of collagen (Ogawa *et al.*, 2004; Jongjareonrak *et al.*, 2005; Song *et al.*, 2006; Heu *et al.*, 2010).

Collagen from marine animals is promising, as it has low risk of transmitting diseases, there are no religious barriers to its consumption, raw materials are abundant, and it has a higher yield of extracted collagen compared with other alternative sources (Senaratne *et al.*, 2006). Collagen has been extracted from many marine species, including black drum (Ogawa *et al.*, 2004), cuttlefish (Nagai *et al.*, 2001), brownstripe red snapper (Jongjareonrak *et al.*, 2005), flatfish (Heu *et al.*, 2010), skate (Hwang *et al.*, 2007), ocellate puffer fish (Nagai *et al.*, 2002), brownbanded bamboo shark (Kittiphattanabawon *et al.*, 2010a), Baltic cod

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(Sadowska *et al*., 2003) and carp (Duan *et al*., 2009). However, there are only few reports on extraction of collagen from jellyfish as for stomolophus meleagris (Nagai *et al*., 1999) and rhizostomous jellyfish (Nagai *et al*., 2000). As compared to collagen from land-based animals and some other marine collagens, jellyfish collagen has been reported to exhibit different characteristics such as amino acid composition, which in turn affects other properties of collagen including thermal behaviours, isoelectric pH, solubility and other properties. Imino acid content, for instance, which is in general low in marine sources, has been reported to be even lower in jellyfish, which results in lower thermal stability of collagen (Kimura *et al*., 1983; Miura & Kimura, 1985; Nagai *et al*., 1999).

Some species of jellyfish, such as rhizostomous jellyfish, esculent jellyfish and *Stomolophus melagris*, have been used as food and as a medicine in China for more than a thousand years (Nagai *et al*., 1999; Zhuang *et al*., 2009). Consumption of these jellyfish reportedly has health benefits and has been used to treat diseases such as arthritis, hypertension, bronchitis, back pain, gastric ulcers, asthma, tracheitis, and burns and to relieve fatigue (You *et al*., 2007; Zhuang *et al*., 2009). Jellyfish contain high amounts of collagen (Nagai *et al*., 2000; Zhuang *et al*., 2009); thus, collagen and collagen derivatives may contribute to these health benefits. Recent reports indicated that jellyfish collagen derivatives were effective in preventing and curing rheumatoid arthritis (Cao & Xu, 2008), osteoarthritis and osteoporosis (Moskowitz, 2000), and high blood pressure (Giménez *et al*., 2009) and also exhibited antifatigue effects (Ding *et al*., 2011).

Jellyfish populations have swelled in recent years as a result of overfishing, eutrophication, climate change and habitat modifications (Purcell *et al*., 2007; Richardson *et al*., 2009). This phenomenon of 'jellyfish blooming' can cause serious problems, including stings to humans (sometimes deadly), declines in coastal tourism, clogging of cooling equipment and disabling of power plants, burst fishing nets, contaminated fish catches, killing of farmed fishes, and consumption of fish eggs and young fish (Dong *et al*., 2010). If substantial amounts of collagen can be obtained from this very abundant and underutilised resource, it may prove to be a valuable source of collagen for industrial use.

The subject of this study was *Chrysaora* sp. (ribbon jellyfish) morphotype 1 and morphotype 2 (class Scyphozoa, order Semaeostomeae). This is a new identified species of jellyfish, and there are no existing reports about its collagen and collagen derivative content. This jellyfish was found along the coastal area of Penang Island, Malaysia. It has a bell diameter of about 8–12 cm, and the flat, ribbonlike oral arms under the bell are 20–50 cm long. Ribbon jellyfish may be totally white (morphotype 1) or have a brown stripe around the bell (morphotype 2). The objective of this study was to extract collagen from the ribbon jellyfish umbrella and to characterise it in terms of molecular mass, subunit composition, primary and secondary structure, ultrastructure, and thermal behaviours.

## Material and methods

### Materials

Ribbon jellyfish were caught along the northern coast of Penang Island, Malaysia, in March 2011. The umbrella was dissected, washed with distilled water, cooled, transported to the laboratory and stored at −80 °C until use. Pepsin (E.C. 3.4.23.1, ≥400 U $mg^{-1}$ pro), V8 protease (EC 3.4.21.19, 800 U $mg^{-1}$) and standard collagens (type II from chicken sternum and type I from calf skin) were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Standard type I collagen from salmon and lysyl endopeptidase (E.C. 3.4.21.50) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). SDS-PAGE chemicals and molecular weight markers were supplied by Bio-Rad Laboratories (Hercules, CA, USA). All chemicals and reagents were of analytical grade.

### Isolation of pepsin-solubilised collagen from jellyfish umbrella (JPSC)

Collagen was extracted according to the method of Nagai *et al*. (1999) with slight modification. All procedures were performed at 4 °C. Jellyfish umbrellas were thawed at 4 °C for 4–5 h, cut into small pieces (0.5 × 0.5 cm) and washed with distilled water. To remove noncollagenous substances, each sample was treated with 0.1 M NaOH at a sample/solution ratio of 1:10 (w/v) with gentle stirring for 2 day (the solution was changed once a day). After being centrifuged at 10 000 ***g*** for 30 min, the remaining insoluble matter was washed with distilled water until neutral pH was achieved. Then, to evaluate the effect of pepsin on extractability of collagen from jellyfish umbrella, different concentrations of pepsin were applied. Different pepsin treatments were created by suspending samples in 0.5 M acetic acid (10 volumes v/w) containing 0%, 5%, or 10% (w/w) pepsin (E.C. 3.4.23.1) with gentle stirring for 3 day. For each treatment, the final viscous liquid was centrifuged at 20 000 ***g*** at 4 °C for 1 h. The supernatant was dialysed against 10 volumes of 0.02 M $Na_2HPO_4$ (pH: 8.8) for 3 day to inactivate the enzyme. The dialysed sample was centrifuged at 20 000 ***g*** at 4 °C for 1 h. The resulting precipitate was dissolved in 0.5 M acetic acid and salted out by adding NaCl to the final concentration of 1 M, followed by

centrifugation at 20 000 ***g*** at 4 °C for 1 h. The resulting precipitate was dissolved in 0.5 M acetic acid and dialysed against 0.1 M acetic acid and distilled water for 2 day. The sample was lyophilised and stored at −80 °C until further analysis.

### Amino acid composition

To determine the amino acid composition of the extracted collagen, a JPSC sample (0.1 g in freeze-dried form) was hydrolysed with 6 N HCl (5 ml) at 110 °C for 24 h under vacuum. Subsequently, 400 μl of 50 μmole $ml^{-1}$ of L-α-amino-*n*-butyric acid (AABA) was added to the resulting hydrolysates (as the internal standard), and distilled deionised water was added to reach a volume of 100 ml. Then, samples were filtered through Whatman No. 1 filter paper followed by a 0.22-μm Millipore filter (Zarkadas *et al*., 2007).

The JPSC hydrolysate amino acids were derivatised by incubating 10 μl of the hydrolysed samples with 20 μl of AccQ·Fluor™ reagent (AQC: 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) for 1 min at room temperature. Samples were then placed on the 150-μl glass insert with poly spring, which was equipped in the screw-capped vial, and the vial was heated for 10 min at 55 °C before being subjected to the analysis. A 10 μl of aliquot sample was injected into the column, and elution was conducted at a flow rate of 1 ml $min^{-1}$.

Determination of amino acid was carried out according to the AccQ·Tag™ method using a Waters HPLC system (USA) with Waters 1525 Binary Pump, Waters 717 plus Autosampler, Waters 2475 Multi λ Fluorescence Detector and a Waters AccQ·Tag™ Amino Acid Analysis Column (3.9 × 150 mm; packing material: silica-based bonded with $C_{18}$). The column was fixed at temperature of 37 °C with fluorescence detection of 250 nm for excitation and 395 nm for emission. AccQ·Tag™ Eluent A and Acetonitrile/Water (60%/40%) was used as eluents, and calibration of the HPLC system was performed using the amino acids standard H (Pierce, Rockford, IL, USA) as reference. Methionine and cysteine were analysed separately by using performic acid procedure of Moore (1963). Breeze Workstation, version 3.20, was used for data analysis. The area under the peak of each amino acid in the chromatogram was calculated and compared with that of the standard. The analysis was carried out in three replicates, and the results were reported as per 1000 amino acid residues.

### SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) using a 7.5% resolving gel and a 4% stacking gel. JPSC samples in freeze-dried form were dissolved in 0.02 M sodium phosphate buffer (pH 7.2) containing 1% SDS (w/v) and 3.5 M urea. They were then mixed with Laemmli sample buffer (Bio-Rad) at the sample buffer ratio of 1:1 (v/v) with and without β-mercaptoethanol and heated for 5 min at 95 °C. Each sample (20 μg protein) was loaded into a well and run at 80 V for 10 min followed by 120 V for 1.5 h. Following electrophoresis, protein bands were stained with Coomassie brilliant blue R-250. A high molecular weight prestained marker (Bio-Rad) was used to estimate the approximate molecular weight of collagen samples. Type I collagen from calf skin (Sigma-Aldrich), type I collagen from salmon (Wako) and type II collagen from chicken sternum (Sigma-Aldrich) were used as references.

### Peptide mapping

Two hundred micrograms of freeze-dried JPSC was suspended in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5% SDS. The sample was heated at 100 °C for 5 min. For proteolytic digestion, 5 μg of *Achromobacter lyticus* lysyl endopeptidase or *Staphylococcus aureus* V8 protease was added to the sample and incubated at 37 °C for 30 min. SDS was added to reach a final concentration of 2%. To stop the reaction, the sample was subjected to boiling for 5 min. SDS-PAGE was performed according to the method of Laemmli (1970) using a 15% resolving gel and a 4% stacking gel. A high molecular weight marker (Sigma-Aldrich) was used to estimate the molecular weight of the peptide fragments. A peptide map of standard type II collagen from chicken sternum (Sigma-Aldrich) was prepared in the same manner and used as a reference.

### Fourier transform infrared (ATR-FTIR) spectroscopy

Fourier transform infrared spectra of collagen were obtained using an Agilent FTIR spectrometer equipped with an attenuated total reflectance system (Agilent technologies, Cary 670 FTIR) (Santa Clara, CA, United States of America). Infrared spectra were recorded in the range of 4000–400 $cm^{-1}$ at an aperture of 1 and sensitivity of 1.5.

### Differential scanning calorimetry

Preparation of the sample and running differential scanning calorimetry (DSC) was performed according to the method of Kittiphattanabawon *et al*. (2010b) with some modification. The freeze-dried collagen samples were rehydrated in 0.05 M acetic acid solution at a sample/solution ratio of 1:40 (w/v). The mixture

was allowed to stand for 2 days at 4 °C. The thermal transition of collagen was measured using a differential scanning calorimeter (Perkin Elmer, Model DSC6, Norwalk, CA, USA). Temperature calibration was performed using the Indium thermogram. Each rehydrated sample (5–10 mg) was accurately weighed in an aluminium pan, sealed and scanned over the range of 10–60 °C at a heating rate of 1 °C $min^{-1}$. The system was equilibrated at 10 °C for 5 min prior to the scan. The empty aluminium pan was used as the reference. The maximum transition temperature (Tmax) was estimated from the maximum peak of the DSC transition curve, and total denaturation enthalpy (ΔH) was estimated by measuring the area of the DSC thermogram.

### Zeta potential of jellyfish umbrella collagen

Zeta potential of JPSC was measured according to the method of Ahmad *et al*. (2010). A freeze-dried JPSC sample was dissolved in 0.5 M acetic acid at a concentration of 0.5 mg $ml^{-1}$. The mixture was stirred at 4 °C until completely solubilised (around 10 h). The zeta potential of each sample was measured using a zeta potential analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, Worcestershire, UK). The pH of the solution was adjusted in the range of 2–12 with 1 M nitric acid or 1 M KOH using an autotitrator (MPT-2, Malvern Instruments). The titration was carried out at 20 °C with decreasing pH intervals of 0.5. Zero zeta potential was obtained from the titration curve. The isoelectric pH (pI) of the sample was identified as the pH value at which the zeta potential was zero.

### SEM/EDX (Energy dispersive X-ray)

The microstructure and EDX spectra of JPSC were studied using scanning electron microscope equipped with energy dispersive X-ray (EDX) detector (EVO I MA 10, Carl Zeiss NTS Ltd., Oberkochen, Germany). Each JPSC sample, in freeze-dried form, was loaded on a stub using double-sided tape, coated with gold and palladium, and scanned. The electron accelerator was operated at 15 kV, and the magnification was from ×100 to ×10000.

### Statistical analysis

All experiments were performed in triplicate, and data are presented as means ± SD. A probability value of ≤0.05 was considered to be significant. Analysis of variance (ANOVA) was performed, and comparisons of means were conducted using Duncan's multiple range tests. Analysis was performed using SPSS, version 20, for Mac OS (IBM, Armonk, NY, USA).

## Results and discussion

### JPSC from *Chrysaora sp.*

Collagen from the ribbon jellyfish umbrella was not easily extracted by 0.5 M acetic acid, and the collagen yield was negligible without pepsin. A similar result was reported for extraction of acid-solubilised collagen from brownstripe red snapper (Jongjareonrak *et al*., 2005), cuttlefish (Nagai *et al*., 2001) and ocellate puffer fish (Nagai *et al*., 2002). This result might be due to intermolecular covalent cross-linking at telopeptide regions that cannot be cleaved by acetic acid (Jongjareonrak *et al*., 2005). When 5% (w/w) pepsin was added, the extraction yield was 9% because the proteolytic effect of pepsin cleaved cross-linked molecules without damaging the triple helix (Jongjareonrak *et al*., 2005). Increasing the pepsin content to 10% (w/w) increased the yield to 19% because more bonds were broken. Therefore, 10% pepsin exhibited the highest yield among three different treatments. The yield of collagen from *Chrysaora* sp. JPSC was higher than that reported for stomolophus nomurai mesogloea (2.2% based on washed dry weight) (Miura & Kimura, 1985), rhizostoma pulmo umbrella and Cotylorhiza tuberculata umbrella (<10% calculated based on 5% dry weigh of jellyfish tissue) (Addad *et al*., 2011). However, this value, even using 10% pepsin, was lower than those reported for rhizostomous jellyfish (Rhopilema asamushi) mesogloea (35.2% on the basis of lyophilised dry weight) (Nagai *et al*., 2000), stomolophus meleagris exumbrella (46% on the basis of lyophilised dry weight) (Nagai *et al*., 1999) and some other marine sources such as cuttlefish skin (35% on the basis of dry weight) (Nagai *et al*., 2001), skate skin (35.6% of dry tissue) (Hwang *et al*., 2007) and ocellate puffer fish skin (44.7% on a dry weight basis) (Nagai *et al*., 2002).

Collagen extracted from the two morphotypes had almost same yield in the 5% pepsin treatment (9% and 8.3% for morphotypes 1 and 2, respectively) and the 10% pepsin treatment (19% for both), but the collagen extracted from morphotype 2 was brownish. Because colour can be an interfering factor, morphotype 1 was selected as the target specimen and all characterisation results are reported for the collagen extracted from this specimen using 10% pepsin.

### Amino acid composition

Glycine (Gly) was the major amino acid in JPSC, with 320 residues/1000 residues. This result agrees with the 'Gly-X-Y' amino acid model in which Gly occurs in every third position (Ahmad *et al*., 2010). Other high content amino acids were glutamic acid (Glu; 101 residues per 1000 residues), alanine (Ala; 87 residues per 1000 residues), proline (Pro; 79

**Table 1** Amino acid composition of different collagens (residues/1000 residues)

| AA | Jellyfish *Chrysaora sp.1* | Jellyfish[a] | Jellyfish[b] | Calf skin[c] | Chicken sternum[d] | Bamboo sharkcartilage[e] | Blacktip shark cartilage[e] | Deep-sea redfish[f] | Unicorn leather Jacket[g] |
|---|---|---|---|---|---|---|---|---|---|
| Hyp | 70 | 57 | 40 | 94 | 117 | 94 | 91 | 61 | 81 |
| Asp | 76 | 71 | 79 | 45 | 46 | 43 | 43 | 46 | 45 |
| Ser | 44 | 45 | 45 | 33 | 22 | 41 | 31 | 64 | 33 |
| Glu | 101 | 94 | 98 | 75 | 85 | 77 | 77 | 69 | 74 |
| Gly | 320 | 344 | 309 | 330 | 310 | 317 | 316 | 357 | 321 |
| His | ND | 1 | 2 | 5 | 4 | 7 | 8 | 6 | 6 |
| Arg | 58 | 57 | 52 | 50 | 52 | 51 | 54 | 53 | 53 |
| Thr | 34 | 28 | 35 | 18 | 26 | 24 | 22 | 22 | 27 |
| Ala | 87 | 77 | 82 | 119 | 104 | 104 | 118 | 104 | 141 |
| Pro | 79 | 79 | 82 | 121 | 115 | 110 | 106 | 99 | 109 |
| Cys | ND | – | – | – | 18 | 1 | 1 | – | ND |
| Tyr | 10 | 5 | 6 | 3 | 5 | 3 | 3 | 2 | 4 |
| Val | 22 | 24 | 35 | 21 | 19 | 25 | 26 | 23 | 21 |
| Met | 16 | 8 | 4 | 6 | 10 | 12 | 14 | 14 | 13 |
| Lys | 17 | 24 | 38 | 26 | 14 | 27 | 26 | 27 | 27 |
| Ile | 23 | 16 | 22 | 11 | 11 | 19 | 20 | 10 | 9 |
| Leu | 31 | 27 | 34 | 23 | 27 | 25 | 26 | 20 | 17 |
| Phe | 14 | 8 | 10 | 3 | 15 | 13 | 13 | 15 | 13 |
| Imino acids | 149 | 136 | 122 | 215 | 232 | 204 | 197 | 160 | 190 |
| Total | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |

ND, not detected.
[a]Stomolophus nomurai mesogloea, Miura & Kimura (1985).
[b]Stomolophus meleagris exumbrella, Nagai *et al.* (1999).
[c]Giraud-Guille *et al.* (2000).
[d]Cao & Xu (2008).
[e]Kittiphattanabawon *et al.* (2010b).
[f]Wang *et al.* (2007).
[g]Ahmad *et al.* (2010).

residues per 1000 residues) and aspartic acid (Asp; 76 residues per 1000 residues). Cysteine and histidine were not found. *Stomolophus nomurai* (Miura & Kimura, 1985) and *Stomolophus melagris* (Nagai *et al.*, 1999) had similar amino acid compositions (Table 1).

A comparison between amino acid composition of JPSC and other sources (Table 1) showed that *Chrysaora* sp. collagen had higher Asp and Glu and lower imino acid (Pro + Hyp) and Ala content. Imino acid content of JPSC was 149 residues per 1000 residues, which is significantly lower than that of chicken sternum and calf skin collagen followed by unicorn leatherjacket and deep-sea redfish collagen (232, 215, 190, and 160 residues per 1000 residues, respectively). Imino acids are involved in hydrogen bonding and affect the stability of the collagen triple helix and its thermal behaviours (Ahmad *et al.*, 2010; Kittiphattanabawon *et al.*, 2010a). The imino acid content value is usually lower in marine collagens than mammalian collagens, resulting in a lower thermal denaturation temperature. The amino acid composition of collagen is one of the key factors affecting the properties of collagens.

## SDS-PAGE patterns

Figure 1 shows the electrophoretic patterns of JPSC under reducing and nonreducing conditions. The JPSC pattern was compared with that of standard type I collagen from calf skin and salmon and standard type II collagen from chicken sternum. No significant difference was observed between patterns of reduced and nonreduced samples, indicating that there is no disulphide bond in JPSC collagen (Kittiphattanabawon *et al.*, 2010b). This result was in agreement with the amino acid analysis (Table 1), which showed that the samples contained no cysteine.

The electrophoretic pattern of JPSC showed that the collagen consisted of α chains ($\alpha_1$) and dimer β chains (with molecular mass values of ~137 and ~241 kDa, respectively), and this pattern was similar to that of type II collagen. The standard type II collagen from chicken sternum was slightly larger than JPSC, probably due to differences in amino acid composition. JPSC did not contain $\alpha_2$ chains; thus, the pattern differed from that of type I collagen from calf skin and salmon. These results suggest that the type II collagen with three identical $\alpha_1$(II)-chains is the main type of

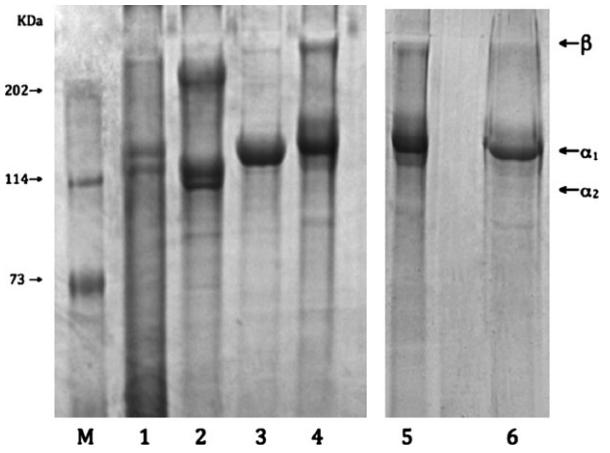


**Figure 1** SDS-PAGE pattern of jellyfish PSC. M: high molecular weight protein marker; 1: type I collagen from calf skin; 2: type I collagen from salmon; 3: type II collagen from chicken sternum; 4 & 5: jellyfish PSC (non-reduced); 6: jellyfish PSC (reduced).

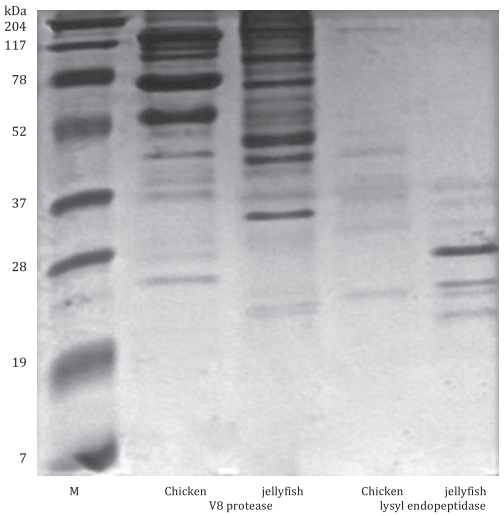


**Figure 2** Peptide maps of jellyfish PSC and type II collagen from chicken digested by V8 protease and lysyl endopeptidase. M: high molecular weight marker.

collagen present in JPSC. Similar results were reported for the cannonball jellyfish (Hsieh, 2005) and *Cyanea nozaki* Kishinouye (Tang *et al*., 2008). However, Nagai *et al*. (2000) demonstrated that collagen from the mesogloea of the jellyfish *Rhopilema asamushi* contains two different α chains, $\alpha_1$ and $\alpha_2$. In addition, Miura & Kimura (1985) found $\alpha_1$, $\alpha_2$, and $\alpha_3$ chains in collagen from the mesogloea of *Stomolophus nomurai*. Collagen from *Stomolophus meleagris* also contains $\alpha_1$, $\alpha_2$, and $\alpha_3$ (Nagai *et al*., 1999). These differences likely are due to species-specific variations in collagen composition.

## Peptide mapping

JPSC collagen and standard type II collagen were digested by both *A. lyticus* lysyl endopeptidase and *S. aureus* V8 protease, and analysed using SDS-PAGE (15% gel) (Fig. 2). Different peptide mapping profiles revealed that the primary structure of JPSC and chicken sternum type II collagen was different. Different peptide patterns result mainly from different amino acid composition, amino acid sequences, cross-links, and accessibility of certain bonds and domains of the protein to the enzyme (Jongjareonrak *et al*., 2005; Kittiphattanabawon *et al*., 2005; Ahmad & Benjakul, 2010). Thus, collagens from different sources (even the same types of collagen) can have different peptide patterns (Nagai *et al*., 2002; Ahmad & Benjakul, 2010).

Both JPSC collagen and standard type II collagen were more susceptible to digestion by lysyl endopeptidase than V8 protease. The α and β components of both samples were totally hydrolysed and degraded to lower molecular weight peptides and almost disappeared following hydrolysis by lysyl endopeptidase. The molecular mass values of resulting peptides were around 20, 24, 30, 36, 42 and 47 kDa for JPSC, and 23, 46, 59 and 132 kDa for standard collagen type II. After digestion by V8, bands were visible at around 131 and 125 kDa for JPSC and type II collagen, respectively, but the intensity of these bands was low. This result suggests that the α and β components of both samples were only partially hydrolysed. Most of the peptide fragments were in the range of 20–131 in JPSC and 44–125 in standard collagen type II. After digestion by V8 protease, JPSC was fully hydrolysed and more peptide fragments were produced compared with type II collagen. The higher susceptibility of JPSC to hydrolysis by V8 protease might be due to its higher content of Glu and Asp (Jongjareonrak *et al*., 2005; Cui *et al*., 2007; Ahmad & Benjakul, 2010).

## FTIR spectra

Fourier transform infrared spectroscopy was used to study the secondary structure of JPSC (Fig. 3) and to compare the secondary structure of different collagens (Table 2). Differences between FTIR spectra are indicative of differences in secondary structure and functional groups (Kittiphattanabawon *et al*., 2010b). The main absorption bands in JPSC were amide A (3314 $cm^{-1}$), amide B (2924 $cm^{-1}$), amide I (1653 $cm^{-1}$), amide II (1551 $cm^{-1}$) and amide III (1239 $cm^{-1}$). Amide A is associated with N-H stretching vibration and the existence of hydrogen bonds (Wang *et al*., 2007). Compared with the free N-H stretching vibration that occurs in the range of

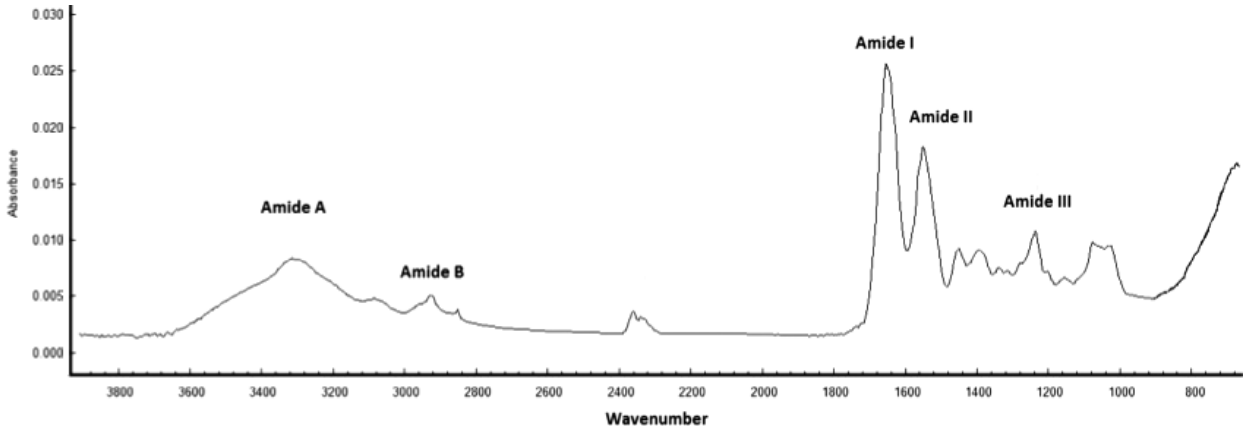


**Figure 3** Fourier transform infrared (FTIR) spectra of jellyfish collagen (the unit of abscissa is $cm^{-1}$).

**Table 2** Fourier transform infrared (FTIR) peak locations and assignments for JPSC and some other different collagens

| | Peak wave number ($cm^{-}$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Region | JPSC | Calf skin[a] | Chicken sternum cartilage[b] | Unicorn leather jacket[a] | Deep-sea redfish[c] | Bamboo shark[d] | Normal range in proteins[e] | Assignment |
| Amide A | 3314 | 3295 | 3309 | 3294 | 3322 | 3294 | 3400–3440 | N-H stretch coupled with hydrogen bond |
| Amide B | 2924 | 2933 | – | 3085 | 2927 | 2925 | 3100 | $CH_2$ asymmetrical stretch |
| Amide I | 1653 | 1635 | 1658 | 1635 | 1655 | 1635 | 1600–1700 | C=O stretch/hydrogen bond coupled with $COO^{-}$ |
| Amide II | 1551 | 1545 | 1552 | 1546 | 1551 | 1541 | 1510–1580 | NH bend coupled with CN stretch, $CH_2$ bend, $COO^{-}$ symmetrical stretch, $CH_2$ wag |
| Amide III | 1239 | 1235 | 1240 | 1236 | 1240 | | 1200–1300 | NH bend coupled with CN stretch, C-O stretch |

[a]Ahmad *et al.* (2010).
[b]Cao & Xu (2008).
[c]Wang *et al.* (2007).
[d]Kittiphattanabawon *et al.* (2010a).
[e]Kittiphattanabawon *et al.* (2010b).

3400–3440 $cm^{-1}$, the amide A band of JPSC was shifted to lower frequencies (3314 $cm^{-1}$). This indicates that the N-H group in JPSC is involved in hydrogen bonding with other groups, which stabilises the helical structure of collagen (Wang *et al.*, 2007; Duan *et al.*, 2009). This peak is shifted even lower in calf skin type I collagen, chicken sternum type II collagen and bamboo shark type I collagen (Table 2). As collagen molecules are mainly stabilised by hydrogen bonds, amide A in all collagens is shifted to lower frequencies (Kittiphattanabawon *et al.*, 2010b).

Amide B is related to the asymmetrical stretch of $CH_2$ and $NH_3^+$ (Ahmad *et al.*, 2010; Kittiphattanabawon *et al.*, 2010b). Higher frequencies of amide B can be attributed to the presence of the free $NH_3^+$ group of lysine or N-terminus (Ahmad *et al.*, 2010). Therefore, the lower frequency of amide B in JPSC (2924 $cm^{-1}$) compared with other sources (except for chicken) can be explained by its lower quantities of lysine (Table 1).

The amide I band is the most intense band in proteins; thus, it is the most sensitive and useful marker for the analysis of secondary structure of proteins in FTIR (Cao & Xu, 2008). Its normal position is in the range of 1600–1700 $cm^{-1}$, which is mainly associated with the C=O stretching vibration coupled with the N-H bending vibration along the polypeptide backbone or with hydrogen bonding coupled with $COO^{-}$, CN stretching and CCN deformation (Kittiphattanabawon *et al.*, 2010a; Pati *et al.*, 2010). Bands around

1630 $cm^{-1}$ indicate imide residues, and bands around 1660 and 1675 $cm^{-1}$ are assigned to intermolecular cross-links and β-turns, respectively (Cao & Xu, 2008). Therefore, amide I in JPSC appears to reflect the presence of intermolecular cross-links. Shifting of this peak to lower frequencies, as in JPSC (1653 $cm^{-1}$), is indicative of higher hydrogen bonding potential (Ahmad *et al.*, 2010), less intermolecular cross-linking and decreased molecular order (Kittiphattanabawon *et al.*, 2010a). The fewer occurrences of cross-links in JPSC might have resulted from pepsin digestion and removal of amino acids such as histidine, hydroxylysine and lysine from telopeptide regions (Kittiphattanabawon *et al.*, 2010b).

The amide II band normally occurs between 1550 and 1600 $cm^{-1}$, and it is associated with the N-H bending vibration coupled with the C-N stretching vibration (Duan *et al.*, 2009). Lower frequencies in this region indicate that the N-H group is involved in bonding with α chains (Ahmad *et al.*, 2010) and that hydrogen bonding in collagen is present (Duan *et al.*, 2009). This amide was found at 1551 $cm^{-1}$ for JPSC.

Amide III represents the combination of the C-N stretching vibration and N-H deformation. Bands between 1200 and 1350 $cm^{-1}$ are referred to as the collagen fingerprint because they are due to the collagen tripeptide (Gly-X-Y) (Cao & Xu, 2008). The absorption intensity ratio between amide III (1239 $cm^{-1}$) and 1454 $cm^{-1}$ was 0.85 for JPSC. A ratio of 1 indicates that the triple helical structure of collagen is intact (Wang *et al.*, 2007; Ahmad *et al.*, 2010; Kittiphattanabawon *et al.*, 2010b; Pati *et al.*, 2010), whereas when the collagen triple helix is affected by cleavage of telopeptides through pepsin digestion, this ratio might be lower (Kittiphattanabawon *et al.*, 2010b). Therefore, the value of 0.85 for jellyfish indicates that the triple helix has not been severely disrupted.

### Thermal stability of JPSC

The $T_{max}$ of JPSC was 37.38 °C (data not shown), which was lower than that of chicken sternum type II collagen (43.8 °C) (Cao & Xu, 2008) and calf skin collagen (40.8 °C) (Kittiphattanabawon *et al.*, 2005) but higher than that of fish collagens such as bamboo shark cartilage (35.98 °C), blacktip shark cartilage (34.56 °C) (Kittiphattanabawon *et al.*, 2010b), unicorn leatherjacket skin (27.2 °C) (Ahmad & Benjakul, 2010), deep-sea redfish (16.1 °C) and cod skin (15 °C) (Kittiphattanabawon *et al.*, 2010a). $T_{max}$ is correlated with imino acid content, body temperature of the specimen and environmental temperature (Nagai *et al.*, 2002; Muyonga *et al.*, 2004). Thermal stability of marine collagens is generally lower than that of land-based collagens and among marine sources, cold-water fishes exhibit lower denaturation temperature due to their lower content of imino acids.

$T_{max}$ of JPSC was higher than that of the aforementioned marine sources, whereas its imino acid content was lower (Table 1). Different conformations of JPSC compared with other sources may explain this difference. For example, JPSC likely has more intramolecular hydrogen bonds and a higher molecular order, which results in a higher thermal stability. A high molecular order was confirmed by the FTIR spectra, which showed a higher frequency for JPSC amide I (1653 $cm^{-1}$) compared with unicorn leatherjacket and bamboo shark amide I (1635 $cm^{-1}$) (Table 2).

JPSC also exhibited a high ΔH value (2.35 J $g^{-1}$), which was higher than that of other marine sources (0.3–1.18 J $g^{-1}$) and close to that of calf skin (2.83 J $g^{-1}$) (Kittiphattanabawon *et al.*, 2005). The enthalpy change (ΔH) can be influenced by molecular stability, which is correlated with the sequence of amino acids in the molecule. Thus, a high ΔH value of JPSC might be related to the high stability of the JPSC molecule, which may be due to the high content of the Gly-X-Y sequence. This might be another explanation for the high value of $T_{max}$ despite the low amount of imino acids and was in agreement with the report for eagle ray and red stingray, where the former had lower imino acid content and degree of prohydroxylation the enthalpy change and subsequently the molecule stability was higher (Bae *et al.*, 2008).

### Zeta potential of JPSC

The pH at which the positive and negative charges on a protein molecule in an aqueous system are equal or the net charge of the protein is zero is called the isoelectric point (pI) (Ahmad *et al.*, 2010; Kittiphattanabawon *et al.*, 2010b). The surface charge of JPSC was measured at different pHs using a zeta potential analyzer. According to the titration curve, the surface charge of JPSC was zero at pH 6.64. This value has been reported to be 6.56 for bamboo shark pepsin-solubilised collagen (Kittiphattanabawon *et al.*, 2010a), 6.96 for blacktip shark and 7.26 for brownbanded bamboo shark (Kittiphattanabawon *et al.*, 2010b). The difference in the pI values of collagen from different source is correlated with the amino acid composition. Amino acids with different charged side chains result in different surface charges in proteins (Kittiphattanabawon *et al.*, 2010b).

### Microstructure and element composition of JPSC

Figure 4 shows the microstructure of JPSC. The JPSC was observed as threads with various diameters along with the collagen sheets, which is combination of sev-

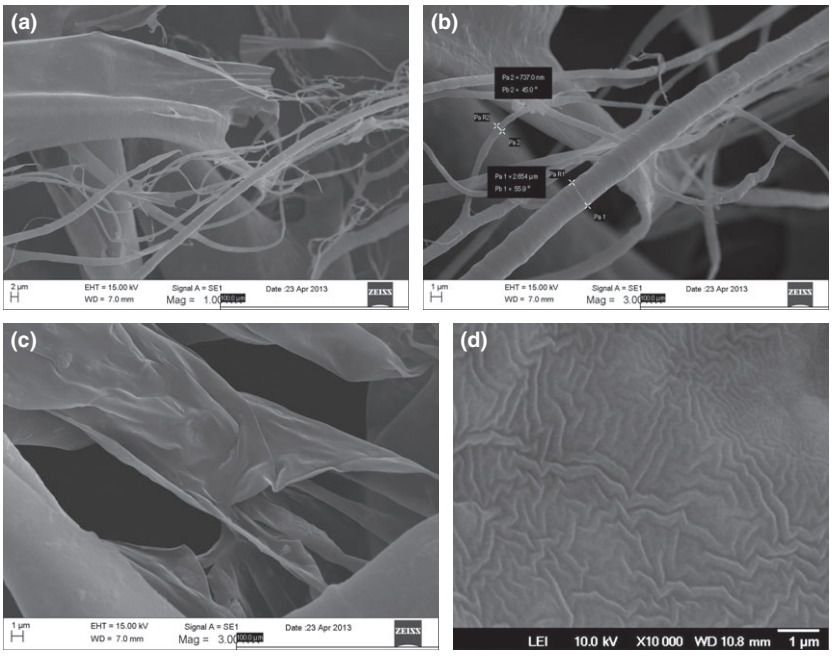


**Figure 4** Scanning electron microscope (SEM) images of JPSC at the magnification of (a) 1000×, (b and c) 3000× and (d): 10000×.

eral collagen fibrils and fibres that are bundled together to form a fibril network and a dense pleated sheetlike structure (Fig. 4a). Collagen fibrils (210–737 nm in diameter) and collagen fibres (1.25–2.65 µm in diameter) were observed in Fig. 4b. Sheets were smoothly wrinkled and folded and appear to be very thin and soft (Fig. 4c). Pleating of the sheets was visible at magnification of ×10000 (Fig. 4d).

The carbon, oxygen, nitrogen, sulphur, chlorine and sodium contents of JPSC were measured using EDX (data not shown). As with other proteins, the main detected elements were carbon (~46%), oxygen (~33%) and nitrogen (~22%). No sulphur was detected, which is in agreement with the amino acid composition result that showed no cysteine or cysteine in the JPSC and also confirms the electrophoretic results (i.e. no difference between the reducing and nonreducing patterns). The trace amount of chlorine presented in the sample might be due to the salting out process.

## Conclusion

Pepsin-solubilised collagen was successfully extracted from ribbon jellyfish umbrella with a maximum yield of 19% (ash-free lyophilised dry weight). The extracted collagen was identified as type II collagen, which is rarely found in marine sources. The amino acid composition and peptide pattern (primary structure) of JPSC was different from that of standard type II collagen from chicken sternum; the secondary structure, however, was similar. Denaturation temperature of JPSC was higher than that of some other marine sources of collagen. These results suggest that this jellyfish species may be a useful source of type II collagen that can be used as an alternative to land-based sources.

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