BORANG ERGS – P3(R)

Code Project : ERGS/1/2013/STG07/USM/02/01

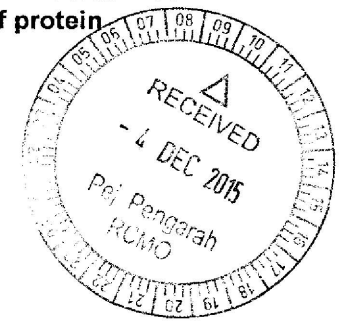
KEMENTERIAN PENDIDIKAN MALAYSIA

FINAL REPORT
EXPLORATORY RESEARCH GRANT SCHEME (ERGS)

Laporan Akhir Skim Geran Penyelidikan Eksploratori (ERGS) IPT
Pindaan 1/2015

A

RESEARCH TITLE: **Development of electropulsation for the extraction of protein from underutilized agro-source, pinto bean, as an alternative solution to the issue of protein malnutrition**



PHASE & YEAR: 4th Phase and 3rd Year

START DATE: 1 June 2013
END DATE: 31 May 2015
EXTENSION PERIOD (DATE): RMC LEVEL: 1 June 2015 until 30 November 2015
KPM LEVEL:

PROJECT LEADER: Dr. Gan Chee Yuen
I/C / PASSPORT NUMBER: 810629-07-5531

PROJECT MEMBERS: (including GRA) 1. **Dr. Lee Chee Keong**
2. **Tan Sze Jack**

PROJECT ACHIEVEMENT *(Prestasi Projek)*

B

ACHIEVEMENT PERCENTAGE			
Project progress according to milestones achieved up to this period	**0 - 50%**	**51 - 75%**	**76 - 100%**
Percentage			/

RESEARCH OUTPUT		
Number of articles/ manuscripts/ books *(Please attach the First Page of Publication)*	**Refereed Journal**	***Non-Refereed Publication***
	6	
Conference Proceeding *(Please attach the First Page of Publication)*	**International**	***National***
	3	
Intellectual Property *(Including Paten, Copyright, Industrial Design, layout Design of Integrated Circuit & Trademarks)*		

HUMAN CAPITAL DEVELOPMENT

Human Capital	Number				Others (please specify)
	On-going		Graduated		
Citizen	Malaysian	Non Malaysian	Malaysian	Non Malaysian	
No. PHD STUDENT	1				
Student Fullname: IC / Passport No: Student ID:	Ngoh Ying Yuan 890613-13-5694 P-CTD0001/14(R) 16 May2014				
No. MASTER STUDENT			1		
Student Fullname: IC / Passport No: Student ID:			Tan Sze Jack 880526-52-5863 P-CTM0001./14(R) 19 June 2014		
No. UNDERGRADUATE STUDENT					
Student Fullname: IC / Passport No: Student ID:					
Total	1		1		

EXPENDITURE *(Perbelanjaan)*

C

Budget Approved *(Peruntukan diluluskan)* : **RM 115,000.00**
Amount Spent *(Jumlah Perbelanjaan)* : **RM 114,999.46**

Balance *(Baki)* : **RM 0.54**
Percentage of Amount Spent : 99.9995 %
(Peratusan Belanja)

ADDITIONAL RESEARCH ACTIVITIES THAT CONTRIBUTE TOWARDS DEVELOPING SOFT AND HARD SKILLS
(Aktiviti Penyelidikan Sampingan yang menyumbang kepada pembangunan kemahiran insaniah)

D

International

Activity	Date (Month, Year)	Organizer
A workflow on the production of bioactive peptides from food sources based on the case study using Pinto bean (Phaseolus vulgaris cv. Pinto)	5-8 October 2015	15th International Nutrition & Diagnostic Conference
Physico-chemical characterization of antioxidative pectic-polysaccharide from mangosteen (Garcinia mangostana) rind	5-8 October 2015	15th International Nutrition & Diagnostic Conference
Cumin seed (Cuminum cyminum), an alternative source of antioxidative and antidiabetic peptides	5-8 October 2015	15th International Nutrition & Diagnostic Conference

E	**PROBLEMS / CONSTRAINTS IF ANY** *(Masalah/ Kekangan sekiranya ada)*
	1. There was a minor change in extraction method due to difficulty in accessing instrument. 2. The distribution of budget had to be adjusted from time to time because we had included more analyses in this project in order to adjust for a PhD project. 3. A PhD project might take up to 3 years, therefore the student (Ngoh Ying Yuan, P-CTD0001/14(R)) has not completed her study within the time frame.
F	**RECOMMENDATION** (*Cadangan Penambahbaikan*)
	1. Other alternative method was used and optimized. 2. Budet Vot was adjusted accordingly. 3. The project should be extended for another year (2016).
	RESEARCH ABSTRACT – Not More Than 200 Words (*Abstrak Penyelidikan – Tidak Melebihi 200 patah perkataan*)
	Pinto bean showed its potential as an alternative source of protein. High amount of protein was successfully extracted from pinto bean. An optimum condition for the extraction was obtained. Therefore, this protein source could be used to overcome protein-malnutrition issue. Apart from that, the protein fragment from this extract exhibited potential in contributing biological activities such as radical scavenging, antioxidative and anti-diabetic properties. Bioactive peptide were therefore produced from this protein using enzyme-assisted approach. Protamex was shown to effectively release the peptides from the protein. Also, the sequences of the bioactive peptide were identified and verified. It was therefore suggested that Pinto bean protein and peptides could be incorporated in food systems as well as nutraceutical products because they could function not just as nutritional product but also has medical properties such as anti-oxidative and anti-diabetic activities. The overall project successfully produced 6 international journals with high impact factor as well as 1 PhD (on going) and 1 MSc (graduated) students which was exceed the outcome that stated in the proposal (i.e.3 journals and 1 MSc student).
	Date : 4/12/15 *Tarikh* — **Project Leader's Signature:** *Tandatangan Ketua Projek*
4	**COMMENTS, IF ANY/ ENDORSEMENT BY RESEARCH MANAGEMENT CENTER (RMC)** *(Komen, sekiranya ada/ Pengesahan oleh Pusat Pengurusan Penyelidikan)*
	Good publication output
	Name: *Nama:* PROF. DR LEE KEAT TEONG Pengarah Pejabat Pengurusan & Kreativiti Penyelidikan Universiti Sains Malaysia — **Signature:** *Tandatangan:* **Date:** *Tarikh:* 4/12/15

Tarikh Cetakan : 02/12/2015

UNIVERSITI SAINS MALAYSIA

JABATAN BENDAHARI

PENYATA PERBELANJAAN SEHINGGA 2 DISEMBER 2015

Projek : DEVELOPMENT OF ELECTROPULSATION FOR THE EXTRACTION OF PROTEIN FROM UNDERUTILIZED AGRO-SOURCE, PINTO BEAN, AS AN ALTERNATIVE SOLUTION TO THE ISSUE
TEMPOH : 01 JUN 2013 HINGGA 31 MEI 2015 (LANJUTAN SEHINGGA 30 NOVEMBER 2015)
DR. GAN CHEE YUEN
PUSAT PERKHIDMATAN ANALITIKAL TOKSIKOLOGI TERMAJU

No. Akaun : 203.CAATS.6730103.

Vot	Nama Vot	Peruntukan Projek	Perbelanjaan Terkumpul Sehingga Thn Lalu	Baki Peruntukan Tahun Lalu	Peruntukan Thn Semasa	Jumlah Peruntukan Thn Semasa	Tanggungan Semasa	Bayaran Thn Semasa	Jum Belanja Thn Semasa	Baki Projek
111	GAJI	18,000.00	9,000.00	27,000.00	-18,000.00	9,000.00	0.00	9,000.00	9,000.00	0.00
221	PERJALANAN DAN SARA HIDUP	862.10	862.10	11,580.90	-11,580.90	0.00	0.00	0.00	0.00	0.00
224	SEWAAN	1,326.00	0.00	1,326.00	0.00	1,326.00	0.00	0.00	0.00	1,326.00
227	BEKALAN DAN BAHAN LAIN	88,561.90	35,053.22	9,617.78	43,890.90	53,508.68	18,152.62	38,686.13	56,838.75	-3,330.07
228	PENYELENGGARAN & PEMBAIKAN KECIL	2,000.00	0.00	2,000.00	0.00	2,000.00	0.00	0.00	0.00	2,000.00
229	PERKHIDMATAN IKTISAS & HOSPITALITI	4,250.00	1,612.77	1,387.23	1,250.00	2,637.23	20.00	2,612.62	2,632.62	4.61
335	HARTA MODAL	0.00	0.00	15,560.00	-15,560.00	0.00	0.00	0.00	0.00	0.00
	Jumlah	**115,000.00**	**46,528.09**	**68,471.91**	**0.00**	**68,471.91**	**18,172.62**	**50,298.75**	**68,471.37**	**0.54**

Penyata ini adalah cetakan komputer tiada tandatangan diperlukan
Penyata ini adalah dianggap tepat jika tiada maklumbalas dalam tempoh masa 14 hari dari tarikh penyata

Food Chemistry 152 (2014) 447–455

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

A comparative study of physicochemical characteristics and functionalities of pinto bean protein isolate (PBPI) against the soybean protein isolate (SPI) after the extraction optimisation

Ee-San Tan [a,b], Ying-Yuan Ngoh [b], Chee-Yuen Gan [b,*]

[a] Bioprocess Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
[b] Centre for Advanced Analytical Toxicology Services, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

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Keywords:
Functionalities
Optimisation
Physicochemical
Pinto bean
Protein isolate

ABSTRACT

Optimisation of protein extraction yield from pinto bean was investigated using response surface methodology. The maximum protein yield of 54.8 mg/g was obtained with the optimal conditions of: temperature = 25 °C, time = 1 h and buffer-to-sample ratio = 20 ml/g. PBPI was found to obtain high amount of essential amino acids such as leucine, lysine, and phenylalanine compared to SPI. The predominant proteins of PBPI were vicilin and phytohemagglutinins whereas the predominant proteins of SPI were glycinin and conglycinins. Significantly higher emulsifying capacity was found in PBPI (84.8%) compared to SPI (61.9%). Different isoelectric points were found in both PBPI (4.0–5.5) and SPI (4.0–5.0). Also, it was found that PBPI obtained a much higher denaturation temperature of 110.2 °C compared to SPI (92.5 °C). Other properties such as structural information, gelling capacity, water- and oil-holding capacities, emulsion stability as well as digestibility were also reported.

1. Introduction

Pinto bean (*Phaseolus vulgaris* cv. *Pinto*) is an underutilised legume containing high amount of protein as well as numerous other phyto nutrients. It has been a stable source of traditional foods in several countries given its high nutritional value. The word "pinto" in Spanish means "painted", referring to its beige background strewn with reddish brown speckles of colour. These beans are often consumed in United States and northwestern Mexico. The top five producers of pinto bean are the United States, China, Brazil, India and Indonesia. Seyam, Banasik, and Breen (1983) had studied the use of this protein source in producing macaroni. Result showed that it improved the cooking quality of the macaroni and increased its lysine content. Also, Ye and Ng (2001) reported that the bean peptides exhibit antifungal activity towards a few fungal species, mitogenic activity toward mouse splenocytes and HIV-1 reverse transcriptase-inhibitory activities. It was also reported that phenolic acids and flavonols extracted from pinto beans, possess significant antioxidant activity (Xu & Chang, 2009), however there is no study on the protein isolation from pinto bean.

In this study, an extraction optimisation of this new protein isolate (i.e. pinto bean protein isolate) will be conducted followed by the comparison of the physicochemical characteristics, and functionalities of the extracted protein isolate against the commercially available soybean protein isolate. Due to the presence of genetic modified soybean, public is concerned about the health issue and its long-term side effects. The reason to compare between PBPI and SPI was to find the possibility of PBPI to be an alternative to SPI. For a successful utilisation of plant proteins in food application or research, it is important to investigate the intrinsic physicochemical characteristics and functionalities such as water and oil holding capacities, protein solubility, gelling properties and foaming properties. These properties manipulate the behaviour of proteins and therefore it is crucial for future protein research.

2. Material and methods

2.1. Materials

Pinto beans (15 kg) were purchased from three different local markets (i.e. Lip Sin Market, Jelutong Market and Bayan Baru Market) located in Penang, Malaysia. These three different sources of pinto bean were used as replicates for the experiment. The beans were separated from the pods and rinsed with distilled water and lyophilised. The dried beans were subsequently milled into powder form, sieved (60 mesh) and stored at 4 °C prior to extraction. All chemicals and reagents used in the experiment were of analytical grade purchased from Sigma–Aldrich (Malaysia) company or otherwise mentioned.

* Corresponding author. Tel.: +604 6534261; fax: +604 6534688.
E-mail address: cygan@usm.my (C.-Y. Gan).

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Enzyme-assisted extraction and identification of antioxidative and α-amylase inhibitory peptides from Pinto beans (*Phaseolus vulgaris* cv. Pinto)

Ying-Yuan Ngoh, Chee-Yuen Gan *

Centre for Advanced Analytical Toxicology Services, Universiti Sains Malaysia (USM), 11800 Penang, Malaysia

ARTICLE INFO

Article history:
Received 28 January 2015
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Antioxidative
Bioactive peptides
Pinto bean

ABSTRACT

Antioxidant and α-amylase inhibitor peptides were successfully extracted from Pinto bean protein isolate (PBPI) using Protamex. A factorial design experiment was conducted and the effects of extraction time, pH and temperature were studied. pH 7.5, extraction time of 1 h, S/E ratio of 10 (w/w) and temperature of 50 °C gave the highest antioxidant activities (i.e., ABTS scavenging activity (53.3%) and FRAP value (3.71 mM)), whereas pH 6.5 with the same extraction time, S/E ratio and temperature, gave the highest α-amylase inhibitory activity (57.5%). It was then fractioned using membrane ultrafiltration with molecular weight cutoffs of 100, 50, 30, 10 and 3 kDa. Peptide fraction <3 kDa, which exhibited the highest antioxidant activities (i.e., ABTS (42.2%) and FRAP (0.81 mM)) and α-amylase inhibitory activity (62.1%), was then subjected to LCMS and MS/MS analyses. Six sequences were identified for antioxidant peptides, whereas seven peptides for α-amylase inhibitor.

1. Introduction

Currently, natural food derived peptides possessing bioactivities is attracting the interest of research globally. Bioactive peptides are defined as specific protein fragments that have a positive impact on the functions and conditions of human health. According to Korhonen and Pihlanto (2006), bioactive peptides are greatly influenced by their amino acid composition and sequences. Depending on these two factors, these peptides are associated with various roles such as opiate-like, mineral binding, immunomodulatory, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic and antihypertensive functions (Sarmadi & Ismail, 2010). These roles are beneficial towards the health of humans.

Bioactive peptides are inactive within the sequence of parent protein and can be released by enzymatic hydrolysis. Enzymatic hydrolysis is the main process that allows biologically active peptides to be obtained from food products by means of the microbial activity of fermented foods or through the action of enzymes derived from proteolytic micro-organisms (Korhonen & Pihlanto, 2006). Apart from that, it was reported that it can improve the functional properties of dietary protein without affecting its nutritive value by converting it into peptides with the desired size, charge and surface properties (Moure, Sineiro, Dominguez, & Parajo, 2006).

Pinto bean, an underutilised legume, is rich in protein content as well as other phyto nutrients. The word "Pinto" in Spanish means "painted", referring to its beige background strewn with reddish brown speckles of colour (Tan, Ngoh, & Gan, 2014). According to Anton, Lukow, Fulcher, and Arntfield (2009), it is high in fibre (15 g/100 g), protein (23 g/100 g) and low in fat (1 g/100 g). In addition, it is accepted as the best source of fat free protein by the World Health Organisation with a potential of providing 347 kcal of energy from 100 g of bean with 21.42% protein content. Due to its high nutritional values, it is a staple food in many countries, especially United States and northwestern of Mexico. Potential values and health benefits of Pinto beans have been revealed in past studies. Kelkar et al. (2012) reported that consumption of Pinto beans has been linked to the reduction in cholesterol levels. In a study conducted by Anton et al. (2009) Pinto bean flour showed a great positive impact on levels of phenols and antioxidant activity besides a significant increase in levels of crude protein. It was also reported that phenolic acids and flavonols extracted from Pinto beans, possess significant antioxidant activities (Xu & Chang, 2009). However, the potential of the Pinto bean peptides in inhibiting oxidative reaction as well as inhibiting hydrolytic function of α-amylase are still remained unknown.

The objectives of the present study were: (a) to extract antioxidant and α-amylase inhibitor peptides from Pinto bean protein

* Corresponding author.
E-mail address: mauganS1@yahoo.com (C.-Y. Gan).

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Functional protein from cumin seed (*Cuminum cyminum*): Optimization and characterization studies

Hwee-Leng Siow, Chee-Yuen Gan*

Centre for Advanced Analytical Toxicology Services, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

ARTICLE INFO

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Keywords:
Bioactive protein
Characterization
Cumin seed
Extraction
Optimization

ABSTRACT

Cumin seed protein isolate (CSPI) was found to be a potential source of bioactive protein. Box-Behnken Design was used in order to optimize the protein extraction parameters: extraction time (X_1: 0.5–1.5 h), extraction temperature (X_2: 20–40 °C) and buffer-to-sample ratio (X_3: 10–30 mL/g). Extraction conditions for maximum protein yield were corresponding to $X_1 = 0.6$ h, $X_2 = 26.3$ °C and $X_3 = 10$ mL/g. A close agreement between experimental (44.98 mg/g) and predicted (45.19 mg/g) values was found. The physicochemical properties (i.e. amino acid composition, protein components, protein structure) and its potential bioactivities (i.e. antioxidant and antidiabetic activity) were also evaluated, giving a better understanding of general structure and properties of CSPI. 2S albumin, 7S globulin, 11S globulin and lectin were found as the components of the extracted seed protein. Structure of CSPI was a mixture of intramolecular β-sheet (1639 cm^{-1}), random coil (1642 cm^{-1}), α-helix (1655 cm^{-1}), β-turns (1660 cm^{-1}) and antiparallel β-sheet aggregates (1690 cm^{-1}) as shown in the FTIR spectra. CSPI was predominant with the Tyr, Glu, Asp, Arg, Leu and Phe, which could be considered as a high quality of natural protein. In addition, CSPI showed appreciable DPPH free radical scavenging activity (47.7 $\%DPPH_{sc}/\mu g$) and reducing power (12.4 mM/μg), which implied that CSPI could be used as a natural antioxidant agent. However, CSPI showed a relatively low α-amylase inhibition activity (6.7%). These findings demonstrated that CSPI could be a used as a potential nutraceutical or ingredient of functional and health-promoting foods.

1. Introduction

The interest of natural plant-derived proteins is expected to grow due to the growing of the consumers' concerns over the food safety as well as the increasing cost of food products derived from animal. In recent years, the increasing demand of continuous improvement regarding the nutritional and functional properties of protein has also contributed to the acceleration of the research on unconventional plant-derived protein (Henry & Kettlewell, 1996). Seeds dietary proteins were reported as an important bioactive protein with significant pharmacology and medicinal values (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008).

Cumin (*Cuminum cyminum*), an annual plant of the family Apiaceae, is one of the traditional medicinal herbs or spices in Asia, Africa and Europe. The seeds have an elongated and oval shape which is similar to fennel seeds. They have an aromatic odor and give a spicy and bitter taste. This makes them as a flavoring agent, which is widely used as a condiment in the food preparation (Allahghadri et al., 2010). The therapeutic properties and physiological effects of aqueous extract, oil or inorganic compounds isolated from cumin seeds have been extensively studied, including antimicrobial (Allahghadri et al., 2010), anti-carcinogenic (Gagandepp, Dhanalakshmi, Mendiz, Rao, & Kale, 2003), antidiabetic (Dhandapani, Subramanian, Rajagopal, & Namasivayam, 2002) and antioxidant (Gagandepp et al., 2003). However, limited information was found on the relative physicochemical and functional attribute of cumin seed protein isolate (CSPI). The properties of proteins are mainly contributed to their application and functionalities (Amza, Amadou, Zhu, & Zhou, 2011; Yu, Ahmedna, & Goktepe, 2007). In this aspect, the study of the physicochemical and properties of the isolated protein (e.g. amino acid composition, molecular weights (MW), protein structure and its potential bioactivities) are required, to provide a better understanding of its characteristics and potential applications in nutraceutical and food industries. To date, there are no relevant study concerning on the preparation and characterization of isolated protein from cumin seed. The aims of the present study were to optimize the extraction conditions using response surface methodology (RSM) in order to

* Corresponding author. Tel.: +60 4 653 4261; fax: +60 4 653 4688.
E-mail addresses: cygan@usm.my, matgan81@gmail.com, matgan81@yahoo.com (C.-Y. Gan).

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0268-005X/

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Journal homepage: http://www.ifrj.upm.edu.my

Optimization study for synthetic dye removal using an agricultural waste of *Parkia speciosa* pod: A sustainable approach for waste water treatment

Ngoh, Y. Y., Leong, Y.-H. and *Gan, C. Y.

Centre for Advanced Analytical Toxicology Services, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

Article history

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Keywords

Coomassie blue
Dye removal
Biosorption
Optimization
Parkia speciosa

Abstract

Parkia speciosa pod showed extremely high potential in dye (Coomassie Brilliant Blue R-250) removal application especially for waste water treatment. Central composite design (CCD) was successfully employed for experimental design and results analysis. The combined effect of pH (X_1: 5.0-8.0), biosorbent dosage (X_2: 0.05-0.10 g) and contact time (X_3: 30-100 min) on dye adsorption was investigated and optimized using response surface methodology (RSM). Analysis of variance (ANOVA) demonstrated that the contribution of the quadratic model was significant for the responses. The optimum adsorption of 83.4% was obtained with the optimal conditions of pH 5.0, dosage of 0.10 g and the contact time of 70 min. Close agreement was found between both the experimental and predicted values. Therefore, it was suggested that *P. speciosa* pod could be a potential natural dye adsorbent.

Introduction

Synthetic dyes are human-made organic dye. They are used globally in the industries of textiles, plastics, rubber, cosmetics, paper and colouration of products (Asgher and Bhatti, 2012). Annual production is found to be over 7 x 10^5 tons with textile industry individually discharging more than 1.5 x 10^8 m^3 of coloured effluents annually (Feng *et al.*, 2011). Production of synthetic dyes is in the trend of increasing due to their inexpensive cost of synthesis, stability, convenient usage and variety of colours choices compared to natural dyes (Aksu and Isoglu, 2006). Extensive use of synthetic dyes has therefore created a major pollution problem. Once being released into the aquatic environment, they are difficult to be decolourized because of their high resistance towards fading on exposure to light (Saad *et al.*, 2010). Both human and aquatic life will be greatly affected as synthetic dyes are known to be carcinogenic, mutagenic and toxic. Apart from that, reduction of sunlight transmission will give negative impacts towards aquatic plants (Salleh *et al.*, 2011).

Coomassie Brilliant Blue (CBB) belongs to the group of synthetic dye. The name CBB refers to the commercial name of two similar triphenylmethane (Raja, 2012). In this study, Coomassie Brilliant Blue R-250 has been used. The term '250' and the suffix 'R' in 'Brilliant Blue R-250' represents the denotation of the purity of the dye and abbreviation for 'red' as the blue colour of the dye has a slight reddish tint respectively. CBB is widely used in biochemical and clinical laboratories for the purification and quantification of proteins. CBB is employed to stain proteins for protein visualisation in a polyacrylamide gel. It is also used in the quantification of electrophoretically separated protein. Recently, CBB was used in the treatment of spinal injuries in rats (Peng *et al.*, 2009) and as a stain to assist surgeons in retinal surgery (Mennel *et al.*, 2008). CBB has shown to be toxic and the potential health effects on human beings include irritation of skin, eye and respiratory difficulty. Their target organs are usually eyes, kidney, liver, heart and central nervous system. The pathway of CBB to human beings is by inhalation, ingestion and skin absorption.

The environmental hazard lies in fact that this toxic substance can ultimately get into the drinking water supplies. Therefore, there is a need to remove these dyes immediately and effectively from the polluted water. Many treatments (chemical, biological and physical) such as oxidation process, photochemical, ozonation, microbial cultures, bioremediation, membrane filtration, and ion exchange have been implemented (Salleh *et al.*, 2011). Among these, adsorption has been considered as an effective decolourization method due to its simplicity, availability and effectiveness in removing non-biodegradable pollutants (including dyes) from wastewater (Feng *et al.*, 2011). Currently, the most promising adsorbent is activated carbon due to its surface area, high adsorption capacity, degree of surface reactivity and microporous structures.

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SHORT COMMUNICATION

Biochemical and radical-scavenging properties of sea cucumber (*Stichopus vastus*) collagen hydrolysates

Md. Zainul Abedin[a], Alias A. Karim[a]*, Aishah A. Latiff[b], Chee-Yuen Gan[c], Farid Che Ghazali[d], Zoha Barzideh[a], Sahena Ferdosh[a], Md. Jahurul Haque Akanda[a], Wahidu Zzaman[a], Md. Rezaul Karim[e] and Md. Zaidul Islam Sarker[f]

[a]*Food Biopolymer Research Group, Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Penang, Malaysia;* [b]*Doping Control Centre, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia;* [c]*Centre for Advanced Analytical Toxicology Services, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia;* [d]*School of Health Sciences, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan Darul Naim, Malaysia;* [e]*Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, Gambang, Kuantan, Pahang, Malaysia;* [f]*Department of Pharmaceutical Technology, Faculty of Pharmacy, International Islamic University Malaysia, Kuantan Campus, 25200 Kuantan, Pahang, Malaysia*

(*Received 5 December 2013; final version received 1 March 2014*)

The molecular mass distribution, amino acid composition and radical-scavenging activity of collagen hydrolysates prepared from collagen isolated from the sea cucumber *Stichopus vastus* were investigated. β and α_1 chains of the collagen were successfully hydrolysed by trypsin. The molecular mass distribution of the hydrolysates ranged from 5 to 25 kDa, and they were rich in glycine, alanine, glutamate, proline and hydroxyproline residues. The hydrolysates exhibited excellent radical-scavenging activity. These results indicate that collagen hydrolysates from *S. vastus* can be used as a functional ingredient in food and nutraceutical products.

Keywords: sea cucumber; integument collagen; trypsin hydrolysis; collagen hydrolysates; biochemical and radical scavenging properties

1. Introduction

The sea cucumber *Stichopus vastus* is a member of the family Stichopodidae in the class Holothuroidea. It is a common species in Malaysia, where it is especially common in seashore regions. It is considered to be an under-utilised species, as only the stomach and intestines are used raw as a salad in some Asian countries; the remaining parts, including the collagen-rich integument, are thrown away as waste. Thus, sea cucumbers are a potential source of collagen (Saito et al. 2002; Cui et al. 2007; Liu et al. 2010) that could be exploited for the production of bioactive protein hydrolysates (i.e. small peptides).

A large number of protein hydrolysates are produced from proteins by controlled enzymatic hydrolysis, and the resultant hydrolysates may enrich the antioxidant and biochemical properties of proteins. Small peptides display bioactive characteristics such as antioxidative and antihypertensive activities (Quian et al. 2008; Giménez et al. 2009; Chen et al. 2012; Li et al. 2012), and the human body can easily absorb them. A number of fish foods, such as lobster, Bombay duck, Northern shrimp, capelin and cod, have been used as a protein source to prepare bioactive hydrolysates through enzymatic hydrolysis (Vieira et al. 1995; Amarowicz & Shahidi 1997; Gildberg & Stenberg 2001; Himaya et al. 2012; Jin et al. 2012).

*Corresponding author. Email: akarim@usm.my

To date, only a few studies have described the peptides prepared from sea cucumber integument collagen following enzymatic hydrolysis (Zhao et al. 2007; Wang et al. 2010; Liu et al. 2011). In this study, the molecular weight distribution, amino acid composition and radical-scavenging activity of collagen hydrolysates obtained via trypsin hydrolysis of *S. vastus* integument collagen were investigated.

2. Results and discussion

2.1 *SDS-PAGE and degree of hydrolysis analysis*

Collagen hydrolysates were produced successfully through trypsin hydrolysis of *S. vastus* integument collagen after 3 h at 50°C. The β and α_1 bands (Figure S1(a)) of the isolated integument pepsin-solubilised collagen were completely destroyed after 3 h of digestion (Figure S1(b)) and were subsequently transformed into a mixture of peptides. The hydrolysates had a molecular mass ranging from 5 to 25 kDa (Figure S1(b)), which is similar to the results reported by Yang et al. (2008). These findings indicate that exogenous enzymes hydrolyse the *S. vastus* integument collagen and might produce bioactive peptides.

2.2 *Amino acid composition analysis*

Table S1 shows the amino acid composition (expressed as residues per 1000 residues) of the hydrolysates obtained from trypsin hydrolysis of the *S. vastus* integument collagen and comparisons with three other fish skin collagen hydrolysates (Saito et al. 2009; Liu et al. 2011). Glycine (Gly), alanine (Ala), glutamate (Glu), proline (Pro) and hydroxyproline (Hyp) were abundant in the hydrolysates and accounted for 322, 108, 97, 91 and 73 residues out of 1000 residues, respectively; in contrast, the content of methionine, tyrosine, histidine, lysine and phenylalanine was very low. The high amino acid content (especially Hyp) is crucial, as it affects the functional properties of collagen (Gómez-Guillén et al. 2011). The Hyp content of *S. vastus* collagen hydrolysates was significantly higher ($p < 0.05$) than that of collagen hydrolysates from the sea cucumber *Parastichopus californicus* and from salmon and trout. In addition, hydrophobic amino acids were dominant (606/1000 residues) in the hydrolysates. Abundant hydrophobic amino acids lead to high solubility of the hydrolysates in lipids, which in turn results in high antioxidant activity (Mendis, Rajapakse, Byun, et al. 2005). These findings suggest that *S. vastus* collagen hydrolysates might have diverse applications.

2.3 *Radical-scavenging activity*

The reducing capability of a particular compound might serve as an indicator for its antioxidant activity. A reducing agent (an electron donor) can provide an electron to a free radical, causing the radical to become neutral and the reducing species to successively obtain protons from the aqueous solution (Wang et al. 2008). In the ABTS assay, collagen hydrolysates obtained via trypsin hydrolysis of *S. vastus* integument collagen were able to quench ABTS radicals up to ~71.3% (Table S2) under designated hydrolysis conditions. This radical-scavenging activity is similar to that of hydrolysates of the jellyfish *Rhopilema esculentum* (Zhuang et al. 2009), which was estimated to be 72.15% under deduced optimised hydrolysis conditions. Further studies are needed to optimise the hydrolysis conditions to predict the exact radical-scavenging activity of the collagen hydrolysates obtained from the *S. vastus* integument collagen.

Collagen hydrolysates likely consist of peptides that act as electron donors to produce more stable products by reacting with free radicals to terminate chain reactions. The antioxidant activity of the hydrolysates obtained from squid skin collagen has been reported (Nam et al. 2008). Mendis, Rakapakse, Kim, et al. (2005) reported that the main antioxidative mechanism of

such peptides consists of amino acids such as Ala, Pro, leucine and valine, which are responsible for quenching free radicals. To date, data on the radical-scavenging activity of the collagen hydrolysates of other sea cucumber species are not available to compare with our findings.

3. Conclusion

Trypsin hydrolysis of integument collagen of the sea cucumber *S. vastus* produces a series of hydrolysates, as reflected by SDS-PAGE analysis, and Gly, Ala, Glu, Pro and Hyp residues are dominant in the hydrolysates. The hydrolysates exhibit superior radical-scavenging activity. Thus, it may be feasible to produce natural functional ingredients from this under-utilised collagen source using commercial enzymes. Further studies are required to identify and isolate the specific biologically active peptides from *S. vastus* collagen hydrolysates that might have potential applications in food and nutraceutical products.

Supplementary material

Experimental details relating to this article are available online, alongside Tables S1 and S2 and Figure S1.

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Original article

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

Zoha Barzideh,[1,2]* Aishah Abd Latiff,[3] Chee-Yuen Gan,[2] Soottawat Benjakul[4] & Alias Abd Karim[1]

1 Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, Minden, Penang 11800, Malaysia
2 Centre for Advanced Analytical Toxicology Services, Universiti Sains Malaysia, Minden, Penang 11800, Malaysia
3 Toxicology and Multipurpose Lab, Anti Doping Lab Qatar, Doha, Qatar
4 Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla university, Hat Yai, Songkla 90112, Thailand

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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

*Correspondent: Fax: +6046534471;
e-mails: zoha_barzideh@yahoo.com; zohabarzideh@gmail.com

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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 (α_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 α_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 α_1(II)-chains is the main type of

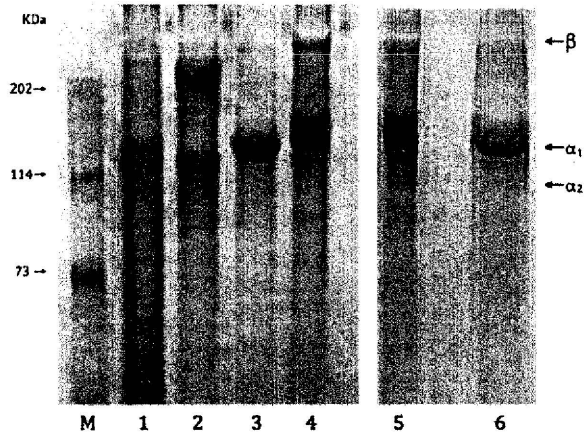

Figure 1 SDS-PAGE pattern of jellyfish PSC. M: high molecular weight protein marker; 1: type I collagen from calf skin; 2: type I collagen from salmon; 3: type II collagen from chicken sternum; 4 & 5: jellyfish PSC (non-reduced); 6: jellyfish PSC (reduced).

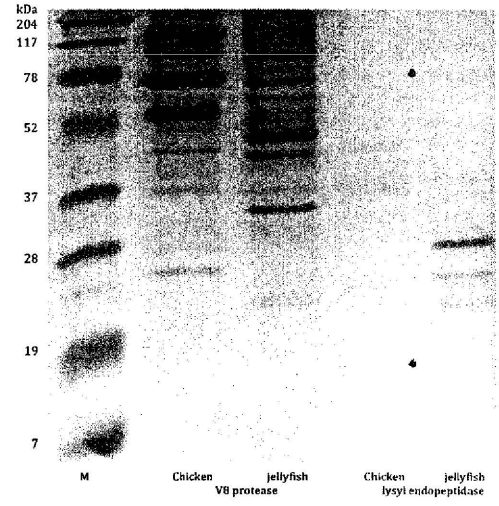

Figure 2 Peptide maps of jellyfish PSC and type II collagen from chicken digested by V8 protease and lysyl endopeptidase. M: high molecular weight marker.

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, α_1 and α_2. In addition, Miura & Kimura (1985) found α_1, α_2, and α_3 chains in collagen from the mesogloea of *Stomolophus nomurai*. Collagen from *Stomolophus meleagris* also contains α_1, α_2, and α_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

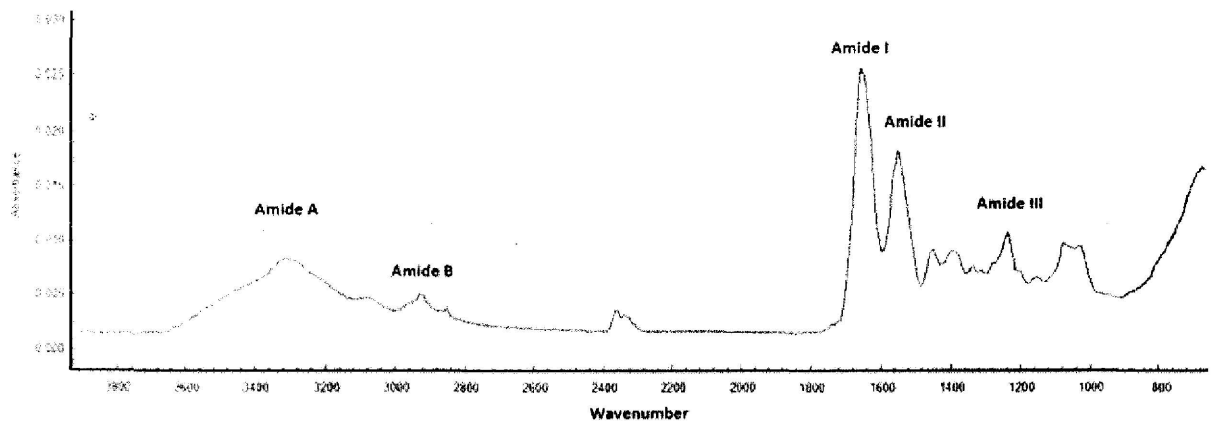

Figure 3 Fourier transform infrared (FTIR) spectra of jellyfish collagen (the unit of abscissa is cm^{-1}).

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

	Peak wave number (cm^{-1})							
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-

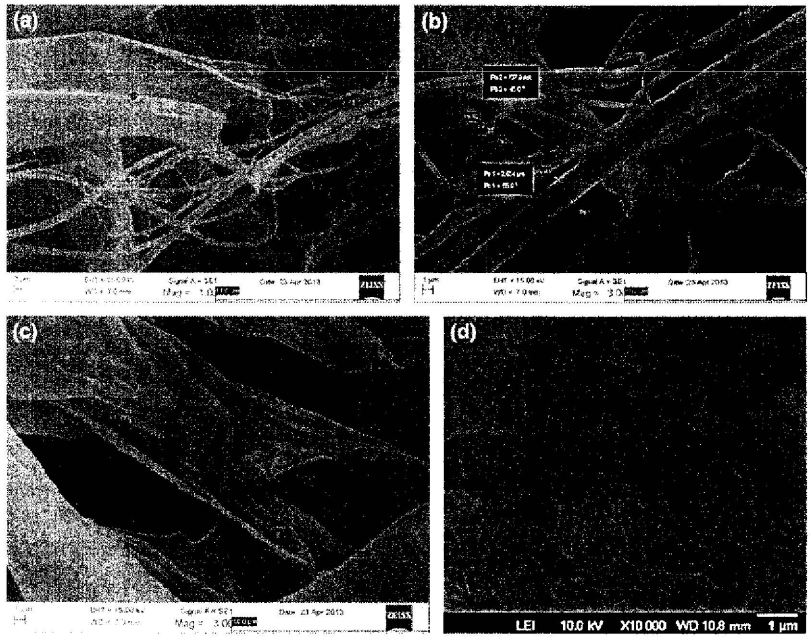

Figure 4 Scanning electron microscope (SEM) images of JPSC at the magnification of (a) 1000×, (b and c) 3000× and (d): 10000×.

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.

Acknowledgments

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BOOK OF PROCEEDINGS

P69 CUMIN SEED (CUMINUM CYMINUM), AN ALTERNATIVE SOURCE OF ANTIOXIDATIVE AND ANTIDIABETIC BIOACTIVE PEPTIDES

Siow H. L. (1), Gan C. Y. (1)

(1) Centre for Advanced Analytical Toxicology Services, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

E-mail: candyshl89@gmail.com

Keywords: Antidiabetic, Antioxidant, Bioactive peptide, Cumin seed

Physiologically active peptides derived from food sources have gained mounting interest due to their therapeutic potential. Such peptides are inactive within the sequence, and however, they could trigger physiological action once released from their intact proteins. Bioactive peptides, which exhibited antioxidative and α-amylase inhibition properties, were successfully extracted from cumin seeds using commercial enzyme. Based on a single factor experiment, hydrolysis parameters including incubation temperature, time and substrate-to-enzyme (S/E) ratio had a significant impact on the bioactivities of peptides. Incubation temperature of 50°C, incubation time of 2 h and the use of S/E ratio of 20 (*w/w*) were demonstrated as the desired parameters in producing peptides with remarkable bioactivities in terms of DPPH scavenging abilities, ferric ion reducing power and α-amylase inhibitory activities. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles were performed to investigate the extent of protein hydrolysis under different conditions. Fractionation based on molecular mass was performed using ultrafiltration and fraction with MW < 3 kDa, exerted the strongest radical scavenging activity (32.26%$DPPH_{sc}$/μg), reducing power (11.56 mM/μg) and α-amylase inhibitory activity (15.14%/μg) than other fractions. A total of 56 peptide sequences composed of 9-23 amino acid residues per molecule, with a molecular weight of 1.0-2.5 kDa, were identified as the most putative antioxidant and antidiabetic peptides using mass spectrometry. The primary structure, amino acid composition and physiochemical properties are important factors in contributing to the bioactivities of cumin seed-derived peptides. In conclusion, the extracted bioactive peptides from cumin seeds showed high commercial nutraceutical and pharmaceutical values, which could be incorporated as active ingredients in functional foods or as food supplements.

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P70 A WORKFLOW ON THE PRODUCTION OF BIOACTIVE PEPTIDES FROM FOOD SOURCES BASED ON THE CASE STUDY USING PINTO BEANS (PHASEOLUS VULGARIS CV. PINTO)

Ngoh Y. Y. (1), Gan C. Y. (1)

(1) Centre for Advanced and Analytical Toxicology Services (CAATS), Universiti Sains Malaysia

E-mail: ngohyy@hotmail.com

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A workflow on the production of bioactive peptides from food sources was discussed in this study with a real case study experiment using Pinto bean (Phaseolus vulgaris cv. Pinto). This workflow proved to be functional and efficient as bioactive peptides derived from Pinto bean protein isolate (PBPI) using Protamex exhibited promising antioxidative and α-amylase inhibition activities. Implementation of this workflow is convenient and consuming less time compared to the previous bioactive peptides studies with high possibility of positive outcome being achieved. Generally, this workflow begins with extraction of peptides from protein isolate by carrying out single factors experiments to identify the trend of the extraction parameters. In the pinto bean case study, four extraction parameters were investigated: contact time (0.5–5 h), S/E ratio (10–100), pH (6.5–9.0) and temperature (30–60 °C). The influential parameters were then studied using a factorial design experiment to determine the most suitable condition in producing peptides with the highest bioactivities. Fractionation using a centrifugal membrane filter with molecular weight cutoffs of 3 kDa was then performed followed by subjecting into LCMS and MS/MS analysis using Thermo LTQ/ Orbitrap Velos for the identification of peptide sequences. Peptide Ranker software was applied for the screening of potential bioactive peptides. Both antioxidant and α-amylase inhibition activities revealed two peptide sequences each. The sequences found were (PLPPHMLP, ACSNHSPLGWRGH) and (PLPPHDLL, FNPFPSPHTP) for antioxidant and α-amylase inhibition activity respectively. The proposed workflow resulting in successful functionality is recommended and applicable in the field of production of bioactive peptides with the hope of exploring and discovering other potential bioactive peptides possessing activities such as antimicrobial, anticancer, antithrombotic, hypocholesterolemic and antihypertensive.

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