Antioxidant Activity of Semen Cassiae Protein Hydrolysate: Thermal and Gastrointestinal Stability, Peptide Identification, and *In Silico* Analysis

CHAI Tsun-thai[1,2], SOO Zhi-yin[1], HSU Kuo-Chiang[3], LI Jen-Chieh[3], ABD MANAN Fazilah[4], WONG Fai-chu[1,2]

(1.Department of Chemical Science, Faculty of Science, Universiti Tunku Abdul Rahman, 31900 Kampar, Malaysia)

(2.Centre for Biodiversity Research, Universiti Tunku Abdul Rahman, 31900 Kampar, Malaysia)

(3.Department of Nutrition, China Medical University. No. 91 Hsueh-Shih Road, Taichung 40402, Taiwan, ROC)

(4.Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Malaysia)

Abstract: *Semen cassiae* (SC), seeds of *Cassia obtusifolia*, is used as treatments for various diseases in Traditional Chinese Medicine. This study evaluated the antioxidant activity and stability of SC hydrolysate fractions and identified peptides in the most active fraction. SC proteins were hydrolyzed by alcalase for 2-6 hours; < 3000 u peptide fractions were produced *via* membrane ultrafiltration. UF 2 h (fraction produced from 2-hour hydrolysate) exhibited the strongest $ABTS^{\bullet+}$ scavenging (EC_{50} = 229 μg/mL) and iron chelating (EC_{50} = 89 μg/mL) activities. Its $ABTS^{\cdot+}$ scavenging activity was retained after thermal treatment and simulated gastrointestinal (GI) digestion, but iron chelating activity was fully retained only after GI digestion. Peptides were purified from UF 2 h by using solid phase extraction, gel filtration chromatography and high performance liquid chromatography analysis, guided by $ABTS^{\cdot+}$ scavenging assay. Four peptides were identified by mass spectrometric analysis: PMPVR (599.29 u), FETLPF (752.34 u), KMRDNL (775.37 u), and LDESKRF (893.50 u). *In silico* analysis predicted that before simulated GI digestion, all four peptides were non-toxic and non-allergenic. After GI digestion, peptide fragments released from the four peptides were predicted to be still non-toxic, whereas fragments of only PMPVR and KMRDNL were predicted to be non-allergenic. Taken together, UF 2 h and the peptides contained in it are promising antioxidants for the development of functional food and nutraceuticals.

Key words: allergenicity; antioxidant peptide; *Cassia obtusifolia*; gastrointestinal stability; thermal stability

Article No.: 1673-9078(2019)09-38-48 DOI: 10.13982/j.mfst.1673-9078.2019.9.004

决明子蛋白水解产物的抗氧化活性：热和胃肠稳定性，肽鉴定和计算机分析

蔡俊泰[1,2]，徐芷茵[1]，徐国强[3]，李仁杰[3]，阿都马南·法希拉[4]，黄怀祖[1,2]

（1.拉曼大学理学院，马来西亚金宝 31900）（2.拉曼大学生物多样性研究中心，马来西亚金宝 31900）

（3.中国医科大学营养系，台湾台中 40402）（4.马来西亚理工大学生物科学系，马来西亚新山 81310）

摘要：决明子乃决明（*Cassia obtusifolia* L.）的干燥成熟种子，被中医界用于治疗各种疾病。本研究探讨决明子水解产物的抗氧化活性和稳定性，并鉴定其中的短肽。决明子蛋白经过碱性蛋白酶水解 2~6 h，以超滤膜技术产生<3000 u 肽组分。UF 2 h（2 h 水解产物分离出来的<3000 u 组分）表现出最强的 $ABTS^{\cdot+}$清除能力（EC50=229 μg/ mL）和铁螯合能力（EC50=89 μg/mL）。经过热处理和模拟胃肠消化后，UF 2 h 的 $ABTS^{\cdot+}$清除能力都得以保留。UF 2 h 仅在模拟胃肠消化胃后保留铁螯合活性。试验通过使用固相萃取，凝胶色谱和高效液相色谱对 UF 2 h 进行分离纯化。通过质谱分析，鉴定了四个肽：PMPVR（599.29 u），FETLPF（752.34 u），KMRDNL（775.37 u）和 LDESKRF（893.50 u）。计算机分析预测，在模拟胃肠消化前，四个肽皆无毒无过敏性。胃肠消化后，四个肽释放的片段仍无毒，仅 PMPVR 和 KMRDNL 释放的肽片段无过敏性的。UF 2 h 及其活性肽可作为抗氧化剂，用于开发功能性食品和营养保健品。

关键词：致敏性；抗氧化肽；决明；胃肠稳定性；热稳定性

Semen cassiae (SC) is known as 'Jue Ming Zi' in Chinese. It is the dry and mature seed of the pantropical species *Cassia obtusifolia* L., cultivated in China, India, Japan and Korea. SC is commonly consumed as a health-promoting tea[1,2]. SC is used in Traditional

Received date: 2019-05-16

Corresponding author: CHAI Tsun-Thai (1973-), Male, Associate professor, Research area: Bioactive peptides

Chinese medicine as remedy for hyperlipidemia, diabetes, Alzheimer's disease, liver injury, inflammation, headache, and high blood pressure. Anthraquinones and naphthopyrones are the key components responsible for the diverse pharmacological effects of SC, which include antioxidant, anti-cancer, neuroprotective, cholesterol-lowering activities[2]. Water-soluble polysaccharides of SC exhibited antioxidant, anti-amylase and anti-lipase activities as well as the ability to bind bile acids[3,4]. In comparison with anthraquinones, naphthopyrones and polysaccharides, very little information is known about the bioactivity of proteins and peptides of SC, despite its notable protein contents, i.e., 18.56~22.93 % by weight[5].

SC crude proteins were reported to exhibit cholesterol-lowering effects in rats; a 19700 u cholesterol-lowering protein has also been purified from SC and structurally characterized[6]. Considering such findings, we hypothesized that SC may be a promising source of bioactive proteins and peptides. This study was undertaken to investigate the potential of SC peptides as natural antioxidants. Owing to the inevitable production of reactive oxygen species in the body, their implication in human diseases, and potential health risks of synthetic antioxidants, there has been constant interest among researchers to search for safe, natural antioxidants, particularly from edible sources[7]. Investigations on SC-derived antioxidant peptides may extend current knowledge on the bioactivity or pharmacology of SC. The SC antioxidant peptides may also have potential applications as functional food or beverage ingredients and nutraceuticals.

Proteases of plant, animal and microbial origins are typically used to release antioxidant peptides from food proteins. Enzyme hydrolysis allows bioactive peptides to be generated from the parental proteins in a fast, safe and easily-controlled manner[7,8]. In this study, alcalase was used to produce hydrolysates of SC proteins. Alcalase is commonly used for generating antioxidant protein hydrolysates and peptides from food materials of plant and animal origins[7,9~12]. Furthermore, the use of microbial proteases, e.g., alcalase, for protein hydrolysate production is relatively cost-effective[9]. When considering the applications of SC protein hydrolysates as functional food or beverage ingredients, characterization of their stability towards potential food processing conditions is pertinent. Many food processing technologies involve thermal treatments, thus we investigated whether SC hydrolysate could retain its activity after heating. On the other hand, functional peptides may not express their activity *in vivo* after oral administration[13]. The resistance of bioactive protein hydrolysates and peptides to gastrointestinal (GI) digestion is key to their *in vivo* bioavailability and bioactivity. Thus, to further characterize the potential of the SC protein hydrolysate as functional food or beverage ingredients or as nutraceuticals, its resistance against simulated GI digestion was assessed. In short, the objectives of this study were four-fold: first, to hydrolyze SC protein isolates (SCPI) by using alcalase and assess the antioxidant activity of the peptide fractions; second, to characterize the stability of the most active peptide fraction under thermal treatments and simulated GI digestion; third, to purify and identify peptides from the most active peptide fraction; and fourth, to predict physicochemical properties, toxicity and allergenicity of the peptide sequences by using *in silico* analysis.

1 Experimental

1.1 Materials and reagents

2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and alcalase were purchased from Calbiochem. Pepsin was purchased from Acros Organics. Ferrozine, glutathione (GSH) and pancreatin were purchased from Sigma-Aldrich. o-Phthalaldehyde (OPA) was purchased from Nacalai Tesque. Macrosep® Advance ultrafiltration centrifugal units (Molecular Weight Cut-off (MWCO) 3000 u) were purchased from Pall Corporation. Solid phase extraction (SPE) cartridges (STRATA C18-E, sorbent mass: 500 mg, volume: 6 mL) were purchased from Phenomenex Inc. All reagents and solvents were of analytical grade or, where appropriate, HPLC grade.

1.2 Protein isolation

SC was purchased from a local commercial supplier of traditional Chinese medicinal herbs. The seeds were pulverized. SCPI was prepared from the seed powder by using the ammonium sulfate precipitation method. The seed powder was added to chilled deionized water at a

mass (g): volume (mL) ratio of 1:8. Proteins were precipitated and dialyzed as previously described[14] to yield the SCPI, which was lyophilized and stored at -20 ℃ until used.

1.3 Protein hydrolysis and preparation of UF fractions

SCPI was hydrolyzed with alcalase for 0, 2, 3, 4, 5 and 6 h at 60 ℃ as previously described[15]. SCPI was added to 50 mM sodium phosphate buffer (pH 8) at a ratio of 1:100 (*W/V*), whereas alcalase was added at an enzyme/substrate ratio of 1:10 (*W/W*). Hydrolysis was terminated by heating a hydrolysate aliquot in boiling water for 10 min. The heated hydrolysate was cooled on ice and then centrifuged at 9000 r/min and 4 ℃ for 10 min to obtain the supernatant. The supernatant collected at each time point was transferred to a pre-wetted ultrafiltration centrifugal unit (MWCO 3000 u). Fractionation was carried out by centrifugation at 6500 r/min and 4 ℃ for 30 min. The<3000 u UF fractions (permeate fractions) collected were designated as UF 0 h, UF 2 h, UF 3 h, UF 4 h, UF 5 h and UF 6 h, respectively. The UF fractions were lyophilized and subsequently tested for peptide content and antioxidant activity as described below.

1.4 Determination of peptide content and antioxidant activity

Peptide content was determined by using the OPA assay[16]. The reaction was carried out by mixing 20 μL of test sample with 200 μL of OPA reagent, followed by 2-min incubation in darkness. Absorbance was measured at 340 nm. Casein peptone was used to generate a standard curve for the assay.

$ABTS^{\cdot+}$ scavenging activity was determined as previously described[17], with modifications. Briefly, the reaction mixture consisted of 30 μL of test sample and 210 μL of $ABTS^{\cdot+}$ working solution. After incubation in darkness for 20 min, the absorbance of the mixture was measured at 734 nm. GSH was used as the positive control. EC_{50} (half-maximal effective concentration), defined as the sample concentration required to achieve 50 % of $ABTS^{\cdot+}$scavenging activity, was determined by using linear regression analysis.

Iron chelating activity of the sample was evaluated based on its ability to intercept the formation of ferrozine-Fe^{2+} complex, following a previously reported protocol[18], with modifications. The reaction mixture consisted of 50 μL of 0.10 mM $FeSO_4 \cdot 7H_2O$, 50 μL of sample, and 100 μL of 0.25 mM ferrozine. After incubation for 10 min, absorbance of the mixture was measured at 526 nm. Disodium salt of EDTA was used as positive control in this assay. EC_{50}, defined as the sample concentration required to achieve 50% of iron chelating activity, was determined by using linear regression analysis.

1.5 Effects of thermal treatments and simulated GI digestion on UF 2 h

The stability of UF 2 h against thermal treatments was determined as previously reported[19], with minor modifications. The samples were dissolved in deionized water and incubated in a temperature-controlled water bath at 25 ℃, 65 ℃, 75 ℃, 85 ℃ and 100 ℃ for 30 min. The stability of UF 2 h against simulated GI digestion was assessed by using a two-stage digestion model (pepsin treatment for 1 h, followed by pancreatin treatment for 2 h)[20]. Antioxidant activity of the treated samples were measured as described above.

1.6 Peptide purification from UF 2 h

UF 2 h was fractionated by using SPE cartridges pre-conditioned with 12 mL of pure methanol and equilibrated with 12 mL of 0.1% trifluoroacetic acid (TFA). Fifty mg of UF 2 h dissolved in 6 mL of 0.1% TFA was loaded. Unbound fraction and fractions eluted with 0%, 20%, 50% and 100% acetonitrile (ACN) were collected and designated as 0 ACN, 20 ACN, 50 ACN and 100 ACN. Solvent in the SPE fractions was removed by rotary evaporation. The SPE fractions were lyophilized before the measurements of peptide contents and $ABTS^{\cdot+}$ scavenging activity as described above.

The 20ACN fraction was purified by using Sephadex G-25 GFC. 2 mL of 20 ACN (10 mg/mL) was pre-filtered with 0.45 μm filter membrane before being loaded onto the GFC column (2×40 cm). Elution was performed by using deionized water at the flow rate of 1.2 mL/min. Eluate was collected at 2-min intervals and

absorbance of each fraction was monitored at 214 and 280 nm with a UV-Vis spectrophotometer. Three pooled fractions, GFP1, GFP2, GFP3, were collected, lyophilized, and tested for peptide contents and $ABTS^{\cdot+}$ scavenging activity.

GFP3 collected from the previous step was dissolved in deionized water (5 mg/mL) and filtered with 0.45 μm filter membrane before it was analyzed with RP-HPLC. 20 μL of sample was injected and separated by RP-HPLC (Shimadzu LC-20D dual binary pumps and Shimadzu Prominence SPD-M20A PDA detector) using a Phenomenex Gemini 5 μm C18 column (150 mm×4.6 mm). The column was eluted with a gradient where solvent A was water containing 0.1% TFA and solvent B was 100% ACN containing 0.1% TFA. Elution was performed with a gradient of solvent B: 0~25 min, 5%~45% B; 26~28 min, 45% B; and 29~30 min, 5%~45% B. The flow rate was set to 1 mL/min. Elution profile was monitored at 214 nm. Data and chromatograms were analyzed by using the LabSolution software (Version 1.25, Shimadzu Corporation, Japan).

1.7 Determination of amino acid sequence and molecular mass

The identification of the peptide sequences in GFP3 and determination of their experimental masses was carried out by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS). Peptides were identified by using a quadrupole-time-of-flight (Q-TOF) mass spectrometer equipped with a nano-electrospray ion source (maXis impact, Bruker Daltonics, Germany). The sample was prepared for analysis in 0.1% formic acid (FA) to obtain a final concentration of 100 ppm. The 0.5% ACN was blended with 0.1% FA for the preparation of solvent A. 100% ACN was blended with 0.1% FA for the preparation of solvent B. After LC-MS/MS analysis, the mass spectral data were searched against the Swiss-Prot database to identify peptides in GFP3. The threshold peptide was set to 25.

1.8 *In silico* analysis of physicochemical characteristics of SC peptides

The theoretical molecular weight, net charge at neutral pH, and isoelectric point (pI) of the four peptides identified from GFP3 were predicted by using the PepDraw tool (http://www.tulane.edu/biochem/ww/pepdraw/index.html). Estimated water solubility of the four peptides was predicted by using an online peptide property calculator (https://pepcalc.com/).

1.9 *In silico* GI digestion and prediction of toxicity and allergenicity

Peptide fragments potentially released from the four SC peptides following *in silico* GI digestion was analyzed as previously described[21]. BIOPEP was used to simulate GI digestion by using pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1). Potential toxicity of the four SC peptides before and after *in silico* GI digestion was predicted by using the ToxinPred web server (http://crdd.osdd.net/raghava/toxinpred/index.html). A support vector machine (SVM)-based method was used for the prediction task, with the threshold value set to 0.0[22]. Potential allergenicity of the four peptides before and after *in silico* GI digestion was analyzed by using AllerTOP v. 2.0 (http://www.ddg-pharmfac.net/AllerTOP/), a bioinformatics tool for allergenicity prediction.

1.10 Data analysis

Analyses were conducted in triplicates and data are presented as mean ± standard errors (SE). Statistical analysis was performed using SAS (version 9.4). Data were analyzed by one-way ANOVA test and means of significant differences were separated by using Fisher's Least Significant Difference (LSD) test. Comparison of two means was performed by using Student's T-test. P-value of less than 0.05 level was considered to be statistically significant.

2 RESULTS AND DISCUSSION

2.1 Peptide contents and antioxidant activity of UF fractions

The yield percentages of UF fractions, on a dry mass basis, were 11.99% (UF 0 h), 12.30% (UF 2 h), 10.75% (UF 3 h), 10.90% (UF 4 h), 10.94% (UF 5 h), and

10.76% (UF 6 h), respectively. Peptide contents of the UF fractions of hydrolysates (UF 2 h-UF 6 h) were about 58 to 102% higher than that of UF0h, the UF fraction of unhydrolyzed SCPI (Table 1). In comparison with UF0h, all other UF fractions had lower EC_{50} values for $ABTS^{\bullet+}$ scavenging and iron chelating activities. For both of the activities, EC_{50} values of all UF fractions were greater than those of their respective positive controls, namely GSH and EDTA. $ABTS^{\cdot+}$ scavenging and iron chelating assays are widely used to evaluate the antioxidant potential of protein hydrolysates and bioactive peptides. The $ABTS^{\cdot+}$ scavenging assay evaluates the capability of the sample to remove free radicals, whereas the iron chelating assay assesses the capability of the sample to bind iron, thus reducing iron-catalyzed radical formation[7,8,23].

Table 1 Peptide contents and antioxidant activities of UF fractions

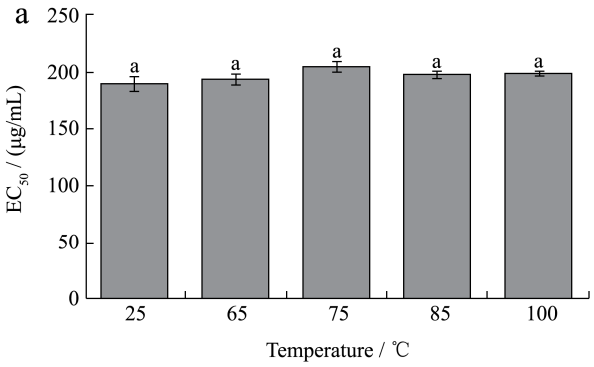
Samples	Peptide content/ (μg/mg)	EC_{50}/(μg/mL)	
		$ABTS^{\cdot+}$ scavenging activity	Iron chelating activity
UF 0 h	132±10[a]	360.71±7.25[a]	172.28±6.27[a]
UF 2 h	209±3[b]	229.71±3.98[b]	88.60±2.96[b]
UF 3 h	228±20[b]	235.97±5.73[b,c]	88.08±4.24[b]
UF 4 h	229±9[b]	236.09±1.63[b,c]	86.98±2.72[b]
UF 5 h	267±4[c]	247.59±5.74[c]	104.67±0.48[c]
UF 6 h	237±15[b,c]	235.23±6.60[b,c]	122.25±9.22[d]
GSH		2.83±0.02[d]	
EDTA			2.48±0.02[e]

Note: Data are mean ± standard errors. Within the same column, data labeled by the same superscript letters are not significantly different (p>0.05), according to Fisher's LSD test.

Alcalase hydrolysis enhanced the antioxidant activities of SCPI, corresponding with increased peptide contents in the resulting<3000 u hydrolysate fractions. Similarly, based on reducing power and radical scavenging assays, alcalase hydrolysates of Chinese chestnut proteins exhibited stronger antioxidant activity than the unhydrolyzed proteins[12]. In our study, we focused on the <3000 u fractions of the protein hydrolysates because food-derived antioxidant peptides are largely less than 3000 u in size[7,9,23,24]. Moreover, the<3000 u fraction represented a peptide-enriched fraction of the hydrolysate; unhydrolyzed proteins, if present, would be more likely retained in the>3000 u fraction. An investigation on cherry seed protein hydrolysates generated by using alcalase and neutrase also showed that among the UF fractions produced, the fraction with the lowest mass range (<3000 u) had the strongest radical scavenging activity[11]. Our results and others' therefore suggest that UF membrane of 3000 u MWCO can be used to effectively generate antioxidant peptide-enriched fractions from plant protein hydrolysates.

2.2 Effects of thermal treatments and simulated GI digestion on UF 2 h

Among the samples analyzed, UF 2 h exhibited the lowest EC_{50} for $ABTS^{\cdot+}$ scavenging activity. Although the EC_{50} of UF 2 h for iron chelating activity was not the lowest, the value did not differ significantly from the lowest EC_{50} of UF4h (86.98 μg/mL) (Table 1). Thus, UF 2 h was chosen for further characterization of stability under thermal treatments and simulated GI digestion. There were no significant differences among the EC_{50} values for $ABTS^{\cdot+}$ scavenging activity of UF 2 h treated at 25, 65, 75, 85 and 100 ℃ (Fig.1a). By contrast, thermal treatments increased the EC_{50} value for iron chelating activity by 38%, from 82 μg/mL (25 ℃ treatment) to 113 μg/mL (100 ℃ treatment) (Fig.1b).



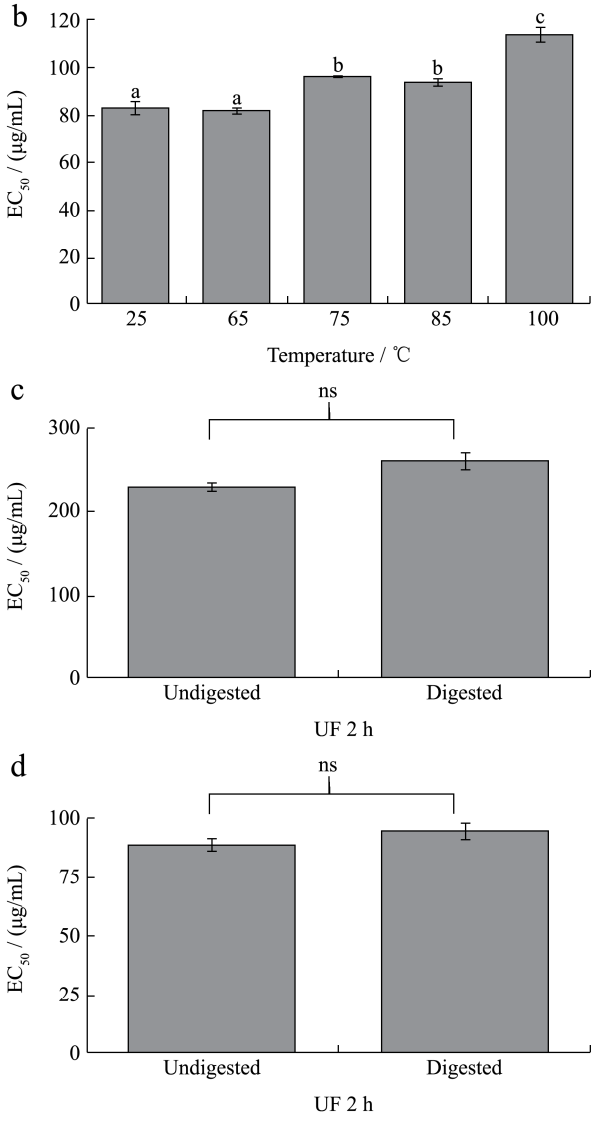


Fig.1 ABTS·+ scavenging activity (a, c) and iron chelating activity (b, d) of UF 2 h after thermal treatments (a, b) and simulated GI digestion (c, d)

Note: Data are mean ± standard errors (n=3). For 'a' and 'b', mean values denoted by the same letters are not significantly different (p>0.05), according to Fisher's LSD test. For 'c' and 'd', "ns" denotes a lack of significant difference (p>0.05) between the two mean values, according to Student's T-test.

ABTS·+ scavenging activity of UF 2 h remained constant after thermal treatments. By contrast, the iron chelating activity of UF 2 h was moderately compromised after thermal treatments at 75 ℃ and higher temperatures. Thermal treatments can cause denaturation and/or aggregation of proteins; it may also induce degradation or aggregation of antioxidant peptides, leading to reduced activity[19,25]. In addition, instability of antioxidant peptides under high temperatures may also be due to alteration of peptide secondary structure[26]. Considering that UF 2 h was likely more enriched in peptides than in proteins, our finding implies that the compromised iron chelating activity of UF 2 h was possibly due to heat-induced peptide degradation/ aggregation or instability of the secondary structure of antioxidant peptides. It is also possible that peptides differing in thermal stability may have accounted for the ABTS·+ scavenging and iron chelating activities of UF 2 h, respectively. Specifically, ABTS·+ scavenging peptides in UF 2 h were possibly mostly heat-stable, whereas some iron chelating peptides in UF 2 h may be non-heat-stable. Considering that UF 2 h retained all of its ABTS·+ scavenging activity and at least 60% of its iron chelating activity after heating, UF 2 h could be used as a source of natural antioxidants in thermally-processed foods.

Following simulated GI digestion, the EC_{50} values for the ABTS·+ scavenging and iron chelating activities of UF 2 h increased marginally by 12% and 6%, respectively. Such differences, nevertheless, were not statistically significant (Fisher's LSD test, p>0.05) (Fig. 1c and 1d). As UF 2 h was a peptide mixture, its unaltered antioxidant activity after simulated GI digestion may be attributed to the GI-resistance of some of the existing antioxidant peptides. Proline-containing antioxidant peptides, for example, are generally resistant to degradation by GI digestive enzymes[27,28]. Moreover, the stability of UF 2 h may also be related to the presence of short antioxidant peptides. According to Chen and Li [29], among casein-derived antioxidant peptides, shorter peptides (<3000 u) exhibited better GI survivability than longer peptides (>3000 u). Besides, generation of new peptide fragments or amino acids with antioxidant activity after GI digestion may also have allowed UF 2 h to maintain its antioxidant activity. *In vitro* simulated GI digestion could serve as an initial screening tool for predicting the potential availability and bioactivity of peptides after *in vivo* GI digestion[30]. In this study, antioxidant activities of UF 2 h were generally stable after simulated GI digestion. Hence, UF 2 h is a promising candidate for functional food ingredient or nutraceutical development.

2.3 Purification of antioxidant peptides

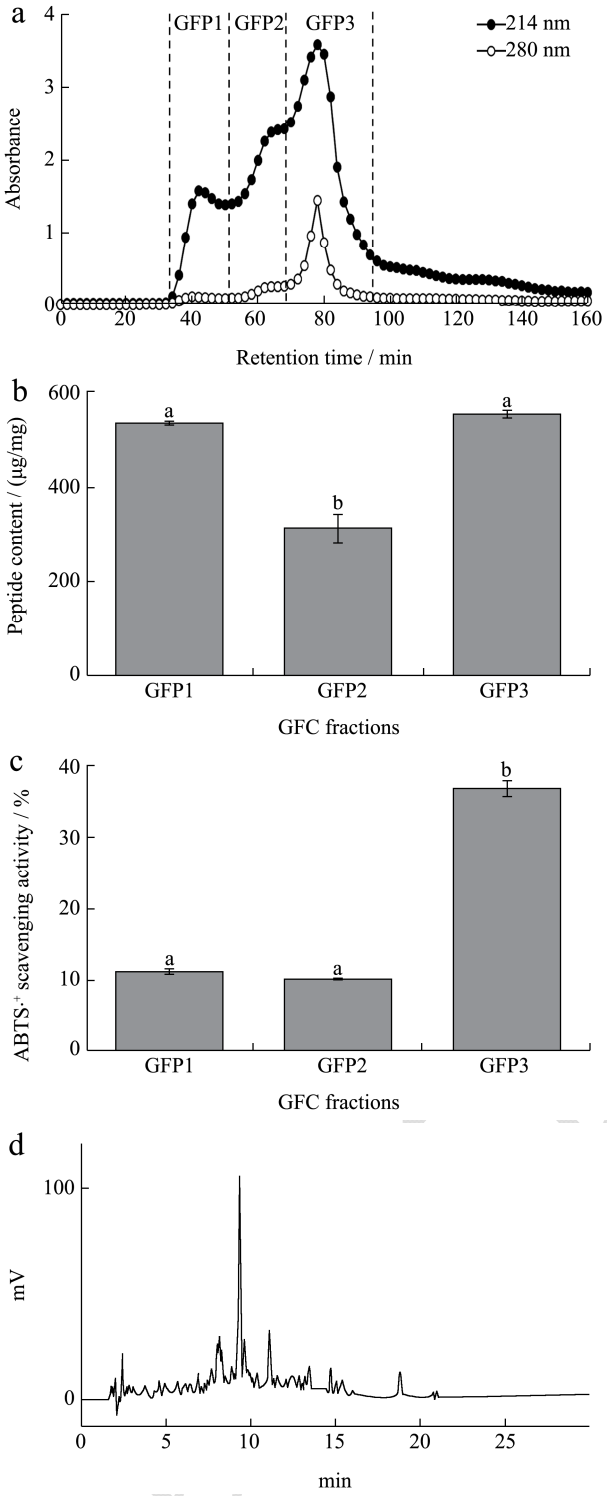


Fig.2 (a) A representative GFC elution profile of 20ACN, monitored at 214 nm and 280 nm; (b) Eluted fractions were pooled to form the GFP1, GFP2 and GFP3 fractions; (c) Peptide contents of GFC fractions; (d) ABTS·+ scavenging activity of GFC fractions tested at 12.5 μg/mL

Note: For (b) and (c), data are mean ± standard errors (n=3). Data denoted by the same letters are not significantly different (p>0.05), according to Fisher's LSD test. (d) RP-HPLC chromatogram of GFP3 monitored at 214 nm.

As UF 2 h stably maintained its ABTS·+ scavenging activity after thermal treatments and simulated GI digestion, we hypothesized that UF 2 h may contain potent antioxidant peptides. Thus, we proceeded to purifying antioxidant peptides from UF 2 h, guided by the ABTS·+ scavenging and iron chelating assays. UF 2 h was first fractionated by using SPE, producing five fractions. Among these, the yields of UB, 0ACN and 20ACN were 65.2%, 24.8% and 5.6%, respectively. The iron chelating activity of UF 2 h declined drastically after SPE fractionation (data not shown). Thus we focused on the ABTS·+ scavenging activity for the rest of the peptide purification process. The 50ACN and 100ACN fractions, owing to their low yields (below 0.2%), were not analyzed. Among the three SPE fractions, 20ACN exhibited the highest ABTS·+ scavenging activity and peptide content (Table 2). Further analysis on 20ACN revealed its EC_{50} for ABTS·+ scavenging activity to be 23.00±0.37 μg/mL, about 10-fold lower than the EC_{50} of UF 2 h.

Table 2 Peptide contents and ABTS·+ scavenging activity of SPE fractions

SPE fractions	Peptide content /(μg/mg)	ABTS·+ scavenging activity /%[a]
UB	110 ± 8[a]	3.32 ± 2.20[a]
0ACN	110 ± 9[a]	9.14 ± 1.81[b]
20ACN	540 ± 16[b]	99.86 ± 0.22[c]

Note: [a] Activity tested at 125 μg/mL. Data are mean ± standard errors. Within the same column, data labeled by the same superscript letters are not significantly different (p>0.05), according to Fisher's LSD test.

The considerably higher ABTS·+ scavenging activity and peptide content of 20ACN than UF 2 h suggest enrichment of antioxidant peptides in our samples. The effectiveness of reversed-phase SPE in peptide enrichment and purification has been reported by others[31,32]. Reversed-phase SPE, pre-packed with C18 sorbents, is an effective tool for fractionating very polar and hydrophobic peptides based on non-polar interaction between the peptides and the sorbents[33]. In this study, SPE procedure resulted in a flow-through or unbound fraction (relatively hydrophilic or polar), 0ACN fraction (relatively hydrophilic bound fraction) and 20ACN fraction (moderately hydrophobic bound fraction) (Table 2). ABTS·+ scavenging activity was higher in the more

hydrophobic fraction (20ACN) than in the more hydrophilic fractions (UB and 0ACN). When a peptide sample is fractionated by reversed-phase SPE, the resulting hydrophobic fraction will contain a greater abundance of hydrophobic amino acids when compared with the hydrophilic fraction[34]. Thus, our results imply the antioxidant activity of UF 2 h and 20ACN may be largely attributed to hydrophobic amino acids and/or peptides containing such amino acids.

Fig.2a shows the elution profile of 20ACN when resolved by GFC. GFC is widely used to separate and purify peptides from food-derived protein hydrolysates based on molecular size[11,12,28]. In our study, the molecular sizes of the three GFC fractions, in descending order, are GFP1>GFP2>GFP3. The low-molecular-weight fraction GFP3 had 3.3-fold stronger $ABTS^{\cdot+}$ scavenging activity than the high-molecular-weight fraction GFP1 (Fig.2c). In general, our finding agrees with the notion that peptides having smaller molecular masses generally exhibit higher antioxidant activity[23]. The EC_{50} of GFP3 for $ABTS^{\cdot+}$ scavenging activity was subsequently determined to be 19.04±0.44 μg/mL, which was 17% lower than EC_{50} of 20ACN. The greater $ABTS^{\cdot+}$ scavenging activity of GPF3 in comparison with that of 20ACN also reflects further enrichment of antioxidant peptides in GFP3 after the GFC experiment. On the other hand, although GFP1 and GFP3 had similar levels of peptide contents (Fig.2b), their peptide constituents were very likely different. The elution profile in Fig.2a shows a distinct peak detected at 280 nm. This implies the presence of aromatic amino acids, i.e., tryptophan and tyrosine, either as free amino acids or as constituents of peptides[35] in GFP3. Thus, the elution profile implies a greater abundance of aromatic amino acids in GFP3 than in GFP1. When analyzed by using RP-HPLC, a prominent peak was detected in the chromatogram of GFP3 at retention time 9.34 min (Fig.2d). The peak accounted for 37.5% of the total peak area in the chromatogram. GFP3 was subsequently subjected to LC-MS/MS analysis for peptide identification.

2.4 Identification of antioxidant peptides and *in silico* analysis

Based on LC-MS/MS analysis and database searching, four peptide sequences of 5~7 residues were identified from GFP3: Pro-Met-Pro-Val-Arg (PMPVR), Phe-Glu-Thr-Leu-Pro-Phe (FETLPF), Lys-Met-Arg-Asp-Asn-Leu (KMRDNL), and Leu-Asp-Glu-Ser-Lys-Arg-Phe (LDESKRF). Experimental molecular masses of the peptides agree well with their theoretical molecular masses (Table 3). PepDraw predicted the four peptides to be different in net charges (between -1 and +1) and isoelectric points (between 3.14 and 10.73). With the exception of FETLPF, the peptides were predicted to have good water solubility (Table 3). Based on our manual calculations, with reference to the classification of A, F, I, L, M, P, V and W residues as hydrophobic amino acids (IARCTP53 database, http://p53.iarc ft/AAProperties.aspx), the percentages of hydrophobic residues in the four peptides were 80% (PMPVR), 66.7% (FETLPF), 33.3% (KMRDNL) and 28.6% (LDESKRF). To the best of our knowledge, the four peptide sequences have not been previously reported for any bioactivities. A search of the BIOPEP database[36] (accessed on 11 October 2018) also revealed no records of these peptides.

The four SC peptides identified in this study exhibit characteristics commonly found in food-derived antioxidant peptides. The four peptides range between 599 and 894 u in size. This is in accordance with the molecular mass range of antioxidant peptides identified from other plant sources, i.e. 500-1000 u[11,12,37]. On the other hand, all four peptides identified in this study contain hydrophobic residues. Likewise, antioxidant peptides identified from corn gluten meal[38], flaxseed[39], hazelnut[40] and Chinese chestnut[12] consist of 25%~100% hydrophobic residues. In addition to the aforementioned characteristics, two of the four peptides identified in this study contain the aromatic amino acid, Phe, in their sequences. The presence of aromatic amino acids can enhance the potency of antioxidant peptides[23]. Aromatic residues, such as Phe, can exert antioxidant activity by donating protons to electron-deficient free radicals[23,41]. Taken together, the low molecular masses, as well as the presence of hydrophobic and aromatic amino acids may have contributed to the antioxidant activity of the four SC peptides identified in this study.

Low-molecular-weight peptides and hydrolyzed proteins are generally non-toxic and less allergenic than

unhydrolyzed native proteins[42,43]. Nevertheless, toxicity and allergenicity, if occurring, may preclude the potential application of bioactive protein hydrolysates and peptides as functional food ingredients or nutraceuticals. Thus, although the four SC peptides identified in this study have low molecular weights and are derived from an edible source, it is still desirable to evaluate their potential toxicity and allergenicity. Assessment of *in vivo* toxicity and allergenicity, although necessary, is costly and time-consuming. Furthermore, for the initial screening of potential allergens in food proteins, the European Food Safety Authority recommended the use of *in silico* analysis[44]. Thus we performed *in silico* analysis to predict the toxicity and allergenicity of the four SC peptides identified in this study. According to the Toxin Pred and Aller Top v. 2.0. servers, the four peptides were non-toxic and likely non-allergenic (Table 3). We found that at least 50 % of the residues in each of the four SC peptides comprise one or more of the following amino acids: Val, Thr, Arg, Met, Leu, Lys, and Phe. Notably, such amino acids are also dominant residues in other non-toxic peptides[22]. Following *in silico* GI digestion, all resulting fragments of the four peptides were still predicted to be non-toxic. However, two of the fragments released, PF and DESK, were predicted to be probable allergens (Table 4). This implies that UF 2 h, from which FETLPF and LDESKRF were derived, may pose a potential allergen risk after consumption and GI digestion. In view of our *in silico* finding, future research is warranted to ascertain the actual toxicity and allergenicity of UF 2 h and the four SC peptides by using *in vivo* models. *In vivo* evaluation will provide more convincing evidence with regards to the safety of UF 2 h as a candidate for use as functional food ingredient or nutraceutical.

Table 3 Physicochemical properties, toxicity and allergenicity of the SC peptides

Peptides	Experimental mass /(g/mol)[a]	Theoretical mass /(g/mol)[b]	Net charge[b]	pI[b]	Estimated Water solubility[c]	Toxicity[d]	Allergenicity[e]
PMPVR	599.2957	598.3251	+1	10.73	Good	Non-toxin	Probable non-allergen
FETLPF	752.3350	752.3732	-1	3.14	Poor	Non-toxin	Probable non-allergen
KMRDNL	775.3652	775.3998	+1	10.14	Good	Non-toxin	Probable non-allergen
LDESKRF	893.5039	893.4592	0	6.76	Good	Non-toxin	Probable non-allergen

Note: [a]Determined by using MS analysis. [b]Predicted by using the PepDraw tool. [c]Predicted by using online peptide property calculator (https://pepcalc.com/). [d]Predicted by using ToxinPred. [e]Predicted by using AllerTOP v. 2.0.

Table 4 Biopeptide fragments released from SC peptides after in silico GI digestion and their potential toxicity and allergenicity

Peptides	Fragments released[a]	Toxicity[b]	Allergenicity[c]
PMPVR	PM, PVR	Non-toxin	Probable non-allergen (all fragments)
FETLPF	F, ETL, PF	Non-toxin	Probable allergen (PF) Probable non-allergen (F, ETL)
KMRDNL	K, M, R, DN, L	Non-toxin	Probable non-allergen (all fragments)
LDESKRF	L, DESK, R, F	Non-toxin	Probable allergen (DESK) Probable non-allergen (L, R, F)

Note: [a]Predicted by using BIOPEP. [b]Predicted by using ToxinPred. [c]Predicted by using AllerTOP v. 2.0.

3 Conclusion

The antioxidant activities of SC peptide fractions were demonstrated for the first time. UF 2 h, the < 3000 u peptidic fraction obtained after 2 hours of hydrolysis by alcalase, exerted the strongest radical scavenging and iron chelating activities among the peptidic fractions analyzed. Notably, UF 2 h retained its radical scavenging activity after simulated GI digestion and thermal treatments. In this study, four novel peptides were purified and identified from UF 2 h. *In silico* analysis predicted the four peptides to be non-toxic before and after GI digestion. Taken together, UF 2 h and the peptides in it are promising candidates for the development of functional food or beverage ingredients and nutraceuticals. Thus future *in vivo* research to confirm their safety for consumption is warranted. Our findings also suggest that SC is a promising source of bioactive peptides which deserves more attention from researchers in future.

References

[1] Feng L, Yin J, Nie S, et al. Fractionation, physicochemical property and immunological activity of polysaccharides from *Cassia obtusifolia* [J]. International Journal of Biological Macromolecules, 2016, 91: 946-953

[2] Dong X, Fu J, Yin X, et al. Cassiae semen: A review of its phytochemistry and pharmacology (Review) [J]. Molecular Medicine Reports, 2017, 16: 2331-2346

[3] Huang Y-L, Chow C-J, Tsai Y-H. Composition, characteristics, and *in-vitro* physiological effects of the water-soluble polysaccharides from *Cassia* seed [J]. Food Chemistry, 2012, 134: 1967-1972

[4] Liu C, Liu Q, Sun J, et al. Extraction of water-soluble polysaccharide and the antioxidant activity from *Semen cassiae* [J]. Journal of Food and Drug Analysis, 2014, 22: 492-499

[5] Vadivel V, Janardhanan K. Agrobotanical traits and chemical composition of *Cassia obtusifolia* L.: A lesser-known legume of the Western Ghats region of South India [J]. Plant Foods for Human Nutrition, 2002, 57: 151-164

[6] Li C, Li M, Chang W, et al. Purification and characterization of a novel cholesterol-lowering protein from the seeds of *Senna obtusifolia* [J]. Science in China Series C: Life Sciences, 2008, 51: 1020-1024

[7] Chai T-T, Law Y-C, Wong F-C, et al. Enzyme-assisted discovery of antioxidant peptides from edible marine invertebrates: A review [J]. Marine Drugs, 2017, 15: 42

[8] de Castro R J S, Sato H H. Biologically active peptides: Processes for their generation, purification and identification and applications as natural additives in the food and pharmaceutical industries [J]. Food Research International, 2015, 74: 185-198

[9] Rani S, Pooja K, Pal G K. Exploration of rice protein hydrolysates and peptides with special reference to antioxidant potential: Computational derived approaches for bio-activity determination [J]. Trends in Food Science & Technology, 2018, 80: 61-70

[10] Chai T-T, Tong S-R, Law Y-C, et al. Anti-oxidative, metal chelating and radical scavenging effects of protein hydrolysates from blue-spotted stingray [J]. Tropical Journal of Pharmaceutical Research, 2015, 14: 1349-1355

[11] Guo P, Qi Y, Zhu C, et al. Purification and identification of antioxidant peptides from Chinese cherry (*Prunus pseudocerasus* Lindl.) seeds [J]. Journal of Functional Foods, 2015, 19: 394-403

[12] Feng Y-X, Ruan G-R, Jin F, et al. Purification, identification, and synthesis of five novel antioxidant peptides from Chinese chestnut (*Castanea mollissima* Blume) protein hydrolysates [J]. LWT-Food Science and Technology, 2018, 92: 40-46

[13] Iroyukifujita H, Eiichiyokoyama K, Yoshikawa M. Classification and antihypertensive activity of angiotensin i-converting enzyme inhibitory peptides derived from food proteins [J]. Journal of Food Science, 2000, 65: 564-569

[14] Quah Y, Mohd Ismail N I, Ooi J L S, et al. Identification of novel cytotoxic peptide KENPVLSLVNGMF from marine sponge *Xestospongia testudinaria*, with characterization of stability in human serum [J]. International Journal of Peptide Research and Therapeutics, 2017: 1-11

[15] Wan Mohtar W A A-Q I, Hamid A A, Abd-Aziz S, et al. Preparation of bioactive peptides with high angiotensin converting enzyme inhibitory activity from winged bean (*Psophocarpus tetragonolobus* L.) DC seed [J]. Journal of Food Science and Technology, 2014, 51: 3658-3668

[16] Church F C, Swaisgood H E, Porter D H, et al. Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins [J]. Journal of Dairy Science, 1983, 66: 1219-1227

[17] Re R, Pellegrini N, Proteggente A, et al. Antioxidant activity applying an improved ABTS radical cation decolorization assay [J]. Free Radical Biology and Medicine, 1999, 26: 1231-1237

[18] Chai T T, Chiam M J, Lau C H, et al. Alpha-glucosidase inhibitory and antioxidant activity of solvent extracts and fractions of *Typha domingensis* (Typhaceae) fruit [J]. Tropical Journal of Pharmaceutical Research, 2015, 14: 1983-1990

[19] Yarnpakdee S, Benjakul S, Kristinsson H G, et al. Antioxidant and sensory properties of protein hydrolysate derived from Nile tilapia (*Oreochromis niloticus*) by one- and two-step hydrolysis [J]. Journal of Food Science and Technology, 2015, 52: 3336-3349

[20] Zheng Y, Li Y, Zhang Y, et al. Purification, characterization and synthesis of antioxidant peptides from enzymatic hydrolysates of coconut (*Cocos nucifera* L.) cake protein isolates [J]. RSC Advances, 2016, 6: 54346-54356

[21] Darewicz M, Borawska J, Pliszka M. Carp proteins as a source of bioactive peptides - An *in silico* approach [J]. Czech Journal of Food Sciences, 2016, 34: 111-117

[22] Gupta S, Kapoor P, Chaudhary K, et al. In silico approach for predicting toxicity of peptides and proteins [J]. PLoS ONE, 2013, 8: e73957

[23] Zou T-B, He T-P, Li H-B, et al. The structure-activity

relationship of the antioxidant peptides from natural proteins [J]. Molecules, 2016, 21: 72

[24] Li Y, Yu J. Research progress in structure-activity relationship of bioactive peptides [J]. Journal of Medicinal Food, 2015, 18: 147-156

[25] Singh B P, Vij S. *In vitro* stability of bioactive peptides derived from fermented soy milk against heat treatment, pH and gastrointestinal enzymes [J]. LWT - Food Science and Technology, 2018, 91: 303-307

[26] Zhu C-Z, Zhang W-G, Kang Z-L, et al. Stability of an antioxidant peptide extracted from Jinhua ham [J]. Meat Science, 2014, 96: 783-789

[27] Segura-Campos M, Chel-Guerrero L, Betancur-Ancona D, et al. Bioavailability of bioactive peptides [J]. Food Reviews International, 2011, 27: 213-226

[28] Wong F-C, Xiao J, Ong M G L, et al. Identification and characterization of antioxidant peptides from hydrolysate of blue-spotted stingray and their stability against thermal, pH and simulated gastrointestinal digestion treatments [J]. Food Chemistry, 2019, 271: 614-622

[29] Chen M, Li B. The effect of molecular weights on the survivability of casein-derived antioxidant peptides after the simulated gastrointestinal digestion [J]. Innovative Food Science & Emerging Technologies, 2012, 16: 341-348

[30] Wu J, Ding X. Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides [J]. Food Research International, 2002, 35: 367-375

[31] Mäkinen S, Johannson T, Vegarud Gerd E, et al. Angiotensin I-converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates [J]. Journal of Functional Foods, 2012, 4: 575-583

[32] Kleekayai T, Harnedy P A, O'Keeffe M B, et al. Extraction of antioxidant and ACE inhibitory peptides from Thai traditional fermented shrimp pastes [J]. Food Chemistry, 2015, 176: 441-447

[33] Herraiz T, Casal V. Evaluation of solid-phase extraction procedures in peptide analysis [J]. Journal of Chromatography A, 1995, 708: 209-221

[34] O'Loughlin I B, Kelly P M, Murray B A, et al. Molecular characterization of whey protein hydrolysate fractions with ferrous chelating and enhanced iron solubility capabilities [J]. Journal of Agricultural and Food Chemistry, 2015, 63: 2708-2714

[35] Lew R A. HPLC in the analysis of peptide metabolism. In HPLC of Peptides and Proteins: Methods and Protocols, Aguilar, M.I., Ed. Humana Press: New Jersey, USA, 2004; pp 275-291.

[36] Minkiewicz P, Dziuba J, Iwaniak A, et al. BIOPEP database and other programs for processing bioactive peptide sequences [J]. Journal of AOAC International, 2008, 91: 965-980

[37] Zhang M, Mu T-H, Sun M-J. Purification and identification of antioxidant peptides from sweet potato protein hydrolysates by Alcalase [J]. Journal of Functional Foods, 2014, 7: 191-200

[38] Wang L, Ding L, Wang Y, et al. Isolation and characterisation of in vitro and cellular free radical scavenging peptides from corn peptide fractions [J]. Molecules, 2015, 20: 3221

[39] Silva F G D e, Hernández-Ledesma B, Amigo L, et al. Identification of peptides released from flaxseed (*Linum usitatissimum*) protein by Alcalase® hydrolysis: Antioxidant activity [J]. LWT-Food Science and Technology, 2017, 76: 140-146

[40] Liu C, Ren D, Li J, et al. Cytoprotective effect and purification of novel antioxidant peptides from hazelnut (*C. heterophylla* Fisch) protein hydrolysates [J]. Journal of Functional Foods, 2018, 42: 203-215

[41] Dhaval A, Yadav N, Purwar S. Potential applications of food derived bioactive peptides in management of health [J]. International Journal of Peptide Research and Therapeutics, 2016, 22: 377-398

[42] Schaafsma G. Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition [J]. European Journal of Clinical Nutrition, 2009, 63: 1161

[43] Lafarga T, Wilm M, Wynne K, et al. Bioactive hydrolysates from bovine blood globulins: Generation, characterisation, and in silico prediction of toxicity and allergenicity [J]. Journal of Functional Foods, 2016, 24: 142-155

[44] Christer H, Andersson S, Arpaia D, et al. Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed [J]. EFSA Journal, 2010, 8: 1700