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Analytical Methods

Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry

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Abstract

Grass carp muscles were hydrolyzed with various proteases (papain, bovine pancreatin 6.0, bromelain, neutrase 1.5MG and alcalase 2.4L) to extract antioxidant peptides. The hydrolysates were assessed using methods of hydroxyl radical scavenging ability and lipid peroxidation inhibition activity. Hydrolysate prepared with alcalase 2.4L was found to have the highest antioxidant activity. It was purified using ultrafiltration and consecutive chromatographic methods including ion-exchange chromatography, multilayer coil high-speed counter-current chromatography, and gel filtration chromatography. The purified peptide, as a potent antioxidant, was identified as Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val (966.3 Da) using RP-HPLC connected on-line to an electrospray ionization mass spectrometry. As well, it was found that basic peptides had greater capacity to scavenge hydroxyl radical than acidic or neutral peptides and that hydrophobic peptides contributed more to the antioxidant activities of hydrolysates than the hydrophilic peptides. In addition, the amino acid sequence of the peptide might play an important role on its antioxidant activity.

Keywords: Grass carp muscle; Antioxidant peptides; High-speed counter-current chromatography; Electrospray ionization-mass spectrometry

1. Introduction

Oxidation is an essential reaction in all living organisms. The formation of free radicals and other reactive oxygen species (ROS) is unavoidable during the oxidative metabolic process. These reactive radicals play an important role in signal transduction (Hancock, Desikan, & Neill, 2001). However, excess free radicals can cause destructive effects on living tissues and foodstuffs (Wang, Zhao, Zhao, & Jiang, 2007). Among all ROS, hydroxyl radical is considered to be the most reactive and capable of damaging almost any compound it comes in contact with in the living cells (Castro & Freeman, 2001). The free radical chain reaction can initiate peroxidation of membrane lipids, which has been shown to be associated with many diseases (Halliwell, 2001; Halliwell & Whiteman, 2004). Lipid peroxidation that occurs in food products is responsible for the development of unacceptable flavour and taste, decrease in shelf life and the formation of potentially toxic reaction products (Pihlanto, 2006). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), t-butylhydroquinone (TBHQ) and propyl gallate have been widely used in foodstuffs to delay the deterioration caused by oxidation (Wanita & Lorenz, 1996). However, they are restricted in some countries due

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to their potential health hazard (Becker, 1993; Branen, 1975). As a result, the demand for safe and naturally occurring antioxidants as alternatives to synthetic ones has grown steadily for many years.

In recent years, the antioxidant activity of bioactive peptides generated from the digestion of various proteins has attracted much attention. The peptides from milk (Blanca, Ana, Lourdes, & Isidra, 2007), soybean (Gibbs, Zougman, Masse, & Mulligan, 2004), rice bran (Parrado et al., 2006), oil seeds (Aluko & Monu, 2003), eggs (Sakanaka, Tachibana, Ishihara, & Juneja, 2004) and porcine (Saiga, Tanabe, & Nishimura, 2003) have all been shown to exhibit antioxidant activity. In addition, aquatic products and by-products have also proven to be good sources of antioxidant peptides. For example, studies on peptides from capelin protein (Amarowicz & Shahidi, 1997), mackerel protein (Wu, Chen, & Shiau, 2003), jumbo squid skin (Mendis, Rajapakse, Byun, & Kim, 2005), hoki frame protein (Kim, Je, & Kim, 2007) and yellowfin sole frame protein (Jun, Park, Jung, & Kim, 2004) have reported the presence of significant antioxidant activities. However, to the best of our knowledge, little research has been done on the antioxidant properties of freshwater fish derived peptides. Grass carp, one of the high yield freshwater fish, currently comprises up to 35–40% of the total freshwater fish species in China. However, the development of value-added products from grass carp has not been fully exploited yet. Therefore, preparation of bioavailable antioxidant peptides from grass carps may be one way of producing high-value food ingredients from this under utilized fish.

The antioxidant activity of peptides is closely related to their amino acid constituents and their sequences (Chen, Muramoto, Yamaguchi, Fujimoto, & Nokihara, 1998). It is believed that the antioxidant peptides possess some metal-chelation or hydrogen/electron donating activity, which could make them interact with free radicals and terminate the radical chain reaction or prevent their formation (Wang et al., 2007). Hydrophobic amino acids and one or more residues of His, Pro, Met, Cys, Tyr, Trp, Phe and Met are believed to enhance the activities of the antioxidant peptides (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Da'valos, Miguel, Bartolome', & Lo'pez-Fandi∼no, 2004; Herna′ndez-Ledesma, Da′valos, Bartolome′, & Amigo, 2005). However, some recent findings have shed new light on this subject. For example, the Glu-Leu residue in peptides was recently reported to play an important role in radical scavenging (Jun et al., 2004). Also, the known antioxidant peptide, Gln-Gly-Ala-Arg, does not contain any of the above mentioned proton-donating amino acid residues in its sequence (Li, Chen, Wang, Ji, & Wu, 2007). It appears that more research is needed to clarify the structure–function relationship of peptides.

As an effective separation technique, high-speed countercurrent chromatography (HSCCC) has been successfully applied to separate natural products and compounds including flavonoid (Peng, Fan, & Wu, 2005), coumarin (Shibusawa, Haqiwara, & Chao, 1997) and saponin (Du, Jerz, & Waibel, 2003). For peptides, the use of HSCCC has been mainly concentrated with the separation of standard mixtures of free peptides (Ma & Ito, 1997), while little has been done on the separation of peptides in hydrolysates.

The objective of this study is to assess and identify or characterize antioxidant peptides from grass carp which is an under utilized freshwater fish. Besides, using HSCCC to separate peptides in hydrolysates can provide useful information for the isolation of peptides derived from other proteins.

2. Materials and methods

2.1. Material

The healthy grass carps (*Ctenopharyngodon idellus*, 822 ± 147 g in weight and 40.8 ± 2.8 cm in length) were obtained from a local market in Guangzhou, China. Grass carps (without head, tail, skin, bone, internal organs and blood) were filleted and minced in a MM12 mincer (Shaoguan Food Machine Co., China). The minced material was frozen and stored at −20 °C for further use. Five food-grade enzymes used for hydrolysis experiments (Papain, bovine pancreatin 6.0, bromelain, neutrase 1.5MG and alcalase 2.4 L) were provided by Novo Nordisk Co. (Beijing, China) and Mingyuan Co. (Guangzhou, China). Protein standard mixtures used for molecular weight calibration by gel filtration chromatography were supplied by AmershamBiotech (GE, Piscataway, NJ, USA). Amino acid standard mixtures, glutathione (GSH), oxidized glutathione (GSSH), methyl tert-butyl ether (MTBE), phenylisothiocyanate (PITC) and acetonitrile were all HPLC-grade and purchased from Sigma (Beijing, China). Alpha-deoxyribose (2-deoxy-D-ribose) was purchased from Fluka (Stockholm, Sweden). All other reagents were analytical-grade.

2.2. Preparation of grass carp muscle hydrolysates

Frozen minced grass carp muscles (1250 g) were thawed and mixed with deionized water (1250 ml). The mixture obtained was divided into five equal fractions. Each of the fractions was adjusted to the required pH with 0.01 M NaOH and heated in a water bath to the required temperatures (Table 1). Papain, bovine pancreatin 6.0, bromelain, neutrase 1.5MG and alcalase 2.4L were dissolved in deionized water, respectively, and added in the proper proportions based on their activities (Table 1). The hydrolysis reaction was performed in a shaking incubator (New Brunswick Scientifics C24, China). At the end of the hydrolysis period, the mixtures were heated in boiling water for 10 min to inactivate the proteases. The hydrolysates were centrifuged in a GL-21M refrigerated centrifuge (Xiangyi Instrument Co. Ltd., Changsha, China) at 4125 g for 30 min and the supernatants were lyophilized and stored in a desiccator for further use.

Table 1
Parameters for enzymatic hydrolysis of grass carp muscles treated by five proteases and the antioxidant activities of the hydrolysates

Samples	Parameters for enzymatic hydrolysis				Antioxidant activities* of the hydrolysates (IC_{50}) (mg/ml)		
	Activity (U/g)	pH	Time (h)	Temperature (°C)	E/S ratio (w/w)	HRSA[A]	LPIA[B]
Papain	2.4×10^4	7.0	4.0	50.0	1.5/1000	3.54 ± 0.21^{a}	5.53 ± 0.37^{a}
Bovine pancreatin 6.0	6.0×10^4	8.0	4.0	50.0	1.0/1000	2.34 ± 0.39^{b}	5.76 ± 0.45^{ab}
Bromelain	3.0×10^4	7.0	4.0	50.0	2.0/1000	5.02 ± 0.35^{c}	7.03 ± 0.48^{b}
Neutrase 1.5MG	3.0×10^4	7.0	4.0	50.0	2.0/1000	4.09 ± 0.39^{ad}	6.67 ± 0.58^{ab}
Alcalase 2.4L	6.9×10^4	8.0	4.0	55.0	1.5/1000	1.81 ± 0.44^{e}	4.60 ± 0.39^{abc}
GSH						1.75 ± 0.20^{e}	3.21 ± 0.47^{c}

* Expressed as means ± SD of triplicates. Values followed by the same letter in the same column are not significantly different ($P > 0.05$) according to paired-samples T-test.
[A] Hydroxyl radical scavenging ability (HRSA).
[B] Lipid peroxidation inhibition activity (LPIA).

2.3. Evaluation of antioxidant activity

For the antioxidation test, sample solutions with different concentrations of the five hydrolysates (1.0, 2.0, 3.0, 4.0 and 5.0 mg/ml) were prepared by dissolving the lyophilized hydrolysates in deionized water.

2.3.1. Hydroxyl radical scavenging ability (HRSA) assay

The HRSA assay was performed as previously described by Chung, Osawa, and Kawakishi (1997) with some modifications. The reaction mixture consisted of 0.1 ml of 10 mM $FeSO_4$, 0.1 ml of 10 mM EDTA, 0.5 ml of 10 mM α-deoxyribose, 0.9 ml of sodium phosphate buffer (pH 7.4) and 0.2 ml of sample were thoroughly mixed in a tube. Hydrogen peroxide (0.2 ml, 10 mM) was then added and the reaction mixture was incubated at 37 °C for 1 h. One milliliter of 2.8% trichloroacetic acid (TCA) and 1.0 ml of 1.0% thiobarbituric acid (TBA) were added to the test tubes and boiled for 15 min. After cooling the mixture, the absorbance was measured at 532 nm. Sodium phosphate buffer (pH 7.4) instead of sample was used as blank. The HRSA was evaluated as the inhibition rate of α-deoxyribose oxidation by hydroxyl radical,

$$\text{HRSA}\ (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 was the absorbance of the blank and A_1 was the absorbance in the presence of the test compound. These values of HRSA were plotted against the concentrations of individual samples, and the concentration to scavenge 50% of radical activity was defined as the IC_{50} value.

2.3.2. Lipid peroxidation inhibition activity (LPIA) assay

The LPLA assay was performed according to the methods described by Ng, Liu, and Wang (2000) and Ohkawa, Ohishi, and Yagi (1979) with some modifications. Male wistar rats weighing 200–250 g were obtained from the Experimental Animal Center of South Medical University (Guangzhou, China). Rats had free access to food and water and sacrificed with sodium pentobarbital (62 mg/kg). Liver tissue was rapidly dissected from the abdomen and homogenized in a mortar in Tris–HCl buffer (40 mM, pH 7.0) to produce a 2.5/10 (w/v) homogenate. The liver homogenate (100 μl) was incubated with sample (200 μl), 30 mM KCl (100 μl), 0.16 mM $FeSO_4$ (100 μl) and 0.06 mM Vitamin C (100 μl) at 37 °C for 1 h. TBA reagent (1 ml of 0.67% TBA and 1 ml of 15% TCA) was then added. The final solution was heated at 100 °C in a boiling water bath for 15 min, cooled on ice for 10 min, and then centrifuged at 6445g for 10 min. The absorbance of the supernatant was read at 532 nm in a UV-754 spectrophotometer (Analytic. Instrumental, Shanghai, China). The blank was performed by substituting Tris–HCl buffer (40 mM, pH 7.0) for sample.

$$\text{LPIA}\ (\%) = (A_{\text{blank}} - A_{\text{sample}}) \times 100/A_{\text{blank}}$$

where A_{blank} and A_{sample} are the absorbance values for the blanks and samples, respectively. The plot of inhibition activity against concentration of hydrolysate was done and IC_{50} (concentration of samples to decrease 50% of the peroxidation) was obtained.

2.4. Purification of antioxidant peptides

The schematic diagram for the purification of grass carp muscle hydrolysates is shown in Fig. 1.

2.4.1. Ultrafiltration

The lyophilized grass carp muscle hydrolysate (GCMH) was dissolved in 100 ml of distilled water and then fractionated through ultrafiltration membranes bioreactor system (Vivaflow 200, Vivascience, Sartorius, Goettingen, Germany) having a range of molecular weight cutoff (MWCO) of 10, 5 and 3 kDa, respectively. GCMH-I, GCMH-II, GCMH-III and GCMH-IV represent the fractions with M_W distribution of >10 kDa, 5–10 kDa, 3–5 kDa and <3 kDa, respectively. All GCMHs recovered were freeze-dried and the antioxidant activity of each fraction determined.

2.4.2. Ion-exchange chromatography

The fraction with the highest antioxidant activity after ultrafiltration separation was dissolved in 15 ml of a 45 mM sodium citrate buffer (pH 5.8) and loaded onto a home-made Amberlite IR-120 (Fisher Scientific Co., Fair Lawn, NJ, USA) cation-exchange column (2.6 × 60 cm), which was previously equilibrated with 45 mM sodium citrate buffer (pH 5.8). The column was then eluted with 20 mM ammonium chloride buffer (pH 8.0) in isocratic

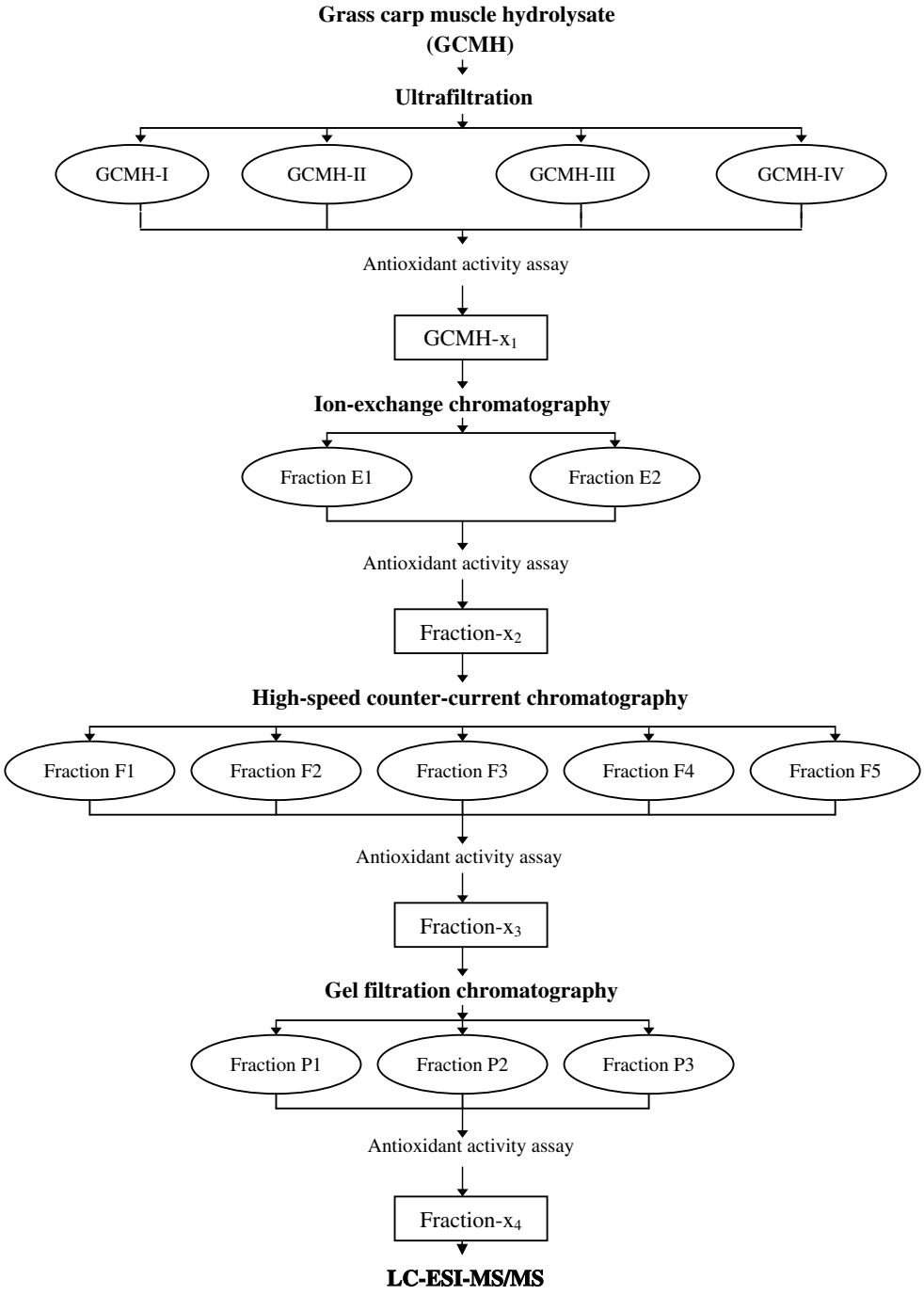

Fig. 1. The schematic diagram for the purification of grass carp muscle hydrolysates.

mode, at a flow-rate of 60 ml/h. Every 3 ml of eluted solution was collected and monitored at 214 nm, which was the characteristic absorbance of random coil monomeric peptides. The fractions with the desire peaks were pooled, concentrated and lyophilized for antioxidant activity test.

2.4.3. High-speed counter-current chromatography (HSCCC)

The fraction exhibiting strong antioxidant activity after ion-exchange chromatography was further separated by multilayer coil HSCCC (TBE300A, TAUTO®, Shanghai, China) according to the experimental conditions described by Ma and Ito, 1997 with minor adjustments. The organic stationary phase and aqueous mobile phase were prepared from a solvent system composed of methyl tert-butyl ether (MTBE)-*n*-butanol-acetonitrile-1% TFA water (2:1:1:4, v/v). The sample solution was prepared by dissolving 1 g of the lyophilized hydrolysate in 10 ml of solvent consisting of equal volumes of the organic phase and aqueous phase. The column was first filled with the organic stationary phase (~400 ml). After injection of the sample solution, the column was eluted with an aqueous mobile phase at a

flow-rate of 2.0 ml/min and an optimum speed of 800 rpm was used throughout. The effluent was continuously monitored at 214 nm for the detection of peptides and collected at 2 min intervals (4 ml/tube). The collection of desire peak was pooled, freeze-dried and tested for antioxidant activity.

2.4.4. Gel filtration chromatography (GFC)

The fraction showing powerful antioxidant activity after HSCCC process was further isolated on a Superdex-peptide-10/300-GL column (Amersham Biotech, GE, Piscataway, NJ, USA) using an ÄKTA GFC purifier (Amersham Biotech, GE, Piscataway, NJ, USA). The column was equilibrated and eluted with 0.25 M phosphate buffer (pH 7.2) in isocratic mode, at a flow-rate of 0.5 ml/min, and random coil monomeric peptides were detected at 214 nm. A protein standard mixture (Globin, 16,949 Da; Globin II, 6214 Da; Globin III, 2512 Da; GSSH, 630 Da; GSH, 307 Da and glycine, 75 Da) was used to calibrate the column. The fractions with different molecular mass distribution were collected, respectively and lyophilized for antioxidant activity evaluation. The active fraction was analyzed for amino acid sequence and molecular mass determination.

2.5. Identification of peptide by on-line LC–ESI-MS/MS

The desirable fraction after GFC purification was dissolved in a buffer of 85% H_2O/15% methanol, then loaded onto an Agilent RP-HPLC system connected on-line to an HCTplus ion trap instrument (Bruker Daltonic, Bremen, Germany). A volume of 5 μl of purified fraction was loaded onto a 150 mm × 2.0 mm Luna 5 μm C_{18} column (Phenomenex, Torrance, CA, USA). The elution was performed using a mobile phase consisting of water–trifluoroacetic acid (1000:0.25, v/v) (eluent A) and methanol (eluent B). Gradient elution was carried out according to the following process: 0–10 min, linear gradient 0–2% B; 10–15 min, linear gradient 2–10% B; 15–30 min, linear gradient 10–40% B; 30–35 min, linear gradient 40–80% B; 35–50 min, isocratic gradient 80% B; 50–55 min, linear gradient 80–2% B; the flow-rate: 0.2 ml/min; the detection wavelength: 254 nm. The flow with a speed of 100 μl/min was directed into the mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA), which was operated in the positive electrospray ionization (ESI^+) mode, via the electrospray interface. The Drying and ESI nebulizing gas used high-purity nitrogen. Spectra were recorded over the mass/charge (m/z) range 200–2000. About five spectra were averaged in the MS and multiple MS (MS/MS) analyses. The peptide sequencing was performed by processing the MS/MS spectra using BioTools (Version 3.0; Bruker Daltonics Inc., Billerica, MA, USA) as well as manual calculation.

2.6. Statistical analyses

All the tests for antioxidant activities of hydrolysates were conducted with three replicates. Data were expressed as means ± standard errors. The statistical analysis was performed using SPSS 10.0 software (SPSS Inc. Chicago, IL, USA). The significant difference was determined with 95% confidence interval ($P < 0.05$).

3. Results and discussion

3.1. Antioxidant activity assessment for grass carp muscle hydrolysates

The obtained five grass carp muscle hydrolysates by five proteases, viz. papain (Mingyuan Co., Guangzhou, China), bovine pancreatin 6.0 (Mingyuan Co., Guangzhou, China), bromelain (Mingyuan Co., Guangzhou, China), neutrase 1.5MG (Novo Nordisk Co., Beijing, China), and alcalase 2.4L (Novo Nordisk Co., Beijing, China), were tested for their antioxidant activities including HRSA and LPIA assays. As was shown in Table 1, alcalase 2.4L hydrolysate revealed the most potent HRSA among the five hydrolysates. There was no significant difference between the HRSA of alcalase 2.4L hydrolysate ($IC_{50} = 1.81 \pm 0.44$ mg/ml) and that of the standard antioxidant GSH ($IC_{50} = 1.75 \pm 0.20$ mg/ml) within 95% confidence interval. Since the chemical activity of the hydroxyl radical was the strongest among the oxygen radicals (You et al., 2002), the powerful HRSA of the alcalase 2.4L hydrolysate was a good indication of its potent antioxidant activity. Besides, alcalase 2.4L hydrolysate also exhibited a stronger capacity for inhibiting lipid peroxidation and there was no significant difference between the LPIA for alcalase 2.4L hydrolysate ($IC_{50} = 4.60 \pm 0.39$ mg/ml) and that of GSH ($IC_{50} = 3.21 \pm 0.47$ mg/ml). This is probably due to the fact that alcalase 2.4L hydrolysate contained antioxidant peptides which rendered their protective actions in lipid peroxidation by scavenging lipid-derived radicals ($R^{\bullet}$, $RO^{\bullet}$ or $ROO^{\bullet}$) to stop chain reactions. Therefore, alcalase 2.4L hydrolysate, having the highest antioxidant activity (both HRSA and LPIA), was further isolated in order to identify the antioxidant peptides.

3.2. Isolation and purification of antioxidant peptides

Ultrafiltration membranes (MWCO = 10, 5 and 3 kDa) was used to separate the alcalase 2.4L hydrolysate into four M_w fractions, GCMH-I ($M_w > 10$ kDa), GCMH-II ($M_w =$ 5–10 kDa), GCMH-III ($M_w = 3$–5 kDa) and GCMH-IV ($M_w < 3$ kDa). As shown in Table 2, among the four fractions, both the strongest HRSA and LPIA were observed in GCMH-IV fraction, with the IC_{50} values of 1.68 ± 0.34 mg/ml and 3.84 ± 0.27 mg/ml, respectively. Therefore, GCMH-IV fraction ($M_w < 3$ kDa) which accounted for 57.83% of total hydrolysates was selected for the identification of the antioxidant peptide. According to the statistic analysis, there was less significant different between the LPIA data for the hydrolysates with different molecular weight distribution, while HRSA was more sensitive to reflect the change of the hydrolysates. Therefore, only

Table 2
Ultrafiltration separation of grass carp hydrolysate treated by alcalase 2.4L and its antioxidant activities evaluation

Sample	Molecular weight (kDa)	Recovery (%)	Antioxidant activities* (IC_{50}) (mg/ml)	
			Hydroxyl radical scavenging ability (HRSA)	Lipid peroxidation inhibition activity (LPIA)
GCMH-I	>10	1.84	5.51 ± 0.34^{a}	4.98 ± 0.22^{a}
GCMH-II	5–10	9.91	4.09 ± 0.18^{b}	4.75 ± 0.20^{a}
GCMH-III	3–5	30.42	2.86 ± 0.35^{c}	4.40 ± 0.25^{a}
GCMH-IV	<3	57.83	1.68 ± 0.34^{c}	3.84 ± 0.27^{b}

* Expressed as means ± SD of triplicates. Values followed by the same letter in the same column are not significantly different ($P > 0.05$) according to paired-samples T-test.

HRSA was chosen to monitor the antioxidant peptides during the following purification process.

GCMH-IV fraction was subjected to Amberlite IR-120 cation-exchange column (2.6 × 60 cm) and two fractions (E1 and E2) were obtained (Fig. 2A). Fraction E1 contained the peptides that were not retained by the column, while fraction E2 represented the bound peptides that were eluted by 0.2 M ammonium chloride buffer (pH 8.0). Since the column used was a cation-exchanger, it signified that E1 was a neutral or weak acidic peptide fraction and E2 was a basic peptide fraction. Fig. 2B showed that fraction E2 ($IC_{50} = 2.11 \pm 0.10$ mg/ml) had stronger HRSA compared with the unbound fraction E1 ($IC_{50} = 4.84 \pm 0.11$ mg/ml). Thus, the conclusion could be made that basic peptides were more effective antioxidants than neutral or weak acidic peptides.

The fraction E2 was then further separated into five fractions (F1–F5) by HSCCC (Fig. 2C). During HSCCC separation, the peptides distributed in the stationary phase were gradually eluted by the countercurrent mobile phase. Most likely, the separation was based on the hydrophobic or hydrophilic property of the peptides. The polar or hydrophilic peaks eluted earlier, while the non-polar or hydrophobic peaks eluted much later. Therefore, the order of the hydrophobic properties for the five fractions was F1 < F2 < F3 < F4 < F5, which was the same order as their antioxidant activities (Fig. 2D). Based on the above results, it appears that the hydrophobic peptides presented in the hydrolysates had a greater impact on the observed antioxidant activities, which was consistent with the results of Rajapakse, Mendis, Byun, and Kim (2005).

Since fraction F5 showed the strongest HRSA ($IC_{50} = 2.29 \pm 0.04$ mg/ml) among all the five fractions (Fig. 2D), it was further fractionated using Superdex-peptide-10/300-GL gel filtration column. As shown in Fig. 2E, three peptide fractions (P1–P3) were isolated and fraction P1 was shown to be very powerful with IC_{50} of 2.86 ± 0.12 mg/ml (Fig. 2F). Comparing the IC_{50} values of fraction F5 and P1, it could be concluded that the purification process caused a decrease in the antioxidant activity of the fractions, which was reported earlier by Chen, Muramoto, and Yamauchi (1995).

3.3. Identification of peptide by LC–ESI-MS–MS

To identify the putative active peptides, fraction P1 was subjected to RP-HPLC coupled on-line to ion trap mass spectrometry. Fig. 3A gave the RP-HPLC–UV chromatographic profile of fraction P1. The MS spectrum of peak 11 (in Fig. 3A) was shown in Fig. 3B and the MS/MS spectrum of a single charged ion with m/z at 966.3 Da was illustrated in Fig. 3C. The fragmentation spectrum contained a major ion at m/z 605.2 Da, which was identified as a b-type fragment ion resulting from the cleavage N-terminal to Pro (Fig. 3C). Pro was related to several unusual fragmentation events, its presence in some of the identified peptides helped with their identification (Blanca, Lourdes, Mercedes, & Isidra, 2004). Although the BioTools (Version 3.0; Bruker Daltonics) failed to obtain an amino acid sequence from the MS/MS spectrum (Fig. 3C), each mass signal and corresponding fragmentation spectra could be matched to a single peptide fragment by manual calculation. In fact, the manual analysis gave five possible sequences, viz. (I) Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val, (II) Pro-Ser-Gly-Ala-Tyr-Glu-Pro-Phe-Val, (III) Pro-Ser-Ala-Gly-Tyr-Glu-Pro-Phe-Val, (IV) Pro-Ser-Lys-Tyr-Gly-Ala-Pro-Phe-Val, (V) Pro-Ser-Lys-Tyr-Ala-Gly-Pro-Phe-Val, due to the fact that the molecular weight of the amino acids inside the peptide chain might represent the sum of two amino acids: (-Glu-) (128) = (-Gly-) (57) + (-Ala-) (71) and (-Lys-) (128) = (-Gly-) (57) + (-Ala-) (71) (Li et al., 2007).

To confirm the peptide sequence, the amino acids composition of fraction P1 was analyzed and Table 3 presented the results. Gly, Pro, Phe, Lys, Glu, Ser, Leu, Tyr and Val were the major constituent amino acids of the peptide. However, the content of Ala was very low, so the sequences (II–V) shown above were impossible because all the four peptides had Ala in their sequences. Thus, the selected amino acid sequence Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val (I) agreed with the amino acids analysis of the peptide. In the sequence of this peptide, three hydrophobic amino acids (Pro, Phe and Val, totally represent 50% of residues) are distributed in two sides of the peptide chain, which is expected to favor oxidation inhibition. Two aromatic amino acids, Tyr and Phe, which have been shown to act positively as direct radical scavengers (Rajapakse et al., 2005), are also present within the peptide sequence. The antioxidant activity of Tyr may be explained by the special capability of phenolic groups to serve as hydrogen donors, which is one mechanism of inhibiting the radical-mediated peroxidizing chain reaction (Jung, Kim, & Kim, 1995; Marcuse, 1960).

It is commonly believed that His, Met and Cys are very important to the radical scavenging activity of peptides due to their special structure of characteristics: the imidazole group in His has the proton-donation ability (Tsuge, Eikawa, Nomura, Yamamoto, & Sugisawa, 1991); Met is prone to oxidation of the Met sulfoxide (Hernaʹndez-Ledesma et al., 2005); Cys donates the sulfur hydrogen (Hernaʹndez-Ledesma et al., 2005). However, the identified pep-

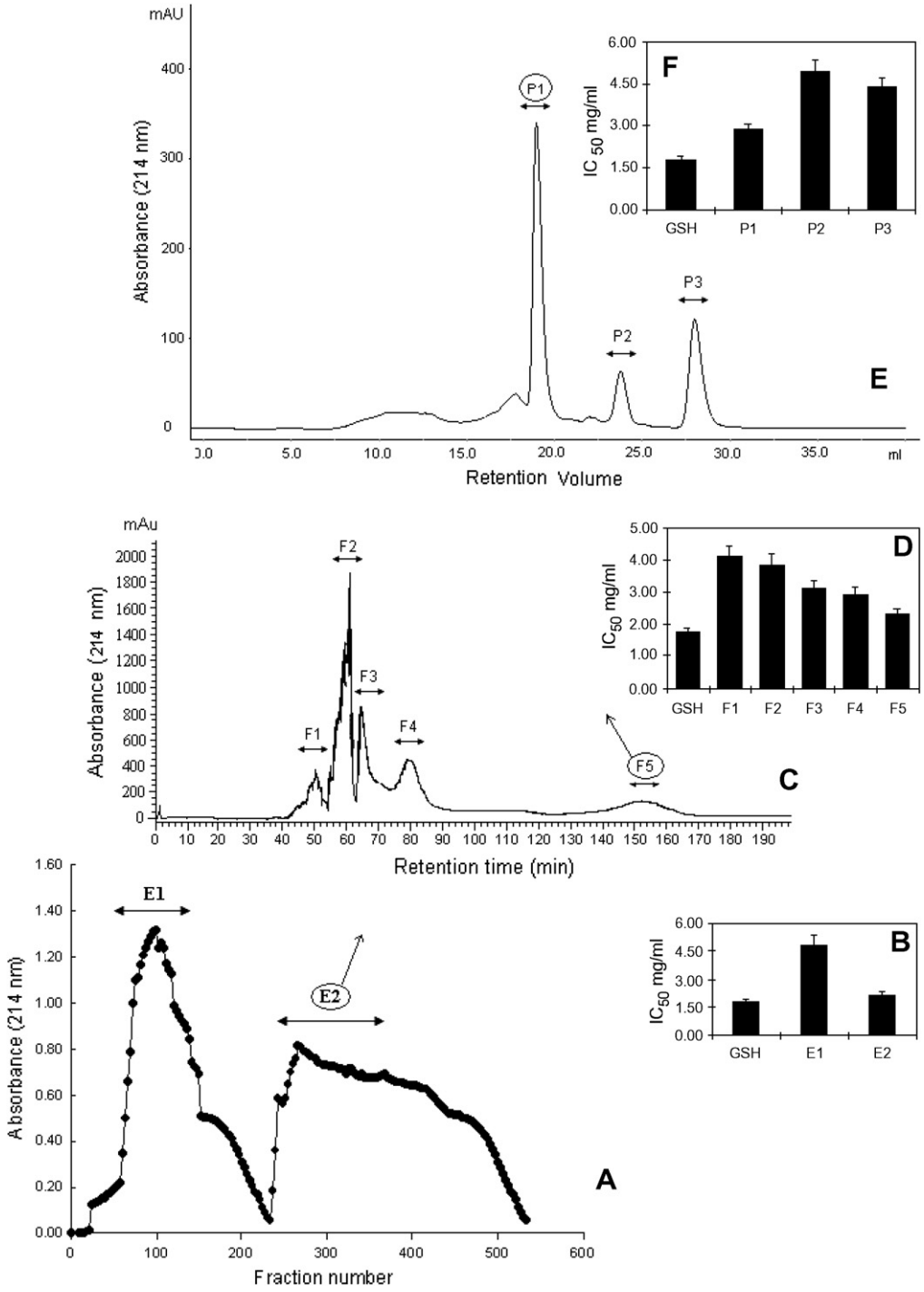

Fig. 2. (A) Separation scheme of antioxidant peptides obtained from hydrolysates of grass carp muscles by ion-exchange chromatography on an Amberlite IR-120 cation-exchange column (2.6 × 60 cm). The column was eluted with 20 mM ammonium chloride buffer (pH 8.0) in isocratic mode and eluted at a flow-rate of 60 ml/h; (B) HRSA assay (indicated by IC_{50} values) for the obtained fractions E1 and E2; (C) separation scheme of antioxidant peptides obtained from hydrolysates of grass carp muscles by HSCCC (TBE300A, TAUTO®). Experimental conditions: sample, 1 g dissolved in 10 ml solvent (5 ml of stationary phase and 5 ml of mobile phase); solvent system, MTBE/*n*-butanol/acetonitrile/1% TFA water (2:1:1:4, v/v); flow-rate, 2 ml/min in the head to tail elution mode; revolution, 800 rpm; (D) HRSA assay (indicated by IC_{50} values) for the obtained fractions (F1–F5); (E) separation scheme of antioxidant peptides obtained from hydrolysates of grass carp muscles by Superdex-peptide-10/300-GL gel filtration column. The column was eluted with 0.25 M phosphate buffer (pH 7.2) in isocratic mode, at a flow-rate of 0.5 ml/min; (F) HRSA assay (indicated by IC_{50} values) for the obtained fractions (P1–P3).

tide (Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val) in this study does not contain any of the three mentioned amino acid residues. This was also reported by other researchers: Li et al. (2007) identified the peptide Gln-Gly-Ala-Arg, which exhibited the highest antioxidant activity from porcine skin collagen hydrolysates; Suetsuna, Ukeda, and Ochi (2000)

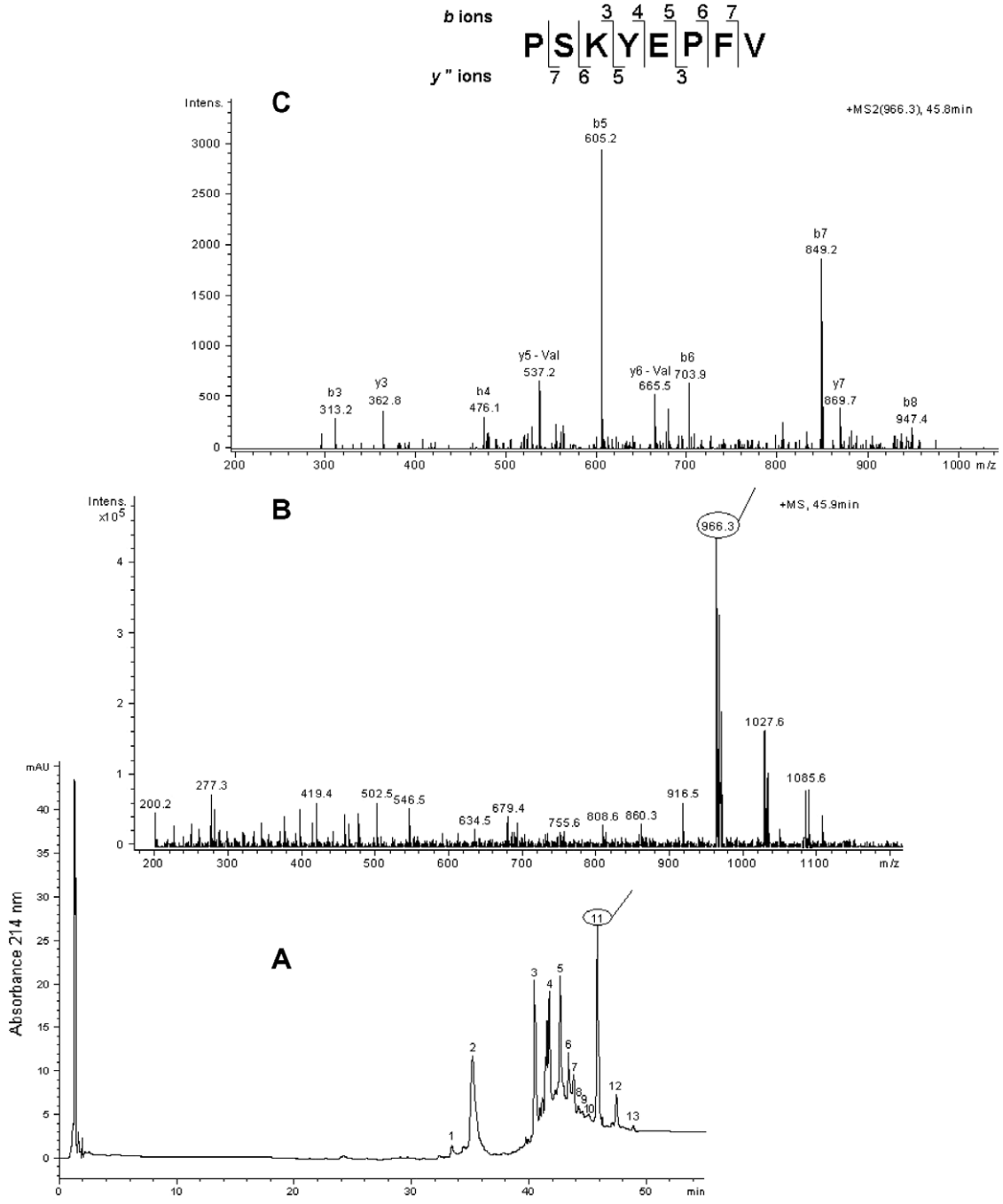

Fig. 3. Identification of the antioxidant peptide: (A) RP-HPLC–UV chromatograms of P1 fraction obtained by GFC from the grass carp protein hydrolysates; (B) mass spectrum of the chromatographic peak 11 in Fig. 3A; (C) MS/MS spectrum of ion *m*/*z* 966.3. By manual calculation, the sequence of this peptide is displayed with the fragment ions observed in the spectrum. For clarity, only *b* and *y*" ions are labeled.

isolated 1 kDa antioxidant peptide from a peptic hydrolysate of casein that possessed a primary structure of Tyr-Phe-Tyr-Pro-Glu-Leu; Rajapakse et al. (2005) reported two antioxidant peptides, Asn-Gly-Leu-Glu-Gly-Leu-Lys (747 Da) and Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala (1307 Da) from giant squid muscle protein. All of the above identified peptides do not contain His, Met or Cys. Besides, eight constituent amino acids (Pro, Ser, Lys, Tyr, Glu, Pro, Phe and Val) of the identified peptide mixed with the same concentration as the peptide did not show antioxidant activity. Therefore, the amino acid sequence of the peptide might play an important role on its activity. The same conclusion was also found by Suetsuna et al. (2000).

Table 3
Amino acids composition of fraction P1 (mg/100 ml)

Amino acids	Contents of amino acids*		
	Total amino acids (TAAs)[a]	Free amino acids (FAAs)[a]	Constituent amino acids of peptides[b] (PAAs)
Aspartic acid	4.0 ± 0.7	0.2 ± 0.3	3.8
Glutamic acid	23.6 ± 7.7	0.8 ± 0.2	22.8
Serine	17.6 ± 5.8	0.6 ± 0.5	17.0
Glycine	48.5 ± 16.4	0.3 ± 0.4	48.2
Histidine	nd[c]	nd	nd
Arginine	12.4 ± 7.4	1.5 ± 1.2	10.9
Threonine	10.9 ± 2.9	0.6 ± 0.2	10.3
Alanine	0.2 ± 0.4	nd	0.2
Proline	32.4 ± 12.5	nd	32.4
Tyrosine	13.2 ± 2.5	0.9 ± 0.7	12.3
Valine	12.5 ± 4.7	0.4 ± 0.5	12.1
Methionine	4.8 ± 1.2	0.1 ± 0.2	4.7
Cysteine	2.2 ± 0.7	0.2 ± 0.3	2.0
Isoleucine	11.1 ± 1.6	nd	11.1
Leucine	15.3 ± 8.3	nd	15.3
Tryptophan	11.1 ± 5.6	0.8 ± 0.6	10.3
Phenylalanine	26.2 ± 4.3	nd	26.2
Lysine	25.9 ± 10.3	0.4 ± 0.5	25.5
Asparagine	2.3 ± 0.9	nd	2.3
Glutamine	0.4 ± 0.3	nd	0.4

* Expressed as means ± SD of triplicates.
[a] Total amino acids (TAAs) and free amino acids (FAAs) were assayed by RP-HPLC.
[b] Contents $_{PAAs}$ = Contents $_{TAAs}$ − Contents $_{FAAs}$.
[c] nd: Not detected.

4. Conclusions

The muscles of freshwater fish, grass carps, can be effectively hydrolyzed by alcalase 2.4L to obtain peptides with strong antioxidant activities. The activity assessment for fractions separated by membrane ultrafiltration showed that low-molecular-weight peptides ($M_w < 3$ kDa) were important for their antioxidant abilities. The eluted basic peptides from cation-exchange chromatography showed greater capacity for scavenging hydroxyl radical than neutral or weak acidic peptides. HSCCC was an effective technique to isolate peptides in the hydrolysates using a solvent system composed of MTBE-*n*-butanol-acetonitrile-1% TFA water (2:1:1:4, v/v). The hydrophobic fractions obtained from HSCCC have more contribution to the observed antioxidant activities of the hydrolysates. The peptide was identified as Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val (966.3 Da) using LC–ESI-MS/MS. The amino acid sequence of the peptide might be responsible for its antioxidant activities, which may exceed the importance of having special amino acid such as His, Met or Cys in their sequences.

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