PAPER

PURIFICATION AND IDENTIFICATION OF ANTIOXIDANT PEPTIDES FROM GELATIN HYDROLYSATES OF UNICORN LEATHERJACKET SKIN

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ABSTRACT

Antioxidant peptides from a gelatin hydrolysate of unicorn leatherjacket skin prepared using a partially purified glycyl endopeptidase were purified using Sephadex G-25 gel filtration, DEAE-cellulose anion-exchange and reverse phase high-performance liquid chromatography. The fractions with the highest ABTS radical scavenging activity were analyzed using UPLC–ESI-MS/MS to identify the peptide sequences therein. Four of the identified peptides, Glu-Pro-Gly-Pro-Val-Gly (555.27 Da), Leu-Pro-Gly-Pro-Ala-Gly (511.29 Da), Leu-Asp-Gly-Pro-Val-Gly (557.30 Da) and Glu-Gly-Pro-Leu-Gly (472.24 Da), were subsequently synthesized. Glu-Gly-Pro-Leu-Gly exhibited the highest antioxidant activity (4.95 μmol TE/g solid). Therefore, peptides from unicorn leatherjacket skin gelatin hydrolysate could be further employed as functional food ingredient.

Keywords: gelatin hydrolysate, unicorn leatherjacket, antioxidant activity, identification, UPLC, mass spectrometry

1. INTRODUCTION

There is an increased interest in the isolation of natural antioxidants from different sources as the use of synthetic antioxidants is restricted due to the potential risks to human health (HRAŠ *et al*., 2000). Antioxidant peptides have been found in several foodstuffs such as milk (PHELAN *et al*., 2009), cereals (MALAGUTI *et al*., 2014) and algae (CORNISH and GARBARY, 2010). Fish protein hydrolysates, especially gelatin hydrolysates, have been shown to have the ability to scavenge free radicals (MENDIS *et al*., 2005), inhibit lecithin liposome peroxidation (KARNJANAPRATUM and BENJAKUL, 2015a; 2015b) and reduce the oxidation of Fe^{3+} to Fe^{2+} (ALEMAN *et al*., 2011). The antioxidant activity of protein hydrolysates is mainly governed by the parent protein sequence, the specificity of enzymes used and the conditions used in the hydrolysate preparation (PEÑA-RAMOS and XIONG, 2011). The amino acid sequences of several antioxidant peptides have been identified (SUETSUNA *et al*., 2000; SAIGA *et al*., 2003; FAN *et al*., 2012), demonstrating an association between bioactivity and the amino acid sequence (SHAHIDI and ZHONG, 2008). Gelatin peptides have an abundance of glycine (Gly), proline (Pro) and hydroxyproline (Hyp), which may contribute to their enhanced bioactivity in comparison with peptides isolated from other sources. Pro residues have a scavenging effect on radicals and the percentage of hydroxylation has been correlated with antioxidant activity (ALEMAN *et al*., 2011).
Antioxidant gelatin hydrolysates from unicorn leatherjacket skin have been produced using different methods. The autolysis-assisted process mediated by indigenous protease in combination with thermal or enzymatic hydrolysis was implemented to prepare antioxidant gelatin hydrolysates from unicorn leatherjacket skin (KARNJANAPRATUM *et al*., 2015b). Glycyl endopeptidase (GE), isolated from papaya latex, was shown to yield gelatin hydrolysates from unicorn leatherjacket skin with higher antioxidant activity, compared to the crude extract from papaya latex (KARNJANAPRATUM *et al*., 2015a). Gelatin hydrolysates from unicorn leatherjacket skin prepared using GE with autolysis-assisted process also demonstrated antioxidant properties in *in vitro* cellular model systems (KARNJANAPRATUM *et al*., 2015). Nevertheless, no information on the structure and sequence of potential antioxidant peptides from skin gelatin hydrolysates of unicorn leatherjacket exists. The aims of this study were to purify and identify the amino acid sequences of antioxidant peptides from gelatin hydrolysate of unicorn leatherjacket skin prepared using glycyl endopeptidase.

2. MATERIALS AND METHODS

2.1. Chemical

2,2′-Azinobis (3-thylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) and other reagents were obtained from Sigma Chemical Co. (Dublin, Ireland). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Fluka Chemie (Buchs, Switzerland). All solvents were high performance liquid chromatography (HPLC) grade and were procured from Merck (Darmstadt, Germany).

2.2. Preparation of gelatin hydrolysate from unicorn leatherjacket skin

2.2.1 Preparation of fish skins

The skins of unicorn leatherjacket (*Aluterus monoceros*) were obtained from a dock in Songkhla, Thailand. The skins were washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm^2). Skins were subjected to alkaline pretreatment to remove non-collagenous proteins as per the method of KAEWRUANG *et al*. (2013). The autolysis of pretreated skin was conducted following the method of KARNJANAPRATUM and BENJAKUL (2015a). The resulting autolyzed skin was used as a substrate for preparation of the gelatin hydrolysate.

2.2.2 Preparation of partially purified glycyl endopeptidase from papaya (*Carica papaya*) latex

The glycyl endopeptidase (GE) was fractionated from the latex using the method of KARNJANAPRATUM and BENJAKUL (2014). An aqueous two phase system (ATPS) with 10% PEG 6000 and 10% ammonium sulphate $(NH_4)_2SO_4$ was used for fractionation of GE. The obtained GE was stored at -40°C until use.

2.2.3 Production of gelatin hydrolysates

The antioxidant gelatin hydrolysate from skin of unicorn leatherjacket was prepared as described by KARNJANAPRATUM and BENJAKUL (2015a). Autolyzed skin solution (3%, w/v) was hydrolysed using GE (8%, w/w based on solid matter) at 40°C for 60 min. The resulting gelatin hydrolysate (GH) was lyophilized using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark) and was then stored at -20°C until use for analysis.

2.3. Purification of antioxidant peptides

From a previous study, it was found that GH demonstrated strong ABTS radical scavenging activity in comparison with ferrous chelating activity and ferric reducing antioxidant power (KARNJANAPRATUM *et al*., 2015a). ABTS radical scavenging activity was therefore selected to monitor antioxidative activity of purified peptide in each step.

2.3.1 Size exclusion chromatography

GH (2 mL, 80 mg/mL) was subjected to gel filtration (2.5 x 50 cm column) using Sephadex G-25 (Product number 17-0032-01, GE Healthcare Bio-Science AB, Uppsala, Sweden) as described by KARNJANAPRATUM and BENJAKUL (2015a). The fractions of 3 mL were collected. The absorbance of the eluent was recorded at 220 and 280 nm. All fractions were measured for ABTS radical scavenging activity.

2.3.2 Anion-exchange chromatography

The fractions exhibiting the highest antioxidant activity obtained from size exclusion chromatography were pooled, lyophilized and further subjected to anion-exchange chromatography (DEAE-cellulose, Whatman, England) column (1.0×50 cm) coupled with a fraction collector (Model 2128, Bio-RAD Laboratories Ltd.). The elution was carried out using a constant flow rate (0.5 mL/min) with a linear gradient of NaCl (0-0.4 M). The

absorbance at 220 and 280 nm was monitored and 3 mL fractions were collected. ABTS radical scavenging activity of all fractions was measured and the fractions exhibiting high antioxidant activity were pooled (Peak B-1, B-2, B-3), desalted into DI water using size exclusion chromatography (Sephadex G-25, 2.5×50 cm column) and subsequently lyophilized.

2.3.3 High performance liquid chromatography (HPLC)

The fraction with the highest ABTS radical scavenging activity obtained from anion-exchange chromatography was further separated using reversed phase- (RP-HPLC) on a 201TP C18 (4.6×250 mm) column (Grace Davision Discovery Science, Epping, Australia). The HPLC system consisted of a spectra system P2000 pump (Thermo Electron corporation, Wisconsin, United State), sample injector (Spectra system AS3000, Thermo Electron corporation) and a detector (Spectra system UV6000LP, Thermo Electron corporation). The column was equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water (solvent A), and a linear gradient was developed using solvent A and solvent B (acetonitrile containing 0.1% (v/v) TFA). To separate the peptides, elution was performed with the following conditions: 0.0-10.0 min, 5% B; 10.0-60.0 min, 5.0-30.0% B; 60.0-70.0 min, 100% B; 70.0-80.0 min, 100% A, at a flow rate of 1.0 mL/min. The fractions were manually collected, based on the peaks of A_{220} and A_{280}. Each fraction was evaporated to dryness in a miVac centrifugal vacuum Quattro concentrator (Genevac Ltd., Ipswich, UK) and ABTS radical scavenging activity of each fraction was then determined. Total activity/A_{220} of each fraction was then calculated.

2.4. ABTS radical scavenging activity

The ABTS radical scavenging activity of gelatin hydrolysates was determined as described by BINSAN *et al*. (2008). The activity was expressed as μmol Trolox equivalent (TE)/g sample.

2.5. Peptide identification using ultra performance liquid chromatography (UPLC)-electrospray ionization (ESI) mass spectrometry (MS) and tandem MS (MS/MS)

Samples were separated on an ACQUITY UPLC (Waters, Milford, MA, USA) and analyzed on an Impact HD™ mass spectrometer (Bruker Daltonics, Bremen, Germany). Mobile phase A was 0.1% formic acid (FA) in MS-grade H_2O and mobile phase B was 0.1% FA in 80% MS-grade acetonitrile. Peptides were separated on an Acquity UPLC BEH 300 C18 RP column (1.7 μm×50 mm, Waters). A flow rate of 0.25 mL/min with isocratic elution was used for 5 min at 100% mobile phase A, followed by gradient elution to 80% mobile phase B from 5 to 25 min.
MS/MS analysis was performed using two different methods: (i) a broad range method targeting peptides having a wide range of molecular masses and (ii) a short peptide method specifically targeting peptides having low molecular masses, as developed by O'KEEFFE and FITZGERALD (2015), was used. The Impact HD™ (Bruker Daltonics) was calibrated using ESI low molecular mass tune mix (Agilent Technologies, Cork, Ireland) for the broad range method while sodium formate (10 mM NaOH, 0.2% formic acid in isopropanol) was used as calibrant for the short peptide method. Mass spectra were acquired in positive ion mode and scans were performed for Auto MS/MS between 100 and 2500 *m/z* for the broad range method and between 50 and 600 *m/z* for the short peptide method. MS/MS conditions were as follows: capillary voltage: 4500 V; collision gas:

nitrogen; nebulizer pressure 1.8 bar; dry heater temperature: 220 ºC and dry gas flow: 8 L/min. Specific broad range method conditions were: collision energy 7.0 eV; commission cell radio frequency (RF): 1500 Vpp and transfer time: 100 μs. Specific short peptide MS/MS conditions were: collision energy 5.0 eV; collision cell RF was stepped between 200 and 350 Vpp (50% of the time each) and transfer time was stepped between 36.1 and 51.1 μs (50% of the time each).
All MS/MS spectra were searched against the SwissProt database, limited to phylum Chordata using PEAKS Studio 7.5 (Bioinformatics Solutions Inc., Waterloo, Canada) and MASCOT (version 2.3, Matrix Science, London, UK). Further peptide identification was carried out by *de novo* sequencing using PEAKS Studio 7.5, Data Analysis (version 4.0, Bruker Daltonics) and Biotools (version 3.2, Bruker Daltonics) software.

2.6. Peptide synthesis

The identified peptides with the typical collagen sequence (Gly-Pro-X motif) and with homology of peptide to collagen proteins ≥75% as well as ≥65% average local confidence (ALC) were selected, in which the presence of Gly at C-terminal, attributed to glycyl endopeptidase cleavage, would be the major criteria. The selected peptides were synthesized by DgPeptides Co., Ltd (Hangzhou, Zhejiang, China). The purity of the synthesized peptides was greater than 95% as determined by HPLC. All peptides were assayed for ABTS radical scavenging activity as described previously and results were expressed as μmol Trolox equivalent (TE)/g peptide.

2.7. Statistical analysis

Experiments were carried out in triplicate. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (STEEL and TORRIE, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Purification of antioxidant peptides

Previous work (KARNJANAPRATUM and BENJAKUL, 2015a), found that ABTS radical scavenging activity had the highest activity in comparison with other antioxidant assays used. Additionally, this assay has been shown to measure the antioxidant activity of both hydrophilic and hydrophobic antioxidants (AGHDAM *et al*., 2011). Therefore, the ABTS assay is suitable for measuring the antioxidant activity of gelatin peptides, which contain both hydrophobic and hydrophilic amino acids in the peptides sequence, as the hydrophilic–hydrophobic property of the peptide is a critical factor affecting its antioxidant activity (MENDIS *et al*., 2005). Thus, the ABTS radical scavenging activity was carried out for screening and selecting the most antioxidant fraction from gelatin hydrolysate.
Initial isolation of the peptides with the highest antioxidant activity from GH was carried out using Sephadex G-25 size exclusion chromatography. Two fractions (A and B) were collected and ABTS radical scavenging activity of each was determined (Fig. 1). Fraction B, which contained smaller lower molecular mass peptides showed the higher antioxidant activity (82.46 μmol/g solid), compared to GH (65.49 μmol TE/g solid) and fraction A

(9.31 μmol TE/g solid) (P<0.05). In general, lower molecular mass peptides possess higher antioxidant activities (SUN *et al*., 2013; INTARASIRISAWAT *et al*., 2013; FAN *et al*., 2012).

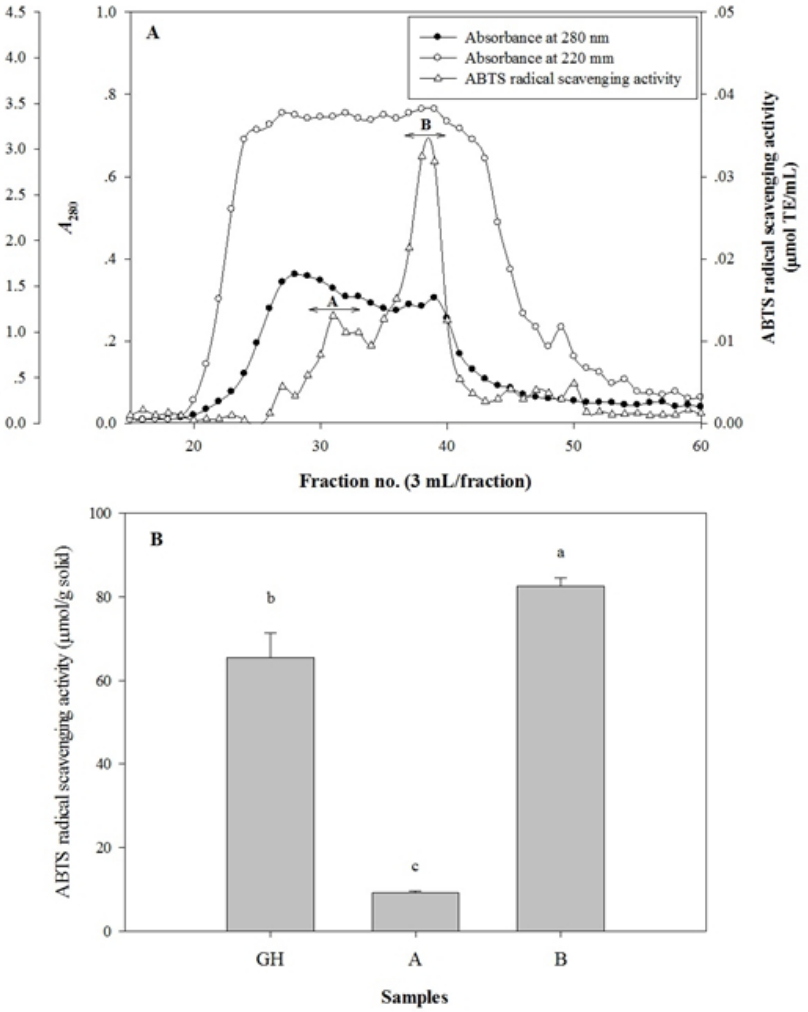

Figure 1. Elution profile of gelatin hydrolysate (GH) from unicorn leatherjacket skin on a Sephadex G-25 column (A) and ABTS radical scavenging activity of GH and its fractions (B). Values represent mean ± SD. Different letters on the bars indicate significant differences ($p < 0.05$) between values.

Fraction B obtained from Sephadex G-25 size exclusion chromatography was further separated by DEAE-cellulose column. Three fractions (B-1, B-2 and B-3) with ABTS radical scavenging activity were obtained from anion exchange chromatography (Fig. 2). The results suggested that most of the peptides in fraction B were negatively charged and those with lower negative charge were dominant (fraction B-1). The peptides with higher negative charge (B-2 and B-3) were eluted with increasing concentrations of NaCl. Fraction B-2 showed the highest ABTS radical scavenging activity (P<0.05) (Fig. 2B). A similar result was reported for antioxidant peptides from tilapia skin gelatin hydrolysates, in which highly charged peptides showed the highest antioxidant activity (ZHANGE *et al*., 2012). Peptide charge can vary depending on hydrolysis conditions such as the type of enzyme and substrate used. When peptides from a skipjack roe hydrolysate were separated by cation exchange, fractions with lower charges had stronger antioxidant

properties (INTARASIRISAWAT *et al*., 2013). In order to remove salt from fraction B-2, a Sephadex G-25 column was used. The desalted fraction was subsequently lyophilized.

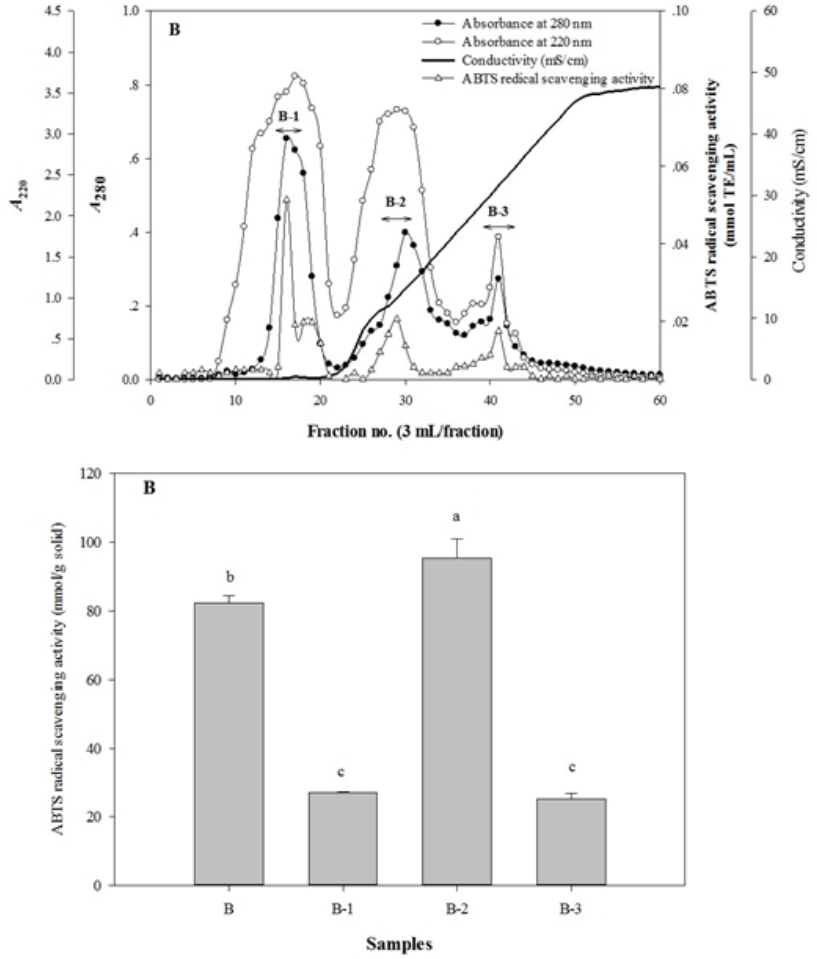

Figure 2. Elution profile of fraction B on a DEAE-cellulose column (A) and ABTS radical scavenging activity of fractions (B). Values represent mean ± SD. Different letters on the bars indicate significant differences ($p < 0.05$) between values.

Fraction B-2 was further fractionated by RP-HPLC on a C-18 column using a linear gradient of acetonitrile containing 0.1% TFA. As shown in Fig. 3, eighteen fractions were obtained from the RP-HPLC column and their ABTS radical scavenging activities were evaluated. The highest antioxidant activity was observed in fraction B-2/4 (0.72 total activity/A_{220}), followed by fraction B-2/8 (0.64 total activity/A_{220}). The fractions with higher hydrophobicity were eluted with increasing acetonitrile concentration. It was reported that the more hydrophobic peptides fractionated from enzymatic hydrolysates of tilapia frame protein using semi-preparative C18 RP-HPLC exhibited the highest antioxidant activity (FAN *et al*., 2012). In contrast, the more hydrophilic peptides obtained from a gelatin hydrolysate of tilapia skin using RP-HPLC column had the stronger antioxidant activity (ZHANG *et al*., 2012). In the present study, fraction B-2/4 and B-2/8, which demonstrated the highest antioxidant, activity were selected for peptide sequence identification using UPLC-MS/MS.

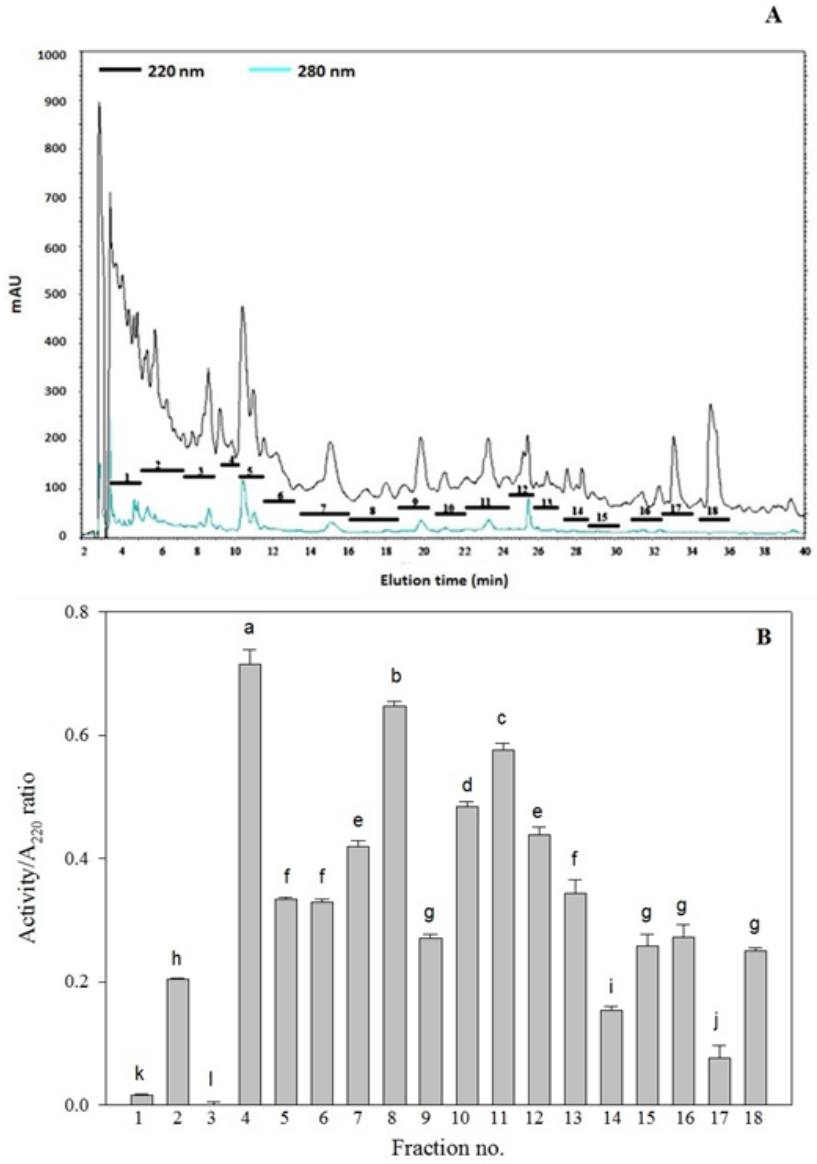

Figure 3. Elution profile of fraction B-2 on RP-HPLC (A) and ABTS radical scavenging activity/A_{220} ratio of fractions (B). Absorbance at 220 nm (-). Absorbance at 280 nm (-). Values represent mean ± SD. Different letters on the bars indicate significant differences ($p < 0.05$) between values.

3.2 Identification of antioxidant peptides

The identification of peptide sequences within selected fractions obtained from RP-HPLC (fraction B-2/4 and B-2/8) were determined by UPLC-MS/MS. Thirty peptides were identified with molecular weight range from 279.17 to 555.28 Da in fraction B-2/4. In fraction B-2/8, twenty six antioxidant peptides observed had MW ranging from 366.20 to 897.37 Da. Two peptides were selected from each fraction followed the criteria as mentioned above (Table 1). Glu-Pro-Gly-Pro-Val-Gly (555.27 Da) and Leu-Pro-Gly-Pro-Ala-Gly (511.29 Da) were identified from fraction B-2/4 (Fig. 4). Another two peptides, Leu-Asp-Gly-Pro-Val-Gly (557.30 Da) and Glu-Gly-Pro-Leu-Gly (472.24 Da), were

identified from fraction B-2/8 (Fig. 5). In this study, each identified peptide possessed a specifically arranged amino acid sequence typical of collagen and gelatin, where glycine strictly represents every third amino acid residue (LI *et al*., 2007). Moreover, the molecular weight of the peptides was similar to the antioxidant peptides isolated from tilapia gelatin hydrolysate (317.33-645.21 Da) (ZHANG *et al*., 2012; SUN *et al*., 2013). In addition, the selected peptides contained Gly at the C-terminal, indicating that the glycyl endopeptidase activity used for preparing the gelatin hydrolysates specifically cleaves peptide bonds with Gly at P_1 position (KARNJANAPRATUM *et al*., 2014).

Table 1. ABTS radical scavenging activity of synthesized peptides

Peptide sequence	Molecular weight (Da)	ABTS radical scavenging activity (μmol TE/g solid)
Glu-Pro-Gly-Pro-Val-Gly	555.28	1.25±0.36^b
Leu-Pro-Gly-Pro-Ala-Gly	511.29	1.22±0.10^b
Leu-Asp-Gly-Pro-Val-Gly	557.30	1.36±0.12^b
Glu-Gly-Pro-Leu-Gly	472.24	4.95±0.65^a

Values represent the mean ± SD for three separated experiment. Different letters in the same column indicate significant differences ($p < 0.05$) between values in the same column.

3.3. Antioxidant activity of synthesized peptides

Selected peptides (Glu-Pro-Gly-Pro-Val-Gly, Leu-Pro-Gly-Pro-Ala-Gly, Leu-Asp-Gly-Pro-Val-Gly and Glu-Gly-Pro-Leu-Gly) were synthesized for further study. All four synthetic peptides had antioxidant activity (Table 1) with Glu-Gly-Pro-Leu-Gly having the highest ABTS radical scavenging activity ($P<0.05$). The remaining three synthetic peptides all demonstrated similar antioxidant activity ($P>0.05$). Therefore, the amino acid sequence played an important role in antioxidant activity. Peptides rich in hydrophobic amino acids (Gly, Pro), which contribute substantially to free radical scavenging, were previously found in Pacific cod skin gelatin hydrolysate (NGO *et al*., 2011). The side chain of Gly consists of a single hydrogen atom and may confer high flexibility on the peptide bond. The pyrrolidine ring of Pro tends to interrupt the secondary structure of the peptide, thus imposing conformational constraints (RAJAPAKSE *et al*., 2005; ALEMAN *et al*., 2011). Glu and Leu acted as direct radical scavengers in a peptide purified from fish skin gelatin (ZHANG *et al*., 2009; MENDIS *et al*., 2005). It was noted that GH herein showed a higher ABTS radical scavenging activity (65.49 μmol TE/g sample) (Fig. 1B), compared with those of the synthesized peptides (1.22-4.95 μmol TE/g sample). This suggests that several peptides and/or free amino acids identified within the antioxidant fractions may exert a synergistic effect on radical scavenging activity.

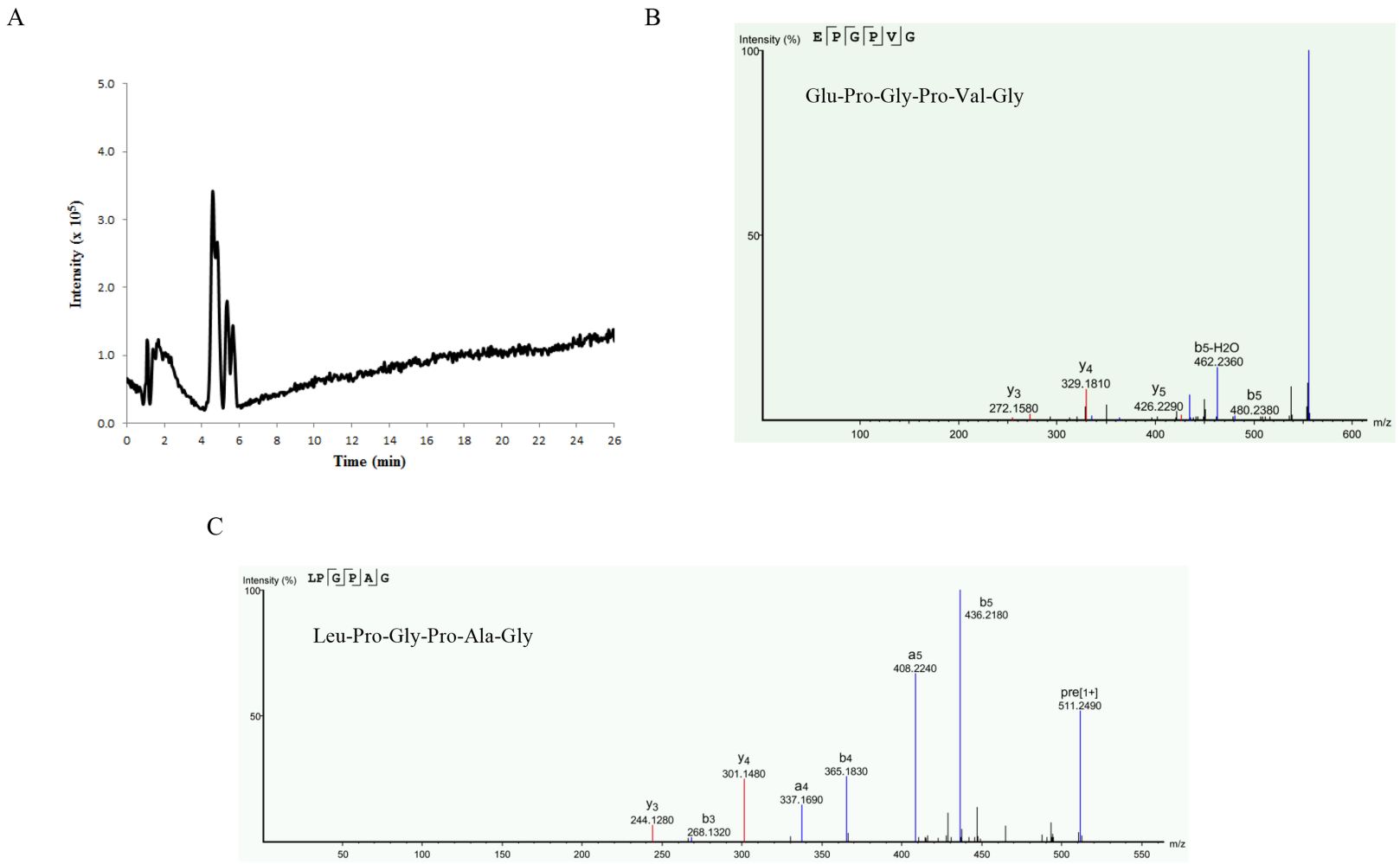

Figure 4. Base peak chromatogram (BPC) of B-2/4 fraction separated by Acquity UPLC BEH C18 column (A) and representative MS/MS spectra of peptides Glu-Pro-Gly-Pro-Val-Gly (B) and Leu-Pro-Gly-Pro-Ala-Gly (C). The x-axis shows the *m/z* of the precursor (pre) and fragment ions while the y-axis shows the relative intensity. The deduced sequence can be seen on the top left.

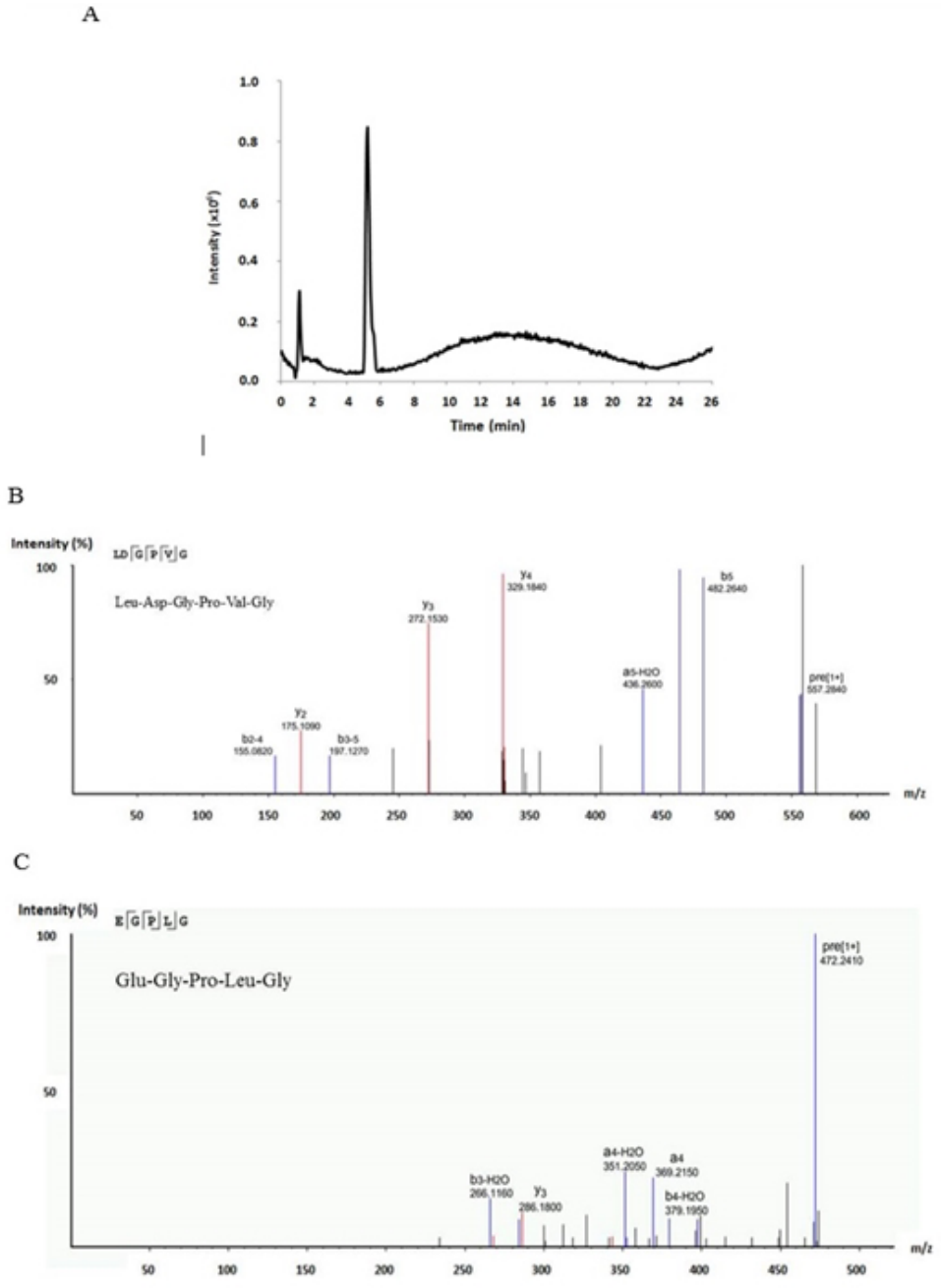

Figure 5. Base peak chromatogram (BPC) of B-2/8 fraction separated by Acquity UPLC BEH C18 column (A) and representative MS/MS spectra of peptides Leu-Asp-Gly-Pro-Val-Gly (B) and Glu-Gly-Pro-Leu-Gly (C). The x-axis shows the *m*/*z* of the precursor (pre) and fragment ions while the y-axis shows the relative intensity. The deduced sequence can be seen on the top left.

A similar result was reported for a patin (*Pangasius sutchi*) protein hydrolysate prepared using Alcalase and papain, in which the purified peptide obtained had a lower antioxidant activity, in comparison with a crude patin hydrolysate (NAJAFIAN *et al*., 2013). Therefore, the combination of peptides present in protein hydrolysates could produce higher bioactivity than the isolated peptides (ALUKO, 2015).

4. CONCLUSIONS

GH from unicorn leatherjacket skin prepared using glycyl endopeptidase contained peptides possessing free radical scavenging activity. Four antioxidant peptides were identified as Glu-Pro-Gly-Pro-Val-Gly (555.27 Da), Leu-Pro-Gly-Pro-Ala-Gly (511.29 Da), Leu-Asp-Gly-Pro-Val-Gly (557.30 Da) and Glu-Gly-Pro-Leu-Gly (472.24 Da). GH and its peptides could be potential candidates for functional food ingredients or nutraceuticals which protect against free radical generation and related diseases.

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Paper Received August 28, 2016 Accepted November 5, 2016