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ORIGINAL PAPER

Purification and Characterization of a Radical Scavenging Peptide from Rapeseed Protein Hydrolysates

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Abstract We previously reported that crude rapeseed peptides (CRPs) and peptide fractions (RP25 and RP55) prepared from aqueous enzymatic extraction of rapeseed exhibited marked antioxidant activities, among which RP55 showed the most potent effects. In the present study, RP55 was further purified using consecutive chromatographic methods for identification of antioxidant peptides. The α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging effects of peptides were measured at each purification step to evaluate the antioxidant activities. RP55 was first fractionated by anion-exchange chromatography, and three fractions (E1, E2, and E3) were obtained. All of them showed significantly lower scavenging activities compared to RP55, which was very probably due to the remarkable loss of tannin during the separation. Next, the active fraction E2 with higher protein content was sequentially purified by gel filtration chromatography (GFC) and reversed-phase high performance liquid chromatography (RP-HPLC). The purified peptide, of which the median effective dose (ED_{50}) value for DPPH radical scavenging was 0.063 mg/mL, was identified to be Pro-Ala-Gly-Pro-Phe (487 Da) using electrospray ionization (ESI) mass spectrometry. The unique amino acid composition and sequence in the peptide might play an important role in expression of its antioxidant activity.

Keywords Rapeseed peptides · Radical scavenger · α,α-diphenyl-β-picrylhydrazyl (DPPH) · Purification · Electrospray ionization mass spectrometry

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Introduction

Aerobic organisms must deal with free radicals that are generated from sequential reduction of oxygen during the normal course of aerobic metabolism. These radicals may cause cellular damage leading to a number of pathological conditions, including atherosclerosis, arthritis, diabetes, and carcinogenesis, if produced in an uncontrolled manner [1]. In addition, radical mediated oxidation of fats and oils is also of a great concern in the food industry, as it leads to the development of undesirable off-flavors and potentially toxic reaction products. Therefore, during the last few decades, there has been a growing interest in the identification and characterization of natural antioxidants. Recently, it has been shown that, upon hydrolysis with enzymes, many food proteins can act as direct scavengers towards diverse free radicals or antioxidants in model systems [2–5]. Furthermore, for elucidating the structure–activity relationship or the antioxidant mechanism, several studies have focused on the structural analysis of the antioxidant peptides derived from various plant and animal origin, such as soybean protein [6], egg yolk protein [7], whey protein [8], fish skin gelatin [9], venison protein[10], and bullfrog skin [11]. It appears that the antioxidant

activity of peptides is closely related to amino acid compositions and their sequences.

Crude rapeseed peptides (CRPs) and three peptide fractions (RP25, RP55, and RP85) could be recovered from the downstream processes for aqueous enzymatic extraction of rapeseed according to our previous studies [12, 13]. Moreover, we found that RP55 showed more potent antioxidant properties, except for hydroxyl radicals scavenging ability, than other fractions, which was attributed to the unique amino acid composition or significantly higher contents of non-protein antioxidant compounds in RP55 [14]. Up to now, little has been known about the structure of rapeseed antioxidant peptides. Therefore, further fractionation studies are necessary to identify and elucidate the compounds in RP55 that play a decisive role in antioxidant activities.

α,α-diphenyl-β-picrylhydrazyl (DPPH) is a stable free radical that shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical would be scavenged and the absorbance reduced. This radical-scavenging assay is quick, convenient and reproducible, and thus widely used in predicting the antioxidant activities of compounds [15–17]. In the previous research [14], the DPPH radical scavenging effects of rapeseed peptides correlated well with their reducing power and inhibitory activities on lipid peroxidation.

The objective of this study is to further purify RP55 and characterize rapeseed antioxidant peptides using consecutive chromatography and tandem mass spectrometry. The scavenging effects on DPPH radical of rapeseed peptides were measured at each purification step to evaluate their antioxidant activities.

Materials and Methods

Materials

RP55 was prepared according to our previous work [12, 13]. α,α-diphenyl-β-picrylhydrazyl (DPPH), Sephadex G-15 and acetonitrile (HPLC-grade) were purchased from Sigma Chemical (St. Louis, MO, USA). Catechin was provided by Taiyo Green Power (Wuxi, China). Macroporous adsorption resin (MAR) and anion-exchange resin were sourced from Jiangsu Suqing Water Treatment Engineering Group (Wuxi, China). All other chemicals used in the experiments were of analytical grade.

Preparation of RP55

Rapeseed peptide fraction RP55 was prepared under optimum conditions according to our previous work [12, 13]. Briefly, the dehulled rapeseeds (2 kg) were boiled for 5 min (seeds-to-water ratio of 1:3, w/v), followed by milling to obtain the uniform rapeseed slurry. Then, part of the rapeseed slurry (containing 800 g original dry rapeseeds) was taken and diluted to seeds-to-water ratio of 1:5 (w/v). The suspension was heated to 48 °C and adjusted to pH 5, followed by addition of the combination of pectinase (1.2×10^5 IU), cellulase (2.2×10^3 IU), and beta-glucanase (4.2×10^3 IU) in the ratio 4:1:1 (v/v/v) at 2.5% (v/w) and further incubation for 4 h. Next, the slurry was heated to 60 °C and the pH was adjusted to 10, with incubation for 30 min followed by readjusting the pH to 9. Alcalase 2.4 L (2.9×10^5 IU) was added at 1.4% (v/w) of the dry rapeseed and the slurry was incubated for 3 h followed by heating at 90 °C for 10 min. After that, the slurry was centrifuged at 1,819*g* for 15 min to obtain the free oil, the aqueous phase, the emulsion and the solid phase.

50 mL of the obtained aqueous phase with a protein concentration of about 40 mg/mL was adjusted to pH 4 with acetate acid and then was pumped through a glass column (180 mL) with MAR at a flow rate of 1 mL/min. Next, stepwise elution was carried out with 1 L deionized water previously adjusted to pH 4, 600 mL of 25% (v/v) ethanol, and 600 mL of 55% (v/v) ethanol at a flow rate of 1.5 mL/min, respectively. The 55% ethanol-eluent was concentrated and freeze-dried to obtain the rapeseed peptide fraction RP55.

Ion-Exchange Chromatography

RP55 (1.2 g) was dissolved in 30 mL of 50 mM sodium borate buffer (pH 9.0) and pumped through a glass column (180 mL) packed with anion-exchange resin at a flow rate of 1 mL/min, which was previously equilibrated with 50 mM sodium borate buffer (pH 9.0). Then, the column was rinsed with deionized water to remove the unbound peptides at a flow rate of 2 mL/min. After rinsing, stepwise desorption with respective 0.1 M sodium phosphate buffer (pH 7.0), 0.2 M sodium acetate buffer (pH 4.0), and 0.2 M HCl was performed at the same flow rate. The complete course was monitored using a UV detector at 220 nm. For desalting, at each desorption step MAR was used to trap the peptides from the ion-exchange eluent, followed by fully rinsing of the MAR with deionized water and finally desorption of the peptides using 85% (v/v) ethanol. By this way, three desalted fractions (E1, E2, and E3, respectively) were obtained and freeze-dried for further analysis.

Gel Filtration Chromatography (GFC)

The fraction (120 mg) from anion-exchange chromatography exhibiting powerful radical scavenging activity was

dissolved in 4 mL deionized water and loaded onto a Sephadex G-15 gel filtration column (1.6 × 150 cm). Separation was obtained with deionized water at a flow rate of 20 mL/h. Each fraction was monitored at 220 nm, collected, concentrated, and freeze-dried for radical scavenging activity assay.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

The fraction showing the highest antioxidant activity after GFC process was further purified using RP-HPLC on a Hedern ODS-2 C_{18} column (10 × 250 mm). The fraction was dissolved in 5% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid (TFA). The elution was performed using a mobile phase consisting of 5% (v/v) acetonitrile containing 0.05% (v/v) TFA (named as eluent A) and 80% (v/v) acetonitrile containing 0.05% (v/v) TFA (named as eluent B). Gradient elution was carried out according to the following process: 0–5 min, 100–80% A; 5–20 min, 80–60% A; 20–25 min, 60–0% A; 25–27 min, 0% A; 27–29 min, 0–100% A; 29–40 min, 100% A. The flow rate was 2 mL/min and the detection wavelength 220 nm. The main peaks were collected and freeze-dried for radical scavenging activity evaluation. For further purification, the potent fraction from the first HPLC was loaded onto a SunFire TM C_{18} ODS analytical column (4.6 × 150 mm). The mobile phase was the same as described above and gradient elution was carried out according to the following design: 0–10 min, 100–80% A; 10–20 min, 80–40% A; 20–23 min, 40–0% A; 23–25 min, 0% A; 25–27 min, 0–100% A; 27–32 min, 100% A. The flow rate was 1 mL/min and the detection wavelength 220 nm. The main peaks were pooled and freeze-dried for radical scavenging activity assay. The final potent fraction was analyzed for amino acid sequence.

DPPH Radical Scavenging Assay

The scavenging effects of peptide fractions on DPPH free radical were measured according to the procedure described previously [14]. The sample solution (2 mL) with various hydrolysate concentrations was added to 2 mL of 0.1 mM DPPH in 95% ethanol (2 mL of 95% ethanol in place of the DPPH solution as sample blank). The mixture was shaken and left for 30 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm. At each concentration of the hydrolysates, triplicate determinations were carried out. The scavenging effects were calculated according to Eq. 1.

$$\text{scavenging effect}, \% = \frac{[C - \text{CB}] - [S - \text{SB}]}{[C - \text{CB}]} \times 100\% \quad (1)$$

where S, SB, C, and CB represent the absorbance of the sample, the sample blank, the control, and the control blank, respectively.

Determination of the Amino Acid Sequence of the Purified Peptide

The molecular mass and amino acid sequence of the purified peptide were determined with a quadrapole time-of-flight (Q-TOF) mass spectrometer (Waters, Milford, MS) coupled with an electrospray ionization (ESI) source in positive mode. The purified peptide was dissolved in methanol/water (1:1, v/v) and then infused into the ESI^+ source. Spectra were recorded over the mass/charge (m/z) range 50–1,000. Following molecular mass determination, the peptide was fragmented using a low energy collision induced dissociation to reveal the peptide fragment for de novo sequencing. Sequence information was obtained by processing the raw spectra with Masslynx software (V4.0; Waters).

Proximate Analysis

The protein content (Kjeldahl N × 6.25) was determined according to the AOAC official methods [18]. The tannin content was analyzed by the vanillin–HCl, 1% HCl in methanol extraction, method, which is specific to determination of condensed tannin [19], using catechin as the standard.

Results and Discussion

The rapeseed peptide fraction RP55 prepared from the downstream processes for aqueous enzymatic extraction of rapeseed exhibited potent antioxidant activities [14]. Therefore, it was selected for further purification to identify the antioxidant peptides and DPPH radical scavenging activity was tested at each purification step. During the first purification, RP55 was fractionated by anion-exchange chromatography, and three distinct bound fractions (E1, E2, and E3) were obtained by the stepwise desorption (Fig. 1). The median effective dose (ED_{50}, meaning the concentration that scavenges 50% of the initial DPPH radical) values for fraction E2 and E3 were 0.159 and 0.143 mg/mL, respectively. E2 and E3 showed higher radical scavenging activities than E1 (ED_{50} = 0.388 mg/mL). However, all of them exhibited significantly lower activities than RP55 (ED_{50} = 0.041 mg/mL, according to the

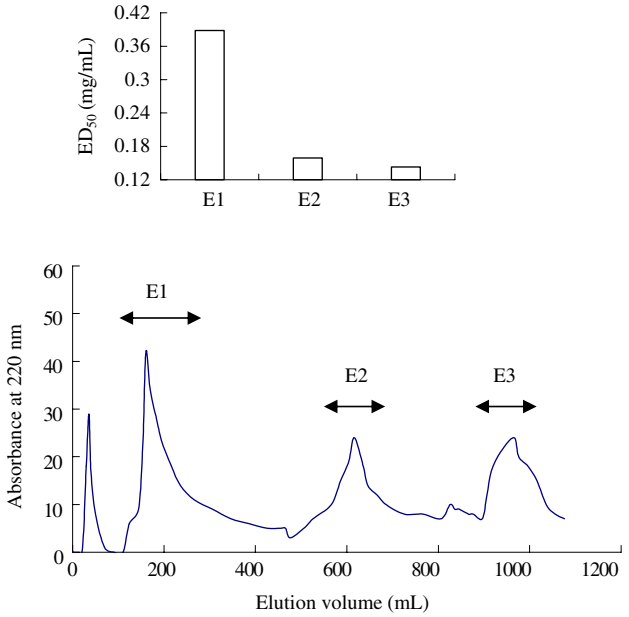

Fig. 1 Anion-exchange chromatography of rapeseed peptide fraction RP55. Stepwise desorption was performed at a flow rate of 2 mL/min with 0.1 M sodium phosphate buffer (pH 7.0), 0.2 M sodium acetate buffer (pH 4.0), and 0.2 M HCl, respectively. The DPPH scavenging effects (indicated by ED_{50} values) of the fractions are shown in the *upper panel*

previous research [14]) by comparison of the ED_{50} values, which indicated that the purification process caused a decrease in the scavenging activities of rapeseed peptides.

As shown in Table 1, compared to RP55, the tannin content of peptide fractions (E1, E2, and E3) was sharply decreased while their protein content remained close. Phenolic compounds like tannin generally carry a lot of negative charges at high pH and can adsorb tightly on the anion resin. Hence, tannin was not easy to be desorbed from the resin in this study, which led to a significant loss of tannin in the resulting peptide fractions. Although the amino acid compositions of the peptide fractions were varied (results not shown), it was speculated that tannin reduction was the predominant factor that resulted in the marked decrease in DPPH scavenging effects. The

Table 1 Proximate analysis of rapeseed peptide fractions (on a dry basis)

	RP55[a]	E1	E2	E3
Protein (%)	72.69 ± 1.24	72.39 ± 1.43	75.18 ± 1.78	69.82 ± 1.14
Tannin (%)	3.08 ± 0.20	ND	0.41 ± 0.03	0.92 ± 0.07

Data were expressed as mean with standard deviations ($n = 3$)

ND Not detected

[a] Values are from the previous study [13]

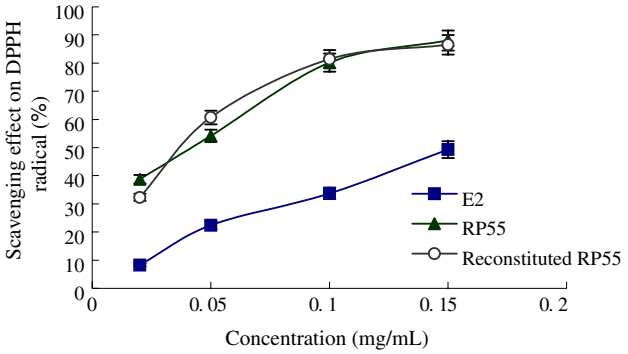

Fig. 2 Scavenging effects on DPPH radical of rapeseed peptides. Data are expressed as mean with standard deviations ($n = 3$). The values for RP55 are from the previous study [14]. Reconstituted RP55 was a mixture of E2 and catechin with an amount equal to the tannin difference between E2 and RP55

experimental results presented in Fig. 2 strongly supported this speculation. It can be seen that reconstituted RP55 (a mixture of E2 and catechin with an amount equal to the tannin difference between E2 and RP55) exhibited close scavenging activity to RP55, indicating that the addition of catechin into E2 effectively restored its antioxidant activity. The added catechin, although the amount was small,

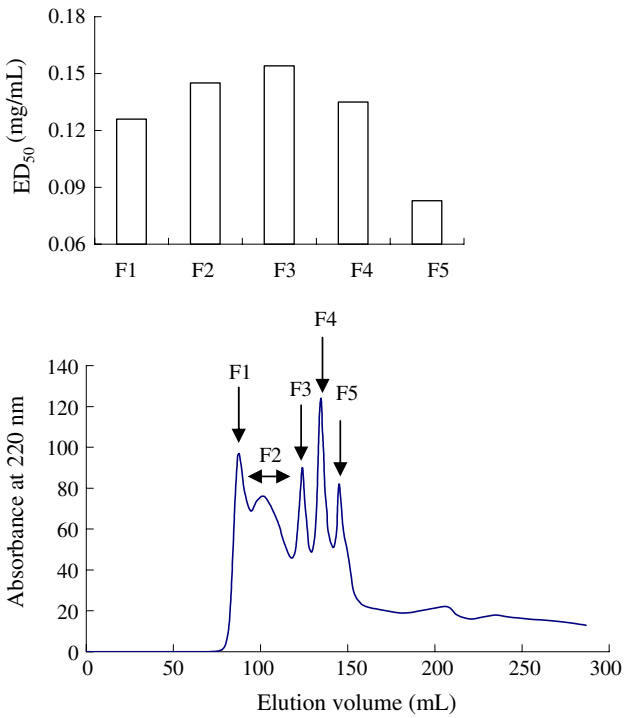

Fig. 3 Gel filtration chromatography of active fraction E2 from anion-exchange chromatography on a Sephadex G-15 column. The column was eluted with deionized water at a flow rate of 20 mL/h. The DPPH radical scavenging effects (indicated by ED_{50} values) of the fractions are shown in the *upper panel*

exerted significantly higher scavenging effect on DPPH than E2 (data not shown). These results suggest that the phenols may play a more important antioxidant role than peptides in rapeseed. The strong antioxidative properties of phenolic compounds in rapeseed were also observed by other researchers. Matthäus [20] reported that the extracts from the fat-free residues of the different oilseeds (including rapeseed) contained considerable amounts of phenolic compounds and had remarkable radical scavenging activities. Yoshie-Stark et al. [21] found that the ED_{50} values for DPPH radical of the de-oiled rapeseeds were much smaller than those of the protein concentrates from which a high proportion of the phenols were removed.

Although the radical scavenging effect of E3 was slightly higher than that of E2, we selected the latter as material for further peptide identification due to its higher protein content and also lower tannin content. Fraction E2 was then applied onto a Sephadex G-15 gel filtration column and eluted with deionized water. The fifth fraction (F5) among five resulting fractions had the highest DPPH scavenging activity (ED_{50} = 0.083 mg/mL) (Fig. 3). Figure 3 shows that small peptides might be more potent radical scavengers. Comparing the ED_{50} values of F5 and E2, it could be concluded that GFC purification resulted in a significantly higher DPPH scavenging activity than that observed after ion-exchange chromatography. Also, the protein content (86.26%, on a dry basis) of F5 was significantly higher and no tannin was detected, which suggests that peptides play a decisive role in expression of the powerful scavenging activity of F5.

The potent fraction F5 obtained from GFC was then further separated on semi-preparative C_{18} HPLC column into 12 different peaks (Fig. 4a). The 8th elution peak exhibited the highest DPPH scavenging activity among the

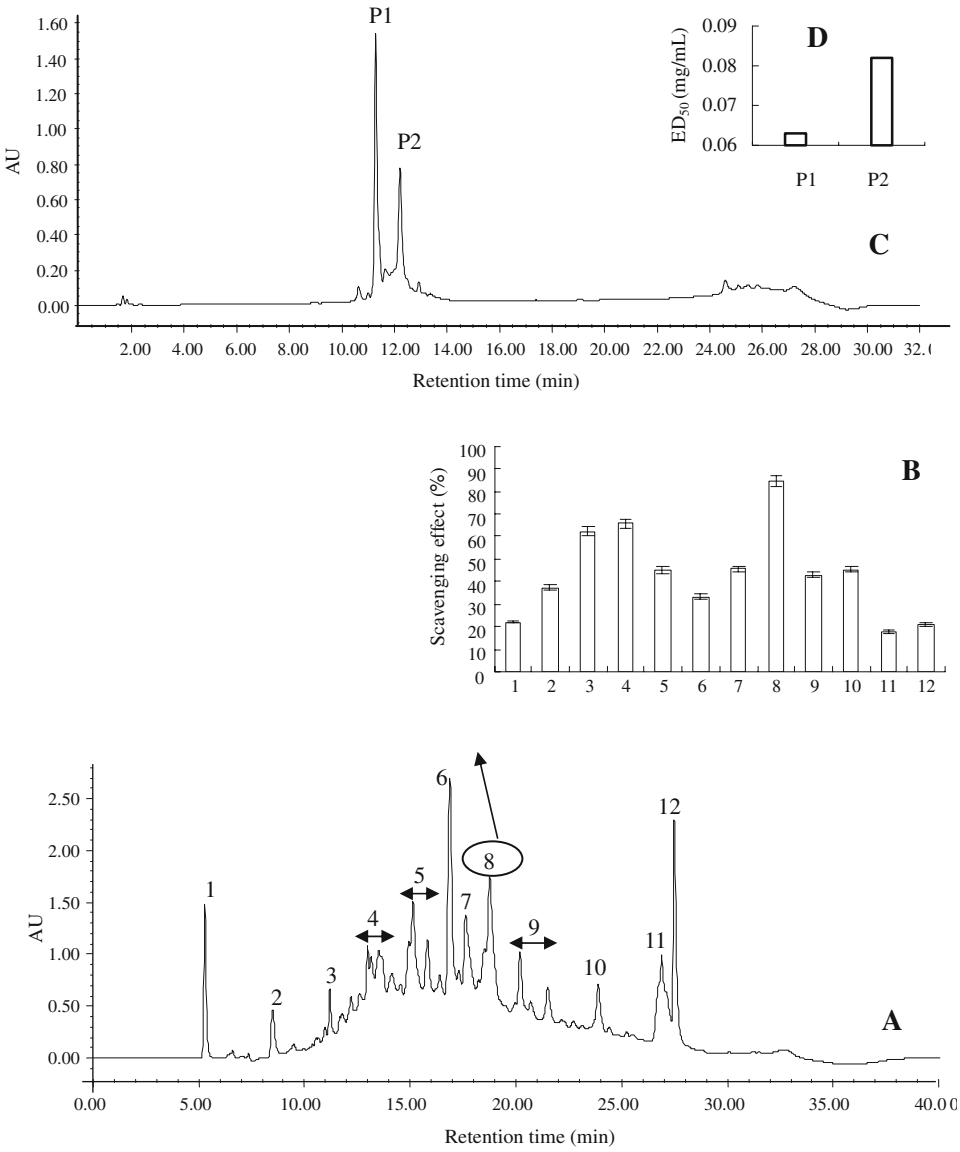

Fig. 4 **a** Reverse-phase HPLC pattern on a C_{18} column (10 × 250 mm) of active fraction F5 from gel filtration chromatography. **b** The DPPH scavenging effects of the fractions at a concentration of 0.1 mg/mL. Data are expressed as mean with standard deviations ($n = 3$). **c** Reverse-phase HPLC pattern on a C_{18} column (4.6 × 150 mm) of active fraction (peak 8) from the first HPLC separation. **d** The DPPH scavenging effects (indicated by ED_{50} values) of the fractions from the second HPLC separation

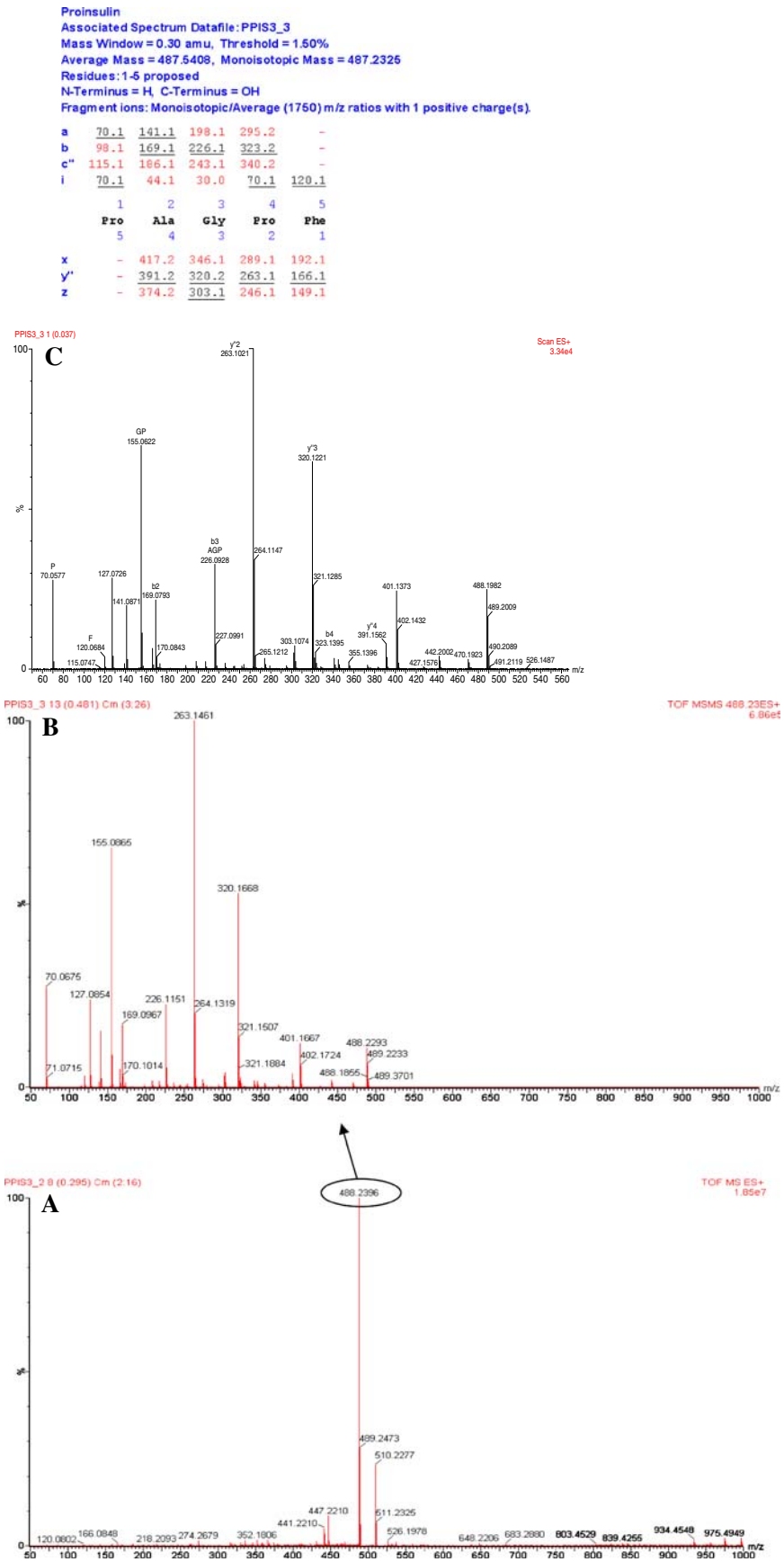

Fig. 5 Identification of the radical scavenging peptide. **a** MS spectrum of active fraction P1 from the HPLC. **b** MS/MS spectrum of ion *m/z* 488.2. **c** The results obtained by processing the MS/MS spectrum of ion *m/z* 488.2 with Masslynx software. The sequence of this peptide is shown in the *upper panel*, as well as the fragment ions which could be found in the MS/MS spectrum (*underlined*) (the peptide matched 38–42 residues of napin [25]). The main fragment ions in the MS/MS spectrum are labeled in the *lower panel*

12 peaks (Fig. 4b) and was finally purified by analytical C_{18} HPLC column into two peptide fractions (P1 and P2) (Fig. 4c). Fraction P1 was more potent to scavenge DPPH radical than P2 (Fig. 4d). However, the scavenging activity of P1 ($ED_{50} = 0.063$ mg/mL) was a little lower than that of its precursor fraction ($ED_{50} = 0.054$ mg/mL), which was attributed to the possible synergistic effects of peptides in the latter. Figure 4 reveals that the scavenging activities of rapeseed peptides had no direct correlation with their hydrophobicity. The similar conclusion was also made by Chen et al. [22] in the case of the His-containing peptides. Recently, it has been widely thought that hydrophobic amino acids act as effective antioxidants in a linoleic acid emulsion system by increasing the affinity to lipophilic fatty acids and thereby facilitating better interaction with free radicals [23, 24]. However, the hydrophobicity of peptides may not be an important factor in expression of their DPPH radical scavenging activities assayed in the aqueous ethanol system. By comparison of the ED_{50} values, the scavenging effect of the finally purified peptide fraction P1 was still slightly lower than that of the raw material (RP55). This was not surprising because RP55 contained considerable amounts of potent antioxidative non-protein compounds such as the phenols. Also, the antioxidant activity of this purified peptide was markedly lower than that of ascorbic acid ($ED_{50} = 7$ μg/mL, according to the previous research [14]).

For peptide identification, the active fraction P1 was subjected to ESI mass spectrometry in positive mode. The MS spectrum of P1 is shown in Fig. 5a. A major single charged ion $[M + H]^+$ with *m/z* at 488.2 in the spectrum indicated that P1 was a purified peptide with the molecular mass being 487 Da. Another obvious single charged ion with *m/z* at 510.2 was highly likely $[M + Na]^+$. The MS/MS spectrum of the ion with *m/z* at 488.2 was illustrated in Fig. 5b. An amino acid sequence (Pro-Ala-Gly-Pro-Phe) for P1 was then proposed by the Biolynx peptide sequencer from the MS/MS spectrum and the main fragment ions were labeled (Fig. 5c). The identified antioxidant peptide Pro-Ala-Gly-Pro-Phe matched 38–42 residues of napin [25].

It should be pointed out that neither the structure–activity relationship nor the antioxidant mechanism of peptides is fully understood. Generally, the scavenging of free radicals was attributed to the donation of hydrogen/electron. Some amino acids were widely believed to be direct radical scavengers due to their special groups in side chains, such as His (imidazole group) [6], Trp (indolic group), and Tyr (phenolic group) [8, 26]. These groups could act as hydrogen donors. Additionally, Met is prone to oxidation of the Met sulfoxide and Cys donates the sulfur hydrogen [8]. Aromatic amino acids (Tyr and Phe) are generally considered as effective radical scavengers, because they can donate protons easily to electron deficient radicals while at the same time maintaining their stability via resonance structures [27]. Therefore, the C-terminus Phe of the identified peptide (Pro-Ala-Gly-Pro-Phe) in this study can be expected as a strong proton-donating residue in the sequence. Moreover, this peptide had two residues of Pro, which has been thought to contribute considerably to the antioxidant activities of soybean peptides [28] and fermented milk peptides [29]. Earlier, Smirnoff et al. [30] reported that Pro was an effective hydroxyl radical scavenger.

Not only amino acid compositions but also special sequences determine the antioxidant activities of peptides. Some identified peptides, although containing few strong proton-donating amino acids as mentioned above, exhibited remarkable antioxidative effects [17, 31]. Recently, a few specific active sequences in the antioxidant peptides have been proposed by researchers. For example, Suetsuna et al. [32] found that the potent radical scavenging activity of the peptide (Tyr-Phe-Tyr-Pro-Glu-Leu) strongly depended on the dipeptide (Glu-Leu), although deletion of N-terminus Tyr, Tyr-Phe, and Tyr-Phe-Tyr caused a loss of activity. Saito et al. [33] screened 40 peptides structurally related to a soybean antioxidant peptide (Leu-Leu-Pro-His-His) and identified Pro-His-His as the active center. In the case of gelatin-derived protein hydrolysates, Kim et al. [34] isolated three antioxidative peptides with one or more repeating sequences (Gly-Pro-Hyp) from bovine skin. Mendis et al. [9] further reported a strong antioxidative peptide (His-Gly-Pro-Leu-Gly-Pro-Leu) from fish skin gelatin, which represented two repeating amino acid residues (Gly-Pro) placing Leu or His at the other position. Therefore, we assume that the Gly-Pro sequence in the identified rapeseed peptide may also play an important role in expression of its antioxidant activity, in addition to the unique amino acid composition of the peptide.

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