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Purification and characterization of high antioxidant peptides from duck egg white protein hydrolysates

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ABSTRACT

The hydrolysate from duck egg white protein (DEWP) prepared by "SEEP–Alcalase" at degree of hydrolysis (DH) value of 21% (namely HSA_{21}) exhibited high antioxidant capacity in different oxidation systems. A consecutive chromatographic method was then developed for separation and purification of HSA_{21}, including ion-exchange chromatography, macroporous adsorption resin (MAR) and gel filter chromatography. The final peptides "$P_{21\text{-}3\text{-}75\text{-}B}$" were obtained with significantly enhanced antioxidant activity ($p < 0.05$). It was further confirmed that the product mainly consisted of five oligopeptides (Mr: 202.1, 294.1, 382.1, 426.3, and 514.4 Da). Furthermore, the antioxidant activity of $P_{21\text{-}3\text{-}75\text{-}B}$ kept stable after *in vitro* digestive simulation. Antioxidant capacity of the purified peptides was closely related to the molecular mass, hydrophobic amino acid residues, acidic amino acid and some antioxidant amino acids. This research provided a valuable route for producing new natural-source peptides with strong antioxidant capacity and high nutritious value for our daily intake.

1. Introduction

Naturally, there is a dynamic balance between free radicals and antioxidant substances in human bodies [1]. Once the antioxidant enzymes, such as superoxide dismutase and catalase, cannot scavenge free radicals promptly, the balance may be disrupted, leading to cells or tissues injury, even cardiovascular diseases and cancer [2]. Thus, it is a present urgent requirement to search a safe, easily absorbed, nutrient and antioxidant edible.

Duck egg is one of the most commonly used eggs in food manufactures in China. A large amount of duck egg white protein (DEWP) was left as byproduct. Considering the quantity and proportion of essential amino acids, DEWP was an ideal edible protein suitable to be absorbed in human intestinal tract [3]. Therefore, it was chosen to prepare peptides with high antioxidant activity and additional value by enzymatic hydrolysis. Although the hydrolysates from DEWP displayed high antioxidant activity [4], abundant impurities and some low antioxidant peptides were included excepted for meritorious peptides. The application of separation and purification procedures was very essential to remove these undesirable components for improving the bioactivity and determining the composition of novel peptides expediently [5,6]. However, there is still little information regarding the isolation and purification of the DEWP hydrolysates.

In our ongoing research related to the preparation of natural-source peptides with high activities [4], we developed a consecutive chromatographic method for separation and purification of an antioxidant hydrolysate from DEWP by macroporous adsorption resin chromatography, ion-exchange chromatography and gel filer chromatography. The antioxidant activities of all the fractions from every purification process were evaluated and compared in different assays, including DPPH·, ·OH, O_2^{-}· radical scavenging activity and reducing power. Furthermore, the molecular mass and amino acids composition of the purified peptides were determined by electron spray ionization tandem mass spectrometry (ESI-MS) and amino acid analyzer.

2. Materials and methods

2.1. Materials and chemicals

Fresh duck egg was provided by Guangzhou Agricultural Institute, China. Alcalase 2.4 L FG (Alcalase) and Pancreatin was purchased from Novo Enzyme Co., Denmark, A hydrolase specific for

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egg protein (SEEP) was purchased from Waldorf Co., China. DPPH was purchased from Johnson Matthey Co., U.K. DEAE sepharose FF was purchased from GE Co. America. Macro absorption resin (MAR) "DA201-C" was purchased from JiangYin Organic Chemical Plant, China. Sephadex G-15 was purchased from Sigma Co., America. All other chemicals and reagents were of analytical grade commercially available.

2.2. Preparation of DEWP hydrolysates

DEWP was isolated from fresh duck eggs, dissolved in distilled water with a final concentration of 50 mg/mL and pretreated at 95 °C for 40 min (pH 10). Then a two-step enzymatic procedure was applied, and the reaction was carried out by sequential addition of the enzymes SEEP and Alcalase. The optimization of specific parameters has been discussed in previous paper [4]. And the final hydrolysates were centrifuged at 10,000*g* for 10 min using a high speed refrigerated centrifuge (Eppendorf Co., Germany). The supernatant was freeze-dried using a freeze dryer (Christ Co., Germany), collected and stored at −18 °C.

2.3. Determination of degree of hydrolysis

When SEEP or Alcalase was used as biocatalyst, the reaction pH value was >7.0 and DH value was measured with "pH-stat" method as follows:

$$\text{Degree of hydrolysis (DH, \%)} = B \times \text{Nb} \times \frac{1}{\alpha} \times \frac{1}{\text{MP}} \times \frac{1}{h_{\text{tot}}} \times 100\% \quad (1)$$

where B is the volume of NaOH solution consumed, mL; Nb is the concentration of NaOH solution consumed, mmol/mL; α is the degree of dissociation of α-amino acid, $\alpha = [10(\text{pH} - \text{pK})]/[1 + 10(\text{pH} - \text{pK})]$ (the average pK of the amino, 7.0; pH, the value of the initial reaction). MP is the total mass content of the substrate protein (g). The h_{tot} value is the total quantity of peptide linkage unit of substrate protein, mmol/g, calculated from amino acid analysis by summing the molar concentrations of each individual amino acid (mmoles) per gram of egg white protein [7].

2.4. DPPH· radical scavenging activity assay [8]

To test tube A, 2 mL ethanol and 2 mL DPPH·-ethanol solution (20 mM) were added and incubated in darkness at room temperature for 30 min. The absorbance of the resulting solution A was measured at 517 nm using a UV 752S spectrophotometer (Lengguang Co., Shanghai, China) with ethanol as control. 2 mL of the sample solution (1 mg/mL) was mixed with 2 mL of 20 mM DPPH·-ethanol solution, giving solution B which was also incubated for 30 min in darkness at room temperature and measured at 517 nm. Determination of VC was as a positive control. The IC_{50} value was used to express the DPPH· radical scavenging activity of samples, which was the concentration of tested samples required for a scavenging rate of 50%. DPPH· radical scavenging activities of the samples were calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{A_0 - (A_x - A_{x0})}{A_0} \times 100\% \quad (2)$$

where A_0 is the absorbance of solution A; and A_x is the absorbance of solution B; A_{x0} is the absorbance of 1 mg/mL sample solution.

2.5. ·OH radical scavenging activity assay

The reaction was initiated by adding 2 mL of 8.8 mM H_2O_2 to the reaction mixture containing 2 mL of 9 mM $FeSO_4$, 2 mL of 9 mM salicylic acid in ethanol and 2 mL of hydrolysate (1 mg/mL). The reaction mixture (solution A) and distilled water (the control, solution B) were incubated at 37 °C for 30 min in a water bath, and their absorbance were measured at 510 nm. Determination of VC was as a positive control. ·OH radical scavenging activities of the samples were calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{A_0 - (A_x - A_{x0})}{A_0} \times 100\% \quad (3)$$

where A_0 is the absorbance of solution B, Ax is the absorbance of solution A; and Ax_0 is the absorbance of 1 mg/mL sample solution.

2.6. Determination of $O_2^{-\cdot}$ radical scavenging activity

The mixture containing 0.2 mL of hydrolysate (1 mg/mL), 4.5 mL of 50 mM Tris–HCl buffer (pH 8.2) and 4 mL of distilled water, was incubated at 25 °C for 10 min and then mixed with 0.3 mL of 3 mM pyrogallic acid solution, forming solution A. When the hydrolyzed sample was replaced by distilled water, the resulted solution was named as solution B. Determination of VC was as a positive control. $O_2^{-\cdot}$ radical scavenging activities of the samples were calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{K_0 - K_1}{K_0} \times 100\% \quad (4)$$

where K_0 is the slope of absorbance variation of solution B in 5 min, and K_1 is the slope of absorbance variation of solution A.

2.7. Determination of reducing power on Fe^{3+}

The reducing power was measured in terms of the absorbance at 700 nm according to "Siddhuraju" method [9]. 2 mL of sample (1 mg/mL) and 1.0% (w/v) $K_4Fe(CN)_6$ were added into 2 mL of 0.05 M phosphate buffer solution (pH 6.6). The mixture was incubated at 50 °C for 20 min, added with 2.5 mL of 10% (w/v) TCA, and centrifuged at 5000 g for 10 min. Then 2.5 mL of the supernatant, 0.5 mL of 0.1% (w/v) $FeCl_3$ and 2.5 mL of distilled water were mixed for a 10 min reaction. Then the absorbance of resulting solution was measured at 700 nm. Determination of VC was as a positive control.

2.8. Ion-exchange chromatography

The hydrolysate (0.5 g) was dissolved in 10 mL of 10 mM Tris–HCl buffer (pH 7.6) and loaded onto an ion-exchange column (2.6 × 60 cm) previously packed with DEAE sepharose FF and equilibrated with the above-mentioned buffer at a flow rate of 48 mL/h continually with the effluent monitored at 214 nm. Then 0–1.0 M NaCl solution in 10 mM Tris–HCl butter (pH 7.6) were loaded onto the chromatography column (2.6 × 60 cm) with a linear gradient at a flow rate of 48 mL/h. 8 mL of each fraction was collected and monitored at 214 nm. The fraction (namely A) with the desired peak was freeze-dried and collected for the following purification.

2.9. Macroporous resin purification

Fraction A of 100 mg/mL was loaded at a flow rate of 60 mL/h onto the chromatography column (2.6 × 60 cm) packed with MAR "DA-201C" continually and the effluent was monitored at 214 nm. The addition of the hydrolysates was stopped, when the absorption value reached higher than 0.1. Then the ethanol solution with the concentration of 25% (v/v) was loaded onto the chromatography column at the same flow rate of 50 mL/h. When the absorbance of the effluent at 214 nm exceeded 0.1, the eluting solvent (25% ethanol) was substituted for 50%, 75% and 100% (v/v)

ethanol in sequence. The fraction (namely B) with the desired peak was freeze-dried, and collected for the further purification.

2.10. Gel filter chromatography

5 mL of fraction B (50 mg/mL) was load onto a gel chromatography column (1.6 × 80 cm) packed with gel Sephadex G-15. Then ultrapure water was added as the eluant at a flow rate of 20 mL/h, and the effluent was monitored at 214 nm. Each fraction was freeze-dried and collected to measure the antioxidant capacity. The fraction (namely C) with the highest antioxidant capacity was freeze-dried and collected for further tests.

2.11. Amino acids composition analysis

To measure the amino acid compositions of different hydrolysates, the samples were digested in 6 M HCl solution containing 1% phenol at 105 °C for 24 h. Subsequently, the digested sample was vacuum-dried at 50 °C twice, dissolved in loading buffer and centrifuged at 10,000*g* for 15 min. The resulting supernatant was analyzed by HPLC on an amino acid column "PICO-TAG" (Hitachi Co., Japan) and monitored at 254 nm using the mobile phase (A solution: acetic acid/sodium acetate buffer solution (pH6.4), B solution: 60% (v/v) acetonitrile) at flow rate of 1.0 mL/min at 38 °C.

2.12. Evaluation of the hydrophobicity of the hydrolysates

The hydrophobic value of the hydrolysates was calculated as follows:

$$\Delta Q = \frac{AA_i}{M_i \sum AA_i/M_i} \times \Delta fti;\ Q = \sum \Delta Q \quad (5)$$

where AA_i is the content of each amino acid in protein (g), M_i is the molar mass of each amino acid, (g/mol), $\Sigma AA_i/M_i$ is the total moles of amino acid in 100 g protein (mol), Δfti is hydrophobic value of side chain of amino acid (cal/mol), and Q is hydrophobic value of the protein (cal/mol).

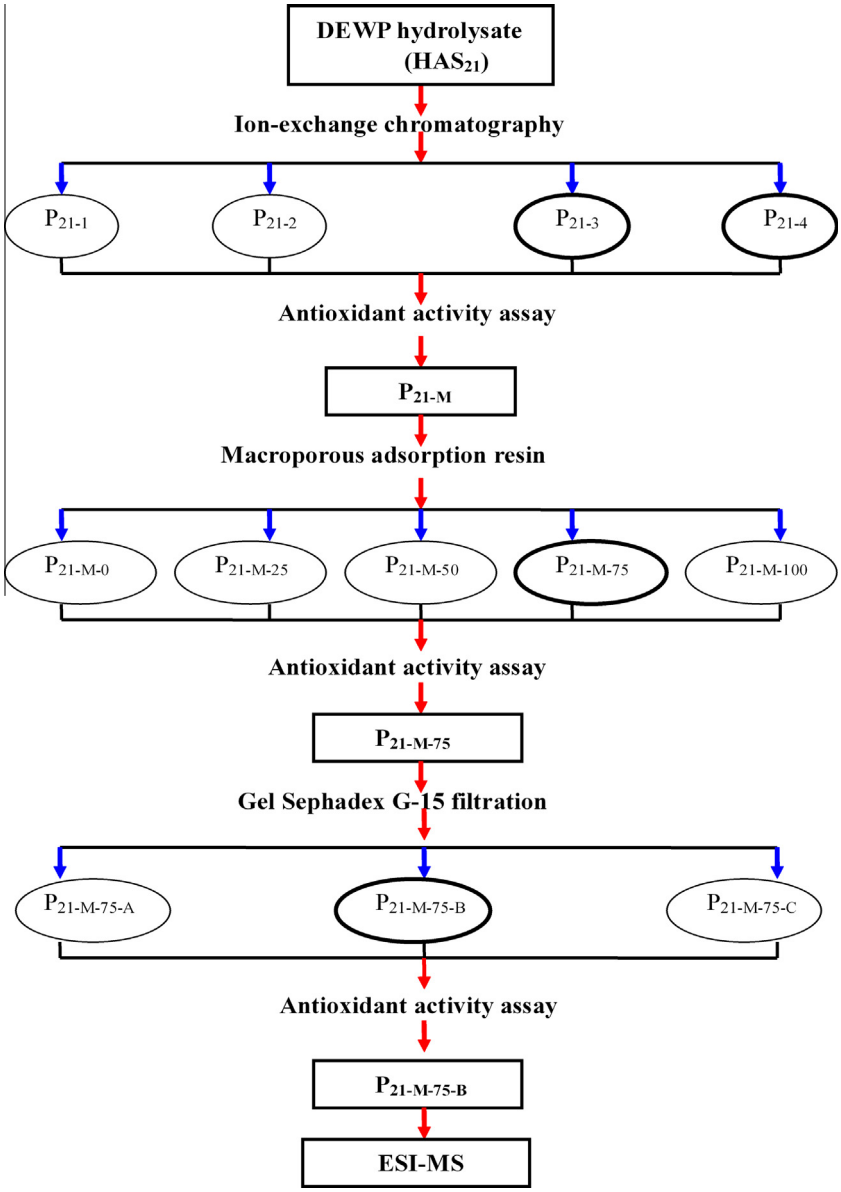

Fig. 1. The flow diagram for the isolation and purification of the antioxidant peptides from DEWP hydrolysates produced by SEEP–Alcalase (DH = 21%; HSA_{21}).

2.13. Determination of molecular mass by ESI-MS

A dose (10 μL) of fraction C was injected into a high capacity ion trap mass spectrometer, and detected using electron spray ionization tandem mass spectrometry (Bruker Daltonics Inc., Billerica, MA). Ionization methods: electrospray ionization; detection mode: positive ion mode; capillary voltage: 3.5 kV; cone voltage: 30 V; source temperature: 80 °C; drying air temperature: 350 °C; collision activated dissociation voltage: 35 V; mass scan range *m*/*z*: 50–1500; dry gas and aerosol: N_2; flow rate: 5.0 L/min; collision gas: Ar.

2.14. Simulated gastrointestinal tract digestion

The pH value of fraction C solution (50 mg/mL) was adjusted to 2.0. Pepsin ([E]:[S] = 1:200) was added as the first enzyme at initial temperature of 37 °C for 3 h, and then the second enzyme, Pancreatin ([E]:[S] = 1:100), was added to restart the reaction at 37 °C (pH 8.0) for 4 h. The digested hydrolysate was obtained by centrifuging at 10,000*g* for 10 min at 4 °C, freeze-dried and collected for the analysis of antioxidant activity.

3. Results and discussion

3.1. Isolation and purification of antioxidant hydrolysates

The hydrolysate prepared by SEEP–Alcalase at DH of 21% (namely HSA_{21}) showed the highest antioxidant activity, and the scavenging rates on DPPH·, ·OH and $O_2^{-}\cdot$, and the reducing power, reached 75.76 ± 0.86%, 81.27 ± 0.99%, 72.73 ± 0.49% and 1.041 ± 0.034 (Reducing power was indicated with the Abs at 700 nm), respectively [4]. Therefore, the hydrolysate HSA_{21} was separated and purified using consecutive chromatographic method. The flow diagram was shown in Fig. 1.

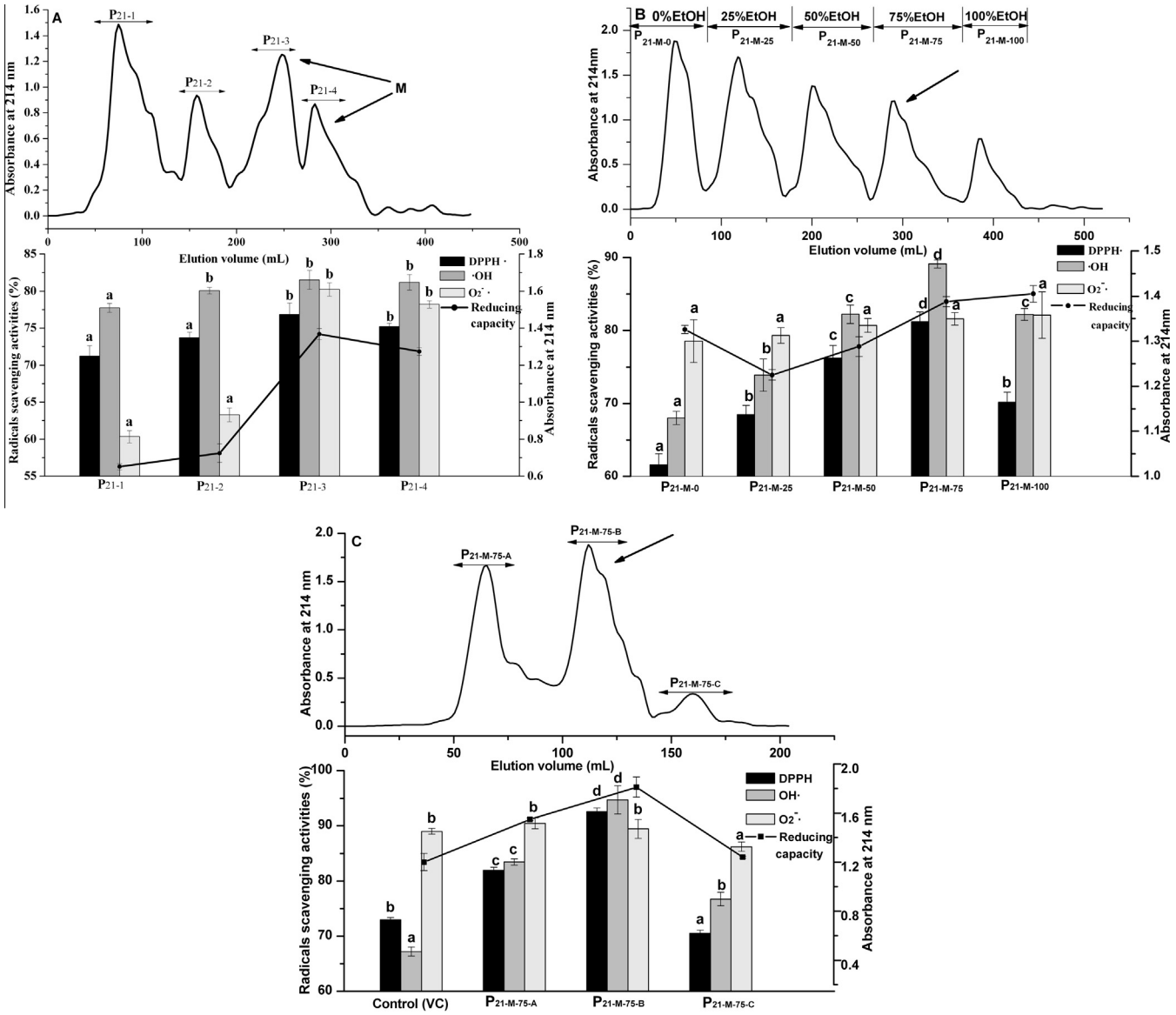

Fig. 2. (A) Elution profile of HSA_{21} separated by DEAE sepharose FF on an ion-exchange column and antioxidant activities of separated fractions ($P_{21\text{-}1}$, $P_{21\text{-}2}$, $P_{21\text{-}3}$ and $P_{21\text{-}4}$), (B) elution profile of $P_{21\text{-}M}$ separated by DA201-C on a macroporous adsorption resin column (MAR) and antioxidant activities of separated fractions ($P_{21\text{-}M\text{-}0}$, $P_{21\text{-}M\text{-}25}$, $P_{21\text{-}M\text{-}50}$, $P_{21\text{-}M\text{-}75}$ and $P_{21\text{-}M\text{-}100}$), and (C) elution profile of $P_{21\text{-}M\text{-}75\text{-}B}$ separated by gel filtration on a Sephadex G-15 column and antioxidant activities of VC and separated fractions ($P_{21\text{-}M\text{-}75\text{-}A}$, $P_{21\text{-}M\text{-}75\text{-}B}$ and $P_{21\text{-}M\text{-}75\text{-}C}$). Data are expressed as the mean value (±SD) of three independent experiments. (a–d) Data marked with are significant differences at $p < 0.05$.

Ion-exchange chromatography was used to separate HSA_{21} into four fractions (namely $P_{21\text{-}1}$, $P_{21\text{-}2}$, $P_{21\text{-}3}$ and $P_{21\text{-}4}$). As showed in Fig. 2A, the scavenging rates of $P_{21\text{-}3}$ on DPPH·, ·OH and O_2^{-}·, and the reducing power were highest, reached 76.85 ± 1.53%, 81.51 ± 1.28%, 80.21 ± 0.90% and 1.368 ± 0.030, respectively, followed closely by $P_{21\text{-}4}$. Compared to the parent HSA_{21}, both the scavenging rates on O_2^{-}· and reducing power of $P_{21\text{-}3}$ and $P_{21\text{-}4}$ increased significantly ($p < 0.01$). Considering that the column used was an anion-exchanger, the last two fractions $P_{21\text{-}3}$ and $P_{21\text{-}4}$ may be acidic peptides fractions. And the acidic peptides may be more effective antioxidants than basic or neutral ones [10,11]. $P_{21\text{-}3}$ and $P_{21\text{-}4}$ were merged as a fraction (namely $P_{21\text{-}M}$) for further isolation.

Five different fractions (namely $P_{21\text{-}M\text{-}0}$, $P_{21\text{-}M\text{-}25}$, $P_{21\text{-}M\text{-}50}$, $P_{21\text{-}M\text{-}75}$ and $P_{21\text{-}M\text{-}100}$) were eluted down by ethanol with different concentrations in Fig. 2B. As ethanol concentrations increased, hydrophobic amino acids (Val, Ile, Leu, Pro, Phe and Met) content of the eluted fractions raised, which was summarized in Table 1. Generally, the hydrophobic value and content of hydrophobic amino acids determined the hydrophobicity of hydrolysates. As showed in Fig. 2B, the scavenging rates of fractions on DPPH· and ·OH raised as the ethanol concentration increased until 75%, which suggested that the hydrophobicity of the fractions contributed greatly to the antioxidant capacity [12].

By gel Sephadex G-15, the fraction $P_{21\text{-}M\text{-}75}$ was further separated to three fractions (namely $P_{21\text{-}M\text{-}75\text{-}A}$, $P_{21\text{-}M\text{-}75\text{-}B}$ and $P_{21\text{-}M\text{-}75\text{-}C}$). As showed in Fig. 2C, the scavenging rates of $P_{21\text{-}M\text{-}75\text{-}B}$ on DPPH·, ·OH, O_2^{-}·, and the reducing power were highest, reached 92.56 ± 0.69%, 94.71 ± 2.57%, 89.45 ± 1.71% and 1.812 ± 0.083, respectively, higher than VC of 73.00 ± 0.37%, 67.20 ± 0.82%, 89.00 ± 0.52% and 1.200 ± 0.070 and the parent $P_{21\text{-}M\text{-}75}$ ($p < 0.01$). Compared with HSA_{21}, $P_{21\text{-}M\text{-}75\text{-}B}$ showed significantly enhanced antioxidant capacity after above-mentioned three consecutive isolation procedures

Table 1
Amino acid compositions, hydrophobic values and yields of the five fractions eluted with ethanol at the concentrations of 0%, 25%, 50%, 75% and 100%.

Amino acids	Δfti (cal/mol)	$P_{21\text{-}M\text{-}0}$[a]	$P_{21\text{-}M\text{-}25}$[a]	$P_{21\text{-}M\text{-}50}$[a]	$P_{21\text{-}M\text{-}75}$[a]	$P_{21\text{-}M\text{-}100}$[a]
Asp	540	10.21 ± 0.68	9.27 ± 0.51	7.23 ± 0.41	5.58 ± 0.23	3.54 ± 0.22
Glu	550	11.89 ± 0.55	11.36 ± 0.34	9.31 ± 0.55	7.82 ± 0.58	6.02 ± 0.35
Ser	40	10.75 ± 0.34	10.68 ± 0.33	8.58 ± 0.46	7.20 ± 0.65	6.01 ± 0.58
Gly	0	4.56 ± 0.18	3.98 ± 0.22	3.87 ± 0.22	3.38 ± 0.27	2.98 ± 0.11
His	500	1.21 ± 0.07	2.56 ± 0.09	2.10 ± 0.08	1.78 ± 0.06	1.09 ± 0.05
Arg	730	4.56 ± 0.21	3.36 ± 0.23	2.21 ± 0.06	2.58 ± 0.14	1.86 ± 0.06
Thr	440	8.42 ± 0.46	7.88 ± 0.36	7.28 ± 0.39	4.10 ± 0.24	4.25 ± 0.27
Ala	730	6.15 ± 0.23	4.02 ± 0.18	5.80 ± 0.22	5.59 ± 0.31	3.24 ± 0.09
Pro	2600	3.12 ± 0.05	2.56 ± 0.06	4.86 ± 0.17	4.98 ± 0.11	5.68 ± 0.23
Tyr	2870	3.21 ± 0.12	3.82 ± 0.14	5.12 ± 0.20	6.12 ± 0.08	7.25 ± 0.28
Val	1690	6.54 ± 0.33	7.02 ± 0.38	6.80 ± 0.35	7.63 ± 0.38	8.75 ± 0.33
Met	1300	4.20 ± 0.26	5.31 ± 0.40	6.56 ± 0.32	6.56 ± 0.27	8.23 ± 0.35
Cys	–	3.89 ± 0.14	2.10 ± 0.08	3.24 ± 0.11	3.45 ± 0.09	3.63 ± 0.15
Ile	2970	3.10 ± 0.03	3.81 ± 0.15	4.64 ± 0.21	7.56 ± 0.24	8.23 ± 0.21
Leu	2420	8.45 ± 0.47	10.24 ± 0.66	11.21 ± 0.75	13.21 ± 0.94	13.56 ± 1.21
Phe	2650	3.56 ± 0.11	4.54 ± 0.25	5.28 ± 0.38	7.01 ± 0.33	8.24 ± 0.66
Lys	1500	3.85 ± 0.17	5.14 ± 0.34	4.50 ± 0.21	5.45 ± 0.34	6.54 ± 0.39
Hydrophobic values (cal/mol)	–	972.71 ± 11.33a	1077.58 ± 18.56b	1204.45 ± 26.36c	1394.60 ± 27.45d	1530.81 ± 38.94e
Yields of fractions (%)	–	17.21 ± 0.82b	22.68 ± 1.06d	20.86 ± 1.25 cd	19.83 ± 0.72c	8.28 ± 0.67a

[a] $P_{21\text{-}3\text{-}0}$, $P_{21\text{-}3\text{-}25}$, $P_{21\text{-}3\text{-}50}$, $P_{21\text{-}3\text{-}75}$, and $P_{21\text{-}3\text{-}100}$, the five fractions eluted with ethanol at various concentrations of 0%, 25%, 50%, 75% and 100%. Data are expressed as the mean value (±SD) of three independent experiments. (a–d) Data marked with in the same line are significant differences at $p < 0.05$.

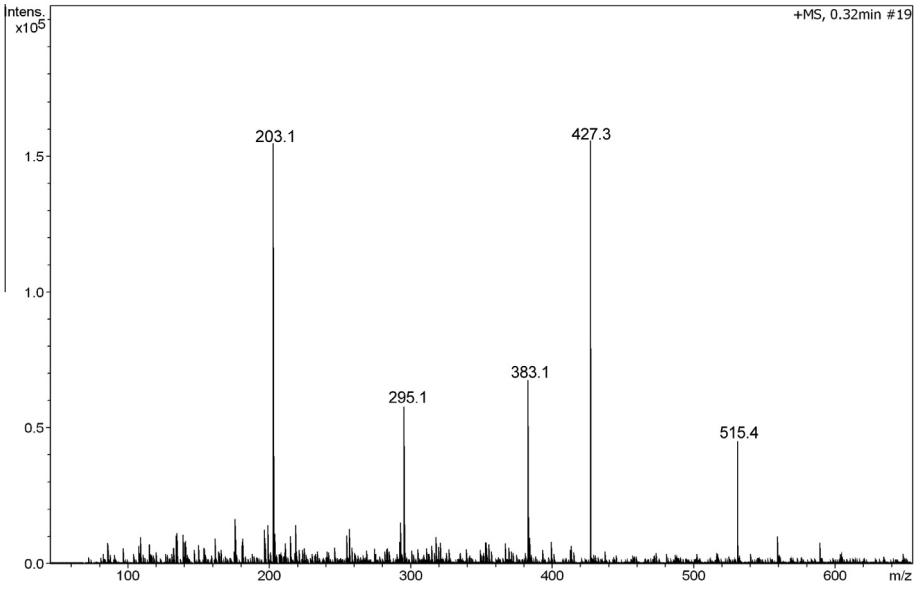

Fig. 3. Mass spectrum of the purified fraction $P_{21\text{-}M\text{-}75\text{-}B}$.

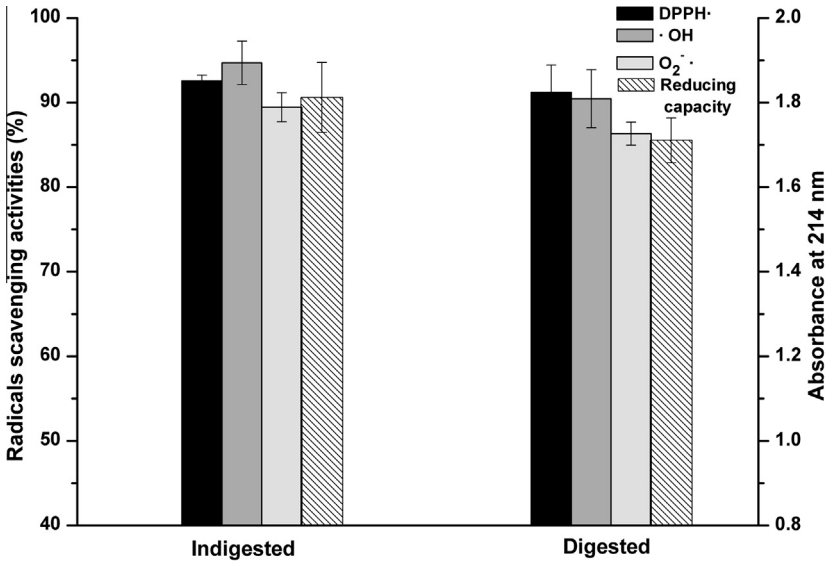

Fig. 4. Comparison of the antioxidant activities of $P_{21\text{-}M\text{-}75\text{-}B}$ and that after Pepsin and Pancreatin hydrolysis.

Table 2
Amino acid compositions of fresh DEWP, the hydrolysate prepared by SEEP–Alcalase at DH 21% and the highest antioxidant fractions from each purification procedure.

Amino acids	HSA_{21}[a] (%)	$P_{21\text{-}M}$[a] (%)	$P_{21\text{-}M\text{-}75}$[a] (%)	$P_{21\text{-}M\text{-}75\text{-}B}$[a] (%)	Fresh DEWP (%)
Ile∗	5.14 ± 0.11	4.86 ± 0.36	7.56 ± 0.24	7.62 ± 0.24	3.60 ± 0.15
Leu∗	11.67 ± 0.46	11.02 ± 0.55	13.21 ± 0.94	13.40 ± 1.04	8.44 ± 0.55
Lys^+	6.70 ± 0.24	5.04 ± 0.38	5.45 ± 0.34	6.11 ± 0.42	7.32 ± 0.36
Met∗	5.93 ± 0.16	5.86 ± 0.29	6.56 ± 0.27	6.85 ± 0.31	4.43 ± 0.12
Phe∗	5.07 ± 0.21	5.48 ± 0.31	7.01 ± 0.33	7.58 ± 0.35	6.47 ± 0.19
Thr	7.39 ± 0.15	7.76 ± 0.66	4.10 ± 0.24	4.26 ± 0.22	5.67 ± 0.35
Val∗	7.36 ± 0.23	7.96 ± 0.68	7.63 ± 0.38	7.74 ± 0.15	6.81 ± 0.52
Cys	3.55 ± 0.08	3.15 ± 0.25	3.45 ± 0.09	2.89 ± 0.11	2.19 ± 0.10
Tyr∗	4.51 ± 0.13	3.23 ± 0.22	6.12 ± 0.08	5.83 ± 0.14	4.72 ± 0.33
Ala∗	4.91 ± 0.22	4.24 ± 0.14	5.59 ± 0.31	4.66 ± 0.26	4.86 ± 0.21
Pro∗	3.92 ± 0.08	5.54 ± 0.48	4.98 ± 0.11	4.65 ± 0.24	3.70 ± 0.15
Arg^+	2.85 ± 0.03	1.80 ± 0.04	2.58 ± 0.14	1.60 ± 0.06	5.89 ± 0.47
His^+	1.41 ± 0.02	1.24 ± 0.33	1.78 ± 0.06	1.04 ± 0.04	2.46 ± 0.08
Asp^-	6.50 ± 0.42	8.36 ± 0.66	5.58 ± 0.23	8.66 ± 0.75	5.98 ± 0.32
Glu^-	10.22 ± 0.88	11.52 ± 0.85	7.82 ± 0.58	8.65 ± 0.68	13.77 ± 1.10
Ser	8.73 ± 0.51	8.55 ± 0.54	7.20 ± 0.65	6.41 ± 0.43	8.94 ± 0.66
Gly	4.14 ± 0.35	4.39 ± 0.27	3.38 ± 0.27	2.05 ± 0.17	4.75 ± 0.26
BCAA[b]	24.17 ± 1.65b	23.84 ± 1.63b	28.40 ± 2.11c	28.76 ± 1.67c	18.85 ± 0.57a
EAA[b]	49.26 ± 2.88ab	47.18 ± 2.76ab	51.52 ± 3.02ab	53.56 ± 2.38b	42.74 ± 2.96a
HAA[b]	48.51 ± 3.66b	48.19 ± 2.34b	58.66 ± 3.25c	58.33 ± 2.43c	43.03 ± 1.84a
PCAA[b]	10.96 ± 0.89b	8.08 ± 0.82a	9.81 ± 0.92ab	8.75 ± 0.34a	15.67 ± 0.56c
NCAA[b]	16.72 ± 1.05b	19.98 ± 1.11c	13.40 ± 0.67a	17.31 ± 0.52b	19.75 ± 0.33c

∗hydrophobic AA; $^+$positively charged AA; $^-$, negatively charged AA; Data are expressed as the mean value (±SD) of three independent experiments. (a–d) Data marked with in the same line are significant differences at $p < 0.05$.

[a] HSA_{21}, the hydrolysates prepared by SEEP–Alcalase at DH 21%; $P_{21\text{-}M}$, the highest antioxidant fractions of HSA_{21} eluted by 0–1.0 NaCl; $P_{21\text{-}M\text{-}75}$, the highest antioxidant fraction of $P_{21\text{-}M}$ eluted by 75% ethanol; $P_{21\text{-}M\text{-}75\text{-}B}$, the highest antioxidant fraction of $P_{21\text{-}M\text{-}75}$ filtrated with gel Sephadex G-15.

[b] BCAA, branched-chain amino acid (AA); EAA, essential AA; HAA, hydrophobic AA; PCAA, positively-charged AA; NCAA, negatively-charged AA.

($p < 0.01$). The last fraction $P_{21\text{-}M\text{-}75\text{-}C}$ may contain a mass of amino acids and salt, resulting in its poor capacity. Thus, $P_{21\text{-}M\text{-}75\text{-}B}$ was chosen for further investigation of the amino acid composition and molecular mass.

3.2. ESI-MS spectrums of $P_{21\text{-}M\text{-}75\text{-}B}$

The high-resolution (+) ESI-MS spectrum was showed in Fig. 3. The excimer ion peaks of $P_{21\text{-}M\text{-}75\text{-}B}$ basically distributed with the m/z of 203.1-515.4. Five peaks presented expressly in the spectrum, which represented five different objective peptides. Two peptides (Mr: 202.1 and 426.3 Da) were conspicuous among them. These small peptides (Mr < 600 Da) played a leading role in antioxidant aspect. It was also reported that low molecular peptides were considered to react easily with oxidation radicals and thereby reducing the damage from radicals [13]. The peptides mixture together produced high antioxidant activity cooperatively, not merely simple one alone [14]. Therefore, this high antioxidant fraction would not be considered to be separated further for obtaining simple small peptide, which was a bit different from other previous reports [15,16].

3.3. Simulated gastrointestinal tract digestion

To exert its antioxidant action *in vivo*, bioactive peptide must go through digestive system and resist the hydrolysis of digestive enzyme *in vivo* to access into blood circulation. After Pepsin and Pancreatin hydrolysis, the scavenging rate of $P_{21\text{-M-75-B}}$ showed slight decrease on DPPH· (from 92.56 ± 0.69% to 91.23 ± 3.21%), ·OH (from 94.71 ± 2.57% to 90.45 ± 3.42%) and O_2^{-}· (from 89.45 ± 1.71% to 86.32 ± 1.37%). And its reducing power also reduced from 1.812 ± 0.083 to 1.711 ± 0.053. Fig. 4, showed that the antioxidant activity of $P_{21\text{-M-75-B}}$ still kept stable after undergoing simulated digestion environment, which might be due to the fact that Pepsin and Pancreatin in gastrointestinal tract were difficult to degrade these antioxidant peptides. These bioactivity peptides could resist our digestion *in vivo*, thereby being able to be absorbed directly in small intestine.

3.4. Amino acid compositions of $P_{21\text{-M-75-B}}$

To gain better understanding of the mechanisms linking amino acids to the antioxidant activity of the peptides, the amino acid compositions of $P_{21\text{-M-75-B}}$ was investigated and compared. As summarized in Table 2, amino acid composition of $P_{21\text{-M-75-B}}$ was obviously different with those of fresh DEWP and HSA_{21}. It took our attention that the contents of several amino acids including Leu, Met and Cys that may contribute greatly to the observed antioxidant activity of peptides obviously increased, compared to those of fresh DEWP ($p < 0.01$). In addition, the total percents of branched chain amino acids (Val, Leu and Ile) of $P_{21\text{-M-75-B}}$ enhanced markedly by 9.91% ($p < 0.01$). In contrast, the positively charged amino acids (Arg, Lys and His) had a significant decrease compared to those of HSA_{21} and fresh DEWP, respectively ($p < 0.05$). The content (58.33 ± 2.43%) of hydrophobic amino acids of $P_{21\text{-M-75-B}}$ showed a remarkable increase compared to that of fresh DEWP (43.03 ± 1.84%) ($p < 0.01$), which was discussed before may contribute effectively to the antioxidant activity [17]. The increase of percentages of branched chain amino acids, hydrophobic amino acids and the decrease of positively charged amino acids may improve crucially the observed antioxidant activity of present peptides.

4. Conclusions

In the present research, the hydrolysates from DEWP hydrolyzed effectively by SEEP–Alcalase at DH 21% (HSA_{21}) representing strong antioxidant capacity was purified by anion-exchange, MAR and gel filter chromatography. The consecutive chromatographic procedure was proven to be an efficient approach to prepare purified peptides and promote antioxidant capacity to a higher level. A final peptides ($P_{21\text{-M-75-B}}$) that consisted of five small peptides (Mr: 202.1, 294.1, 382.1, 426.3 and 514.4 Da) was obtained with the scavenging rates on DPPH·, ·OH, O_2^{-}·, and the reducing power of 92.56 ± 0.69%, 94.71 ± 2.57%, 89.45 ± 1.71% and 1.812 ± 0.083, respectively, higher than VC. The digestive test further confirmed the stability of the peptide in a digestive environment. Acidic and hydrophobic peptides with low molecule mass were considered to express potentially better antioxidant capacity. This research proved a new bioprocessing for preparing high antioxidant peptides ingredients for producing functional foods. Further researches on antioxidant capacity *in vivo* and scale-up production of purified DEWP peptides are in progress.

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