Characterization and Antioxidant Properties of Hemp Protein Hydrolysates Obtained with Neutrase®

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Summary

Hemp protein hydrolysates with various yields of trichloroacetic acid (TCA)-soluble peptides (Y_{sp}) and surface hydrophobicity (H_o) were obtained by Neutrase® hydrolysis from hemp protein isolate (HPI). The peptide profiles, amino acid composition and antioxidant activities (DPPH radical scavenging ability, reducing power and Fe^{2+} chelating ability) of the hydrolysates, obtained at 60–240 min, were evaluated. Higher DPPH radical scavenging (IC_{50}, 2.3–2.4 mg/mL) and Fe^{2+} chelating (IC_{50}, 1.7–1.8 mg/mL) abilities were observed for the hydrolysates with Y_{sp} in the range of 28–30 and 18–28 %, respectively, while the high reducing power was only observed for the hydrolysate with Y_{sp} of 18 %. The DPPH radical scavenging and Fe^{2+} chelating abilities were closely correlated with the peptide profiles and H_o of the hydrolysates. The peptide profiles of the hydrolysates with higher hydrophobic amino acids exhibited higher DPPH radical scavenging and Fe^{2+} chelating abilities.

Key words: hemp protein, hydrolysate, enzymatic hydrolysis, antioxidant activity, Neutrase®

Introduction

Hemp (*Cannabis sativa* L.) seed, a byproduct during commercial utilization of valuable hemp fibre, is a rich source of high quality oil and protein. The seed usually contains over 30 % of oil and about 25 % of protein. The oil is rich in polyunsaturated fatty acids, especially linoleic (ω-6) and α-linolenic (ω-3) acids, while the storage protein in the seed has superior essential amino acid composition and is easily digested (*1–3*). Hemp protein mainly consists of edestin (globulin) and albumin, and like the hexamer of soy glycinin, edestin is usually composed of six identical subunits, each consisting of an acidic subunit (AS) and a basic subunit (BS) linked by one disulphide bond (*4*). This protein can be efficiently obtained by alkali solubilization/acid precipitation from defatted hemp seed meal (*2*). In hemp protein isolate (HPI), edestin usually composes about 70 % of total protein. The solubility of this protein isolate is much poorer relative to soy protein isolate (SPI), since in this case, the protein aggregation is very obvious, possibly due to free sulphydryl group and disulphide bond exchange between individual protein components (*2*). The poor solubility greatly impairs surface-related functional properties of HPI. In our previous work, it was shown that the poor solubility of HPI could be effectively improved by limited enzymatic hydrolysis with trypsin; however, the hydrolysis would result in further decrease in the functional properties, *e.g.* emulsifying activity index, foaming capacity and foam stability, water holding and fat adsorption capacities (*5*).

The enzymatic hydrolysis could be applied not only as a technique to modify the properties of food proteins, but also as a means to bestow these proteins with added

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value, *e.g.* potential health effects. The antioxidant properties of enzymatic hydrolysates or the purified peptides of food proteins, including bovine caseins and whey proteins (*6*), soy proteins (*7–9*), wheat protein (*10*), chickpea protein (*11*), porcine haemoglobin, collagen and myofibrillar protein (*12–14*), and fish proteins (*15,16*), have been widely characterized. This kind of investigation has greatly enlarged the utilization of food proteins for human nutrition. Thus, it will be interesting to investigate the antioxidant potential of the hydrolysates from hemp proteins.

In our previous work, we investigated enzymatic hydrolysis of HPI using six kinds of proteases, including Alcalase®, Flavourzyme®, Neutrase®, Protamex®, pepsin and trypsin, and found that the hydrolysates obtained by Neutrase exhibited much better antioxidant potential than those by other proteases (*17*).

The main objective of the present work is to further investigate the antioxidant activities of hemp protein hydrolysates obtained by Neutrase, using the same *in vitro* antioxidant assays. The peptide profiles and H_o of hydrolysates obtained at various periods of hydrolysis time, as well as the amino acid compositions of these hydrolysates (and its peptide groups) have been analyzed. Additionally, possible relationships between peptide profiles (or H_o) of hydrolysates and their antioxidant activities are also evaluated.

Materials and Methods

Materials

Defatted hemp meal was kindly supplied by Yunnan Industrial Hemp Co. Ltd. (PR China). Hemp protein isolate (HPI) was prepared from this meal, according to the same process as described by Tang *et al.* (*2*). The protein content of this sample was about 87 g per 100 g of fresh matter. Neutrase 1.5 MG® (1.5 AU/g), produced from *Bacillus* sp. was kindly supplied by Guangzhou Office (PR China) of Novozymes, Bagsvaerd, Denmark. All the chemicals were of analytical or better grade.

Enzymatic hydrolysis of HPI and determination of the yield of TCA-soluble peptide (Y_{sp})

A mass of 10 grams of freeze-dried HPI was dispersed in 200 mL of deionized water at room temperature. The dispersions were preincubated at 55 °C, prior to adjusting the pH of the dispersion to 7.0. The mixture of protein and enzyme (Neutrase®) with a mass ratio of 100:5 was incubated in a temperature-controlled water bath at 55 °C. The pH of the mixture was kept constant during hydrolysis by the addition of 1.0 mol/L of NaOH. At the end of the reaction, the mixture was stopped by heat treatment in boiling water for 10 min, and cooled immediately in ice water to room temperature. The resulting digests were centrifuged at 4000×*g* for 20 min to remove insoluble residues. The supernatants were then lyophilized to produce the hydrolysates, which were stored at –20 °C before further analysis.

The Y_{sp} was determined from peptide solubility of enzyme-digested hydrolysates in 10 % (*m/V*) TCA solution. Aliquots (10 mL) of the enzyme digests of HPI, obtained after hydrolysis for 0, 30, 60, 90, 120 and 240 min respectively, were fully mixed with the same volumes of 20 % (*m/V*) TCA solution. The mixtures were placed at room temperature for 10 min, and centrifuged at 3000×*g* for 20 min. The nitrogen content of the supernatants was determined by conventional Kjeldahl method (N×6.25) (*18*). The Y_{sp} was defined as:

$$Y_{sp}/\% = (N_2 - N_1) \cdot 100 / (N_0 - N_1) \qquad /1/$$

where N_0 is total nitrogen content in undigested HPI (g), N_1 and N_2 are TCA-soluble nitrogen content (g) of undigested HPI and enzyme-treated digests, respectively.

Size exclusion chromatography (SEC)

The SEC experiment was performed using a Sephadex® G-50 column (2.0×100 cm) at room temperature. The hydrolysate samples (2 mL) at a concentration of 50 mg/mL were applied to the column, eluted with 50 mmol/L phosphate buffer (pH=7.0), at a rate of 1 mL/min. The effluent was collected at 3 mL per tube. The UV absorbance of the collected effluent was recorded at 280 nm.

Surface aromatic hydrophobicity (H_o)

H_o was determined with the fluorescence probe 1--anilino-8-naphtalene-sulphonate (ANS) according to the method of Alizadeh-Pasdar and Li-Chan (*19*). Serial dilutions in 0.01 mol/L phosphate buffer (pH=7.0) were prepared with the hydrolysates to a final mass fraction of 0.005–0.2 %. ANS solution (8.0 mmol/L) was also prepared in the same phosphate buffer. A volume of 20 μL of ANS solution was added to 4 mL of each dilution and fluorescence intensity (FI) of the mixture was measured at 365 nm (excitation) and 484 nm (emission) using F4500 fluorescence spectrophotometer (Hitachi Co., Japan). The initial slope of the FI *vs.* protein concentration (mg/mL) plot (calculated by linear regression analysis) was used as an index of H_o.

α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity was determined by the method described by Shimada *et al.* (*20*). A volume of 2 mL of the sample solution with various solid concentrations was mixed with 2 mL of 2·10⁻⁴ mol/L DPPH methanolic solution (freshly prepared) and vortexed for about 10 s. The resulting solution was then left to stand for 30 min, prior to being spectrophotometrically measured at 517 nm. A low absorbance at 517 nm indicates a high DPPH scavenging activity. Methanol was used as the blank. The DPPH scavenging activity is calculated according to the following equation:

$$(1 - A_{\text{test sample}} / A_{\text{blank sample}}) \cdot 100 \qquad /2/$$

where $A_{\text{test sample}}$ and $A_{\text{blank sample}}$ are the absorbance for the test and blank samples, respectively.

Reducing power

The reducing power of the hydrolysates was evaluated using the method developed by Oyaizu (*21*), with slight modifications. The sample solution (10 mL) in

ionized water with a solid concentration in the range of 0–5 mg/mL was mixed with 2.5 mL of 0.2 mol/L phosphate buffer (pH=6.6) and 2.5 mL of 1 % potassium ferric cyanide solution. The mixture was then kept in a water bath at 50 °C for 20 min. The resulting solution was cooled rapidly, and then mixed with 2.5 mL of 10 % TCA (*m/V*), and centrifuged at 3000×*g* for 10 min. Finally, the supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1 % ferric chloride solution. The absorbance of the resulting mixture was measured at 700 nm after the reducing reaction for 10 min. High absorbance indicates strong reducing power.

Fe^{2+} chelating ability

The Fe^{2+} chelating ability was determined by the method of Decker and Welch (*22*). Volumes of 5 mL of the sample solutions with different solid concentrations were mixed with 0.1 mL of 2 mmol/L $FeCl_2$ and 0.2 mL of 5 mmol/L ferrozine solutions. After 10 min, the absorbance of the resulting solutions was recorded at 562 nm. A complex of $FeCl_2$/ferrozine had a strong absorbance at 562 nm. High ferrous ion chelating ability in the test sample resulted in low absorbance. The Fe^{2+} chelating ability as a percentage is calculated using Eq. 2.

Amino acid analysis

The amino acid composition of protein hydrolysate samples was determined by an automatic amino acid analyzer (Waters M510, USA), using PicoTag® column. The determination was carried out at 38 °C, the detection wavelength of 254 nm and flow rate of 1.0 mL per minute. The samples were hydrolyzed with 6 mol/L HCl at 110 °C for 24 h in a sealed tube. The amino acid composition was reported as g per 100 g of protein. The amino acid tryptophan was not determined.

Statistics

All determinations were done in at least triplicate, and data of means and standard deviations were given. An analysis of variance (ANOVA) of the data was performed, and the least significant difference (LSD) with a confidence interval of 95 or 99 % was used to compare the means. The fitting of the data with the correlated equations was carried out with Microbial Origin 7.5 (OriginLab Co., Northampton, MA, USA).

Results and Discussion

SEC analysis

The enzymatic hydrolysis of HPI with Neutrase for up to 240 min had been investigated by monitoring the release of TCA-soluble peptides (Y_{sp}) in our previous work (*17*), where it was shown that a high rate of hydrolysis occurred for the first 120 min, followed by a gradual decrease in the rate, and at the end of hydrolysis (240 min), the Y_{sp} reached 30.2 %. At specific periods of hydrolysis time, *e.g.* 60, 120, 180 and 240 min, HPI hydrolysates with various Y_{sp} values of about 18.6, 26.2, 28.2 and 30.2 %, respectively, were obtained.

Fig. 1 shows the size exclusion chromatography (SEC) analysis using Sephadex G-50 of HPI hydrolysates, ob-

tained in 60–240 min. In the chromatogram of untreated HPI, there was a major peak eluting at a close void volume of about 90 mL (or tube no. 30) (data not shown). This major peak was clearly attributed to the edestin component in HPI. In order to elucidate the influence of enzymatic hydrolysis on the protein solubility of HPI, total integrated area of all the peaks in the SEC chromatogram was compared. The total integrated area of the hydrolysates (obtained in 60–240 min) was remarkably higher than that of the untreated HPI (data not shown). This data suggest that protease-induced hydrolysis could effectively improve solubility of hemp proteins (*5*).

In the elution chromatograms of all the hydrolysates, there were four similar major peaks, eluting at tube nos. 30, 75, 85 and 100, respectively (Fig. 1). The peaks on the chromatograms of the hydrolysates were divided into five groups. Groups I–V were collected from tube nos. 25–35, 36–60, 61–80, 81–94 and 95–115, respectively. The integrated area percentages of individual groups in the SEC chromatograms of HPI and its hydrolysates are listed in Table 1. In untreated HPI, the area percentage of group I was the largest, about 80.2 %, while the area percentage of other groups was much lower. The enzymatic hydrolysis for 60 min resulted in remarkable decline in the area percentage of group I and concomitant increases in the area percentages of groups III and IV (Table 1). The increases in the area percentages of groups III and IV indicated the formation of low molecular mass (MM) peptides. However, the area percentage of group I gradually increased at the expense of the area percentage of group IV or III, when the hydrolysis time was increased from 120 to 240 min (Figs. 1b–d and Table 1). A similar phenomenon was observed in Flavourzyme-induced hydrolysis of haemoglobin, where it was supposed to be due to the peptide formation reaction (plastein reaction) by Flavourzyme (*13*). In the present study, the increase in the area percentage of group I after extensive hydrolysis could be attributed to aggregation of hydrophobic peptides or fragments (especially those from basic subunits of edestin), released during the enzymatic hydrolysis.

Surface hydrophobicity (H_o)

The H_o values of HPI and its hydrolysates obtained by Neutrase® for 60–240 min are shown in Table 2. The H_o of the hydrolysates obtained in 60 min was significantly higher than that of control. However, the H_o of the hydrolysates gradually decreased with hydrolysis time increasing from 60 to 240 min. The data showed that enzymatic hydrolysis for a shorter time (*e.g.* 60 min) by Neutrase resulted in structural cleavage of edestin component molecules and subsequent exposure of hydrophobic groups (thus leading to increase in H_o). The exposed hydrophobic peptides seemed to be further associated into soluble aggregates, during extensive incubation with this enzyme. As a consequence, the H_o of the hydrolysates decreased. This phenomenon is consistent with SEC analysis (Fig. 1), further indicating the occurrence of aggregation of hydrophobic peptides.
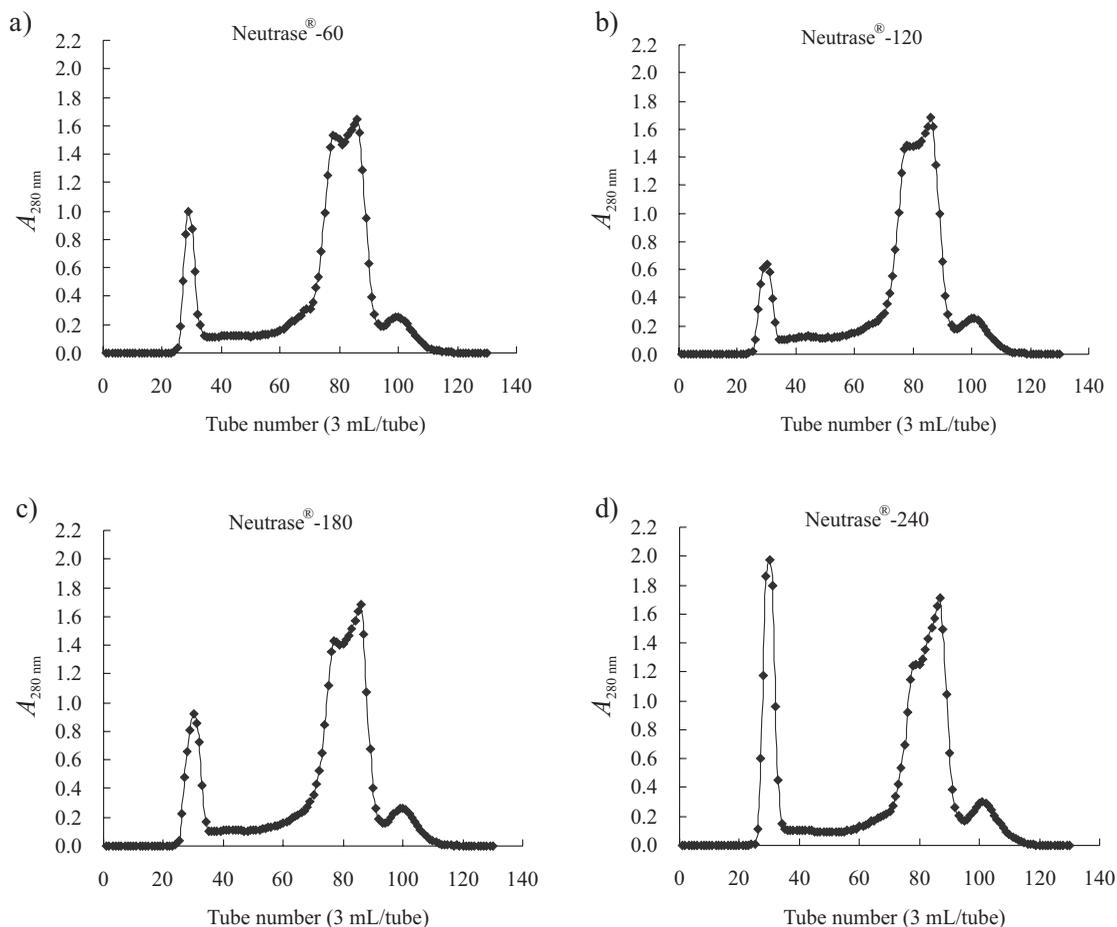
Fig. 1. Sephadex® G-50 gel filtration chromatograms of HPI hydrolysates obtained by Neutrase® in (a) 60, (b) 120, (c) 180 and (d) 240 min, respectively

Table 1. The area percentages of the peak groups of HPI (control) and its hydrolysates as analyzed by gel filtration chromatography on Sephadex® G-540

Sample	Area/%				
	Group I (tube no. 25–35)	Group II (tube no. 36–60)	Group III (tube no. 61–80)	Group IV (tube no. 81–94)	Group V (tube no. 95–115)
Control	80.2	2.5	9.0	8.0	0.3
Neutrase®-60	12.5	8.2	29.6	42.2	7.4
Neutrase®-120	9.8	8.3	29.5	44.5	7.9
Neutrase®-180	14.5	7.8	30.6	39.6	7.5
Neutrase®-240	24.0	6.3	21.4	40.0	8.3

Table 2. The surface hydrophobicity (H_o) and antioxidant activities of HPI and its hydrolysates obtained by Neutrase® in 60, 120, 180 and 240 min, respectively

Hydrolysate sample	H_o	Antioxidant activity		
		DPPH radical scavenging activity, IC_{50}/(mg/mL)	Reducing power ($A_{700\ nm}$)	Fe^{2+} chelating ability, IC_{50}/(mg/mL)
Control	$(89\pm15)^*$	n.d.	n.d.	n.d.
Neutrase®-60	154±20	$(3.5\pm0.2)^a$	$(0.32\pm0.02)^a$	$(1.8\pm0.1)^b$
Neutrase®-120	96±15	$(3.3\pm0.1)^a$	$(0.29\pm0.04)^b$	$(1.7\pm0.1)^b$
Neutrase®-180	70±16	$(2.4\pm0.1)^b$	$(0.29\pm0.03)^b$	$(1.7\pm0.3)^b$
Neutrase®-240	65±7	$(2.3\pm0.1)^b$	$(0.28\pm0.02)^b$	$(2.0\pm0.1)^a$

*The value is determined from soluble components; n.d.=not determined, due to poor protein solubility; letters in superscripts indicate significant difference at p<0.05 level in the same column

Antioxidant activities

The DPPH radical scavenging ability, reducing power and Fe^{2+} chelating ability of hemp protein hydrolysates are also included in Table 2. The stable DPPH radical in ethanol has been widely used to test the ability of some compounds to act as free radical scavengers or hydrogen donors (*20,23*). In the present case, IC_{50} value (mg/mL; meaning the concentration that causes a decrease in initial DPPH concentration by 50 %) is used to evaluate the scavenging activity. The lower the IC_{50} value, the higher the free radical scavenging ability. The IC_{50} value of the hydrolysates gradually decreased with increasing hydrolysis time from 60 to 240 min (Table 2). The DPPH radical scavenging ability of the hydrolysates obtained for 180 or 240 min was significantly higher than that for 60 or 120 min. Previous studies had pointed out that high DPPH or other radical scavenging activities for the protein hydrolysates or peptides are usually associated with high hydrophobic amino acid or hydrophobicity (*11,15*). Thus, the data showed that more extensive enzymatic hydrolysis by Neutrase might result in the formation of more hydrophobic peptides that could scavenge the DPPH radical. The DPPH radical scavenging ability (IC_{50}=2.3–2.4 mg/mL) of the hydrolysates (*e.g.* obtained after 180 or 240 min) was close to the highest value of haemoglobin hydrolysate obtained by Alcalase after 4–10 h (*13*) and canola protein hydrolysate obtained by Alcalase and/or Flavourzyme (*24*), but much lower than that of wheat germ protein hydrolysate (IC_{50}=1.3 mg/mL) and chickpea protein hydrolysate (IC_{50} about 1.0 mg/mL) obtained by Alcalase (*10,11*).

The reducing capacity of a compound can be used to evaluate its potential antioxidant activity (*25*). In the present work, the reducing power is denoted as absorbance at 700 nm to monitor the amount of Fe^{3+} reduced to Fe^{2+} (*26*). The hydrolysates exhibited similar reducing power values of 0.28–0.32 (Table 2). The hydrolysate obtained after 60 min had higher reducing power than that after 120 min or longer. The reducing power of these hydrolysates (at a solid concentration of 3 mg/mL) was much higher than that of haemoglobin hydrolysates even at a concentration of 5 mg/mL (*13*), and comparable to the highest reducing power of purified fractions of chickpea protein hydrolysate (*14*). This suggests that the hydrolysates obtained by Neutrase exhibit excellent reducing power.

The antioxidant activity of some compounds is related to their Fe^{2+} chelating ability. In the present work, this Fe^{2+} chelating ability was estimated by the reduction of absorbance (red) of ferrozine-Fe^{2+} complexes after the addition of the hydrolysates, and also expressed as IC_{50} value (meaning the concentration that causes a decrease in red colour absorbance by 50 %). The hydrolysates obtained in 60–180 min exhibited similar Fe^{2+} chelating ability (IC_{50}=1.7–1.8 mg/mL), while the Fe^{2+} chelating ability of the hydrolysates significantly decreased when the hydrolysis time was increased up to 240 min (Table 2). The Fe^{2+} chelating ability of these protein hydrolysates obtained by Neutrase is much stronger than that of haemoglobin hydrolysates (except for the hydrolysates obtained by pepsin in 2 hours) (*13*), but much weaker than that of wheat germ protein hydrolysate (*10*).

Relationships between peptide profiles and the antioxidant activities of the hydrolysates

The antioxidant activities of the hydrolysates may be correlated with their H_o and peptide profiles. The relationships between the peptide profiles (area percentages of individual groups in the SEC elution profiles (Table 1) and the antioxidant activities of HPI hydrolysates (Table 3) were analyzed. In Table 3, the correlations between antioxidant activities (and area percentages of individual groups in SEC chromatograms) and H_o were also included. It was found that DPPH radical scavenging ability of the hydrolysates was significantly and positively correlated with the amount of groups II and IV at $p<0.10$ or $p<0.05$ level, and significantly and negatively correlated with the amount of group I at $p<0.10$ level (Table 3). The Fe^{2+} chelating ability was very significantly and positively correlated with the amount of group I, and very significantly and negatively correlated with the amount of groups II and III at $p<0.01$ level. However, the reducing power was insignificantly correlated with the relative amount of the peak groups at $p<0.10$ level. Similar relationships between the antioxidant activities and the area percentage of peak groups in the SEC chromatograms have been observed for porcine haemoglobin hydrolysates (*13*).

Interestingly, the H_o of the hydrolysates was significantly and positively correlated with the amounts of groups II and IV at $p<0.05$, and significantly and negatively correlated with the amount of group I (Table 3). As expected, a positive correlation with a high correlation coefficient of 0.973 was observed between DPPH radical scavenging ability and H_o of the hydrolysates.

Table 3. Correlation coefficients between the area percentages of peak groups in the SEC chromatograms (or H_o) and antioxidant activities of HPI hydrolysates

	Group I	Group II	Group III	Group IV	Group V	DPPH radical scavenging ability	Reducing power	Fe^{2+} chelating ability
DPPH radical scavenging ability	−0.732*	0.760*	0.517	0.786**	−0.385			
Reducing power	−0.522	0.621	0.529	0.298	−0.684	0.822**		
Fe^{2+} chelating ability	0.923***	−0.907***	−0.954***	−0.472	0.697*	−0.421	−0.291	
H_o	−0.865***	0.874***	0.657	0.847**	−0.445	0.973***	0.740*	−0.606

*significant at $p<0.10$ level; **significant at $p<0.05$ level; ***significant at $p<0.01$ level

The reducing power of the hydrolysates was also positively and significantly ($p < 0.10$) correlated with their H_o.

Comparison of amino acid compositions and antioxidant activities of HPI hydrolysates and their peptide groups

In order to confirm the contribution of hydrophobic peptides to antioxidant activities, amino acid compositions of HPI hydrolysates and their peptide groups in the SEC chromatograms were analyzed (data not shown). From the determined amino acid compositions, relative contents of hydrophobic amino acids (including Ala, Pro, Val, Met, Ile, Leu and Phe) of the hydrolysate (obtained at 4 h; control) and its four peptide groups (I–IV), relative to total amino acids were obtained, as displayed in Fig. 2. The hydrophobic amino acid composition of groups I and III was similar to that of control. The hydrophobic amino acid content of group II (relative to total amino acid content) was significantly lower than that of control, while that of group IV was significantly higher (Fig. 2). The data suggest that hydrophobic peptides released during enzymatic hydrolysis are concentrated in the amount of group IV in the SEC chromatogram.
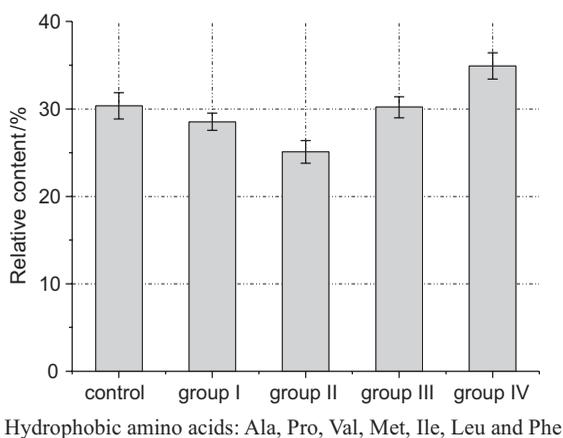

Hydrophobic amino acids: Ala, Pro, Val, Met, Ile, Leu and Phe

Fig. 2. Hydrophobic amino acids of HPI hydrolysates (obtained with Neutrase® in 240 min) and their four peak groups in the SEC chromatograms. Each value is the mean value with standard deviation of duplicate measurements

The DPPH radical scavenging ability, reducing power and Fe^{2+} chelating ability of four peptide groups (groups I–IV), at a solid concentration of 3 mg/mL, were also analyzed and compared. It was found that at this concentration, the DPPH radical scavenging ability (40.5 %) of group IV was slightly but not significantly higher than control (40.2 %), and that of groups I–III (30–32 %) was significantly ($p < 0.05$) lower. The reducing power of groups II (0.361) and IV (0.334) was significantly higher than that of control (0.285), while groups III and IV exhibited significantly higher Fe^{2+} chelating ability (data not shown). The data confirmed that the DPPH radical scavenging ability and Fe^{2+} chelating ability of the hydrolysate were largely associated with the hydrophobic amino acids or peptides, while the reducing power was unrelated to the hydrophobic amino acids.

Conclusion

Hemp protein isolate could be efficiently hydrolyzed by Neutrase® to produce hydrolysates with various Y_{sp} (10–30 %) and H_o (65–150) values. The aggregation of the released hydrophobic peptides became more obvious upon further hydrolysis (*e.g.* 240 min). The hydrolysates exhibited antioxidant activities (DPPH radical scavenging ability, reducing power and Fe^{2+} chelating ability) to various extents, depending on their Y_{sp} values or the periods of hydrolysis time. The DPPH radical scavenging and Fe^{2+} chelating abilities of the hydrolysates were closely related to their peptide profiles and/or H_o values. The hydrolysates with higher contents of hydrophobic amino acids seem to have higher DPPH radical scavenging and Fe^{2+} chelating abilities.

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