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The Purification of Free Radical Scavenging Peptides from Naked Oats

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Abstract The present study conducted enzymatic hydrolysis of naked oats glutelin by Alcalase (2.4L FG).Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) result showed that the molecular weight of naked oats glutelin ranged from 14 to 97 kDa. Ion exchange chromatography was used to isolate and purify naked oats glutelin hydrolysates. The results showed that hydrolysates were purified into 4 fractions by ion exchange chromatography. Fraction D showed higher scavenging effect against hydroxyl radical (IC_{50} 1.40 mg mL^{-1}), and 2,2-Dipheyl-1-picrylhydrazyl (DPPH) radical (IC_{50} 1.26 mg mL^{-1}) than the other 3 fractions. Moreover, fraction D exhibited higher scavenging effect against hydroxyl and DPPH radical than the hydrolysates. Free radical scavenging capacity of naked oats glutelin hydrolysates was improved by isolation and purification.

Keywords: *naked oats, glutelin, enzymatic hydrolysis, purification, free radical*

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1. Introduction

Bioactive peptides refer to peptides with special biological functions, which also have metabolic and physiological functions to humans, besides being digestible and easy to absorb. Due to their hormonal regulation, immune improvement, hypotensive, hypolipidemic, antifatigue, antiviral and/or antioxidant activities, bioactive peptides have become a promising functional ingredient in pharmaceutical and functional food industry. Free radicals can attack cell wall and macromolecules, which lead to certain diseases and aging [1]. Under normal physiological conditions, the human body produces free radicals and free radical scavenging enzymes in dynamic equilibrium state. However, in adverse environment or with ageing, this balance can hardly be maintained. Either human body produces excess amount of free radicals or is not capable of scavenging those free radicals. Free radical scavengers are also known as antioxidants, which can remove free radicals and lower the risks of certain diseases.

Since the nineteen forties, proteolysis was utilized by individuals those can not digest protein [2]. Typically, protein modification related to physical, chemical or enzymatic action change the structure, which could cause physicochemical and structural characteristic changes [3]. Compared to strong acid or alkali hydrolysis, protein enzymatic hydrolysis has minimal formation of by-products due to selective protease and milder process condition [4,5].

The peptides produced by proteolysis have smaller molecular size and less secondary structure than proteins isolate and may be expected to increased solubility near the isoelectric point, decreased viscosity and significant changes in the foaming, gelling, and emulsifying properties from those of original proteins [6].

Owing to the growing market demand, lots of protein isolated from various sources appeared these years [7]. Research of antioxidant peptide mainly focuses on plant protein. Antioxidant bioactive peptides were isolated from soy protein [8,9,10]. Kou *et al.* [11] got antioxidant peptides from chickpea albumin. Cheng *et al.* [12] studied the wheat germ antioxidant peptide. Researchers also evaluated the free radical scavenging activity of buckwheat globulin and albumin [13,14] as well as naked oats globulin [15] extracts, and confirmed that they have certain antioxidant activity.

Oats protein was shown to have good nutritional value and functional properties [16] (Christina and Elke, 2012). The major protein fraction of naked oats is globulin, but glutelin content is also relatively high (20%~25%) [17].The objective of the present study is to isolate and purify naked oats glutelin hydrolysates, evaluate the free radical scavenging activity of the peptides.

2. Materials and Methods

2.1. Materials and Reagents

Naked oats, harvested in 2012 in department of agriculture, Inner Mongolia Agricultural University, China, stored at 0 ~ 4°C. 732 strong acid ion exchange resin (Resin Co., Ltd of Linhai, Jiangsu). DPPH (Sigma-Aldrich, USA). Coomassie brilliant blue G-250, lauryl

sodium sulfate (SDS), acrylamide, bisacrylamide, tris-base (Sigma), N,N,N,N′-Tetramethylethylenediamine (TEMED), Alcalase 2.4L FG(Novozymes, Beijing, China). Peroxide, hydrochloric acid, ammonium persulfate, ammonium ferrous sulfate, salicylic acid, sodium hydroxide, ammonia water, glacial acetic acid, ethanol, ammonium acetate, the above are all provided from Tianjin third chemical reagent factory, China and all analytical grade.

2.2. Instrumentation

DYCZ-24D vertical electrophoresis tank, DY-602 steady flow regulator electrophoresis, WD-9405A rockers (Beijing Liuyi instrument); UV-Vis spectrophotometer (Beijing general instrument Co., Ltd.); H2500R-2 High-speed refrigerated centrifuge (Hunan Xiangyi laboratory instrument development Co., Ltd.); FD-2 freeze dryer, nucleic acid protein chromatography (Beijing Boyikang experimental instruments Inc.); chromatographic column(2.6 × 60 cm) (Shanghai Jinhua chromatography equipment factory).

2.3. Methods

2.3.1. Preparation of Naked Oats Glutelin

Naked oats glutelin was prepared by Osborne [18] method. Briefly, the flour (through 80 mesh sieve and defatted) was fully dispersed in 12-fold volume of 0.1 mol L^{-1} NaOH for 2 h at room temperature. The dispersion was centrifuged at 10000g for 15 min, and the resultant supernatant was adjusted to pH 4.8 using 1.0 mol L^{-1} HCl to precipitate the glutelin. The precipitate was obtained by centrifugation at 10000g for 15 min and the precipitate was freeze-dried to obtain naked oats glutelin.

2.3.2. Determination of Molecular Weight of Glutelin by Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

15% separation gel and 5% spacer gel was used to analyze glutelin and standard protein by SDS-PAGE electrophoresis 10 μL of sample was loaded on the SDS-PAGE and ran for 5h with constant current at 30 mA, followed by comassie brilliant blue R-250 staining for 1h, then decolorated by acetic acid, ethanol overnight, and photographed in the Gel Electrophoresis System Imager.

2.3.4. Enzymatic Hydrolysis of Naked Oats Glutelin and Preparation of the Hydrolysates

Five grams of naked oats glutelin were dispersed in 500 mL of deionised water at room temperature. The dispersions were pre-incubated at 55°C, prior to adjusting pH of the dispersion to 8.5. The mixture of protein and enzyme (Alcalase) at various enzyme-to-substrate (E/S) ratios of 1:40, 1:20 and 1:10 (v/w) was incubated in a temperature-controlled water bath at 55°C. The pH of the mixture was kept constant during hydrolysis, by addition of 0.5 mol L^{-1} NaOH. The change in degree of hydrolysis (DH) during the enzymatic hydrolysis was followed by pH-stat method [19].

The percent DH was calculated according to the following equation:

$$DH\ (\%) = \frac{V_{NaOH} \cdot N_{NaOH}}{\alpha \cdot M_p \cdot H_{tot}} \times 100$$

where V_{NaOH}, N_{NaOH}, Mp and H_{tot} are the base consumption in mL, the normality of the base, the mass of protein being hydrolysed (g), and the total number of peptide bonds in the protein substrate (meqv g^{-1} protein), respectively. The H_{tot} was calculated from the amino acid composition of naked oats. In the present study, the H_{tot} of naked oats was calculated to be 7.31mmol $g^{-1}$of protein. The 1/α is the calibration factor for pH- stat, and also the reciprocal of the degree of dissociation of the α- NH_2 groups. The α was calculated as the following equation:

$$\alpha = \frac{10^{(pH - pK)}}{1 + 10^{(pH - pK)}}$$

where pK is the average dissociation value for the α-amino groups, calculated according to the Gibbs-Helmholz equation[19]. At 55 °C (in the present study), the average dissociation value pK can be calculated to be 7.1.

The hydrolysates were prepared using Alcalase at an E/S ratio of 1:20 (v/w). At specific periods of hydrolysis time, aliquots of the digested mixture were taken out, and heated at 100 °C for 10 min, and then cooled immediately in ice water to room temperature. The resulting digests were centrifuged at 10000g for 15 min to remove insoluble residues. The supernatants were then adjusted to pH 4.8, and lyophilised to produce the hydrolysates samples, which were stored at -20°C before further analysis.

2.3.5. Isolation of Naked Oats Hydrolysates by Ion Exchange Chromatography

Pretreatment of ion exchange resin (strong acid type)

The resin was soaked in deionized water for 2~3 h. After the impurities were removed and water was clear, two volumes of 2% sodium hydroxide solution were added and mixed for 4 h. Then the sodium hydroxide was washed out to pH8~10. Two volumes of hydrochloric acid solution were added and mixed for 4 h. At the end, the acid was washed out until it become neutral. At this point, the resin has been converted into H type.

Static adsorption experiment

4 g of resin was weighed, and pretreated as the steps above. Then put the resin into 250mL erlenmeyer flask, added 50 mL 10 mg/mL naked oats glutelin hydrolysates, vibrated 12 h at room temperature. The filtrate was taken and the volume was recorded. The peptide content was determined by folin phenol colorimetric method [20].

Absorption rate = (initial concentration of the sample solution- concentration of the sample solution when equilibrium)/ initial concentration of the sample solution

Purification of naked oats hydrolysates by ion exchange chromatography

Component, which exhibited the strongest free radical scavenging activity, was dissolved in ammonia acetate buffer (pH4.5), at concentration of 24 mg mL^{-1}. Column was packed with prepared resin on a column sized 2.6×60 cm, then conditioned with ammonia acetate buffer (pH4.5) for 12 h. The sample was eluted with 0.2 mol/L ammonia water at constant speed (1.0 mL min^{-1}). The eluant was collected with fraction collector. After the absorbance was

measured by protein nucleic acid detection instrument at 220 nm, the same peak was collected and lyophilized for further analysis.

2.3.6. Free Radical Scavenging Activity of Naked Oats Glutelin Hydrolysates

Scavenging effect against hydroxyl radical

1mL of ammonium ferrous sulfate (7.5×10^{-3}mol L^{-1}), 1mL of salicylic acid (7.5×10^{-3}mol L^{-1}), 1mL of 0.3% peroxide, and 1mL of the extract were added in order into a colorimetric tube, and the volume was set to 10 mL with the deionized water. After 30 min, taking extract as reference, the absorbance was measured with a spectrophotometer at 510 nm, and free radical scavenging capacity was calculated according to the following formula [21]:

$$\text{Scavenging capacity}(\%) = \left(A_{compare} - A_{sample}\right) / A_{compare} \times 100\%$$

$$= \frac{\left[\left(A_{compare} - A_{reference}\right) - \left(A_{sample} - A_{sample\ reference}\right)\right]}{\left(A_{compare} - A_{reference}\right)} \times 100\%$$

where: $A_{reference}$—the absorbance of added Fe^{2+} and H_2O_2; $A_{compare}$—the absorbance for hydroxyl radical system of added Fe^{2+}, H_2O_2 and salicylic acid; $A_{sample\ reference}$—the absorbance of added Fe^{2+}, H_2O_2 and different extracts; A_{sample}—the absorbance for hydroxyl radical system of added Fe^{2+}, H_2O_2, different extracts and salicylic acid.

Evaluation of DPPH radical scavenging activity

DPPH radical scavenging capacity assay was carried out according to Brand-Williams *et al*., [22], with slight modifications. 2 mL of deionized water and 2 mL of sample were added to 2 mL of DPPH ethanol solution (0.1 mmol/L, freshly prepared) respectively, vertex to dissolve. After 30 min of reaction at room temperature, the optical density (OD) values were measured at 517 nm by spectrophotometer to get A_0 and A_1. 2 mL of sample was added to an equal volume of absolute ethanol and measure at same condition to get A_2.

$$\text{Free radical scavenging capacity}(\%) = [1-(A_1-A_2)/A_0] \times 100\ \%$$

2.4. Statistical Analysis

Each experiment was performed at least 3 times, and data were expressed as the mean± standard deviation (SD). One way analysis of variance (ANOVA) and Duncan's new multiple range test were used to determine the differences among these means at $p < 0.05$.

3. Results and Discussion

3.1. Naked Oats Glutelin SDS-PAGE Electrophoresis

As shown in Figure 1, left band is the sample and the right is molecular mass markers. Naked oats glutelin molecular weight distributes between 14~97 kDa, mainly between 29~66 kDa. This result is the same as oats glutelin molecular weight distribution reported by Yu-Wei *et al*., [23].

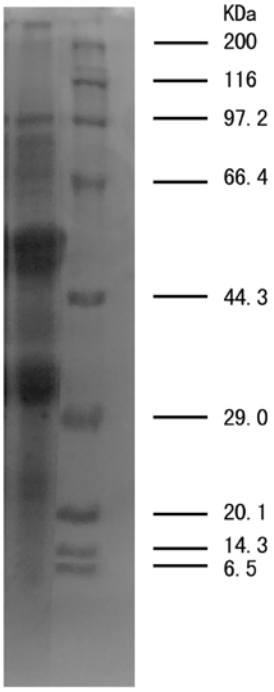

Figure 1. SDS–PAGE patterns of naked oats glutelin

3.2. Enzymatic Hydrolysis

The DH changes during enzymatic hydrolysis of naked oats glutelin using Alcalase with E/S ratios of 1:40, 1:20 and 1:10 (v/w), respectively, were monitored by pH-stat method for up to 4 h, as shown in figure 2. As expected, the DH increased with hydrolysis time, indicating gradual release of peptide fragments during the hydrolysis. The rate of the hydrolysis or the release of peptide fragments was fast during initial hydrolysis (e.g., <0.5 h) and gradually decreased with the hydrolysis time increasing. Upon further hydrolysis, e.g., >3 h, the rate of hydrolysis was nearly unchanged. The change of DH with hydrolysis time is similar to that of defatted buckwheat protein treated by Alcalase [24].

The DH of naked oats glutelin was closely dependent upon the concentration of applied enzyme, namely the E/S ratio. When the E/S ratio is 1:40, the DH is lower than that of E/S ratio of 1:20 and 1:10. However, there were no significant differences between the DH when the E/S ratios were 1:20 and 1:10, respectively (Figure 2). Finally, the optimum hydrolysis conditions, in terms of hydrolysis time and E/S ratio, were 3 h and 1:20 (v/w).

3.3. Free Radical Scavenging Activity of Naked Oats Glutelin Hydrolysates

Hydroxyl and DPPH radical scavenging effect of naked oats glutelin hydrolysates are shown in figure 3. The scavenging effect against two kinds of free radicals showed quadratic upward trend with the concentrate of hydrolysates was increased. The 50% inhibiting concentration (IC_{50}) of scavenging effect against hydroxyl and DPPH radical is 4.02 mg mL^{-1} and 2.33 mg mL^{-1} respectively.

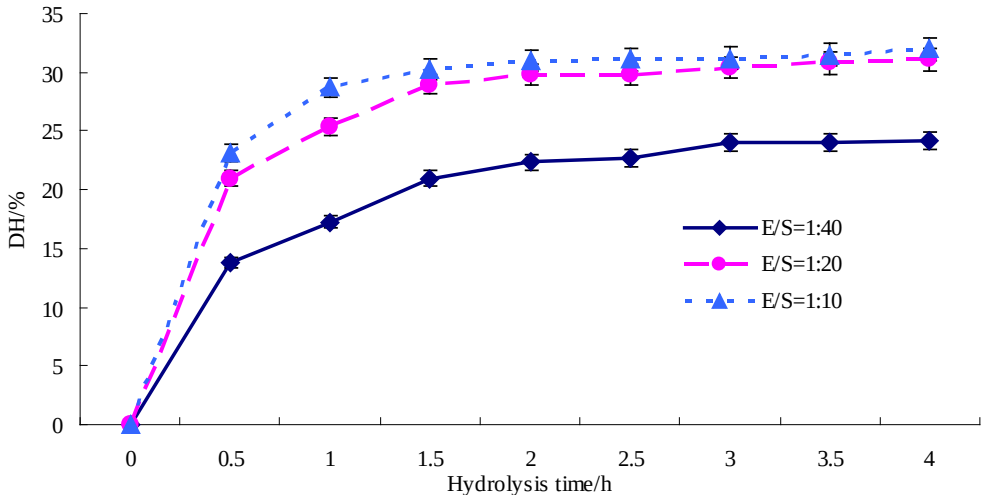

Figure 2. DH changes of naked oats glutelin during hydrolysis by Alcalase at various E / S ratios

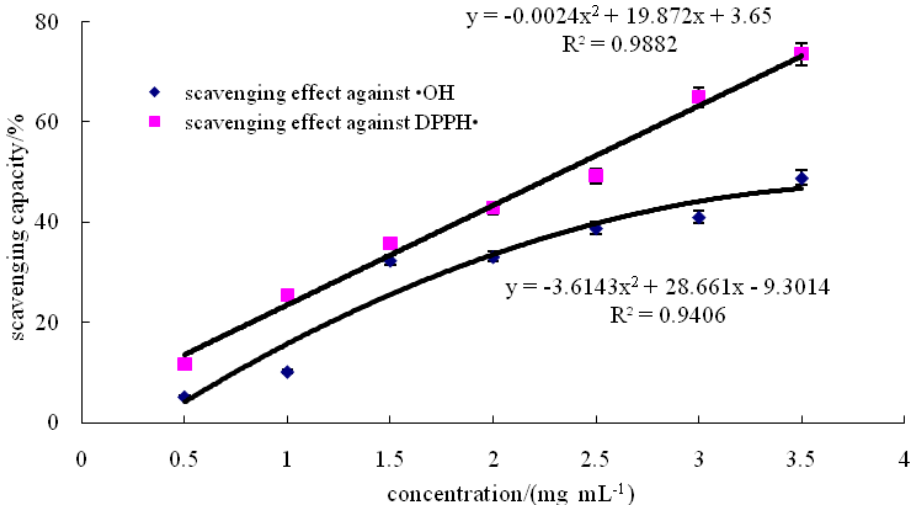

Figure 3. Free radical scavenging activity of naked oats glutelin hydrolysates

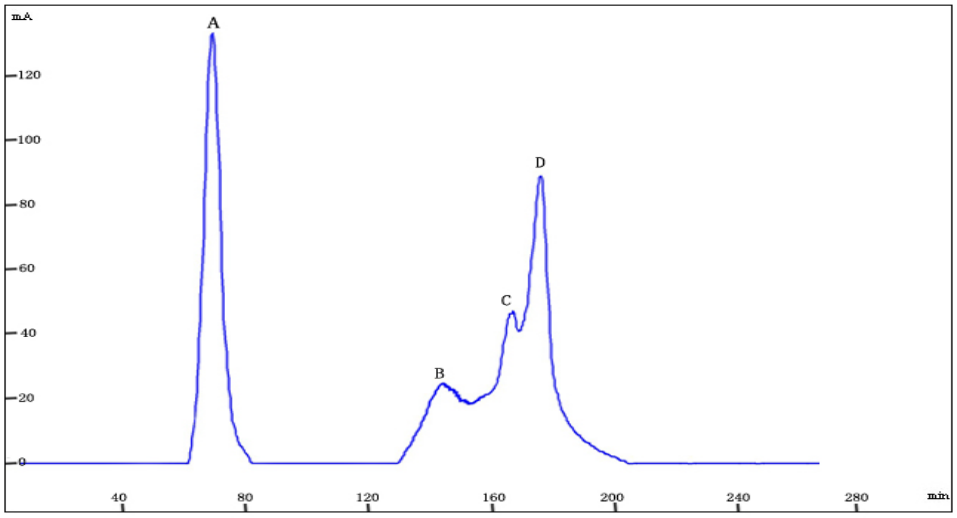

Figure 4. Purification of glutelin hydrolysates by ion exchange chromatography

3.4. Isolation and Purification of Naked Oats Hydrolysates by Ion Exchange Chromatography

The absorption rate of 732 strong acid ion exchange resin is 89.26%, it showed that absorption to the naked oats glutelin peptide is relatively strong. As shown in Figure 4, glutelin hydrolysates were further purified into A, B, C, D four fractions. Fraction A had the shortest retention time, which is not easily absorbed by cation resin, indicating an acidic amino acid. However, the retention time of fraction D is the longest, which indicates a basic amino acid [25].

3.5. Evaluation of Free Radical Scavenging Activity of Each Fraction

The hydroxyl radical scavenging activity of each fraction was evaluated. The results indicate that fraction D has the strongest hydroxyl radical scavenging activity as shown in Figure 5-a. IC_{50} of hydroxyl radical scavenging activity of fraction D was 1.40 mg mL^{-1} and fraction A IC_{50} was 2.39 mg mL^{-1}. Hydroxyl radical scavenging activity of fraction C and fraction B were weak.

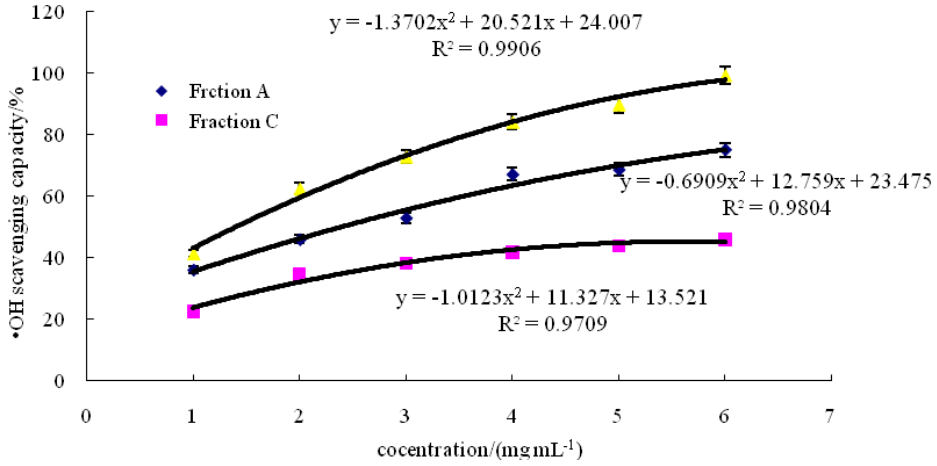

Figure 5-a. hydroxyl radical scavenging activity of each fraction after ion exchange purification

As shown in Figure 5-b, DPPH radical scavenging activity of each fraction showed the same trend as the hydroxyl radical scavenging assay. IC_{50} of fraction D was 1.26 mg mL^{-1} and 2.08 mg $mL^{-1}$for IC_{50} of fraction A. Fraction D showed stronger DPPH radical scavenging activity than fraction A. The DPPH radical scavenging activity of fraction C and fraction B were weak. Therefore, fraction D was the best candidate for further purification. Generally, basic amino acids have stronger antioxidant than neutral and acidic amino acids [25]. Chen *et al.*, [8] purified VNPHDHQN, LVNPHDHQN, LLPHH, LLPHHADADY and LNSGDALRVPSGTTYY peptide from soy protein hydrolysate, which are all basic peptides with strong antioxidant activity. Tsuge *et al.* [26] purified albumen hydrolysate and collected AH, VHH and VHHANEN peptide. They also found basic amino acids have strong antioxidant properties.

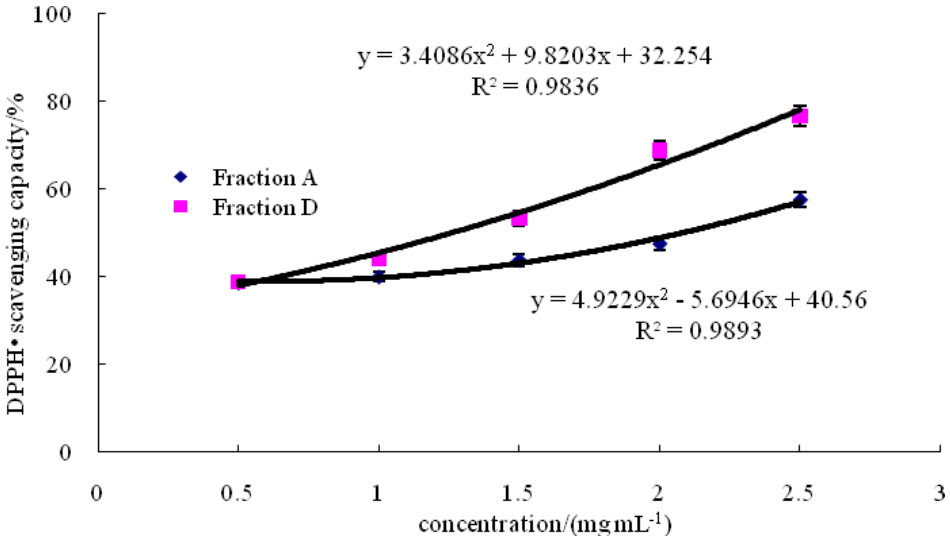

Figure 5-b DPPH radical scavenging activity of fraction A and D after ion exchange purification

3.6. Comparison of Free Radical Scavenging Activity of Naked Oats Glutelin Hydrolysate before and after Purification

According to Table 1, the scavenging effect against hydroxyl and DPPH radicals are both in the order of Fraction D> Fraction A> glutelin hydrolysates. Free radical scavenging activity of naked oats glutelin hydrolysates depends on the distribution of the molecular size. It indicates that short peptides may be composed of various amino acid sequences which have biological activity.

Table 1. IC_{50} of naked oat glutelin hydrolysates and purified fractions in free radical scavenging assays

Fraction	IC_{50}/(mg mL^{-1})	
	·OH	DPPH·
Hydrolysates	4.02±0.04	2.33±0.03
Fraction A	2.39±0.02	2.08±0.02
Fraction D	1.40±0.01	1.26±0.02

4. Conclusions

The present study conducted the process of enzymatic hydrolysis of naked oat glutelin to obtain the bioactive peptides at the optimal condition. The naked oat glutelin hydrolysates were isolated and purified by ion exchange chromatography into four different fractions. These four different fractions were subjected to evaluation of free radical scavenging activities. The result showed that the naked oat glutelin hydrolysates which extracted from naked oats and hydrolyzed by Alcalase has strong antioxidant activity evaluated by two different free radical scavenging systems in vitro. The free radical scavenging ability of the peptides was significantly improved after the hydrolysates were isolated and purified by ion exchange chromatography. In conclusion, the peptide derived from naked oat possesses strong antioxidant activity. These results provided valuable references to understand the naked oats health benefits since the naked oats is a less common cereal compared to wheat and barley. Further studies should be conducted on the naked oats peptide structures and the mechanisms of their health benefits.

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