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Antioxidant properties of papain hydrolysates of wheat gluten in different oxidation systems

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Abstract

Enzymatic hydrolysis was used for preparing hydrolysates from wheat gluten which is by-product during production of wheat starch. The enzyme used for the hydrolysis was papain. The hydrolysate was separated based on the molecular weight of the peptides by membrane ultrafiltration (UF) with a molecular weight cut-off of 5 kDa into permeate (P) and retentate (5-K) fractions. The antioxidative activities of the hydrolysate and its UF fractions were investigated by using the TBA method and scavenging effect of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The three fractions showed strong antioxidative activities in the linoleic acid oxidation system, and exhibited DPPH radical scavenging activity. The antioxidative activity of the P fraction was almost the same as that of vitamin E at pH 7.0. The molecular weight distribution of the P fraction was concentrated in 4.2 kDa (86.5%) after gel permeation chromatography fractionation using an HPLC system.The P and 5-K fractions had higher surface hydrophobicities (H_0) at pH7.0 compared with the hydrolysate. The resulting UF fractions were superior to the hydrolysate in terms of antioxidative activities.

Keywords: Wheat gluten hydrolysate; Antioxidative activity; 2-Thiobarbituric acid; 1,1-Diphenyl-2-picrylhydrazyl radical scavenging; Molecular weight distribution

1. Introduction

Lipid or fatty acid oxidation will result in quality deterioration and shorten the shelf-life of food products. The main cause is that oxidation can generate some free radicals that lead to fatty acid decomposition and development of undesirable rancid odors and flavors (Akoh & Min, 2002; Nawar, 1996). Numerous lines of evidence have indicated that free radicals play a critical role in a variety of pathological conditions including the processes of aging, cancer, multiple sclerosis, inflammation, coronary heart and cardiovascular diseases, senile dementia, arthritis and atherosclerosis (Blake & Winyard, 1995; Halliwell & Gutteridge, 1990; Halliwell & Gutteridge, 1999). Following the growing realization that a wide range of herbal medicines and foodstuffs may be credited for preventive effects on chronic diseases due to their radical scavenging or antioxidant properties, although the overall function in vivo has yet to be clarified, increasing attention has been directed to the development of safe and effective functional foods and the extraction of novel potential antioxidants from medicinal plants (Gordon, 1996; Potterat, 1997). Synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, may be used to prevent food products from deterioration during storage, and to extend the shelf-life of the food products. However, the demand for natural antioxidants has recently increased because of questions about the long-term safety and negative consumer perception of synthetic antioxidants (Yu et al., 2002).

Recently, some protein hydrolysates have been reported to exhibit antioxidant activity. The protein hydrolysates

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have effective antioxidative activities against the peroxidation of lipids and/or fatty acids (Amarowicz & Shahidi, 1997; Diaz & Decker, 2004; Pena-ramos & Xiong, 2002; Sakanaka, Tachibana, Ishihara, & Juncja, 2004).

Wheat gluten and wheat starch are economically important coproducts produced during wet processing of wheat flour. Wheat gluten includes two main components, glutenins and gliadins. They are highly polymorphic polypeptides, consisting of more than 60 different molecular weight species ranging in M_r from 30,000 to 90,000 kDa (Payne, Nightingale, Krattiger, & Holt, 1987; Shewry, Halford, & Tatham, 1992). In the food industry, wheat gluten is traditionally used as an additive to improve the baking quality of flour. It is readily available in large quantities and at low prices. The use of wheat gluten in food and non-food applications is gaining much interest. However, little information about antioxidants derived from wheat gluten proteins can be found.

In this present study, the antioxidant activities of wheat gluten hydrolysate and its UF fractions obtained by papain hydrolysis and membrane ultrafiltration were investigated in comparison with that of commercial antioxidant VE in two different oxidation systems. Meanwhile, amino acid composition and surface hydrophobicity of the hydrolysate and its UF fractions derived from wheat gluten were evaluated.

2. Materials and methods

2.1. Raw materials

Wheat gluten, produced by the Martin process, was obtained from Lianhua Co., China. Gluten contained 71.5% (m/m, dry basis) protein, 6.8% moisture. Papain (600,000 U/g) was purchased from Guangzhou Enzyme Co. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was of analytical grade from Nacalai Tesque (Kyoto, Japan). Linoleic acid (about 99%) and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals were of analytical grade.

2.2. Preparation of wheat gluten hydrolysate and its UF fractions

Eight percent of an aqueous dispersion of wheat gluten was heated in a water bath at 90 °C for 30 min prior to enzymatic hydrolysis.The enzymatic hydrolysis was carried out at 50 °C, constant pH 6.5 with an enzyme to substrate ratio[E/S] of 6000 U/g for 6 h. The enzyme was inactivated by heating at 100 °C for 10 min.The resulting hydrolysate was then rapidly cooled to ambient temperature in the ice bath, and was passed through a 5 kDa molecular weight cut-off (A/G Technology Co., model UFP-5-C, Needham, MA, USA) membrane. The hydrolysate and its UF fractions (Permeate, P fractions; retentate, 5-K fraction) were freeze-dried and stored at −20 °C until use.

2.3. Measurement of antioxidant activity

2.3.1. Scavenging effect of DPPH radical

The free radical scavenging activity was measured using the method of Chen, Muramoto, Yamauchi, Fujimoto, and Nokihara (1998). The dried hydrolysate and its UF fractions were dissolved in 4 ml distilled water at protein concentration with 0%, 0.04%, 0.08%, 0.12%, 0.16%, 0.2% and 0.24% (m/v,). A 1 ml of 40 μM DPPH radical in ethanol was added to the sample solution. The absorbance at 517 nm was measured after 30 min of incubation at 25 °C. The residual radicals in the samples were calculated according to the following equation:

$$\text{Residual DPPH radicals } (\%) = 100 - [(\text{DPPH blank} + \text{control sample}) - \text{DPPHsample}/(\text{DPPH blank})] \times 100$$

where DPPH blank is the value for 4 ml of water/1 ml of ethanol including 40 μM DPPH, the DPPH sample is the value of 4 ml of sample solution/1 ml of ethanol including 40 μM DPPH, and the control sample is the value of 4 ml of sample solution/1 ml of ethanol.

2.3.2. TBA method

The hydrolysate and its UF fractions at 250 mg of protein dissolved in 4.87 ml of distilled water, 0.13 ml of linoleic acid, 10 ml of ethanol, and 10 ml of 50 mM phosphate buffer (pH 7.0) were mixed in glass flasks. The flasks were sealed tightly with silicone rubber caps and kept at 40 °C in the dark. At regular intervals, aliquots of the reaction mixtures were withdrawn with a microsyringe for measurements of the oxidation using the thiobarbituric acid (TBA) method with minor modification (Ohkawa, Ohishi, & Yagi, 1979). The reaction mixture (50 μl) was added to a mixture of 0.8 ml of 20% acetic acid (pH 3.5), and 1.5 ml of 0.8% TBA solution in water. The mixture was cooled in ice bath, it was centrifuged (5000 rpm) for 10 min. The absorbance of the supernatant was determined at 532 nm and antioxidative activity was expressed as malondialdehyde (MDA) concentration.

2.4. Molecular size distribution analysis

Molecular size distribution profile of the hydrolysate and its UF fractions was determined by HPLC (Water's 1525, USA) on a GPC column. GPC column (7.8 mm × 30 mm, Protein-Pak60) with exclusion limits of 1–20 kDa was connected. The elution buffer was 0.05 M Tris–HCl (pH 7.2), flow rate 0.7 ml/min, and monitored at 280 nm.

2.5. Amino acid analysis

The PICO TAG method, with modification, was used for measuring the amino acid profile of the hydrolysate and its UF fractions (Bildlngmeyer, Cohen, Tarvin, & Frost, 1987). The dry sample(weight equivalent to 4%

protein) was added with 6 N HCl (15 ml) and placed in the oven at 110 °C for 24 h. Internal standard (10 ml) was added to the mixture. After derivatisation, 100 μl PICO TAG diluent was added and mixed. Sample (100 μl) was then injected into the HPLC and analysed with a Water's PICO TAG amino acid analyzer.

2.6. Surface hydrophobicity (H_0)

Values of H_0 were determined by the hydrophobicity fluorescence probe [with 1-anilino-8naphthalene sulfonate (ANS) according to the method reported by Kato and Nakai (1980). Protein dispersions (1 mg/ml) were prepared in 0.01 M phosphate buffer (pH7.0) with or without NaCl (0.1–1 M), stirred for 2 h at 20 °C, and centrifuged for 20 min (8000 rpm). Protein concentration in the supernatants was measured by the micro-kjeldahl procedure. Each supernatant was serially diluted with the same buffer to obtain protein concentrations ranging from 0.5 to 0.005 mg/ml; a volume of 3 ml of each diluted sample was then added with 40 ml of ANS (8.0 mM in 0.1 M pH 7.0 phosphate buffer solution). Fluorescence intensity (FI) was measured at 365 nm (excitation) and 484 nm (emission) and the reading was calibrated by adjusting it to a value of 80 (1×) with 15 ml of ANS in 3 ml of methyl alcohol (Parker HPLC grade).The initial slope of the FI versus protein concentration plot (calculated by linear regression analysis) was used as an index of protein surface hydrophobicity.

2.7. Statistical analysis

All the tests were done in triplicate and data were averaged. Standard deviation was also calculated. Duncan's multiple-range test (Steel & Torrie, 1980) was used to evaluate significant differences ($P < 0.05$) between the means for each sample.

3. Results and discussion

3.1. Preparation of the gluten hydrolysate and its fractions

The wheat gluten hydrolysate was prepared by means of enzymatic hydrolysis with papain. The resultant hydrolysate was fractionated by ultrafiltration performed with a 5 kDa molecular weight cut-off membrane. The hydrolysate and its UF fractions (P and 5-K fractions) were collected and freeze-dried. The P fraction included 26.4% of protein, whereas 5-K fraction had 53.8% of protein.

3.2. Antioxidative activities of the hydrolysate and its UF fractions

DPPH is a stable free radical which has commonly been used in antioxidant activity analysis. This test system can be used for the primary characterization of the scavenging potential of compounds (Hatano et al., 1989; Yamaguchi, Takamura, Matoba, & Terao, 1998; Yoshida et al., 1989). As shown in Fig. 1, the hydrolysate and its UF fractions had the ability to quench the DPPH radical. Obviously, the scavenging effect of these fractions increased with increasing concentrations of the protein in the samples used in the test. Significant ($P < 0.05$) increase in the DPPH scavenging activity of P fraction compared to the hydrolysate and 5-K fraction at the range of concentrations used was found. The DPPH scavenging activity of P fraction at low concentration of protein was almost the same as that of V_E (α-tocopherol), the commercial antioxidant used in the food industry. It indicated that the wheat gluten hydrolysate and its UF fractions were excellent antioxidant compounds with radical scavenging activity. The results revealed that the wheat gluten hydrolysate and its UF fractions possibly contained some substrates, which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

Antioxidative activities of wheat gluten hydrolysate and its UF fractions were determined by means of TBA method and compared with those of α-tocopherol. As shown in Fig. 2, the oxidation activities of linoleic acid were markedly inhibited by the addition of the hydrolysate and its UF fractions. Among the three fractions, the highest antioxidative activity was found in the P fraction, which exhibited significant ($P < 0.05$) inhibition of linoleic acid peroxidation. The antioxidative activity of P fraction was

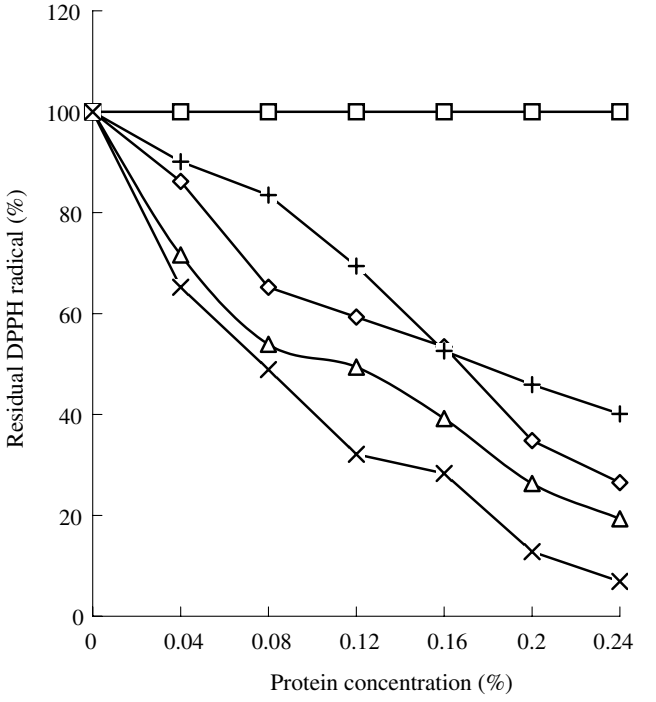

Fig. 1. DPPH radical scavenging activity of the hydrolysate and its UF fractions. □, Control (distilled water alone); ◇, the hydrolysate; △, P fraction; +, 5-K fraction; ×, α-tocopherol. The values are the means of triplicates.

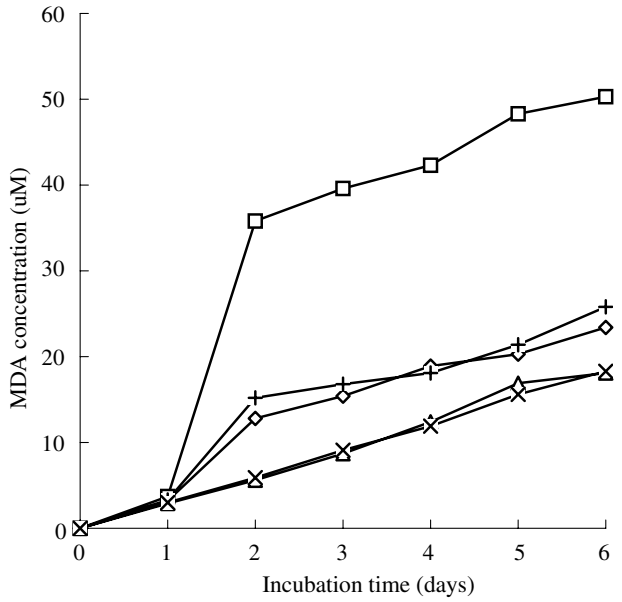

Fig. 2. Antioxidative activities of the hydrolysate and its UF fractions. The activity was determined by the TBA method. The symbols are the same as Fig. 1. The values in figure are the means of triplicate.

Table 1
Amino acid composition of the hydrolysate and its UF fractions

Amino acid	Composition (mg/g protein)[a]		
	Hydrolysate	P fraction	5-K fraction
Aspartic[a]	81	23	50
Glutamic acid[b]	412	342	320
Serine	49	52	38
Glycine	37	39	29
Histidine	75	85	78
Arginine	34	36	23
Threonine	27	29	21
Alanine	21	21	42
Proline	100	102	90
Tyrosine	48	36	75
Valine	28	29	21
Methionine	4	9	7
Cysteine	2	2	1
Isoleucine	22	23	17
Leucine	48	56	54
Phenylalanine	50	44	33
Lysine	11	10	7
Total AA	939	928	906

[a] Aspartic + asparagines.
[b] Glutamic acid + glutamine.

similar to that of α-tocopherol. The hydrolysate and 5-K fraction also showed inhibition of the oxidation, but the inhibition of the oxidation was not significant ($P < 0.05$).

These results, therefore, indicated that the hydrolysates and its UF fractions seemed to contain some antioxidative peptides.

3.3. Amino acid composition change of the hydrolysate and its UF fractions

Some amino acids, such as His, Tyr, Met, and Cys, had been reported to show antioxidant activity (Marcuse, 1960). Especially, histidine exhibited strong radical scavenging activity due to the decomposition of its imidazole ring (Yong & Karel, 1978). Carnosine was a famous antioxidant peptide in muscle protein, and its activity had been suggested to be due to the radical scavenging activity (Decker, Crum, & Calvert, 1992) and the quenching of singlet oxygen species (Dahl, Midden, & Hartman, 1988) by His. Table 1 presents the amino acid profiles of the hydrolysate and its UF fractions. Although they were rich in glutamic acid, the hydrolysate and its UF fractions contained His, Leu, Val, and Ala. Therefore, the antioxidative activities of the wheat gluten hydrolysates seemed to be caused by these amino acids in the hydrolysate peptides. It was presumed that the amino acids such as His, Leu, Val, and Ala present in the sequence of the hydrolysate peptides had favored the radical scavenging properties of the wheat gluten hydrolysates. Moreover, these amino acids were reported to be effective in inhibiting oxidation of fatty acids tested in a linoleic acid model system (Marcuse, 1962). The antioxidative activity of the peptides isolated from protein hydrolysates depended upon the amino acid sequence of the peptides in the hydrolysates (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Kim et al., 2001; Suetsuna, Ukeda, & Ochi, 2000).

3.4. Change in molecular size distribution profile of gluten hydrolysates

The gel permeation chromatography (GPC) using an HPLC system was used to study molecular weight distribution profiles of the hydrolysates. Table 2 shows the molecular size distribution profiles of the hydrolysate and its UF fractions. The chromatogram indicated that major peaks of the hydrolysate was located at >20, 10–20, 5–10, 5, 4.2, 2–4, and <2 kDa, respectively. The molecular weight distributions of the P fraction were concentrated in 5, 4.2 (86.5%), and <2 kDa. While the molecular weight distributions of the 5-K fraction were in >5 kDa, 71.5% was that of molecular weight >20 kDa. According to their antioxidative

Table 2
Molecular weight distribution profile of the hydrolysates and its UF fractions[a]

Molecular weight (kDa)	Peak area (%)		
	Hydrolysate	P fraction	5-K fraction
>20	60.8 ± 5.2c	–	75.1 ± 8.2d
10–20	8.1 ± 2.4b	–	18.3 ± 1.6a
5–10	7.9 ± 0.5b	–	6.6 ± 0.4ab
5	2.8 ± 0.1ab	3.9 ± 0.3d	–
4.2	12.4 ± 0.7a	86.5 ± 6.4a	–
2 to 4	3.7 ± 0.1da	5.4 ± 0.3cd	–
<2	4.3 ± 0.2cd	2.2 ± 0.1b	–

[a] Values are means of two determinations ± standard deviation. Means in the same columns with different letters are significant difference ($P < 0.05$).

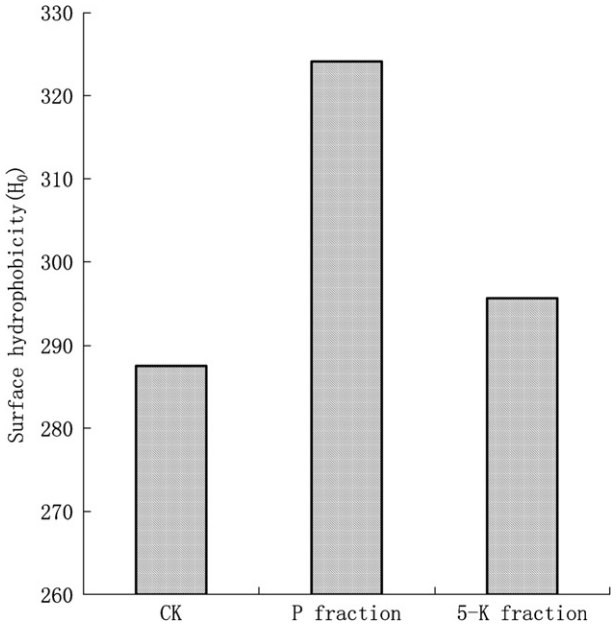

Fig. 3. Surface hydrophobicity (H_0) of the hydrolysate and its UF fractions at pH 7.0 (sample concentration, 0.1 mg/mL). The values are the means of at least two determinations.

activities, the strongest activity occurred in the P fraction in this study, it indicated that the antioxidative activity of the hydrolysates depended on their molecular weight distribution. The peptide fraction of 4.2 kDa in the P fraction was probably associated with higher antioxidative activity.

3.5. Change in the surface hydrophobicity of the hydrolysate and its fractions

The surface hydrophobicity of a protein is an indicator of the number of hydrophobic groups on the surface of a protein in contact with the polar aqueous environment. Surface hydrophobicity of a protein is associated with its capacity for intermolecular interaction, thereby influencing its functionality.

The surface hydrophobicity (H_0) of the hydrolysate and its UF fractions is shown in Fig. 3. The P and 5-K fractions showed higher surface hydrophobicities (H_0=324.1 ± 26.5 and 295.6 ± 23.7, respectively) at pH 7.0 in comparison with the hydrolysate ($H_0 = 287.5 \pm 16.3$). The P fraction increased significantly ($P < 0.05$) H_0 compared with the other two fractions. No significant ($P < 0.05$) difference in Ho between the hydrolysate and 5-K fraction. Significant difference between the results indicated that the peptides obtained by ultrafiltration had a high proportion of hydrophobic groups at pH 7.0 which would confer surface properties.

4. Conclusion

The gluten hydrolysate and its UF fractions showed strong antioxidative activities in the linoleic acid oxidation system and DPPH radical scavenging activity. The UF fractions obtained were superior to the hydrolysate in terms of the antioxidative activities. The molecular weight distribution of the P fraction was concentrated in 4.2 kDa (86.5%) after GPC fractionation. P fraction and 5-K fraction, especially the former, showed higher surface hydrophobicities (H_0) at pH 7.0 in comparison with the hydrolysate.

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