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ORIGINAL ARTICLE

Evaluation of wheat gluten hydrolysates as taste-active compounds with antioxidant activity

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Abstract Wheat gluten was subjected to enzymatic hydrolysis with various proteases (Alcalase, Flavourzyme, Protamex) and the taste-enhancing properties and antioxidant activities of the resulting wheat gluten hydrolysates (WGHs) were characterized. The contents of the hydrophobic amino acid of the WGHs were highly correlated with the degree of hydrolysis by Flavourzyme and Protamex, except Alcalase. The taste profiles of the Alcalase-treated WGHs showed decreased bitterness while umami and overall acceptability increased. On the other hand, the WGHs produced by Flavourzyme and Protamex showed increased bitterness with increasing hydrolysis duration. However, taste profiles, such as umami, kokumi, and overall acceptability of the WGHs by Flavourzyme and Protamex were unaffected by the degree of hydrolysis. The WGH treated by Alcalase for 24 h (A24h) exhibited taste-enhancing property and its antioxidant effects were concentration-dependent. As a result, the A24h may be used as a multi-functional seasoning ingredient having potential antioxidant activity.

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Introduction

Since late 1900s, chemical seasonings such as monosodium glutamate (MSG) and nucleotide flavors have been widely used as a taste enhancer in various dishes (Sand 2005). However, due to the recent exposure to bird flu, bovine spongiform encephalopathy (BSE), swine fever, and genetically modified organism (GMO), consumers have begun purchasing naturally-made and healthy seasoning compounds. Therefore, the food seasoning manufacturers have made a lot of efforts to meet the consumer preference for natural products. For example, Japanese manufacturers were able to develop a naturally-made ingredient from fermented products, enzymatic hydrolysate, vegetable and fishery extracts, and yeast extracts (Ueda et al. 1990, 1997; Tomohiko 2006). Hedwig and Amado (2002) also reported that peptides derived from vegetable proteins, such as soybeans, wheat, and corn, by enzymatic hydrolysis were associated with umami, a savory taste produced by glutamic acid or pyroglutamic acid. The taste of hydrolyzed vegetable protein (HVP) comes from amino acids, especially glutamic acid, smaller peptides, salt, and various volatile compounds (Aaslyng et al. 1998; Hedwig and Amado 2002).

Among these flavor ingredients, umami and kokumi are considered as major taste factors. Umami, which means “delicious” in Japanese, has shown the ability to strengthen the flavor (aroma and taste) and mouth-feel of savory dishes (Hedwig and Amado 2002). Umami compounds have been studied in various sources, such as Japanese soy sauces (Lioe et al. 2006), Japanese green tea (Kaneko et al.

2006), and fermented salmon fish sauce (Indoh et al. 2006). Noguch et al. (1975) isolated four acidic peptides, Glu-Asp-Glu, Asp-Glu-Ser, Thr-Glu, and Ser-Glu-Glu from an enzymatic fish protein hydrolysate, showing similar umami intensity to sodium glutamate. Hedwig and Amado (2002) also reported that pyroglutamyl peptides from deamidated wheat gluten yielded a glutamate-like taste and their sequences were determined to be pGlu-Pro-Ser, pGlu-Pro, pGlu-pGlu-Pro-Glu, and pGlu-Pro-Gln. Furthermore, Lioe et al. (2006) described that free Glu and Asp were the main contributors to the development of umami in soy sauces. On the other hand, kokumi means "rich," and taste continuity derived from amino acids and peptides (Hedwig and Amado 2002). Dunkel et al. (2007) reported γ-glutamyl peptides, such as γ-L-gluyamyl-L-leucine, γ-L-gluyamyl-L-valine, and γ-L-gluyamyl-L-cysteinyl-β-alanine isolated from edible beans as kokumi contributors. In addition, kokumi-active glutamyl peptides in cheeses were identified as γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His (Toelstede and Hofmann 2009; Toelstede et al. 2009).

Wheat gluten, which incidentally is produced through the wheat starch industry, is a very economic protein source (Kong et al. 2007). Wheat gluten, including gliadin and glutenin, is composed of polymorphic polypeptides, showing more than 60 different molecular weights ranging from 30,000 to 90,000 kDa (Wang et al. 2007a, b). Thus, trials for improving the functional properties of wheat gluten have been conducted in order to expand its applications. Among them, enzymatic hydrolysis was shown to be the most effective way in the amelioration of wheat glutens' functionalities, such as solubility, emulsifying, and foaming capacity (Kong et al. 2007; Wang et al. 2007a, b). In addition, Wang et al. (2007a, b) and Kong et al. (2008) have reported that wheat protein hydrolysate inhibited linoleic acid autoxidation and quenched DPPH radical. However, there is little information on the taste-enhancing effect and biological activity of wheat gluten hydrolysates which were prepared under different conditions of enzymatic hydrolysis.

Thus, wheat glutens were treated with different types of protease (Alcalase, Flavourzyme, Protamex) for 1 to 24 h to prepare for wheat gluten hydrolysates (WGHs) with various degree of hydrolysis. Then, their hydrophobic amino acid contents, sensory properties, and antioxidant activities were characterized.

Materials and methods

Materials Wheat gluten was obtained from Sempio Co. Ltd. (Korea). Alcalase 2.4L (EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g), Flavourzyme (EC 3.4.11.1, from *Aspergillus oryzae*, 500 LAPU/g), and Protamex (EC 3.4.28.24, from *Bacillus* sp., 1.5 AU/g) were purchased from Novozymes (Denmark). Trinitrobenzene-sulphonic acid (TNBS), DPPH, ABTS, trolox, ascorbic acid, thiobarbituric acid (TBA), trichloroacetic acid (TCA), and potassium ferricyanide were purchased from Sigma Chemical Co. (USA). All other reagents were of analytical grade.

Preparation of wheat gluten hydrolysates Wheat gluten was dispersed in distilled water (5%, *w*/*v*) and incubated at 50 °C for 30 min while stirring continuously. The pH of wheat gluten suspension was adjusted to 7.0 with 4 M NaOH. The reaction was initiated by the addition of the enzyme to yield a final enzyme-to-substrate ratio of 1:100. After the predetermined reaction time, the mixture was heated at 100 °C for 10 min to inactivate the enzyme and centrifuged at 3,000× g for 10 min to separate the hydrolysates. The supernatant was freeze-dried and stored at −80 °C before further analysis.

Degree of hydrolysis Degree of hydrolysis (DH) was measured by TNBS method according to Adler-Nissen (1979). Wheat gluten hydrolysates were diluted in 1% (*w*/*v*) sodium dodecyl sulfate (SDS) solution. Diluted sample solutions or standard solutions (0.05 ml) were mixed with 0.2125 M sodium phosphate buffer (pH 8.0, 0.4 ml) and 0.1% TNBS (0.4 ml), and then incubated at 50 °C for 60 min. After the reaction was stopped by adding 0.1N HCl (0.8 ml), the resultant solutions were cooled at room temperature for 30 min. The absorbance was measured at 340 nm by UV spectrophotometer (DU 650, Beckman Coulter Inc., USA) and L-leucine was used as a standard. DH values were calculated using the following formula.

$$\mathrm{DH}(\%) = (\mathrm{H}/\mathrm{Htot}) \times 100$$

H is the milli-equivalent/g of protein of α-amino group generated by the protease after a given hydrolysis time, and Htot is 8.3 milli-equivalent/g of total α-amino group, which can be generated from wheat gluten by hydrolysis (Nielsen et al. 2001).

Amino acid composition The total and free amino acid composition of WGHs were analyzed using an AccQ-Tag C_{18} column equipped HPLC system composed of 1,525 binary Pump, 717 plus auto sampler, and 474 fluorescence detector (Waters, USA). The eluent A was 0.14 M sodium acetate and 10% triethylamine adjusted to pH 5.02 with 1% phosphoric acid, while the eluent B was 40% water in acetonitrile. For total amino acid analysis, the hydrolysates were hydrolyzed with 6 M HCl at 100 °C for 22 h in sealed glass tubes under N_2 atmosphere and evaporated to remove HCl, followed by HPLC analysis.

Sensory analyses The panels were composed of ten trained assessors from Sempio Co. Ltd. (Korea). They were trained with reference solutions for the following taste attributes: sucrose (5%) for sweet taste, lactic acid (0.08%) for sour taste, NaCl (1%) for salty taste, caffeine (0.05%) for bitter taste, and sodium L-glutamate (0.3%) for umami taste in sensory experiments at regular intervals for at least 6 months. During the training for kokumi activity, the panels were asked to compare the gustatory impact of chicken broth (control) with serial dilutions of KOJI-AJI (0.3%) in chicken broth (both at pH 6.5). Sensory analyses were performed in a sensory panel room at 19–22 °C under red light by using a rating scale from 0 (not detectable) to 6 (intensely detectable) (Hedwig and Amado 2002; Toelstede and Hofmann 2009).

DPPH radical scavenging activity The DPPH radical scavenging activity was measured according to method described by Shimada et al. (1992). Sample solution (2 ml) was mixed with 0.1 mM DPPH in 95% ethanol (2 ml), left for 60 min at room temperature, and then the absorbance was measured at 517 nm. The DPPH scavenging activity was calculated using the following formula.

$$\text{Scavenging activity } (\%) = \frac{(C - CB) - (S - SB)}{C - CB} \times 100$$

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

ABTS radical scavenging activity The ABTS radical scavenging activity was measured according to the method described by Re et al. (1999). The ABTS stock solution was prepared with final concentration of 7 mM ABTS with 2.45 mM potassium persulphate. The ABTS solution was left in darkness at room temperature for 12 h before use. Then 40 uL of sample (10, 20, 30, 40, 50 mM) was added to 4 ml of diluted ABTS solution and then placed in darkness at room temperature for 15 min, followed by absorbance measurement at 734 nm. The results were expressed in trolox (1, 2, 3, 4 mM) using the relevant calibration curve.

Hydroxyl radical scavenging activity The 2-Deoxyribose oxidation method (You et al. 2009) was used for determining hydroxyl radicals scavenging activity of WGHs. 10 mM FeSO4-EDTA (0.2 ml), 10 mM α-deoxyribose (0.5 ml), sample solution (0.2 ml), 0.2 M sodium phosphate buffer (pH 7.4, 0.2 ml), and 10 mM hydrogen peroxide (0.2 ml) were mixed and incubated at 37 °C for 1 h. Then, 1.0 ml of 2.8% TCA and 1.0 ml of 1.0% TBA were added to stop the reaction. After the resulting mixture was boiled for 15 min and cooled in ice, the absorbance was measured at 532 nm with a spectrophotometer (DU 650, Beckman Coulter Inc., USA). The hydroxyl radical scavenging activity was calculated as the inhibition rate of α-deoxyribose oxidation by hydroxyl radicals using the following formula (Chung et al. 1997).

$$\text{Scavenging activity } (\%) = (1 - \text{control absorbance}/\text{sample absorbance}) \times 100$$

Reducing power The reducing power was measured according to the method described by Oyaizu (1986). The sample solution (10 ml) was mixed with 0.2 M phosphate buffer (pH 6.6, 2.5 ml) and 1% potassium ferric cyanide solution (2.5 ml). The mixture was then kept in a 50 °C water bath for 20 min. The resulting solution was cooled, mixed with 10% TCA (*w*/*v*, 2.5 ml), and then centrifuged at 3,000 g for 10 min. After the supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.1% ferric chloride solution (0.5 ml), it was placed at room temperature for 10 min. The absorbance of the resulting mixture was measured at 700 nm.

Statistical analysis Statistical analysis was carried out using the SPSS (Statistical Package for Social Science, SPSS Inc., USA). One-way analysis of variance (ANOVA) and *t*-test were performed ($p<0.05$). All experiments were done in at least triplicate and presented as mean ± standard deviation (SD) values. Also, the relationship between degree of hydrolysis and the hydrophobic amino acid composition of WGHs was expressed by correlation coefficients.

Results and discussion

Enzymatic hydrolysis The hydrolytic patterns of wheat gluten treated by Alcalase, Flavourzyme, or Protamex were monitored for 1 h, 4 h, 12 h, and 24 h by the TNBS method (Fig. 1). Overall, the degree of hydrolysis (DH) sharply increased at the early stage of the reaction, gradually increased for 12 h, and then reached a plateau. However, the hydrolytic patterns appeared to be varied depending on the type of enzymes used. The WGHs hydrolyzed by Flavourzyme showed the fastest hydrolysis rate, followed by those hydrolysed by Protamex and Alcalase. These results would be attributed to the mode of hydrolytic action of Flavourzyme which is involved in both endoprotease and exoprotease activities (Lee et al. 2008; Seo et al. 2008). In addition, the final DH values of WGHs treated with Alcalase, Flavourzyme, or Protamex were 26%, 54%, and 36% during 24 h, respectively. Seo et al. (2008) reported that the final DH values of soy protein hydrolysates by Alcalase, Flavourzyme, and Protamex for 3 h were 23%,

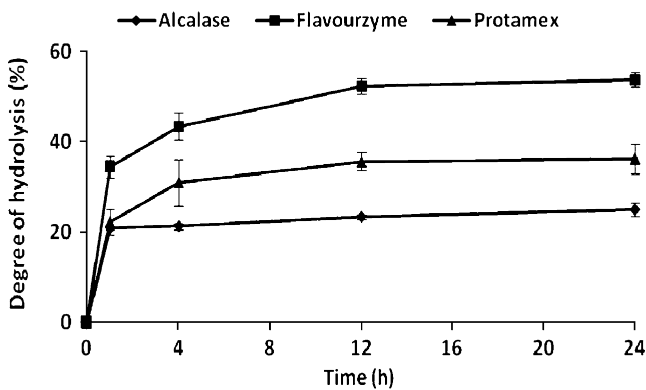

Fig. 1 Hydrolysis curves of wheat gluten using Alcalase, Flavourzyme, or Protamex for 24 h. Reaction conditions: pH, 7.0; temperature, 50 °C; enzyme to substrate ratio, 1:100; wheat gluten suspension concentration, 50% (*w*/*v*)

23%, and 28%, respectively. Wang et al. (2007a, b) indicated that wheat gluten hydrolysates having a DH of 11% by papain for 6 h. In addition, pea protein was hydrolyzed by Alcalase, Flavourzyme, Papain, Trypsin, and α-Chymotrypsin for 4 h, resulting in final DH values of 28%, 55%, 19%, 18%, and 17%, respectively (Humiski and Aluko 2007). Thus, it seems likely that the DH values of protein hydrolysates are highly dependent on protein source, enzyme type, and hydrolysis conditions.

Amino acid compositions of the six WGHs obtained through various hydrolysis conditions are shown in Table 1. The contents of total and free amino acids were 29–42% and 26–55% in all six WGHs, respectively. The WGH treated by Alcalase for 24 h showed the highest glutamic acid content among the six hydrolysates. Specially, the free glutamic acid content was increased from 1.3 up to 12-fold when WGH was hydrolyzed by Alcalase for 24 h, indicating its potential as a taste-enhancing ingredient. In particular, hydrophobic amino acids such as Pro, Gly, Ala, Val, Met, Ile, Leu, and Phe in the WGHs treated with Flavourzyme or Protamex increased as the hydrolysis time was prolonged to 24 h. In general, glutamic acid is an important compound of umami taste (Aaslyng et al. 1998). Dunkel et al. (2007) indicated that savory compounds such as sodium chloride and L-glutamic acid were able to increase the kokumi. Nishiwaki et al. (2002) also proposed that the hydrophobic peptides contribute to bitter taste. Lioe et al. (2006) isolated umami taste- and amino acid-rich fractions from Japanese soy, containing 20% Glu and 16% Asp. Indoh et al. (2006) also reported that strong umami taste depended on the higher content of free glutamic acid

Table 1 Contents of total and free amino acids of enzymatic wheat gluten hydrolysates (g/100 g hydrolysate)

Amino acid	A1h		A24h		F1h		F24h		P1h		P24h	
	TAA[a]	FAA[b]	TAA	FAA	TAA	FAA	TAA	FAA	TAA	FAA	TAA	FAA
Asp	3.11	1.01	2.95	0.00	2.98	1.22	1.80	0.00	2.35	0.00	2.60	0.00
Thr	2.30	11.11	2.40	18.53	2.35	31.22	2.35	23.14	1.92	8.11	2.15	9.01
Ser	4.85	4.04	4.41	2.35	4.84	6.73	3.08	2.75	3.87	0.68	3.69	1.88
Glu	40.15	9.60	40.96	12.06	39.41	4.49	37.72	4.05	31.96	0.00	38.37	4.50
Pro	12.94	0.00	12.63	0.00	12.46	0.00	13.38	3.83	10.33	0.00	13.71	0.00
Gly	3.81	0.00	3.65	0.00	3.56	1.84	4.28	2.82	2.96	0.00	3.81	1.50
Ala	2.47	0.00	2.31	5.00	2.32	2.45	4.37	8.39	1.79	0.00	3.54	12.38
Cys	1.31	0.00	1.28	0.00	1.30	0.00	1.44	0.65	0.91	0.00	1.48	0.00
Val	2.79	15.66	3.01	0.00	3.22	0.00	3.63	6.36	2.07	0.00	3.15	0.00
Met	1.22	3.54	1.28	5.29	1.19	3.27	1.19	2.24	0.93	5.41	1.30	4.13
Ile	2.09	1.52	2.28	2.94	2.52	4.08	3.24	6.15	1.59	2.70	2.60	7.32
Leu	6.05	9.09	6.18	17.35	6.14	14.29	6.54	13.16	4.70	12.84	6.66	21.39
Tyr	1.34	0.00	0.55	0.00	1.45	0.00	0.46	5.13	1.39	0.00	1.03	1.50
Phe	4.91	0.00	5.51	0.00	5.39	0.00	5.74	6.87	4.19	0.00	5.33	8.44
His	2.53	26.26	2.50	18.53	2.55	12.04	2.72	4.12	2.10	46.62	2.24	11.82
Lys	1.37	1.52	1.40	2.35	1.36	3.67	1.25	1.81	1.26	0.00	1.24	1.88
NH3	4.33	12.63	4.75	15.59	4.35	6.33	5.89	7.59	5.41	15.54	5.08	12.57
Arg	2.44	4.04	1.95	0.00	2.61	8.37	0.92	0.94	20.26	8.11	2.03	1.69
THAA[c]	36.28	29.80	36.85	30.59	36.80	25.92	42.36	49.82	28.58	20.95	40.09	55.16

[a] Total amino acid

[b] Free amino acid

[c] Total hydrophobic amino acids, Pro, Gly, Ala, Val, Met, Ile, Leu, and Phe

and peptide-type amino acids in fish sauce. As mentioned above, the production of glutamic acid during hydrolysis could play an important role in enhancing taste potential. Thus, the WGH treated by Alcalase for 24 h which showed the highest glutamic acid and the lowest hydrophobic amino acids was selected, implying potential for high kokumi taste and low bitter taste.

Figure 2 shows the relationship between degree of hydrolysis and hydrophobic amino acid content of wheat gluten treated with Alcalase, Flavourzyme, and Protamex for 1 h, 4 h, 12 h, and 24 h. The WGHs by Flavourzyme and Protamex showed positive correlation coefficients of 0.76 and 0.53, respectively, indicating that hydrophobic amino acids had a general tendency to increase with increasing degree of hydrolysis. However, the hydrophobic amino acid content in WGHs treated by Alcalase was not related to relate to their degree of hydrolysis. Thus, the WGHs treated with Alcalase, Flavourzyme, or Protamex for 1 h or 24 h were selected for the evaluation of sensory properties, which were designated as A1h, A24h, F1h, F24h, P1h, and P24h, respectively.

Sensory property The taste qualities of the WGHs were investigated using a model chicken broth, and the intensities of bitterness, umami, kokumi, and overall acceptability were rated on a scale from 0 (not detectable) to 6 (intensely detectable) by trained sensory panels. In Fig. 3, the taste profiles of the Alcalase-treated WGHs showed decreased bitterness (3.1→2.5) upon more extensive hydrolysis while umami (4.3→5.0) and overall acceptability (4.2→5.2) increased. On the other hand, the WGHs produced by Flavourzyme and Protamex exhibited increased bitterness from 2.4 to 3.0 and from 2.6 to 3.1, respectively, with increasing hydrolysis time. However, it seemed that taste profiles, such as umami, kokumi, and overall acceptability of the WGHs by Flavourzyme and Protamex were unaffected by hydrolysis time.

Antioxidant property Antioxidants, an important part of the human defense system against diseases, inhibit reactive oxygen species, including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and harmful free radicals (Wang et al. 2007a, b). Therefore, the WGH produced by Alcalase for 24 h (A24h) was selected, and its DPPH, ABTS, and hydroxyl radical scavenging effects were investigated. As can be seen in Fig. 4, the DPPH, ABTS, and hydroxyl radical scavenging activities of A24h were increased by 15–50%, 23–71%, and 15–48%, respectively, in a concentration-dependent manner. The IC_{50} value for A24h (1 mg/ml) in the presence of DPPH radicals was similar to that of wheat germ protein hydrolysate by Alcalase treatment for 6 h (1.3 mg/ml) (Zhu et al. 2006). Tang et al. (2009a, b) reported that the IC_{50} value of the DPPH

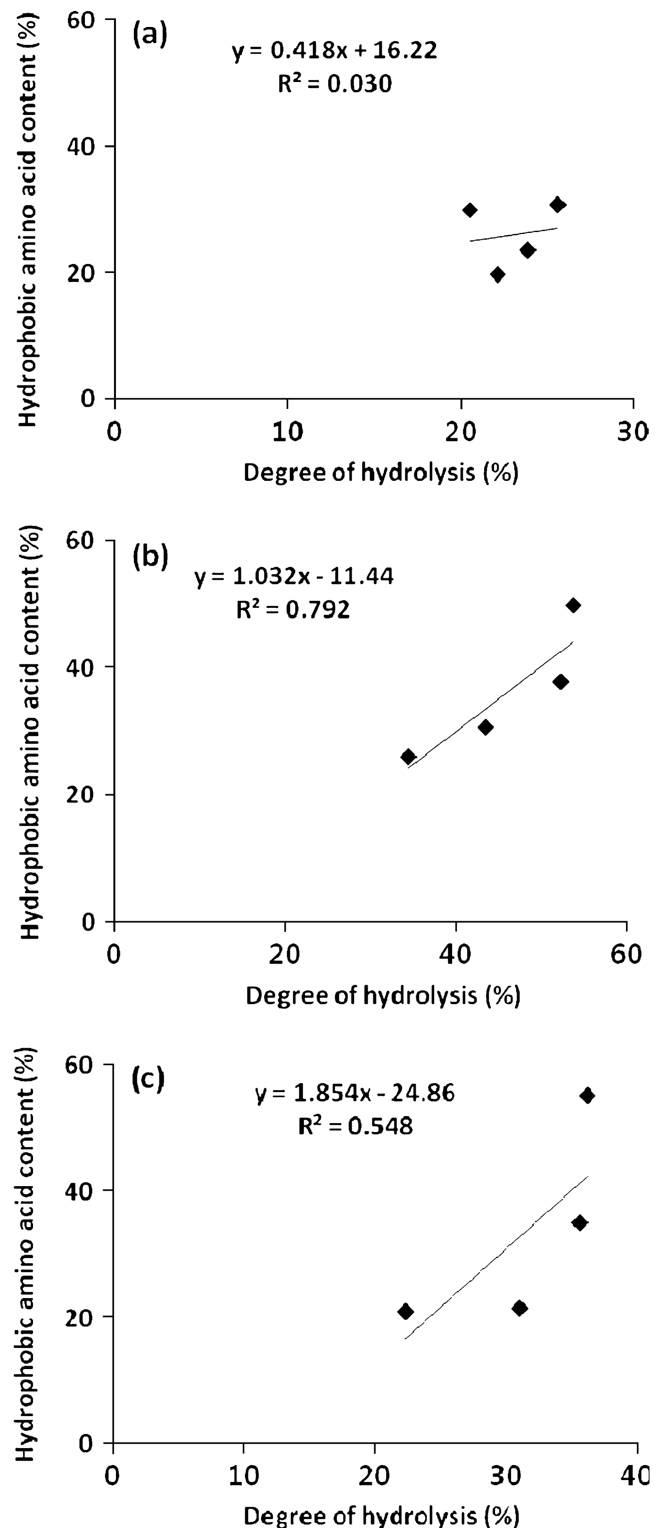

Fig. 2 Relationships between degree of hydrolysis and hydrophobic amino acid content of wheat gluten hydrolysates with the use of Alcalase (**a**), Flavourzyme (**b**), or Protamex (**c**)

radical scavenging capacity of hamp protein hydrolysate by Protamex treatment for 2 h was 2.8 mg/ml, which was lower than the results obtained in this study. In case of

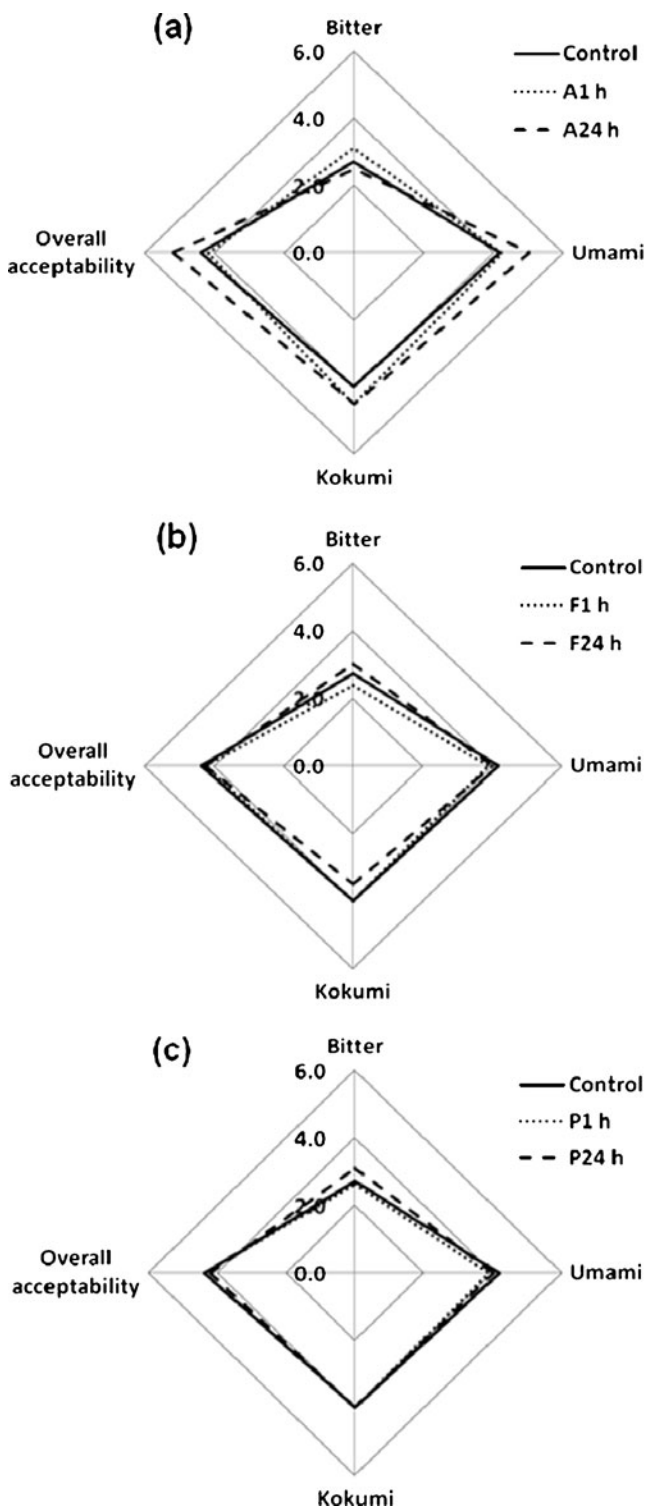

Fig. 3 Taste profiles of enzymatic wheat gluten hydrolysates with the use of Alcalase (**a**), Flavourzyme (**b**), or Protamex (**c**) in model chicken broth. A1h, Alcalase-treated for 1 h; A24h, Alcalase-treated for 24 h; F1h, Flavourzyme-treated for 1 h; F24h, Flavourzyme-treated for 24 h; P1h, Protamex-treated for 1 h; P24h, Protamex-treated for 24 h

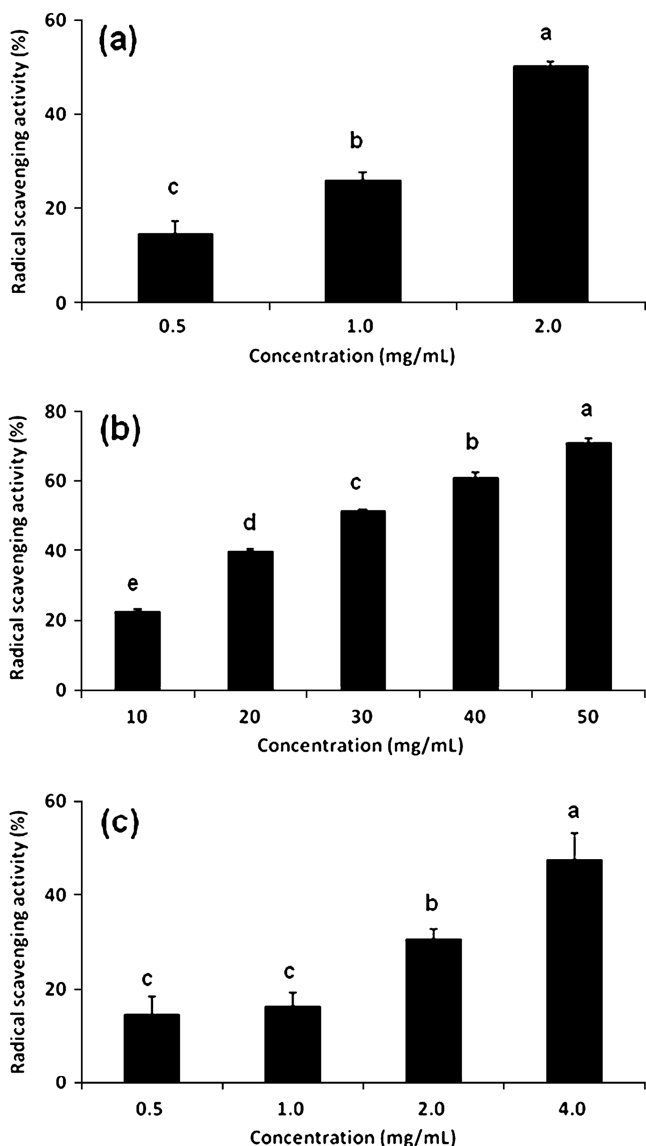

Fig. 4 Anti-radical effect of wheat gluten hydrolysate with Alcalase for 24 h against DPPH (**a**), ABTS (**b**), or hydroxyl (**c**) radical

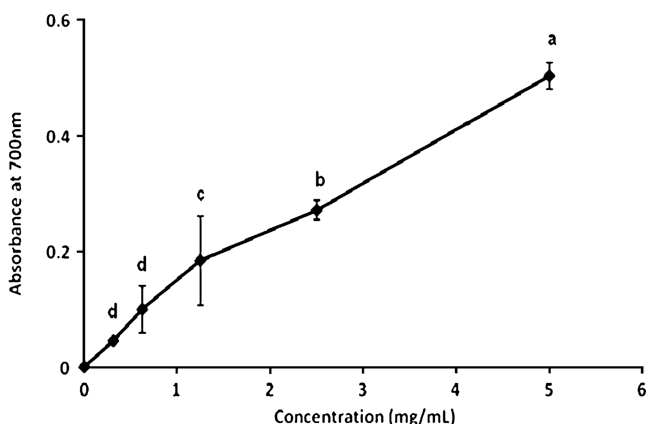

Fig. 5 Reducing power of wheat gluten hydrolysate with Alcalase for 24 h

ABTS radicals, A24h quenched 23% at a concentration of 10 mg/ml, being 4-fold lower than that from zein

hydrolysate by Alcalase at the same concentration (Tang et al. 2010). On the other hand, Kong et al. (2008) described that the fraction of wheat gluten hydrolysate by Pepsin and Pancreatin inhibited hydroxyl formation by 85.98% at a concentration 1.5 mg/ml. Moreover, A24h at a concentration of 4.0 mg/ml showed a hydroxyl radical quenching effect of 48%, being 1.7-fold lower than that from chickpea protein hydrolysate by Alcalase at a concentration of 1.5 mg/ml (Li et al. 2008).

As shown in Fig. 5, A24h exhibited a concentration-dependent reducing power. The reducing power of A24h at a concentration of 5 mg/ml was higher than haemoglobin and its hydrolysates (Chang et al. 2007), while it is lower than buckwheat protein hydrolysates (Tang et al. 2009a, b).

In spite of pro-oxidative effects, Tyr, Met, His, Lys, and Trp played a role as antioxidants (Marcuse 1960). Yamaguchi et al. (1975) described that peptides, including hydrophobic amino acids, such as Ala, Tyr, His, and Met, at the N-terminus, exhibited an antioxidative effect in an oil system. They suggested that the interaction between amino acid residues of peptides and fatty acids of linoleic acid was related to their antioxidant activity. Additionally, Chan and Decker (1994) reported histidine-containing peptides as important antioxidant factors due to their metal-chelating ability and free radical-trapping effect. Since Ala, Met, and His were enriched in A24h (Table 1), methionine and histidine appeared to play an important role in the observed antioxidant activity of A24h. As mentioned above, the antioxidant activity might vary with different hydrolysis conditions, such as protein source, protease type, and reaction time. Specially, radical scavenging effects are strongly correlated with the high content of total hydrophobic amino acids (Li et al. 2008).

Conclusions

Our findings revealed that there was a possible general relationship between the degree of hydrolysis and the hydrophobic amino acid content of wheat gluten hydrolysates obtained by Flavourzyme or Protamex. In addition, the content of hydrophobic amino acids was mainly responsible to the taste qualities of WGHs, specifically bitterness. Specially, A24h with a favorable taste-enhancing property showed a concentration-dependent antioxidant effect. Thus, from an overall point of view, wheat gluten hydrolysate could be a useful functional seasoning ingredient with biological activities.

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