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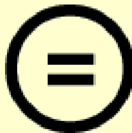

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생활과학석사학위논문

Application of Two- or Three-Step Enzymatic Hydrolysis for Efficient Production of Wheat Gluten Hydrolysates

밀 글루텐 가수분해물의 효율적 생산을 위한
단계적 효소 처리 방법 탐색

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화 열

ABSTRACT

Application of Two- or Three-Step Enzymatic Hydrolysis for Efficient Production of Wheat Gluten Hydrolysates

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Wheat gluten, a coproduct in wheat starch industry, possesses poor water solubility, which limits its broad application in the food industry. Enzymatic hydrolysis has been used to enhance functional properties of proteins and to produce bioactive peptides. In the food industry, it is prerequisite to produce protein hydrolysates in an economical and efficient approach. The objective of this study was to develop an efficient production method of wheat gluten hydrolysates (WGH) with desirable properties to be applied in beverages by sequential enzymatic hydrolysis.

In the present study, limited conditions, such as high solid loading (25%, w/w), low protease concentration (1:300, w:w) and limited hydrolysis time (6 h), were employed to produce WGH. Endo-proteases (Protamex, P; Alcalase, A; and

Neutrase, N) and endo- and exo-protease mixture (Flavourzyme, F) were used. In order to explore suitable sequences for enzymatic hydrolysis of wheat gluten, various combinations of endo-proteases were tested.

Yields of water-soluble WGH were 59.3-65.7%. Sequential hydrolyses by combination of A with other endo-proteases produced WGH with significantly ($p<0.05$) higher proportions (more than 50%) of fractions below 1 kDa than the others. The WGH produced by N-P sequence showed the lowest bitterness among the WGH. A5F1, N5F1, A4P1F1, N4P1F1, A3N2F1 and N3A1F1 (numbers representing hydrolysis time) possessed lower turbidity, better thermal stability and higher L^* values than the others. The WGH produced by P-A and A-P sequences exhibited higher DPPH radical scavenging activities than the others. The A4P1F1 showed better properties to be applied in beverages than the others. *In vitro* digests of the A4P1F1 exhibited antioxidant activities, especially reducing power and copper chelating activity.

Keywords: Wheat gluten; Sequential enzymatic hydrolysis; Small peptides; Antioxidant activity

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CONTENTS

ABSTRACT I

CONTENTS III

LIST OF TABLES V

LIST OF FIGURES VI

INTRODUCTION 1

MATERIALS AND METHODS 4

1. Materials 4

2. Preparation of WGH 4

3. *In vitro* pepsin-pancreatin SGID 5

4. Determination of MW distribution 7

5. Sensory evaluation of bitterness 8

6. Turbidity and thermal stability 8

7. Color 9

8. Antioxidant activities 9

8.1. DPPH radical scavenging activity 9

8.2. Ferric reducing antioxidant power (FRAP) 10

8.3. Metal chelating activity 10

9. Statistical analysis 11
RESULTS AND DISCUSSION..........12
1. Development of an efficient production method of WGH....12
1.1. Yield of the WGH 12
1.2. MW distribution of the WGH 14
1.3. Bitterness of the WGH solution 16
1.4. Turbidity and thermal stability of the WGH solution 19
1.5. Color of the WGH solution..........23
1.6. DPPH radical scavenging activity of the WGH 23
1.7. Summary 27
2. Antioxidant activities of the WGH digests..........28
2.1. MW distribution of the WGH digests 28
2.2. Antioxidant activities of the WGH digests..........28
2.3. Summary 31
CONCLUSION..........33
REFERENCES..........34
국문초록..........41

LIST OF TABLES

Table 1. Sampling scheme of wheat gluten hydrolysates (WGH) by two- or three-step enzymatic hydrolysis 6
Table 2. Yields of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis 13
Table 3. Bitterness of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis 18
Table 4. Color values of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis 24
Table 5. DPPH radical scavenging activities of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis 26
Table 6. Molecular weight distribution of wheat gluten hydrolysate (WGH) digests 29
Table 7. Antioxidant activities of wheat gluten hydrolysate (WGH) digests 30

LIST OF FIGURES

Figure 1. Molecular weight distribution of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis. 15

Figure 2. Turbidity and thermal stability at pH 3 (A) and pH 5 (B) of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis. 21

INTRODUCTION

Wheat gluten, a coproduct in wheat starch industry, has been readily supplied at low price with the expansion of wheat starch production. Poor water solubility of wheat gluten, however, greatly limits its broad application in the food industry (Kong et al., 2007a). Enzymatic hydrolysis using various proteases has been used to enhance functional properties of proteins and to produce bioactive peptides (Sarmadi and Ismail, 2010; Tavano, 2013). In previous studies, enzymatic hydrolysis was also applied to prepare wheat gluten hydrolysates (WGH) which possessed improved solubility, foaming and emulsifying capacities (Wang et al., 2006; Kong et al., 2007b; Wouters et al., 2016) and exhibited antioxidant (Wang et al., 2007; Kong et al., 2008; Koo et al., 2014) and anti-inflammatory activities (Sato et al., 2013).

It has been reported that short-chain peptides (2-20 amino acids (AA)) generated from protein hydrolysis were more likely to be responsible for bioactivities than intact proteins or free AA (de Castro and Sato, 2015). At the same time, hydrophobic AA residues in the small peptides may inevitably cause undesirable bitterness of the protein hydrolysates (Saha and Hayashi, 2001), which is one of the challenges for their application in foods, especially in beverages.

In the food industry, the most concerned issue is to process in economic strategy, which is another challenge in production of protein hydrolysates (Agyei and Danquah, 2011). In the view of this point, it is prerequisite to employ proper processing conditions, such as high solid loading, low enzyme concentration and limited hydrolysis time, which are suitable for mass production and wide

application of the protein hydrolysates. To the best of our knowledge, previous studies generally conducted enzymatic hydrolysis of wheat gluten under the conditions of 5-15% (w/w) solid loadings (Wang et al., 2006; Kong et al., 2007a; Deng et al., 2016), more than 1:100 ratios of enzyme to solid and 6-24 h hydrolysis times (Berends et al., 2014; Merz et al., 2015). However, since these conditions may not be preferable for industry-scale production of WGH, an efficient method of enzymatic hydrolysis should be investigated.

A sequential enzymatic hydrolysis has been considered as a prospective solution to efficiently produce WGH. Sequential hydrolysis with multiple proteases, which provides various cleavage patterns of peptide bonds, can be more efficient to obtain peptides with low molecular weights (< 1 kDa) compared to hydrolysis with a single protease (Nchienzia et al., 2010; Zhang et al., 2012). Moreover, use of exo-proteases in combination with endo-proteases has been reported to reduce bitterness of protein hydrolysates (FitzGerald and O'Cuinn, 2006; Liu et al., 2016a). Protease type, treatment sequence and time are crucial factors for sequential hydrolysis, which can affect physicochemical properties and bioactive activities of protein hydrolysates (Jin et al., 2016).

Food-grade microbial proteases, such as Protamex (P), Alcalase (A), Neutrase (N) and Flavourzyme (F), have been commonly used in the industry, since they have advantages of relatively lower cost and greater diversity compared to plant or animal-derived proteases (Agyei and Danquah, 2011). P and A are endo-proteases with broad specificities to hydrophobic amino acids, while N is a neutral endo-protease that hydrolyzes internal peptide bonds. F, a mixture of endo- and exo-proteases, contains aminopeptidase and carboxypeptidase (Waglay and Karboune,

2016). With high exo-activity, F can cleave off the hydrophobic residues at the end of a peptide chain, and thus be applied for debittering of the protein hydrolysates (Liu et al., 2016a). Hydrolytic capacities of those individual proteases for wheat gluten have been reported in previous studies. However, their combination efficacies for sequential hydrolysis of wheat gluten are yet unclear.

Gastrointestinal digestion is a key process to be considered in developing bioactive protein hydrolysates, since bioactivities of the hydrolysates may be influenced by action of digestive proteases. Simulated gastrointestinal digestion (SGID), an *in vitro* model, is a rapid and simple approach to mimic gastrointestinal digestion and has been employed to evaluate the effect of digestive proteases over the hydrolysates (You et al., 2010; Espejo-Carpio et al., 2016). However, as for the WGH produced by enzymatic hydrolysis, no studies on their antioxidant activities following the gastrointestinal digestion have been published.

The objective of this study was to develop an efficient production method of WGH with desirable properties to be applied in beverages by sequential enzymatic hydrolysis using different combinations of endo-proteases (P, A and N). High solid loading (25%, w/w), low protease concentration (1:300, w:w) and limited hydrolysis time (6 h) were employed for the production of WGH.

MATERIALS AND METHODS

1. Materials

Wheat gluten product (78.9±0.58% crude protein (N × 5.7), 4.83±0.15% water, 4.36±0.20% crude fat and 0.60±0.02% crude ash) was obtained from Anhui Ruifuxiang Co. (Anhui, China). Commercial food-grade P (EC 3.4.24.28, from *Bacillus subtilis*, 1.5 AU/g), A (EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g), N (EC 3.4.24.28, from *Bacillus amyloliquefaciens*, 0.8 AU/g) and F 1000 L (EC 3.4.11.1, from *Aspergillus oryzae*, 1000 AU/g) were purchased from Novozymes (Bagsvaerd, Denmark). Pepsin (EC 3.4.23.1, from porcine gastric mucosa, powder, ≥250 units/mg solid), pancreatin (EC 232-468-9, from porcine pancreas, 8 × USP specifications), cytochrome C, aprotinin, bacitracin, Gly-Gly-Tyr-Arg, Gly-Gly-Gly, 1,1-diphenyl-2-picrylhydrazyl (DPPH), pyrocatechol violet (PV) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p´-disulfonic acid monosodium salt hydrate (ferrozine) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium bicarbonate and ferric chloride were purchased from Duksan Pure Chemical Co., Ltd. (Ansan, Korea). Acetonitrile used for liquid chromatography was purchased from Avantor Performance Materials (Center Valley, PA, USA). Other reagents were of analytical grade and purchased from Samchun Chemical Co. (Seoul, Korea).

2. Preparation of WGH

WGH were prepared by two- or three-step sequential hydrolysis with the proteases (P, A, N and F). Enzymatic hydrolyses were under the limited conditions

as follows: (1) wheat gluten product (solid) was suspended in distilled water (DW) at 25% (w/w); (2) enzyme to solid ratio (E/S) was 1:300 (w:w); and (3) total hydrolysis time was 6 h. Under the limited conditions, the suspension was pre-incubated for 10 min using an overhead stirrer at 200 rpm and a water bath at 50±1 ˚C prior to the addition of the proteases. Then sequential hydrolyses were carried out at 50±1 ˚C with continuous stirring at 200 rpm under floating pH condition. Sampling scheme is shown in Table 1. Two-step hydrolysis was performed with one of the endo-proteases (P, A or N) followed by F. For three-step hydrolysis, wheat gluten product was sequentially hydrolyzed with one of the endo-proteases (P, A or N), another endo-protease (P, A or N) and F. At the end of the hydrolyses, the mixtures were heated at 95 ˚C for 10 min and then cooled down to 50±1 ˚C. The mixtures were centrifuged at 12,000×g for 10 min at 20 ˚C. The supernatants were lyophilized and stored at -20 ˚C for further analysis. The samples were abbreviated as the proteases used in sequences and the corresponding hydrolysis times, such as A4P1F1. Yield of the WGH was calculated as percentage of weight of the lyophilized WGH to weight of the used wheat gluten product (dry basis).

3. *In vitro* pepsin-pancreatin SGID

In vitro SGID was carried out according to Espejo-Carpio et al. (2016). The WGH were dissolved in distilled water (3%, w/v) and pre-incubated in a shaking incubator (C-SKI-2, Chang Shin Scientific Co., Seoul, Korea) at 100 rpm and 37 ˚C for 30 min before adjusting pH to 2.0 with 1.0 M HCl. Pepsin (4%, weight/weight of WGH powder) was added to the WGH solution, and the mixture

Table 1. Sampling scheme of wheat gluten hydrolysates (WGH) by two- or three-step enzymatic hydrolysis

Sequential hydrolysis	Proteases sequence	Hydrolysis time (h)	Solid:DW (w:w)	Enzyme:solid (E:S, w:w)
Two-step hydrolysis	P→F A→F N→F	5→1	1:3	1:300
Three-step hydrolysis	P→A→F A→P→F P→N→F N→P→F A→N→F N→A→F	1→4→1 2→3→1 3→2→1 4→1→1	1:3	1:300

Alcalase, A; Protamex, P; Neutrase, N; Flavourzyme, F; and distilled water, DW.

was incubated at 100 rpm and 37 ˚C for 1 h. pH was then adjusted to 5.3 with 0.9 M $NaHCO_3$ solution, and further to pH 7.5 with 1.0 M NaOH. Pancreatin was added (4%, weight/weight of WGH powder), and the mixture was further incubated at the same conditions for 2 h. The SGID was terminated by heating at 95 ˚C for 10 min to inactivate the enzymes. After lyophilization, the digests of the WGH were stored at -20 ˚C until analyzed further. To investigate the changes in MW distribution and antioxidant activity of WGH digests during the SGID, digests were obtained at 1 h (gastric digest, GD), 2 h (gastrointestinal digest-1, GID-1) and 3 h (gastrointestinal digest-2, GID-2), respectively.

4. Determination of MW distribution

MW distribution of the WGH or *in vitro* digests of A4P1F1 was determined by size exclusion chromatography (SEC) on a TSK gel G2000 SWXL column (7.8 × 300 mm; applicable for globular protein samples up to 150,000 Da; Tosoh Co., Tokyo, Japan), using Waters 2695 liquid chromatography system (Waters Co., Milford, MA, USA) equipped with Waters 2996 PDA detector and Empower work station (Waters Co.). A mobile phase consisting of acetonitrile/water/trifluoroacetic acid (30/70/0.1, v/v/v) was delivered at a flow rate of 0.5 mL/min. Sample solutions (2.5 mg/mL) were filtered through low protein binding filters (Acrodisc syring filters with 0.2 μm Supor membrane, Pall Co., Ann Arbor, MI, USA) before injection. Absorbance was determined at 214 nm. Cytochrome C from equine heart (12,500 Da), aprotinin from bovine lung (6,500 Da), bacitracin (1,450 Da), Gly-Gly-Tyr-Arg (451 Da) and Gly-Gly-Gly (189 Da) were used as standards to obtain a calibration curve between retention time (RT) and log (MW). Based on the curve,

the chromatograms were divided into MW ranges of 0-0.5 kDa, 0.5-1 kDa, 1-3 kDa, 3-5 kDa, 5-10 kDa and above 10 kDa. Peak area (%) indicated the amount of fractions of a given MW range in the analyzed sample.

5. Sensory evaluation of bitterness

Bitterness of the WGH was quantified by sensory evaluation described by Meinlschmidt et al. (2016) with some modification. Ethics approval was obtained from Institutional Review Board (IRB) of Seoul National University (IRB No. 1702/001-008). A sensory panel consisting of 6 females and 2 males was screened based on their interest, availability to take part in training and nine-session evaluation, and ability to differentiate various bitterness intensities. A solution (2%, w/v) of the WGH produced by A (production conditions: solid:DW=1:3 (w:w), E:S=1:100 (w:w), 50˚C, 6 h without pH adjustment, centrifugation at 12000×g) was used as a reference solution. During each session (three samples per session), the reference solution was first given to the panel and then the sample solutions (20 mL each, 5%, w/v) coded with three-digit numbers were randomly presented. The panel evaluated bitterness of the samples in relation to that of the reference solution using a 15-cm line scale. Water and plain crackers were provided to the panel for rinsing their mouths.

6. Turbidity and thermal stability

Considering the effect of pH on turbidity, the WGH were dissolved at 50 mg/mL in buffer solution prepared by 0.2 M sodium phosphate and 0.1 M citric acid at pH 3 and 5. The WGH solutions were incubated at 90 ˚C for 10 min and

then cooled down to room temperature. Turbidity of the solutions was measured as the optical density (OD) at 600 nm before and after heat treatment using a spectrophotometer (Optizen 2120 UV; Mecasys, Daejeon, Korea). Distilled water was used as blank.

7. Color

Color of the sample solutions (50 mg/mL) was measured by a colorimeter (CM-5, Konica Minolta Co., Tokyo, Japan). Hunter L* (lightness), a* (redness) and b* (yellowness) values were recorded. Distilled water was used for calibration.

8. Antioxidant activities

8.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method described by Kong et al. (2008) with some modification. Two hundred μL of the sample (WGH or its *in vitro* digest) solutions (5 mg/mL) was mixed with 200 μL 0.2 mM DPPH dissolved in 95% (v/v) ethanol. The mixture was allowed to stand in the dark for 20 min and then centrifuged at 8,000×g for 10 min. Two hundred μL of the supernatant was transferred to a 96-well plate and absorbance was monitored at 517 nm using a microplate reader (SpectraMax 190, Molecular Devices Corp., CA, USA). The same volume of distilled water instead of the sample solution was used as blank. DPPH radical scavenging activity was calculated as follows:

DPPH radical scavenging activity (%) = $[(A_{blank}-A_{sample})/A_{blank}]\times100$,

where A_{blank} is the absorbance of the blank and A_{sample} is the absorbance of the sample solution.

8.2. Ferric reducing antioxidant power (FRAP)

FRAP was determined by the method of Cian et al. (2015) with some modification. The sample (WGH or its *in vitro* digest) solutions (250 μL, 50 mg/mL) were mixed with 250 μL 0.2 M phosphate buffer (pH 6.6) and 250 μL 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then 250 μL 10% (w/v) trichloroacetic acid was added and the mixture was centrifuged at 1,500×g for 10 min. Thereafter, 250 μL of the supernatant was mixed with 250 μL distilled water and 50 μL 0.1% (w/v) ferric chloride. After standing for 10 min at room temperature, 200 μL of the mixture was transferred to a 96-well plate and absorbance was determined at 700 nm using the microplate reader.

8.3. Metal chelating activity

Iron chelating activity was measured according to Torres-Fuentes et al. (2012) with a slight modification. A WGH or its *in vitro* digest (2.5 mg) was mixed with 250 μL 100 mM sodium acetate buffer (pH 4.9) and 30 μL ferrous chloride (0.01%, w/v). After incubation for 30 min at room temperature, ferrozine (12.5 μL, 40 mM) was added. The mixture was then centrifuged at 8,000×g for 5 min and 200 μL of the supernatant was transferred to a 96-well plate to measure absorbance at 562 nm using the microplate reader.

Copper chelating activity was measured according to Torres-Fuentes et al. (2011) with some modification. Sample (WGH or its *in vitro* digest) solution was prepared in 50 mM sodium acetate buffer (pH 6.0) at 10 mg/mL. Two hundred μL of the sample solution was mixed with 200 μL copper sulphate (0.1 μg/μL in 50

mM sodium acetate buffer (pH 6.0)) and 50 μL PV (4 mM in 50 mM sodium acetate buffer (pH 6.0)). After incubation for 5 min, the mixture was centrifuged at 8,000×g for 5 min. Then 200 μL of the supernatant was transferred to a 96-well plate to measure absorbance at 632 nm using the microplate reader. The same volume of the buffer solution was used as blank. Chelating activity was calculated as follows:

$$\text{Chelating activity (\%)} = [(A_{blank} - A_{sample})/A_{blank}]\times 100,$$

where A_{blank} is the absorbance of the blank and A_{sample} is the absorbance of the sample solution.

9. Statistical analysis

All results were expressed as means±standard deviations. Independent t-test and one-way analysis of variance (ANOVA) with Duncan's new multiple range test were performed with SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

1. Development of an efficient production method of WGH

1.1. Yield of the WGH

When considering mass production of protein hydrolysates in the food industry, yield of the hydrolysates has been known as one of the most crucial factors. Yields of the WGH ranged from 59.3 to 65.7% under the condition of 25% (w/w) solid loading (Table 2), indicating that the sequential hydrolyses employed in the present study produced more than 59% water-soluble hydrolysates from water-insoluble wheat gluten. Previous studies reported that hydrolysates with 9.7-76.7% yields were produced by enzymatic hydrolysis from food proteins (He et al., 2013; Chalamaiah et al., 2010; Ko et al., 2012). Comparing the WGH produced by the two-step hydrolysis, yield of P5F1 was significantly ($p<0.05$) higher than those of A5F1 and N5F1, indicating that P could be more likely to hydrolyze wheat gluten into soluble peptides than the other endo-proteases. Ko et al. (2012) also reported that the highest yield (43.2%) was noted in hydrolysates produced by P from *Styela clava* fresh tissue compared to those produced by A, N, papain and so on. As for the three-step hydrolysis, the WGH produced by P-A and A-P sequences, except for A1P4F1, showed significantly ($p<0.05$) higher yields than those produced by the other sequences. This may be because the hydrolyses with different sequences of protease treatments led to cleavage on different sites and consequently produced hydrolysates with different water-solubilities (Nimalaratne et al., 2015). Based on the results, combination of P and A could be more efficient to obtain the WGH with higher yields than any other sequences. Nevertheless, there were no great

Table 2. Yields of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis

Sequential hydrolysis	Hydrolysis time (h)	Yield (%)					
Two-step hydrolysis	5→1	P→F		A→F		N→F	
		64.2±0.04^{abcd}		60.0±1.36^{hij}		61.3±0.79^{jk}	
Three-step hydrolysis		P→A→F	A→P→F	P→N→F	N→P→F	A→N→F	N→A→F
	1→4→1	65.1±0.25^{ab}	60.0±1.49^{jk}	61.4±0.64^{hij}	59.3±0.80^{k}	61.4±0.95^{ghij}	60.3±0.85^{ijk}
	2→3→1	64.6±0.35^{abc}	64.4±0.88^{abcd}	61.5±0.91^{ghij}	63.8±1.66^{bcde}	61.7±0.76^{ghi}	61.8±0.10^{fghi}
	3→2→1	65.6±0.30^{a}	64.6±1.00^{abc}	63.4±1.12^{bcdef}	63.1±0.94^{cdefg}	62.8±1.12^{defgh}	60.6±0.25^{ijk}
	4→1→1	65.0±0.31^{ab}	65.7±0.61^{a}	63.8±1.37^{bcde}	62.7±1.16^{defgh}	63.9±0.66^{bcde}	62.5±0.58^{efgh}

Alcalase, A; Protamex, P; Neutrase, N; and Flavourzyme, F.

All data represent the means and standard deviations (n=3).

Different small letters indicate significant differences among the WGH ($p<0.05$; one-way ANOVA and Duncan's multiple range test).

differences among the yields of the WGH produced by various sequences of endo-protease treatments. Therefore, other important factors in evaluating the method of WGH production under the limited conditions, such as MW distribution, bitterness, and turbidity, should be also considered in addition to the yields.

1.2. MW distribution of the WGH

MW distribution has been known as a parameter related to not only the degree of hydrolysis (DH) of protein hydrolysates, but also their physicochemical properties and bioactive potencies (Chi et al., 2014; Chen and Li, 2012). As shown in Fig. 1, the A5F1 significantly ($p<0.05$) possessed the highest proportion (50.0%) of small fractions (<1 kDa) among the WGH produced by the two-step hydrolysis, followed by the P5F1 and N5F1. This indicates that A may be more efficient to cleave the peptide bonds of wheat gluten and produce WGH with low MW within a limited hydrolysis time than P and N, in agreement with the results of Kong et al. (2007a) and Liu et al. (2016a).

With exceptions of the WGH produced by P-N and N-P sequences, the WGH produced by the three-step hydrolysis had significantly ($p<0.05$) higher amounts of the fractions below 1 kDa than those produced by the two-step hydrolysis. More specifically, the WGH produced by the combination of A and N significantly ($p<0.05$) contained the highest amount of the fractions less than 1 kDa, followed by those produced by the combination of P and A. This may result from the fact that the proteolysis by the first treated protease, which exposed cleavage sites to the second treated protease, could facilitate the hydrolysis of the next step. Jin et al.

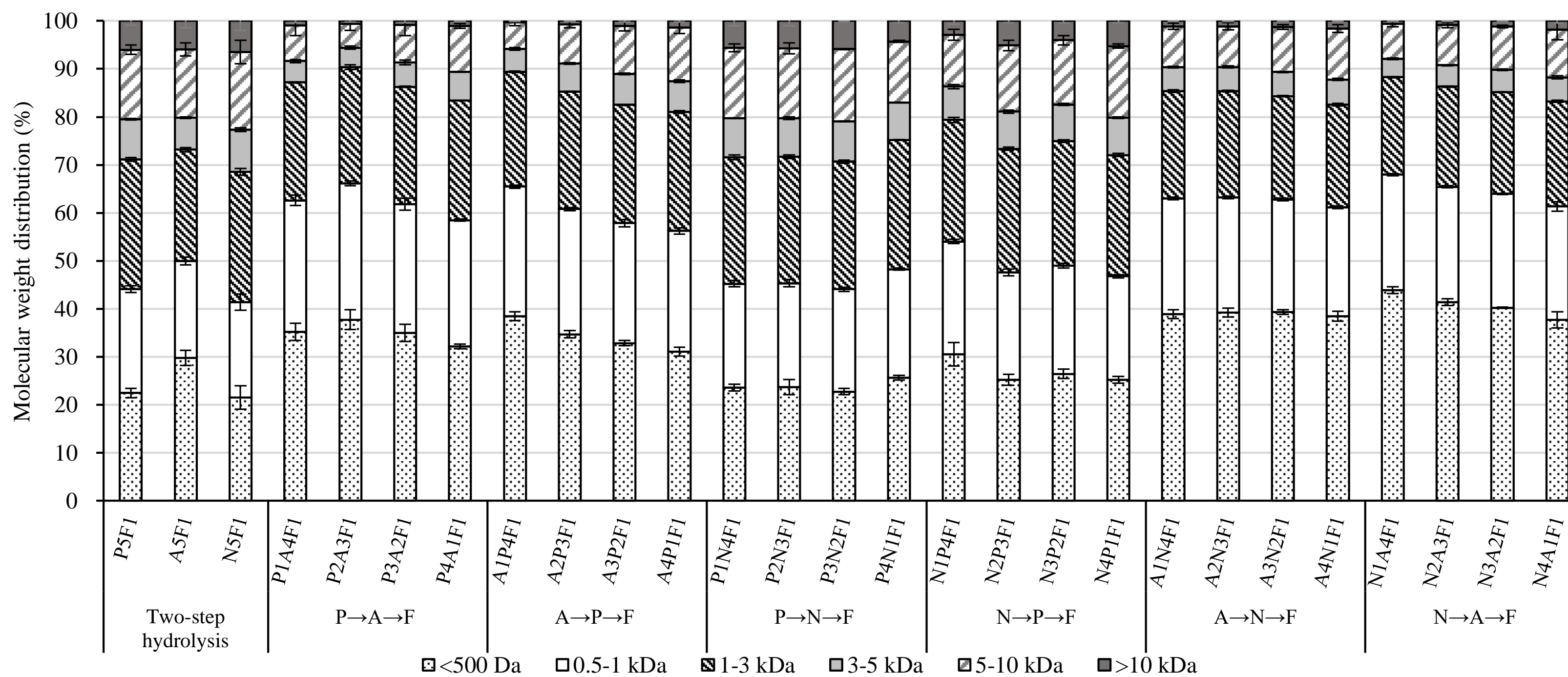


Figure 1. Molecular weight distribution of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis.

Alcalase, A; Protamex, P; Neutrase, N; Flavourzyme, F; and number represents hydrolysis time (h).

All data represent the means and standard deviations (n=3).

(2016) also reported that F effectively hydrolyzed the residual peptides of core gluten meal which had been exposed by A's action. On the other hand, the P-N and N-P sequences produced significantly ($p<0.05$) less fractions under 1 kDa. This might be attributed to the reduced cleavage sites of P and N, which were damaged by each other during the process of sequential hydrolysis. Moreover, the WGH produced by the same protease sequences with different treatment times also showed different MW distributions. Overall, these results demonstrate that protease type, treatment sequence and time are important in sequential hydrolysis, exerting critical effects on the MW distribution of the produced hydrolysates.

Moreover, it was reported that high solid concentrations (>20%) of wheat gluten in enzymatic hydrolysis resulted in an increase of fractions with MW of 10-25 kDa and >25 kDa and a decrease of fractions <10 kDa (Hardt et al., 2013). A similar result was also observed by Spellman et al. (2005), who found a much slower rate of proteolysis and a decrease in the percentage of low MW peptides (<500 Da) in whey protein hydrolysates at high solid level (300 g/L) than low solid level (50 g/L). However, the sequential hydrolysis employed in the present study seemed to be suitable to produce low-MW WGH under high solid loading. Especially the three-step hydrolysis was more efficient than the two-step hydrolysis. Combinations of A and other endo-proteases could be preferable to produce WGH with high proportions of low MW fractions under the limited conditions.

1.3. Bitterness of the WGH solution

As hydrophobic AA residues might be exposed in the process of hydrolysis (Matoba and Hata, 1972), the WGH possessed bitterness without exception (Table

3). Among the WGH produced by the two-step hydrolysis, the A5F1 showed the most intense bitterness. A, an endo-protease with broad specificity for peptide bonds, may be responsible for the generation of more hydrophobic AA residues than either P or N. Previous studies (Humiski and Aluko, 2007; Spellman et al., 2009; Meinlschidt et al., 2016) also reported that protein hydrolysates prepared with A showed more bitterness than those produced by the other tested proteases such as P and F. As for the three-step hydrolysis, A1N4F1 showed the most intense bitterness followed by the WGH produced by P-A and A-P sequences, while the WGH produced by P-N and N-P sequences showed less bitterness than the others. The results indicate that to some extent the bitterness of the WGH seems to be in connection with their MW distributions. Small hydrophobic peptides, especially peptides with MW less than 6 kDa, have been shown to be a predominant factor for the bitter taste of the protein hydrolysates (Saha and Hayashi, 2001; Liu et al., 2016a). In the view of this point, less amounts of short-chain peptides present in the WGH produced by P-N and N-P sequences may contribute to their relatively low bitterness. On the other hand, some WGH produced by A-N and N-A sequences, such as A3N2F1 and N3A2F1, had even more low-MW fractions than the others, but they did not exhibit stronger bitterness. This result indicates that MW may not be an only factor that affects bitterness of protein hydrolysates. Other factors such as AA composition and sequence of small hydrophobic peptides also play roles in bitterness (Tavano, 2013; Liu et al., 2016b). Hou et al. (2011) also reported that increased DH, representing increased amounts of low-MW peptides, did not lead to a significant ($p<0.05$) difference in bitterness of Alaska Pollock frame hydrolysates prepared with mixed enzymes.

Table 3. Bitterness of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis

Sequential hydrolysis	Hydrolysis time (h)	Bitterness (15-cm line scale)					
Two-step hydrolysis	5→1	P→F		A→F		N→F	
		7.76 ± 3.15^{bcde}		8.30 ± 1.49^{abcde}		7.60 ± 2.68^{bcde}	
Three-step hydrolysis		P→A→F	A→P→F	P→N→F	N→P→F	A→N→F	N→A→F
	1→4→1	10.5 ± 3.35^{ab}	9.53 ± 1.99^{abcd}	6.80 ± 2.43^{de}	8.31 ± 2.56^{abcde}	11.1 ± 2.25^{a}	10.5 ± 2.77^{ab}
	2→3→1	9.64 ± 1.69^{abcd}	9.53 ± 2.90^{abcd}	6.00 ± 2.86^{e}	6.63 ± 2.77^{de}	10.0 ± 2.10^{abc}	8.88 ± 1.68^{abcde}
	3→2→1	9.85 ± 3.14^{abc}	9.53 ± 2.76^{abcd}	7.76 ± 2.92^{bcde}	6.66 ± 2.88^{de}	8.60 ± 1.33^{abcde}	8.04 ± 1.90^{bcde}
	4→1→1	10.6 ± 1.78^{ab}	9.19 ± 2.44^{abcd}	7.34 ± 2.63^{cde}	6.09 ± 2.40^{e}	8.81 ± 2.14^{abcde}	8.35 ± 2.89^{abcde}

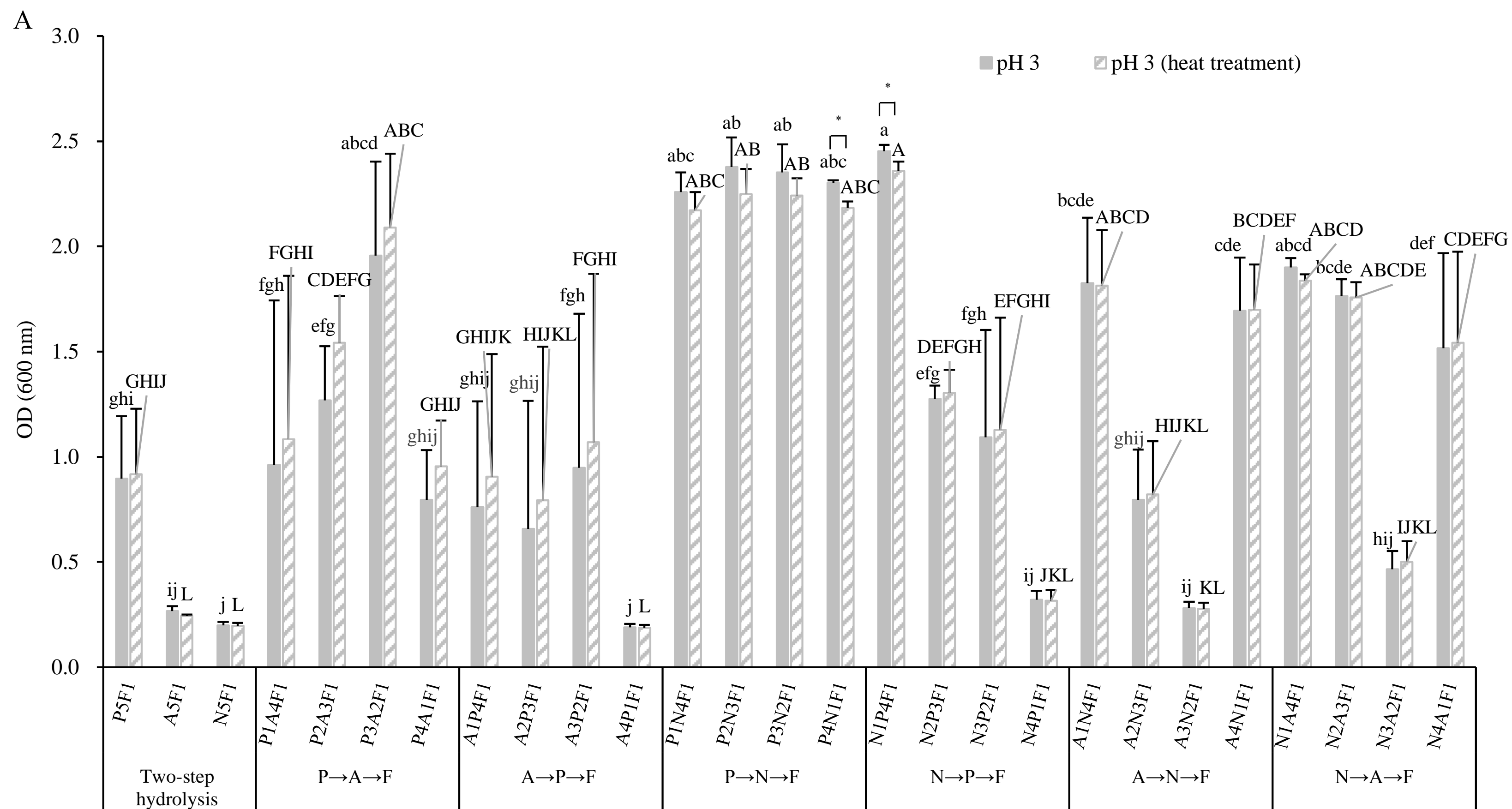
Alcalase, A; Protamex, P; Neutrase, N; and Flavouzyme, F.

All data represent the means and standard deviations (n=8).

Different small letters indicate significant differences among the WGH ($p<0.05$; one-way ANOVA and Duncan's multiple range test).

1.4. Turbidity and thermal stability of the WGH solution

A WGH solution with lower turbidity and higher thermal stability may be more likely to be applied in commercial beverages. Turbidity was measured at pH 3 and 5 in the present study, as the pH of most commercial beverages are near these values (Reddy et al., 2016). The A5F1, N5F1, A4P1F1, N4P1F1, A3N2F1 and N3A1F1 were significantly ($p<0.05$) less turbid at both pH 3 and 5 than the others (Fig. 2). All the WGH with lower turbidity mentioned above exhibited apparent thermal stability at pH 3 as their turbidity did not significantly ($p>0.05$) change after heat treatment. On the other hand, although heat treatment led to their higher turbidity at pH 5, they were significantly ($p<0.05$) less turbid than the others. It is worth noting that protease type and hydrolysis time employed for the first step hydrolysis may play important roles in turbidity of the WGH. More specifically, compared to P, use of A or N for the first step hydrolysis led to production of less turbid WGH. The WGH produced by first step hydrolyses for more than 3 h, such as the A4P1F1 and N3A2F1, were less turbid. Moreover, a previous study (Barca et al., 2000) reported that enzymatic hydrolysates of soy protein isolate, especially fractions below 10 kDa, were less turbid at pH 4.0, 5.5 and 7.0. However, in the present study, some of the WGH, such as the P4N1F1 in which fractions below 10 kDa accounted for even 90% (Fig. 1), showed relatively highly turbid. These results may be attributed to the fact that protein aggregation induced by hydrophobic peptides and pH environment, in addition to MW, could also affect turbidity of a protein hydrolysate solution (Agyare et al., 2008).



A
pH 3
pH 3 (heat treatment)
OD (600 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P5F1
A5F1
N5F1
P1A4F1
P2A3F1
P3A2F1
P4A1F1
A1P4F1
A2P3F1
A3P2F1
A4P1F1
P1N4F1
P2N3F1
P3N2F1
P4N1F1
N1P4F1
N2P3F1
N3P2F1
N4P1F1
A1N4F1
A2N3F1
A3N2F1
A4N1F1
N1A4F1
N2A3F1
N3A2F1
N4A1F1
Two-step hydrolysis
P→A→F
A→P→F
P→N→F
N→P→F
A→N→F
N→A→F

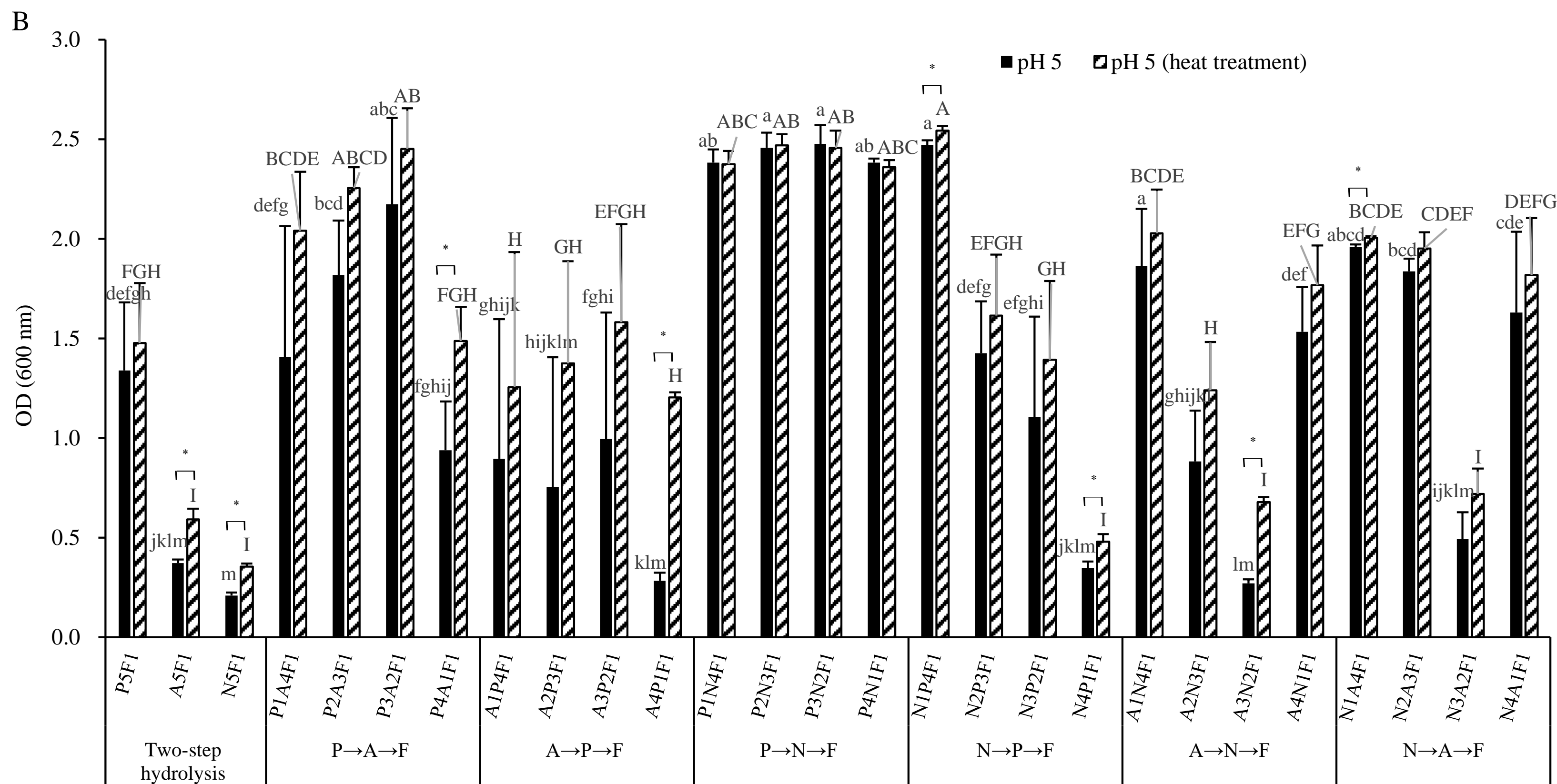


Figure 2. Turbidity and thermal stability at pH 3 (A) and pH 5 (B) of wheat gluten hydrolysates (WGH) produced by two- or three-step

enzymatic hydrolysis.

Alcalase, A; Protamex, P; Neutrase, N; Flavourzyme, F; and number represents hydrolysis time (h).

All data represent the means and standard deviations (n=3).

Different small letters indicate significant differences among the WGH before heat treatment ($p<0.05$; one-way ANOVA and Duncan's multiple range test). Different capital letters indicate significant differences among the WGH after heat treatment ($p<0.05$; one-way ANOVA and Duncan's multiple range test). *$p<0.05$; independent t-test.

1.5. Color of the WGH solution

As shown in Table 4, the N5F1, A5F1, N4P1F1, A3N2F1, N3A2F1, P5F1 and A4P1F1 showed significantly ($p<0.05$) higher L* values and lower a* values than the others. Moreover, the P4A1F1 significantly ($p<0.05$) showed the highest b* value, followed by the P1A4F1, A2P3F1 and P2A3F1, implying that they appeared more yellow than the other WGH. It should be noted that relevance between color and turbidity has been observed in the WGH, suggesting that the WGH with lower turbidity appeared lighter, less red and yellow than those with higher turbidity. Overall, as the apparent colors of the WGH were affected more by L* values than by a* and b* values in the present study, lighter WGH may be more acceptable for beverage development.

1.6. DPPH radical scavenging activity of the WGH

As shown in Table 5, the WGH produced by P-A and A-P sequences exhibited significantly ($p<0.05$) higher DPPH radical scavenging activities than those produced by the other sequences, while the WGH produced by P-N and N-P sequences showed significantly ($p<0.05$) lower radical scavenging activities than the others. Kong et al. (2008) reported that WGH fractions less than 3 kDa showed significantly ($p<0.05$) higher DPPH radical scavenging activity than the other fractions (3-5 kDa and 5-10 kDa). In the present study, however, the WGH produced by A-N and N-A sequences, which also possessed higher proportions of low-MW fractions (Fig. 1), showed relatively low DPPH radical scavenging activities. These results suggest that more presence of the fractions below 500 Da

Table 4. Color values of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis

Sequential hydrolysis	Proteases sequence	Hydrolysis time (h)	Color values		
			L*	a*	b*
Two-step hydrolysis	P→F	5→1	86.7±3.76^{abc}	-0.14±0.43^{hi}	12.7±0.58^{defg}
	A→F		90.8±0.33^{ab}	-1.00±0.01^{k}	10.7±0.06^{klm}
	N→F		92.7±0.22^{a}	-1.19±0.11^{k}	10.1±0.26^{m}
Three-step hydrolysis	P→A→F	1→4→1	62.4±5.50^{g}	1.67±0.43^{ab}	14.1±1.28^{b}
		2→3→1	57.8±2.42^{gh}	1.86±0.30^{a}	14.0±0.53^{b}
		3→2→1	54.0±4.16^{h}	1.26±0.41^{bc}	13.1±0.93^{cde}
		4→1→1	70.0±2.87^{f}	1.45±0.04^{abc}	15.2±0.44^{a}
	A→P→F	1→4→1	78.8±6.50^{de}	0.54±0.61^{defg}	13.9±0.64^{bc}
		2→3→1	78.9±5.59^{de}	0.49±0.64^{defg}	14.1±0.36^{b}
		3→2→1	77.8±8.14^{de}	0.52±0.70^{defg}	13.3±0.70^{bcd}
		4→1→1	86.5±0.48^{abc}	-0.47±0.11^{ij}	12.1±0.22^{ghi}
	P→N→F	1→4→1	58.2±2.28^{gh}	0.68±0.10^{def}	11.5±0.19^{ijkl}
		2→3→1	54.5±2.03^{h}	0.56±0.12^{defg}	11.5±0.21^{ijkl}
		3→2→1	54.4±2.13^{h}	0.63±0.16^{def}	11.6±0.14^{ijk}
		4→1→1	57.3±0.67^{gh}	0.53±0.08^{defg}	11.7±0.15^{hij}
	N→P→F	1→4→1	55.0±0.55^{h}	0.22±0.07^{fgh}	10.7±0.21^{lm}
		2→3→1	82.4±2.15^{cd}	0.67±0.15^{def}	12.5±0.43^{defgh}
		3→2→1	85.3±2.10^{bc}	0.43±0.24^{efg}	12.2±0.17^{fghi}
		4→1→1	89.9±0.53^{ab}	-0.44±0.07^{ij}	11.1±0.40^{jkl}

Table 4. (continued)

	Proteases sequence	Hydrolysis time (h)	Color values		
			L*	a*	b*
Three-step hydrolysis	A→N→F	1→4→1	69.5±6.33^{f}	0.58±0.03^{defg}	12.4±0.75^{efghi}
		2→3→1	85.5±0.99^{bc}	0.06±0.12^{gh}	12.7±0.11^{defg}
		3→2→1	89.9±0.37^{ab}	-0.82±0.08^{jk}	10.8±0.15^{jklm}
		4→1→1	76.7±5.83^{de}	0.59±0.23^{defg}	12.6±0.28^{defg}
	N→A→F	1→4→1	69.5±0.95^{f}	0.63±0.03^{def}	12.0±0.19^{ghi}
		2→3→1	69.6±1.91^{f}	0.96±0.05^{cde}	12.2±0.20^{fghi}
		3→2→1	88.2±0.36^{abc}	-0.15±0.10^{hi}	11.5±0.10^{ijkl}
		4→1→1	73.4±2.29^{ef}	0.99±0.03^{cd}	13.0±0.48^{def}

Alcalase, A; Protamex, P; Neutrase, N; and Flavourzyme, F.

All data represent the means and standard deviations (n=3).

Different small letters in the same columns indicate significant differences among the WGH ($p<0.05$; one-way ANOVA and Duncan's multiple range test).

Table 5. DPPH radical scavenging activities of wheat gluten hydrolysates (WGH) produced by two- or three-step enzymatic hydrolysis

Sequential hydrolysis	Hydrolysis time (h)	DPPH radical scavenging activity (%)					
Two-step hydrolysis	5→1	P→F		A→F		N→F	
		$28.6±1.94^{defghi}$		$30.7±2.04^{cdef}$		$24.4±0.23^{jk}$	
Three-step hydrolysis		P→A→F	A→P→F	P→N→F	N→P→F	A→N→F	N→A→F
	1→4→1	$43.7±1.86^{a}$	$44.4±2.82^{a}$	$23.3±1.24^{k}$	$25.9±2.30^{ghijk}$	$28.1±0.64^{defghij}$	$31.1±0.96^{cde}$
	2→3→1	$42.2±1.30^{a}$	$38.3±0.66^{b}$	$28.4±2.73^{defghij}$	$22.7±0.34^{k}$	$30.0±1.59^{defg}$	$26.7±1.59^{fghijk}$
	3→2→1	$42.2±2.56^{a}$	$34.1±1.30^{c}$	$25.4±2.34^{hijk}$	$17.7±3.66^{l}$	$30.3±1.48^{cdef}$	$24.7±2.35^{ijk}$
	4→1→1	$38.0±0.62^{b}$	$32.2±1.10^{cd}$	$29.2±3.57^{defgh}$	$22.8±1.19^{k}$	$27.4±2.65^{efghij}$	$25.2±4.88^{hijk}$

Alcalase, A; Protamex, P; Neutrase, N; and Flavourzyme, F.

All data represent the means and standard deviations (n=3).

Different small letters indicate significant differences among the WGH ($p<0.05$; one-way ANOVA and Duncan's multiple range test).

in the WGH produced by A-N and N-A sequences might dilute the radical scavenging activities to some extent. Li et al. (2008) also reported that 500-1,500 Da peptides and total hydrolysates obtained from corn gluten meal exhibited higher DPPH radical scavenging activities than the peptides above 1,500 Da and below 500 Da. Moreover, significant ($p<0.05$) differences in DPPH radical scavenging activity were also observed among the WGH produced by the same protease sequences with different treatment times.

1.7. Summary

Protease type, treatment sequence and time might play critical roles in the yield, MW distribution, bitterness, turbidity, thermal stability, color and free radical scavenging activity of the WGH. Yields of the water-soluble WGH were 59.3-65.7%. The sequential hydrolyses by combination of A with other endo-proteases produced WGH with significantly ($p<0.05$) higher proportions (more than 50%) of fractions below 1 kDa than the others. Meanwhile, the higher contents of low-MW fractions in the WGH led to relatively stronger bitterness. The A5F1, N5F1, A4P1F1, N4P1F1, A3N2F1 and N3A1F1 possessed lower turbidity, better thermal stability and higher L* values than the others, indicating that they may be applicable in the food industry, especially in beverages. On the other hand, the WGH produced by P-A and A-P sequences exhibited higher DPPH radical scavenging activities than the others, suggesting that they may possess higher antioxidant potencies.

In summary, based on the results, the A4P1F1, possessing better properties to be applied in beverages than the others, was selected to carry out the further study on

antioxidant activities of *in vitro* digests of the WGH.

2. Antioxidant activities of the WGH digests

2.1. MW distribution of the WGH digests

As shown in Table 6, gastric digestion by pepsin led to a significant ($p<0.05$) increase in fractions below 500 Da and decrease in factions above 5 kDa. Further digestion by pancreatin drastically ($p<0.05$) increased the fractions less than 1 kDa, especially fractions below 500 Da, which may be because that the pancreatin broke peptides in the gastric digests into smaller fractions and even AA. After the *in vitro* SGID, the digests contained more than 88% fractions below 1 kDa. You et al. (2010) also reported that following *in vitro* SGID of loach protein hydrolysates, a significant ($p<0.05$) decrease in fractions of 1-3 kDa was observed and the final gastrointestinal digests were rich in fractions less than 500 Da (about 77.7%).

2.2. Antioxidant activities of the WGH digests

As the action of gastrointestinal proteases may affect *in vivo* bioactivities of protein hydrolysates, an SGID model was employed in the present study to evaluate the antioxidant activity of the WGH following the gastrointestinal digestion (Espejo-Carpio et al., 2016). Three kinds of antioxidant assays with different mechanisms were employed to evaluate the antioxidant activities of the WGH digests (Table 7).

Gastric digestion of the WGH by pepsin significantly ($p<0.05$) decreased its DPPH radical scavenging activity from 32.2% to 25.9%. And during the further digestion, a sharp ($p<0.05$) decrease in DPPH radical scavenging activity of the

Table 6. Molecular weight distribution of wheat gluten hydrolysate (WGH) digests

Samples	Molecular weight distribution (%)					
	<500 Da	0.5-1 kDa	1-3 kDa	3-5 kDa	5-10 kDa	>10 kDa
WGH	31.1±0.92[d]	25.2±0.65[b]	24.8±0.29[b]	6.44±0.32[b]	11.2±1.27[a]	1.33±0.20[a]
GD	33.9±0.54[c]	23.4±0.64[c]	25.9±0.59[a]	7.59±0.43[a]	8.77±0.24[b]	0.38±0.05[b]
GID-1	55.8±0.12[b]	27.3±0.06[a]	14.6±0.10[c]	1.58±0.06[c]	0.65±0.05[c]	0.03±0.01[c]
GID-2	61.2±0.40[a]	27.3±1.25[a]	11.1±0.20[d]	0.68±0.07[d]	0.37±0.03[c]	0.01±0.01[c]

WGH is produced by sequential hydrolysis with Alcalase for 4 h, Protamex for 1 h and Flavourzyme for 1 h.

GD, gastric digest produced by pepsin; GID-1, gastrointestinal digest produced by pepsin for 1 h and pancreatin for 1 h; and GID-2, gastrointestinal digest produced by pepsin for 1 h and pancreatin for 2 h.

All data represent the means and standard deviations (n=3).

Different small letters in the same columns indicate significant differences among the samples ($p<0.05$; one-way ANOVA and Duncan's multiple range test).

Table 7. Antioxidant activities of wheat gluten hydrolysate (WGH) digests

Samples	DPPH radical scavenging activity (%)	Ferric reducing antioxidant power	Metal chelating activity (%)	
			Iron chelating activity	Copper chelating activity
WGH	32.2±1.10[a]	0.40±0.01[a]	15.8±1.47[a]	73.8±0.27[b]
GD	25.9±2.57[b]	0.34±0.02[b]	10.9±1.97[b]	76.3±0.51[a]
GID-1	5.13±2.88[d]	0.34±0.02[b]	7.21±2.44[c]	56.6±1.56[d]
GID-2	10.4±2.52[c]	0.36±0.02[b]	8.12±0.95[bc]	60.3±1.30[c]

WGH was produced by sequential hydrolysis with Alcalase for 4 h, Protamex for 1 h and Flavourzyme for 1 h.

GD, gastric digest produced by pepsin; GID-1, gastrointestinal digest produced by pepsin for 1 h and pancreatin for 1 h; and GID-2, gastrointestinal digest produced by pepsin for 1 h and pancreatin for 2 h.

All data represent the means and standard deviations (n=3).

Different small letters in the same columns indicate significant differences among the samples ($p<0.05$; one-way ANOVA and Duncan's multiple range test).

digests was observed with additional 1 h pancreatin digestion followed by a slight ($p<0.05$) increase with the final 1 h digestion. After the pepsin-pancreatin digestion, the DPPH radical scavenging activity of the final digest was only 10.4%. You et al. (2010) also reported that following SGID, DPPH radical scavenging activity of loach protein hydrolysates decreased from 80.8% to 4.1%.

On the other hand, digestion by pepsin led to a significant ($p<0.05$) decrease in reducing power of the WGH, while further digestion by pancreatin led to no significant ($p>0.05$) changes in the reducing power. Teixeira et al. (2016) also reported that *in vitro* digestion caused a decrease in reducing power of Cape hake hydrolysates.

Moreover, as for metal chelating activity, changes in iron chelating activity of the WGH during the SGID showed similar trends with those in the DPPH radical scavenging activity. The iron chelating activity of the final digest was 8.12%, while the activity of the WGH was 15.8%. Digestion by pepsin slightly ($p<0.05$) increased copper chelating activity of the WGH, and further digestion by pancreatin significantly ($p<0.05$) decreased it to 60.3%.

From an overall point of view, the changes in antioxidant activities of the WGH during the *in vitro* SGID may result from the fact that peptides responsible for those activities might be damaged or generated by the action of the gastrointestinal proteases (You et al., 2010; Zhu et al., 2008).

2.3. Summary

In vitro digestion of the WGH by pepsin and pancreatin led to a significant ($p<0.05$) increase in fractions below 1 kDa (88.5%), especially fractions below 500

Da. Although some decreases were observed in antioxidant activities of the WGH following *in vitro* SGID, they still exhibited antioxidant activities, especially reducing power and copper chelating activity.

CONCLUSION

Protease type, treatment sequence and time might play critical roles in yield, MW distribution, bitterness, turbidity, thermal stability, color and free radical scavenging activity of the WGH. The A4P1F1 showed better properties to be applied in beverages than the others. Following *in vitro* SGID, the A4P1F1 still exhibited antioxidant activities, especially reducing power and copper chelating activity. In conclusion, sequential hydrolysis, especially the A4-P1 sequence, may be a method to efficiently produce WGH, which is a potential candidate to be applied in beverages.

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국문초록

밀 글루텐 가수분해물의 효율적 생산을 위한 단계적 효소 처리 방법 탐색

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밀 글루텐은 밀 전분을 생산 중에 얻을 수 있는 단백질 부산물이며 낮은 수용성으로 인하여 식품에서 적용하기 힘들다. 효소 가수분해는 이러한 단백질의 한계점을 극복할 수 있는 방법이며, 생리 활성이 있는 펩타이드를 생산할 수 있어 널리 활용된다. 하지만 그 동안 단백질 가수분해물을 경제적이며 효율적인 방법으로 생산하는 것에 대한 연구는 제한적으로 수행되었다. 따라서 본 연구의 목적은 단계적 효소 처리를 통하여 음료에 적용하기에 적합한 밀 글루텐 가수분해물을 생산할 수 있는 효율적인 가수분해 방법을 개발하는 것이다.

본 연구에서는 높은 고체비율(25%, w/w), 낮은 효소 농도(1:300, w;w), 짧은 가수분해 시간(6 h)과 같은 산업 조건을 이용하여 밀 글루텐 가수분해물을 생산하였다. Endo-protease (Protamex, P;

Alcalase, A; Neutrase, N)와 endo-와 exo-protease의 혼합효소(Flavourzyme, F)을 사용하였다. 또한 가수분해에 적합한 효소 처리 순서를 탐색하기 위하여 다양한 endo-protease의 조합을 시험하였다.

효소 처리 후 얻은 밀 글루텐 가수분해물의 수율은 59.3-65.7%였다. A와 다른 endo-protease들을 조합하여 얻은 밀 글루텐 가수분해물은 다른 조합의 가수분해물보다 유의적으로 1 kDa 이하의 펩타이드를 더 많이(50% 이상) 함유하였다. N-P순서로 처리하여 생산한 밀 글루텐 가수분해물은 쓴맛이 가장 낮았다. A5F1, N5F1, A4P1F1, N4P1F1, A3N2F1 및 N3A1F1(숫자는 각 효소의 처리 시간)은 다른 효소 처리로 생산한 밀 글루텐 가수분해물보다 물에 녹였을 때, 탁도가 낮았고 열 안정성이 우수하며 L^*값이 높았다. P-A 및 A-P순서로 처리하여 생산한 밀 글루텐 가수분해물은 다른 가수분해물보다 DPPH 라디칼 소거능이 높았다. 이러한 결과를 통하여 A4P1F1이 전반적으로 음료에 적용하기에 우수하다고 판단한다. A4P1F1를 체외 위장 소화 모델을 이용하여 소화 효소로 처리한 후의 소화산물도 소화전과 비교하였을 때 항산화능이 큰 차이가 없었다.

주요어: 밀 글루텐; 단계적 효소 가수분해; 저분자 펩타이드; 항산화능

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