**The potential of papain and alcalase enzymes and process optimizations to reduce
allergenic gliadins in wheat flour**

Running Title: Wheat gliadin as affected by different proteases and enzymatic process

Ying Li^{a, b}, Jianmei Yu^{a, *}, Ipek Goktepe^c, Mohamed Ahmedna^c

^aDepartment of Family and Consumer Sciences, North Carolina A&T State University, Greensboro,
NC 27411, USA

^bInstitute of Agricultural Products Processing, Jiangsu Academy of Agricultural Science, Nanjing
210014, P.R. China

^c Department of Health Sciences, College of Arts and Sciences, Qatar University, Doha,
Qatar

*Corresponding author: jyu@ncat.edu (Jianmei Yu).

ABSTRACT

The objectives of this study were to select effective enzymes that catalyze the hydrolysis of allergenic proteins, gliadins, in wheat flour and to optimize the enzymatic treatment conditions. Six proteases were tested. Hydrolyzed samples were tested for residual gliadin concentrations and *in vitro* allergenicity. The hydrolysis conditions of wheat protein by the effective enzymes were optimized by central composite design. Results showed that alcalase from *Bacillus licheniformis*, and papain from latex of papaya fruit had greater ability to reduce gliadin content of wheat flour than had flavourzyme, pepsin, trypsin or α -chymotrypsin. The sequential-treatment of wheat flour by alcalase-papain was more effective in reducing gliadin content than single enzyme treatment. Under the optimal conditions of sequential enzymatic treatment, gliadin was almost completely removed, resulting in the flour extract showing lowest IgE-binding. Therefore, this could be a promising biotechnology for preparing low allergenic wheat products.

Keywords: Wheat allergen reduction, Enzymatic treatment, Digestive protease, Non-digestive protease, Gliadin content, IgE-binding

1. Introduction

Wheat is one of the three most harvested cereals and a most important part of the daily diet of human beings throughout the world. Many foodstuffs, such as breads, noodles and baked goods, are made from wheat flour. But wheat contains antigenic proteins that are capable of inducing allergic reactions. The rate of sensitization is 0.4–1.3% in children and 0.2–0.9% among adults (Prandi, Faccini, Tedeschi, Galaverna, & Sforza, 2013). Ingestion of extremely small amounts of wheat flour might affect highly sensitive patients, with allergic reactions. For instance, gluten-sensitive enteropathy, known as celiac disease, is induced by ingestion of cereals containing gluten (Kottgen, Volk, & Gerok, 1982; Di Sabatin & Corazza, 2009). Bakers' asthma and rhinitis, a well-defined immunoglobulin E (IgE)-mediated allergy to inhaled flour, is a common disease, primarily among workers with occupational exposure (Suarthana et al., 2010; Bittner, Grassau, Frenzel, & Baur, 2008; Salcedo, Quirce, & Diaz-Perales, 2011). Wheat-dependent exercise-induced anaphylaxis is a life-threatening allergy in adults, whereby ingestion of wheat, in connection with physical exercise, induces symptoms of anaphylaxis (Morita, Matsuo, Mihara, Morimoto, Savage, & Tatham 2003; Palosuo et al., 2001).

Several wheat allergens have been identified in the different fractions of wheat grain. Among them, gliadins (ω -, α -, β - and γ -), a polymorphic group of ethanol-soluble proteins, containing regions of repeated sequences consisting of Pro- and Gln-rich peptide motifs, are major allergens due to the presence of an allergenic epitope in the native proteins. The epitope refers to a part of the macromolecule that is recognized by the immune system. IgE antibody in the sera of sensitive patients reacts with the epitope peptide of wheat, which is capable of inducing immune responses and functioning as an allergen in humans. The structural features of the allergenic gluten were investigated and it was found that the pentapeptide repeat motif, Gln-Gln-Gln-Pro-Pro, of a low molecular weight glutenin subunit was an IgE-binding epitope (Tanabe, Arai, Yanagihara, Mita, Takahashi, & Watanabe, 1996). It was found that the N-terminal glutamine residue and two proline residues are essential for the binding to IgE. Repeat peptides, such as (Ser-Gln-Gln-Gln

-(Gln-)Pro-Pro-Phe)₄ might be favourable in cross-linking IgE antibodies and triggering the release of chemical mediators from mast cells in the body (Tanabe, 2008). Another element of gluten protein structure, related to its allergenicity, is the presence of disulphide bonds (SS). SS bonds in gliadins are primarily intra-chain. This increases amino acid chain folding (Müller & Wieser, 1997). Specific reduction of SS bonds to free SH groups resulted in a considerably decreased affinity for specific antibodies (Kączkowski, 2000). This suggests that the presence of disulphide bonds may relate to gliadin allergenicity. Another element of protein structure that can relate to their allergenicity is glycosidic bonds resulting from the addition of saccharides to the amino acid chain. Carbohydrate-protein complexes have always been found to bind strongly to IgE antibodies (from patients showing symptoms of bakers' asthma), whereas detachment of saccharides inhibited the immunological reaction (Waga, 2004; Altmann, 2007).

Enzymatic treatment has the potential to destroy allergenic epitopes and reduce the antigenicity of many food products by hydrolyzing proteins into small peptides. Our previous studies have shown that hydrolysis of peanut protein by animal digestive proteases, trypsin and α -chymotrypsin, significantly reduced peanut allergens in peanut kernels and peanut butter (Yu, Ahmedna, Goktepe, Cheng, & Maleki, 2011; Yu, Ahmedna, & Goktepe, 2013; Li, Yu, Ahmedna, & Goktepe, 2013). The reduction in antigenicity of protein depends on both the specificity of proteolytic enzymes used and the degree of hydrolysis. Different enzymes have different specific cleavage sites of protein. Therefore, they reduce the allergenicity of a protein to different extents. Papain is a cysteine protease preparation, produced from latex of papaya fruit, which has broader specificity than have pancreatic proteases (Grzonka, Kasprzykowski & Wicz, 2007). Alcalase is a protease preparation of bacterium *Bacillus licheniformis*, which contains subtilisin as the dominating protease (Donlon, 2007). Because papain and alcalase are endopeptidases and are less specific than are trypsin or α -chymotrypsin, we hypothesized that papain and alcalase could hydrolyze allergenic proteins more-completely than could animal digestive enzymes, such as pepsin, trypsin and α -chymotrypsin. Furthermore,

sequential enzyme treatment may enhance the allergen reduction by proteases of plant and bacterial origin.

Response surface modelling has proved to be a valuable tool for simultaneous optimization of several process parameters in biological and chemical sciences (Zhao et al., 2012; Poonkuzhali&Palvannan, 2011; Jouki, Mortazavi, Yazdi, & Koocheki, 2014). Hydrolysis parameters, e.g. pH, temperature, and enzyme-to-substrate ratio and their interactive effects, could greatly influence the antigenicity of protein hydrolysate (Zheng, Shen, Bu, & Luo, 2008).

The purpose of this study was to (1) select the effective enzymes from commonly used food proteases to reduce the wheat allergen, (2) establish a predictive model that can be used to predict the degree of gliadin reduction based on hydrolysis parameters, (3) provide the optimal hydrolysis conditions under which low antigenicity hydrolysates would be obtained, (4) determine the effectiveness of enzyme treatments on IgE binding of treated wheat extracts.

2. Materials and methods

2.1. Materials

The Gold Medal all-purpose wheat flour (General Mills Incorporation, Minneapolis, MN), purchased from a local market (Greensboro, NC), was used as enzyme substrate. Enzymes used in this study, including alcalase (EC3.4.21.14, 22868 units/ml liquid) from *Bacillus licheniformis*, papain (EC3.4.22.2, 3662 units/g solid, lyophilized powder) from papaya latex, flavourzyme (EC3.4.11.1, 4471 units/g protein) from *Aspergillus oryzae*, α -chymotrypsin (EC3.4.21.1, 4342 units/g protein) from bovine pancreas, trypsin (EC 3.4.21.4, 6362 units/g protein) from bovine pancreas, and pepsin (EC3.4.23.1, 3060 units/g protein) from porcine gastric mucosa, were purchased from Sigma-Aldrich (St. Louis, MO). Enzyme activities presented were determined by universal protease activity assay, using casein as substrate and expressed as U/g enzyme preparation. In this study, the proteases from animal source are defined as digestive enzymes, while the proteases from bacterium, fungi and plant are defined as non-digestive enzymes. ELISA kits for

gliadin residue quantification were purchased from ELISA Systems (Queensland, Australia). The ELISA kit contains antibody-coated strips, standards, dilution buffer, conjugate, TMB substrate, gliadin extraction solution and wash buffer. Bicinchoninic acid (BCA) Protein Assay Kit was purchased from Pierce (Rockford, IL, USA). The pooled human plasma obtained from 10 patients with gliadin allergy was purchased from Plasma Lab International (Everett, WA, USA). Superblock and hydrogen peroxide (30%) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Anti-human IgE (ϵ -chain specific)-peroxidase antibody, produced in goat, and *o*-phenylenediamine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Enzyme treatment of wheat flour

Wheat flour was weighed into a set of beakers (20 grammes per beaker), and then 45 ml of phosphate buffer (10 mM), containing protease, were added to each beaker and mixed (with a spatula) quickly. The enzyme to substrate (dry flour) ratio (E/S) was 25430U/100g. The pHs of buffers varied, depending the type of enzyme: papain and flavourzyme = 7.0, for α -chymotrypsin; alcalase = 8.0 and trypsin = 6.5. The optimal pH of each enzyme was obtained from the product datasheet provided by the vendor. For pepsin treatment, glycine/hydrochloric acid buffer (10 mM, pH 2.5) was used. Samples containing trypsin, α -chymotrypsin and pepsin were incubated in a Gyrotory Water Bath Shaker G76 (New Brunswick Scientific Co., Edison, NJ, USA) for 1h at their optimum temperature (37 °C, as shown on their product datasheets) with gentle shaking, while samples mixed with other enzymes were incubated in a Gyrotory Water Bath Shaker G76 for 1h at temperatures within their optimal ranges: alcalase = 50 °C, papain = 65 °C and flavourzyme = 37 °C. The hydrolysis process was terminated by heating the samples at 95 °C for 10 min in a preheated water bath and then cooled to room temperature immediately in a cold water bath. The hydrolysates were sealed with para film in sterile centrifuge tubes and stored at 4 °C for further analysis. Each treatment was conducted in duplicate.

2.3. Extraction of wheat gliadin

The gliadin of enzyme-treated and untreated wheat was extracted, using the extraction solution in the ELISA kits. One part of sample was weighed into a suitable test tube and 9 parts of the extraction solution were added. Samples were mixed for 1-2 min, using a vortex mixer, and then incubated in a water bath at 60°C for 15 min under gentle shaking. After cooling, samples were centrifuged at 500 x g for 15 min and supernatants were collected and stored at 20°C. The volumes of the supernatants were recorded. Insoluble protein in the precipitate fractions were extracted, using standard electrophoresis sample treatment buffers containing glycerol, SDS and dithiothreitol (pH6.8), as described by Schmitt, Nesbit, Hurlburt, Cheng & Maleki (2010).

2.4. Determination of soluble protein by BCA method

The concentration of soluble proteins in each extract was determined by the bicinchoninic acid (BCA) method, using analytical grade bovine serum albumin (BSA) as a standard to evaluate the effect of enzyme treatment condition on the solubility of wheat protein.

Samples were diluted 5-10 times with deionized water to bring protein concentration in the test sample down to the linear range of the BSA calibration curve (0–1.0 mg/ml). The soluble protein contents in wheat extract were expressed as mg protein/g flour.

2.5. Determination of gliadin residue in wheat flour samples using a sandwich ELISA

The gliadin concentration of protein extract was used as an indicator to evaluate the efficiency of allergen reduction by enzyme treatment and was determined by the ELISA kits, following the procedure provided by the vendor of the kit. (1) Properly diluted samples were added to the primary antibody-coated wells (100 µl/well) and mixed by moving the strip holder gently sideways for 10 seconds. The mixtures were incubated at 22-23°C for 30 min, then washed three times with wash buffer (phosphate buffer saline containing Tween-20, PBST). (2) The enzyme conjugate (100 µl/well) was added and mixed as in step (1). Mixtures were incubated at 22-23°C for 30 min, then washed three times with wash buffer. (3) The substrate solution was added (100µl/well) and the mixtures were incubated at 22-23°C for 15 min. (4) Finally, 100 µl/well of the stop solution were added and the absorbance was read, using a Synergy HT Microplate Reader (Bio-Tek, Winooski, VT) and using a bichromatic reading at 450nm (primary) and

620-650nm (reference). The concentration of gliadin of each sample was calculated, using standard curves developed with gliadin standards (0-25 ppm). The final results were calculated as µg gliadin per gramme of flour, and were expressed as means ± standard deviation of **triplicate analyses**. The percentage reduction of gliadin was calculated as follows:

$$\% \text{ Gliadin Reduction} = (C_0 - C_t) \times 100 / C_0$$

Where C_0 is the concentration of gliadin in the untreated wheat flour sample, C_t is the concentration of gliadin in enzyme-treated wheat flour sample.

2.6. SDS-PAGE of soluble protein fractions

SDS-PAGE was performed to visualize the change of target protein due to enzyme treatment. The protein extracts of enzyme-treated and untreated wheat flour were adjusted to 1 mg/ml with phosphate buffer (10 mM, pH 7.2). They were mixed with the same volume of sample treatment buffer (pH 6.8, Tris buffer containing glycerol, SDS and dithiothreitol), then heated in boiling water bath for 10 min. After cooling to room temperature, samples (10µl/well) were loaded onto 4/15% polyacrylamide gel and run, using a Bio-Rad Mini Protein III system (Bio-Rad, Hercules, CA). Pre-stained SDS-PAGE Standard (Bio-Rad, Hercules, CA) was used as marker. The gel was resolved under the power conditions recommended by Bio-Rad (200 V, 20 mA, 6 Wand 60 min) and stained with Coomassie Brilliant Blue R-250 solution (Bio-Rad, Hercules, CA) overnight. After destaining, the gels were photographed with a Gel Doc XR imaging system (Bio-Rad, Hercules, CA).

2.7. Determination of IgE binding of treated wheat extracts with human plasma

A competitive inhibition ELISA (ciELISA) was used to evaluate the effect of enzyme treatment on the IgE-binding potential of wheat protein extracts. The assay was carried out in quadruplicate (n=4) **by a slightly modified method of Chung & Champagne (2009).** Briefly, a microplate was coated with untreated wheat extract (diluted to 2 µg/ml with

PBS) and incubated at 37°C for 2 h. After washing wells 3 times with PBST, the plate was blocked 3 times (5 min each time) with Superblock (200 µl/well) at room temperature, then washed 3 times with PBST. Fifty microlitres of wheat extracts, diluted to protein concentrations of 0, 0.01, 0.1, 1.0, 10, 100, 1000 µg/ml, were mixed with 50 µl of properly diluted pooled human plasma from wheat-allergic individuals. The mixtures were added to the plate (100 µl/well) and incubated for 45 min at room temperature, followed by 3 washes. The goat anti-human IgE-peroxidase conjugate (1:1000 diluted with superblock) was added (100µl/well) and incubated at room temperature for 30 min. Following three washes, the substrate solution (100 µl), containing *o*-phenylenediamine (0.5 mg/ml) and 0.03% hydrogen peroxide in 0.1 M citrate buffer (pH 5.5), was added. The enzyme reaction was stopped by adding 100 ml/well of stop solution after 10 min of incubation at room temperature. The absorbance was read at 490 nm with a Synergy HT Microplate Reader. The absorbance values of samples containing IgE and the wheat extract were expressed by B, while the absorbance of a control, containing IgE only, was expressed as B₀. The % IgE-binding inhibition of a sample was expressed as (B/B₀)*100.

2.8. Experimental design and statistical analysis

Single factor (type of enzyme)-one level design was used to select effective enzymes. The single enzyme experiments were conducted at the optimal pH and temperature of each enzyme, with an enzyme to substrate ratio of 25430U/100g flour. Central composite design, using Design Expert 7.0 Trial (Stat Ease, Minneapolis, MN) was applied to optimize the hydrolysis conditions for the selected enzyme. The enzyme treatment time (X1), enzyme-to-substrate ratio E/S (X2), and temperature (X3) were chosen as independent variables (k=3). For the optimization experiments, the E/S was expressed as percentage of enzyme preparation to substrate (flour). The enzyme treatments were conducted at the optimal pH of each enzyme. Therefore, pH was not considered as an independent variable. The percentage of gliadin reduction was used as the dependent variable, Y, to evaluate the efficiency of enzyme treatment.

The independent variables were optimized, using a central composite design containing five levels for each independent variable, coded as -1.682, -1, 0, +1, +1.682.

The actual values of independent variables corresponding to these coded values are given in Tables 1, 2, and 3. The complete central composite design consisted of 2^3 experiments for a full factorial design plus $2 * 3$ star experiments and six centre experiments, resulting in 20 experiments (Table 1, 2, 3). Each hydrolysis experiment was conducted in duplicate. The final results were tested in triplicate, and were expressed as means $\pm$ standard deviation. The statistical analyses of the data were performed by ANOVA (analysis of variance), using Design Expert 7.0 Trial Software to determine the variables that have significant effects on gliadin reduction at a $P < 0.05$ level of significance.

3. Results and discussion

3.1. Effectiveness of different proteases on protein solubility and allergen content of wheat flour

Proteinase hydrolysis has been proven an effective method to reduce the antigenicity of food protein (Lakshman, Toyokawa, Tachibana, Toyama, Taira, & Yasuda., 2011; Latif, Pfannstiel, Makkar, & Becker, 2013; Shimakura, Tonomura, Hamada, Nagashima, & Shiomi, 2005). Taking gliadin as an example, the major epitope, Gln-Gln-Gln-Pro-Pro, should be hydrolyzed by the enzyme that can cleave the bonds near Pro to reduce the antigenicity of wheat. Bromelain, with preferential cleavage on Pro and low amylase activity, was used to hydrolyze the proteins in wheat flour, resulting in hypoallergenic protein hydrolysates (Tanabe et al., 1996). Nevertheless, it was found that bromelain was allergenic itself. Therefore, it is not safe to use bromelain for food allergen reduction (Zorzi, Curioni, Simonato, Giannattasio, & Pasini, 2007).

In this study, six proteases, including alcalase, papain, flavorzyme, pepsin, trypsin, and α -chymotrypsin, at E/S of 25430U/100g flour, were tested to select effective enzymes to hydrolyze the allergenic proteins, gliadins, in wheat flour. Results show that the soluble protein concentrations of wheat flour treated by six different proteases, under the optimal pH and temperature of each enzyme, varied greatly, reflecting the variations in hydrolyzing capacity of the proteases. The flour sample hydrolyzed by papain showed the highest soluble protein, followed by those hydrolyzed by α -chymotrypsin, flavourzyme,

alcalase, trypsin and pepsin.

The gliadin residue of wheat flour treated by different proteases is shown in Fig. 1. The flour treated with papain and alcalase exhibited lower gliadin levels than did that treated with other proteases (Fig.1). Papain was the most effective enzyme in gliadin reduction, followed by alcalase and flavourzyme. The results indicate that the protease preparations, such as papain, alcalase and flavourzyme, from plant, bacteria and fungi, respectively, had greater ability to reduce allergenic protein content of wheat flour than had digestive enzymes, such as pepsin, trypsin and α -chymotrypsin. These results confirmed that food allergens are sometimes characterized by their high stability against digestive enzymes, with their epitope structures remaining unchanged (Watanabe, Suzuki, Ikezawa, & Arai, 1994), but they can be hydrolyzed by other food grade proteases. Therefore, papain and alcalase were selected for the process optimization to determine the best treatment condition for wheat allergen reduction.

3.2. Effects of alcalase treatment on the allergen of wheat flour

The experimental design and results of alcalase treatments of wheat flour are presented in Table 1. Variations of soluble protein and gliadin reduction, under different hydrolysis conditions, were observed. The protein solubility increased, due to more small peptides being generated from protein by protease. The concentration of residual gliadin decreased as soluble protein concentration increased. A similar correlation was reported for enzyme-treated peanuts (Li, Yu, Ahmedna, & Goktepe, 2013).

ANOVA and regression analysis of data show that enzyme to substrate ratio ($[E]/[S]$) and treatment time had significant effects on reduction of gliadin ($P < 0.05$), and there were significant interactions between $[E]/[S]$ and treatment time ($P < 0.05$), and between $[E]/[S]$ and temperature ($P < 0.05$). Based on the experimental data, a model that correlates dependent variable Y (gliadin reduction, %) and the independent variables A (alcalase treatment time, min), B (alcalase to substrate ratio, %) and C (temperature, °C) was obtained by response surface modelling, using Design Expert 7.0.

$$Y = 99.93 + 11.87A + 6.12B + 8.47C - 3.42AB + 17.25AC - 9.84BC - 8.33A^2 - 11.06B^2 - 10.45C^2$$

$$(R^2 = 0.9049, P = 0.0004)$$

The very small P value ($P=0.0004$) and high R^2 indicate that the model fitted the data well and could represent the actual relationship between responses (Y) and independent variables. The optimal hydrolysis conditions for maximum gliadin reduction, obtained according to the regression model, were [E]/[S] of 0.17% at pH 8.0, treatment time 62 min and temperature 53°C. The dominating enzyme in the alcalase preparation is subtilisin A which has optimal pH of 7-8, isoelectric point at pH 9.04 and optimal temperature range of 50-65 °C according to the Sigma-Aldrich datasheet. The optimized pH and temperature obtained in this study for gliadin hydrolysis are within the optimal pH and temperature range of alcalase.

3.3. Effects of papain treatment on the allergen reduction of wheat flour

Table 2 shows the experimental design and results of papain treatment of wheat flour. Similarly to the treatment with alcalase, papain treatment significantly increased the soluble protein content and reduced the gliadin content of wheat flour. The papain exhibited great effects on gliadin reduction at very low enzyme concentration. Among 20 experiments, 12 of them resulted in more than 99% of reduction of gliadin. ANOVA and regression analysis results show that [E]/[S] was the significant factor which affects the protein hydrolysis ($P < 0.0001$), while treatment time and temperature were not. The correlation between gliadin reduction (Y, %), treatment time (A, min) and papain to substrate ratio (C, %) was expressed as:

$$Y = 99.55 + 3.80A + 21.08B - 0.43C - 2.31AB + 0.22AC + 0.77BC - 17.42A^2 - 15.35B^2 + 1.54C^2$$

$$(R^2 = 0.9120, P = 0.0003)$$

From the regression model, the optimal hydrolysis conditions for papain treatment of wheat flour were [E]/[S] of 0.26%, hydrolysis time of 32 min, and temperature 48°C. Although the optimal hydrolytic temperature shown on the product datasheet from Sigma-Aldrich is 65 °C, the optimal temperature obtained in this study is the same as that

reported by Liu, Lo & Li (2012) and is in the optimal range (35-60°C) reported by the Bio-Cat company (<http://www.bio-cat.com/papain/>).

3.4. Effects of sequential enzymatic treatments on allergen reduction in wheat flour

Due to the differences in protein cleavage sites of different proteases, we tested a sequential enzyme treatment of wheat flour, expecting some complimentary effect of two selective enzymes on gliadin reduction. From the results presented in sections 3.2 and 3.3, alcalase to substrate ratio, alcalase treat time and papain to substrate ratio were the significant factors for gliadin reduction. These three factors were selected as dependent variables, and other treatment parameters were the optimal conditions obtained from sections 3.2.and 3.3. Wheat flour was hydrolyzed with alcalase, then papain. Table 3 shows the experimental design and the results of sequential enzymatic treatment of wheat flour. This treatment increased the soluble protein and reduced gliadin much more effectively than did the single enzyme treatment. Among 20 experiments, 7 of them showed almost complete removal of gliadin (100%). The regression analysis of data in Table 3 shows that alcalase to substrate ratio, alcalase treat time and papain to substrate ratio were the significant factors ($P < 0.05$) and there was a significant interaction between alcalase to substrate ratio and alcalase treat time ($P < 0.05$). **Correlation between gliadin reduction (Y) and the independent variables was expressed as.**

$$Y = 100.05 + 2.47A + 5.66B + 2.91C - 3.94AB - 2.15AC - 3.82BC - 0.63A^2 - 3.64B^2 - 1.18C^2$$
$$(R^2 = 0.8775, P = 0.0016)$$

where A is alcalase treatment time (min), B is alcalase to substrate ratio (%) and C is papain to substrate ratio (%).

From the regression model above, the optimal hydrolysis conditions of sequential enzymatic treatment were obtained. Wheat flour was first incubated with 0.06% alcalase for 62 min at 53°C and at pH 8.0, followed by the treatment with 0.07% papain for 32 min at 48°C and pH7.0 to complete the hydrolysis of the allergens in wheat flour. Total

enzyme used to achieve 100% reduction of gliadin is 0.13%. This suggests that sequential treatment of wheat flour by alcalase and papain is more cost-effective than is single enzyme treatment for gliadin reduction.

3.5. SDS–PAGE of proteins in soluble and insoluble fractions of wheat flour

Fig. 2 presents a visual illustration of the protein profile of soluble and insoluble fractions of wheat flour samples. Multiple protein bands, with molecular weights (MW) between 6.9 and 101 kDa, were observed in the protein extracts and precipitates of untreated sample (control)(lane 2). For flour samples treated with 0.002% digestive proteases, protein bands with MW around 38.5 and 10 kDa were observed in both extracts and precipitates (Fig. 2 lanes 3, 5, and 7), but their intensities were significantly lower compared with the control; when the concentrations of digestive proteases increased to 0.01%, all protein bands disappeared from the SDS-PAGE of the extracts (Fig. 2A, lanes 4, 6 and 8), but bands with $MW \geq 38.5$ kDa, and more bands with MW between 6.9 and 21 kDa, showed up on the SDS-PAGE of precipitates (Fig.2C lanes 4, 6, and 8). This visually illustrated that digestive proteases could not effectively reduce the allergens of wheat flour. For flour samples treated with non-digestive proteases (Fig. 3B and 3D), only the extract of 0.002% alcalase-treated sample showed protein bands with MW between 29 and 56.2 kDa (Fig. 2B, lane 7). The precipitates of papain or alcalase-treated samples still had proteins with MW of 6.9-38.5 kDa and proteins smaller than 6.9 kDa in the precipitates, depending on the enzyme concentration (Fig. 2D, lanes 5-8), but samples obtained from sequential treatment of alcalase and papain did not contain any protein with MW larger than 6.9 kDa in either soluble or insoluble fractions (Fig. 2B and 2D, lanes 3 and 4). The wheat proteins recognized by sensitized individuals corresponded to multiple bands from 14-98 kD ([Beyer, Bardina, & Sampson, 2005](#)). There were no protein bands within this MW range in the soluble and insoluble fractions of flour samples hydrolyzed with 0.01% of alcalase/papain. This suggests the high effectiveness of sequential enzymatic treatment in reducing gliadin of wheat flour.

3.6. Effects of enzymatic treatment on IgE-binding of wheat protein extracts

IgE-containing plasma, from 10 patients with clinically confirmed wheat allergy, was used to test the IgE-binding capacities of wheat protein extracts obtained from different treatments. To compare the allergenicity reducing potential of different proteases, the samples treated with low enzyme dose (total 0.002%, w/w) were selected. The flour sample from sequential enzymatic treatment was hydrolyzed with 0.001% alcalase, and then 0.001% papain. The untreated wheat extract was used as the control. At the same protein concentration, the higher the IgE-binding inhibition, the lower was the allergenicity, and vice versa (Chung & Champagne, 2009). Fig. 3 shows that the protein extracts of wheat flour treated with digestive proteases exhibited lower IgE-binding inhibition than did the extracts of samples treated with non-digestive proteases. For single enzyme treatment, alcalase and papain were the most effective, while pepsin was the least effective in reducing IgE-binding. The results were in accordance with those in Fig.1. The alcalase-papain sequential treatment resulted in extracts with highest IgE-binding inhibition. These results indicate that the alcalase-papain sequential treatment was the most effective treatment in reducing allergen of wheat flour. This is because two enzymes with different preferred cleaving sites should cleave more peptide bonds than a single enzyme, thus transforming the protein to peptides with fewer epitopes.

4. Conclusion

This study focussed on the reduction of major wheat allergens by enzymatic treatment. The protease preparations, papain and alcalase, were found to be more effective in increasing protein solubility, and reducing allergenic protein content of wheat flour than were the digestive proteases. The sequential enzymatic treatment of wheat flour with alcalase and papain almost completely removed gliadin under optimal conditions. The IgE-binding test showed that the treatment of wheat with alcalase-papain significantly reduced the *in vitro* allergenicity of wheat flour. It is clear that the sequential hydrolysis of wheat flour with alcalase-papain, under proper conditions, is a promising method for preparation of low allergenic wheat products. However, skin prick test or oral challenge are needed to confirm if the wheat flour with reduced *in vitro* IgE-binding is

hypoallergenic. In addition, sensory and functional properties of the product treated with alcalase-papain need to be investigated to determine the feasibility of the treatment.

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References

- Altmann, F. (2007). The Role of Protein Glycosylation in Allergy. *International Archive of Allergy and Immunology*, 142, 99–115.
- Beyer, K., Bardina, L., & Sampson, H. A. (2005). Differences in recognition of water-soluble and -insoluble wheat allergens in two varieties of wheat flour between wheat-allergic and wheat-sensitized but clinically tolerant patients. *The Journal of Allergy and Clinical Immunology*, 115, S245.
- Bittner, C., Grassau, B., Frenzel, K., & Baur, X. (2008). Identification of wheat gliadins as an allergen family related to baker's asthma. *Journal of Allergy and Clinical Immunology*, 121, 744-749.
- Chung, S. Y., & Champagne, E. T. (2009). Reducing the allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds. *Food Chemistry*, 115, 1345 – 1349.
- Di Sabatino, A., Corazza, G.R. (2009). Coeliac disease. *Lancet*, 373, 1480–1493
- Donlon, J. (2007). Subtilisin. In J. Polaina and A. P. MacCabe (Eds), *Industrial Enzymes-structure, function and applications* (pp: 197-206), Dordrecht, The Netherlands: Springer.
- Grzonka, Z., Kasprzykowski, F., & Wiczak, W. (2007). Cysteine Proteases. In J. Polaina and A. P. MacCabe (Eds), *Industrial Enzymes-structure, function and applications* (pp: 181-196), Springer Dordrecht, The Netherlands: Springer.

431 Jouki, M., Mortazavi, S. A., Yazdi, F. T., & Koocheki A. (2014). Optimization of
 432 extraction, antioxidant activity and functional properties of quince seed mucilage by
 433 RSM. *International Journal of Biological Macromolecules*, 66, 113-124.

434 Kàczkowski, J. (2000). New aspects of the cereal storage protein synthesis and
 435 degradation including the role of thioredoxin, as well as its regulatory properties. *Acta*
 436 *Physiologiae Plantarum*, 22, 483–494.

437 Kottgen, E., Volk, B., & Gerok, W. (1982). Gluten, a lectin with oligomannosyl
 438 specificity and the causative agent of gluten-sensitive enteropathy. *Biochemical and*
 439 *Biophysical Research Communications*, 109, 168 – 173.

440 Lakshman, P.L. N., Toyokawa, Y., Tachibana, S., Toyama, H., Taira T., & Yasuda M.
 441 (2011). Reducing the antigenicity of milk whey protein using acid proteinases from
 442 *Monascus pilosus*. *Process Biochemistry*, 46, 806–810.

443 Latif, S., Pfannstiel, J., Makkar, H.P.S., & Becker, K. (2013). Amino acid composition,
 444 antinutrients and allergens in the peanut protein fraction obtained by an aqueous
 445 enzymatic process. *Food Chemistry*, 136, 213 – 217.

446 Li, H., Yu, J., Ahmedna, M., & Goktepe, I. (2013). Reduction of major peanut allergens
 447 Ara h 1 and Ara h 2, in roasted peanuts by ultrasound assisted enzymatic treatment.
 448 *Food Chemistry*, 141, 762-768.

449 Liu, X., Luo, Y., & Li, Z. (2012). Effects of pH, temperature, enzyme-to-substrate ratio
 450 and reaction time on the antigenicity of casein hydrolysates prepared by papain. *Food*
 451 *and Agricultural Immunology*, 23, 69-82.

452 Morita, E., Matsuo H., Mihara, S., Morimoto, K., Savage, A.W.J., & Tatham A.S. (2003).
 453 Fast ω -gliadin is a major allergen in wheat-dependent exercise-induced anaphylaxis.
 454 *Journal of Dermatological Science*, 33, 99 – 104.

455 Müller S., Wieser H. (1997). The location of disulphide bonds in monomeric γ -type
 456 gliadins. *Journal of Cereal Science*, 1997, 26, 169–176.

457 Palosuo, K., Varjonen, E., Kekki O. M., Klemola, T., Kalkkinen, N., Alenius, H., & Timo,
 458 R. (2001). Wheat ω -5 gliadin is a major allergen in children with immediate allergy to
 459 ingested wheat. *Journal of Allergy and Clinical Immunology*, 108, 634-638.

460 Poonkuzhali, K., &Palvannan, T.(2011). Thermo stabilization of laccase by
 461 polysaccharide additives: Enhancement using central composite design of RSM.
 462 *Carbohydrate Polymers*, 86, 860-864.

463 Prandi, B., Faccini, A., Tedeschi, T., Galaverna, G., & Sforza, S. (2013). LC/MS analysis
 464 of proteolytic peptides in wheat extracts for determining the content of the allergen
 465 amylase/trypsin inhibitor CM3: Influence of growing area and variety.*Food*
 466 *Chemistry*, 140, 141-146.

467 Salcedo, G., Quirce, S., & Diaz-Perales, A. (2011). Wheat allergens associated with
 468 baker's asthma. *Journal of Investigational Allergology and Clinical Immunology*, 21,
 469 81-92.

470 Schmitt, D. A., Nesbit, J. B.,Hurlburt, B. K., Cheng, H.,&Maleki, S. J.(2010). Processing
 471 can alter the properties of peanut extract preparations. *Journal of Agricultural*
 472 *andFood Chemistry*, 58, 1138–1143.

473 Shimakura, K., Tonomura, Y., Hamada, Y., Nagashima, Y., & Shiomi, K.(2005).
 474 Allergenicity of crustacean extractives and its reduction by protease digestion. *Food*
 475 *Chemistry*, 91, 247–253.

476 Suarthana, E., Vergouwe, Y., Moons, K. G., Monchy, J.D., Grobbee, D., Heederik, D., &
 477 Meijer E. (2010). A diagnostic model for the detection of sensitization to wheat
 478 allergens was developed and validated in bakery workers. *Journal of Clinical*
 479 *Epidemiology*, 63, 1011-1019.

480 Tanabe, S., Arai, S., &Watanabe, M. (1996). Modification of wheat flour with bromelain
 481 and baking hypoallergenic bread with addedingredients. *Bioscience, Biotechnology,*
 482 *and Biochemistry*, 60, 1269-1272.

483 Tanabe, S., Arai, S., Yanagihara, Y., Mita, H., Takahashi, K., &Watanabe, M. (1996). A
 484 major wheat allergen has a Gln-Gln-Gln-Pro-Pro motif identified as an IgE-binding
 485 epitope. *Biochemical and Biophysical Research Communications*, 219, 290-293.

486 Waga, J. (2004). Structure and allergenicity of wheat gluten proteins – a review. *Polish*
 487 *Journal of Food and Nutrition Sciences*, 13/54, 327-338.

- Watanabe, M., Suzuki, T., Ikezawa, Z., & Arai, S. (1994). Controlled enzymatic treatment of wheat proteins for production of hypoallergenic flour. *Bioscience, Biotechnology, and Biochemistry*, 58, 388-390.
- Yu, J., Ahmedna, M., Goktepe, I., Cheng, H., & Maleki, S. (2011). Enzymatic treatment of peanut kernels to reduce allergen levels. *Food Chemistry*, 127, 1014-1022.
- Yu, J., Ahmedna, M., & Goktepe, I. (2013). Enzymatic treatment of peanut butter to reduce the concentration of major peanut allergens. *International Journal of Food Science and Technology*, 48, 1224-1234.
- Zhao, X. J., Liu, J. G., Hu, Y. L., Fan, Y. P., Wang, D. Y., Yuan, J., Xu, L., Cui, L., & Jing, Z. (2012). Optimization on condition of glycyrrhetinic acid liposome by RSM and the research of its immunological activity. *International Journal of Biological Macromolecules*, 51, 299-304.
- Zheng, H., Shen, X. Q., Bu, G. H., & Luo, Y. K. (2008). Effects of pH, temperature and enzyme-to-substrate ratio on the antigenicity of whey protein hydrolysates prepared by alcalase. *International Dairy Journal*, 18, 1028-1033.
- Zorzi, M. D., Curioni, A., Simonato, B., Giannattasio, M., & Pasini, G. (2007). Effect of pasta drying temperature on gastrointestinal digestibility and allergenicity of durum wheat proteins. *Food Chemistry*, 104, 353-363.

Legends of Figures

Fig.1 Residual gliadin contents of wheat flour samples treated with different proteases (enzyme concentration was 25430U/100g flour. Control was untreated flour)

Fig. 2 SDS-PAGE of extracts (A, B) and precipitates (C, D) of wheat flour samples treated with different proteases. A and C: Lane 1– molecular marker; lane 2 – control; lane 3 – 0.002% Trypsin; lane 4 – 0.01% Trypsin E; lane 5 – 0.002% pepsin; lane 6 – 0.01% pepsin; lane 7 – 0.002% α -chymotrypsin; lane 8 – 0.01% α -chymotrypsin. B and D: Lane 1– marker; lane 2 – control; lane 3 – 0.002% alcalase + papain; lane 4 – 0.01% alcalase + papain; lane 5 – 0.002% papain; lane 6 – 0.01% papain; lane 7 – 0.002% alcalase; lane 8 – 0.01% alcalase.

Fig.3 IgE-binding of protein extracts of wheat flour samples treated with different proteases. (Total enzyme concentration was 0.002% (w/w). In the sequential enzymatic treatment, the flour was treated with 0.001% alcalase, followed by 0.001% papain. Control was untreated wheat extract.)

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524 Fig. 1

Fig. 2

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530 Fig. 3

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Table 1 Effects of alcalase treatment on soluble protein and gliadin contents of wheat flour - Full factorial central composite design and results

Assay	Independent variables			Dependent variables		
	Time (min)	E/S (%)	Temperature (°C)	Soluble proteins (mg/g flour)	Gliadin (µg/g)	Reduction of gliadin(%)
control	15	0	37	35.0±1.09	17086±606	
1	15 (-1.682)	0.18(0)	48.5(0)	59.1±0.51	7654±171	55.2
2	74.8 (1)	0.11(-1)	41.66(-1)	59.0±0.88	8484±92.7	50.3
3	52.5 (0)	0.18(0)	37(-1.682)	66.2±1.35	5984±26.0	65.0
4	52.5(0)	0.18(0)	48.5(0)	77.7±1.10	20.9±0.05	99.9
5	30.2(-1)	0.25(1)	41.66(-1)	61.7±1.52	6136±37.1	64.1
6	52.5(0)	0.30(1.682)	48.5 (0)	79.2±0.70	10.5±0.12	99.9
7	52.5(0)	0.18(0)	48.5(0)	75.0±1.14	32.4±0.11	99.8
8	74.8(1)	0.25(1)	55.34(1)	77.9±0.86	19.3±0.07	99.9
9	74.8 (1)	0.11(-1)	55.34(1)	67.4±1.02	5084±38.31	70.3
10	74.8(1)	0.25(1)	41.66(-1)	77.8±0.84	21.7±0.16	99.9
11	30.2(-1)	0.11(-1)	55.34(1)	70.6±1.30	70.5±0.17	99.6
12	52.5(0)	0.06(-1.682)	48.5 (0)	56.6±0.98	9697±243	43.3
13	52.5 (0)	0.18(0)	48.5(0)	76.0±0.84	28.8±0.32	99.8
14	30.2(-1)	0.25(1)	55.34(1)	59.9±1.18	10122±133	40.8
15	52.5(0)	0.18(0)	48.5(0)	73.1±0.84	20.5±0.25	99.9
16	30.2(-1)	0.11(-1)	41.66(-1)	55.3±1.34	9135±267	46.5
17	90(1.682)	0.18(0)	48.5(0)	79.4±0.92	8.7±0.14	100
18	52.5(0)	0.18(0)	60(1.682)	68.2±0.84	4312±144	74.8
19	52.5(0)	0.18(0)	48.5(0)	76.2±0.73	26.3±0.17	99.9
20	52.5(0)	0.18(0)	48.5(0)	78.0±1.59	19.1±0.23	99.9

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536 **Table 2** Effects of papain treatment on soluble protein and gliadin contents of wheat flour
537 - Full factorial central composite design and results

Assay	Independent variables			Dependent variables		
	Temperature (°C)	E/S (%)	Time (min)	Soluble proteins (mg/g flour)	Gliadin (µg/g)	Reduction of gliadin(%)
control	37	0	15	35.0±1.09	17086±606	
1	55.34(1)	0.1(-1)	16.15(-1)	55.8±0.88	8840±21.5	48.3
2	48.5(0)	0.001(-1.682)	32.5(0)	52.1±1.43	11136±202	34.8
3	41.66(-1)	0.4(1)	16.15(-1)	72.0±1.34	5.17±0.21	100
4	48.5(0)	0.25(0)	60(1.682)	73.1±0.92	1.14±0.11	100
5	48.5(0)	0.25(0)	5(-1.682)	65.3±1.26	17.7±0.40	99.9
6	41.66(-1)	0.4(1)	48.85(1)	65.9±0.79	51.5±1.34	99.7
7	55.34(1)	0.1(-1)	48.85(1)	61.7±1.55	9260±97.8	45.8
8	48.5(0)	0.25(0)	32.5(0)	70.0±1.26	38.5±0.32	99.8
9	41.66(-1)	0.1(-1)	16.15(-1)	52.2±1.65	10315±136	39.6
10	48.5(0)	0.25(0)	32.5(0)	71.1±0.86	38.5±0.32	99.8
11	37 (-1.682)	0.25(0)	32.5(0)	67.7±0.96	10875±81.0	36.4
12	48.5(0)	0.25(0)	32.5(0)	71.2±0.87	38.5±0.32	99.8
13	48.5(0)	0.25(0)	32.5(0)	73.9±1.21	38.5±0.32	99.8
14	60 (1.682)	0.25(0)	32.5(0)	67.3±1.11	7465±44.5	56.3
15	48.5(0)	0.25(0)	32.5(0)	71.0±0.84	38.5±0.32	99.8
16	55.34 (1)	0.4(1)	16.15(-1)	70.9±0.67	63.9±3.28	99.6
17	48.5(0)	0.25(0)	32.5(0)	71.1±1.18	38.5±0.32	99.8
18	48.5(0)	0.5(1.682)	32.5(0)	72.8±0.82	5203±105	69.6
19	41.66 (-1)	0.1(-1)	48.85(1)	59.5±0.92	10935±75.9	36.0
20	55.34(1)	0.4(1)	48.85(1)	68.7±1.01	8.26±0.30	100

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Table 3 Effects of 2- enzyme sequential treatment on soluble protein and gliadin contents of wheat flour - Full factorial central composite design matrix

Assay	Independent variables			Dependent variables		
	Alcalase time(min)	Alcalase E/S (%)	Papain E/S (%)	Soluble proteins (mg/g flour)	Gliadin (µg/g)	Reduction of gliadin(%)
Control	15	0	0	35.0±1.09	17086±606	
1	22.23(-1)	0.15(1)	0.08(1)	89.2±0.88	19.8±1.14	99.9
2	72.77(1)	0.15(1)	0.08(1)	90.2±1.16	16.6±0.21	99.9
3	47.50(0)	0.001(-1.682)	0.05(0)	76.6±1.07	3217±20.1	81.2
4	47.50(0)	0.10(0)	0.05(0)	89.1±1.35	0.17±0.06	100
5	47.50(0)	0.10(0)	0.05(0)	93.0±0.84	0.22±0.09	100
6	72.77(1)	0.04(-1)	0.08(1)	86.2±1.53	96.9±6.03	99.4
7	72.77(1)	0.04(-1)	0.02(-1)	85.3±1.26	47.3±0.27	99.7
8	90.00(1.682)	0.10(0)	0.05(0)	94.1±1.39	0.67±0.04	100
9	72.77(1)	0.15(1)	0.02(-1)	86.2±1.65	116±14.2	99.3
10	5.00(-1.682)	0.10(0)	0.05(0)	85.8±0.88	319±20.1	98.1
11	47.50(0)	0.10(0)	0.05(0)	92.0±0.84	0.22±0.05	100
12	47.50(0)	0.10(0)	0.001(-1.682)	84.1±1.26	829±17.7	95.2
13	47.50(0)	0.19(1.682)	0.05(0)	92.2±1.20	4.66±0.17	100
14	22.23(-1)	0.04(-1)	0.02(-1)	69.1±0.88	5411±49.5	68.3
15	47.50(0)	0.10(0)	0.05(0)	92.9±1.20	0.26±0.06	100
16	22.23(-1)	0.04(-1)	0.08(1)	82.2±1.53	1231±25.3	92.8
17	47.50(0)	0.10(0)	0.05(0)	91.2±1.00	0.32±0.02	100
18	47.50(0)	0.10(0)	0.05(0)	89.3±1.71	0.11±0.06	100
19	22.23(-1)	0.15(1)	0.02(-1)	89.0±1.84	29.1±1.81	99.8
20	47.50(0)	0.10(0)	0.10(1.682)	91.3±2.17	19.3±0.33	99.9