

Structural Modification of Wheat Gluten by Dry Heat-Enhanced Enzymatic Hydrolysis

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Summary

In this paper, a dry heat method is used to modify wheat gluten, and the effects of dry heat on the enzymatic hydrolysis and structure of wheat gluten are investigated. The results show that the degree of hydrolysis of wheat gluten after dry heating increased from 20 to 30 % within 1 h of hydrolysis. Meanwhile, the percentage of the fraction with $M_r < 1000$ Da in the obtained hydrolysate increased from 17.6 to 30.7 %, which was revealed by size-exclusion high-performance liquid chromatography (SE-HPLC). Further analysis of the structure of dry-heated gluten was done and it showed disappearance of extended structures with the concomitant increase in β -sheet for secondary structure, exhibited by Fourier transform infrared spectroscopy (FTIR), a decrease in the hydration and increases from 56.7 to 74.5 of surface hydrophobicity and from 4.74 to 16.18 $\mu\text{mol/g}$ of the sulphhydryl content. The higher β -sheet proportion and surface hydrophobicity as well as sulphhydryl content of dry-heated wheat gluten was assumed to be the reason for the enhancement of its enzymatic hydrolysis.

Key words: wheat gluten, dry heat, FTIR, SE-HPLC, enzymatic hydrolysis

Introduction

Wheat gluten, a by-product of wheat starch industry, is available in large amounts and at relatively low cost. The advantage of low cost has stimulated manufacturers to explore ways of converting wheat gluten into value-added products. The most attractive way is enzymatic hydrolysis of wheat gluten to produce value-added peptides with a wide range of bioactivities.

However, the enzymatic hydrolysis of wheat gluten is challenged by its limited solubility and poor dispersion. Several methods including physical and chemical pretreatments have been developed to modify the wheat gluten in order to facilitate proteolysis. From both environmental and industrial point of view, physical pretreatment is the most promising technique because it is safe and does not have side-effects. Physical pretreatments

most frequently used are heat treatment (1), high-pressure processing (2) and ultrasonic treatment (3).

Heat treatment is classified into aqueous heating and dry heating according to the heat medium used. Heating in aqueous solution is a traditional treatment used to enhance proteolysis by altering the structure and physicochemical properties of a protein, which results in protein denaturation, aggregation and gelation through disruption and reformation of hydrogen bonds and hydrophobic interactions (4,5). These thermal and structural changes have been evaluated by Fourier transform infrared spectroscopy (FTIR) (6–8). However, heating of wheat gluten in aqueous medium as a pretreatment of enzymatic hydrolysis did not give good results due to the formation of wheat gluten viscoelastic polymers. This could be avoided by using dry heating instead. To the best of our knowledge, dry heat has not been used as a pretreatment in proteolysis.

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In this paper, dry heat method was developed as a pretreatment of wheat gluten for enzymatic hydrolysis, and it was found that it enhances the hydrolysis. Further research on the structure of dry-heated wheat gluten needs to be done to see if there is any correlation between wheat gluten enzymatic hydrolysis and its structural alteration affected by dry heating pretreatment. This paper may give a new idea for efficient industrial preparation of low molecular mass peptides ($M_r < 1000$ Da) although this research was done on a laboratory scale.

Material and Methods

Materials and chemicals

Wheat gluten (protein 73 % wet base) was kindly donated by Lian Hua Ltd. Co. (Henan, PR China), and Alcalase® 2.4 L (enzyme activity of 2.4 AU/mL, optimal parameters: pH=8.0 and 55 °C) was purchased from Novozyme Ltd. Co. (Shanghai, PR China). DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) and ANS (1-anilino-8-naphthalene sulphonate) were purchased from Sigma-Aldrich (Beijing, PR China). All other chemicals used in the experiment were of analytical grade or the highest purity available from National Chemical Co. (Shanghai, PR China).

Preparation of wheat gluten by dry heating

Wheat gluten powder was pretreated by dry heat. A mass of 500 g of wheat gluten powder was evenly distributed on an enamel plate (length by width by height of 40×25×3 cm) and then put into an oven at 120 °C for 5 min, then taken out and stirred for 1 min in order to distribute the heat evenly. Heating was continued for 20 min.

Hydrolysis of dry-heated wheat gluten using Alcalase 2.4 L

Commercial Alcalase 2.4 L was used to hydrolyze wheat gluten. Briefly, 2.5 mL of Alcalase 2.4 L were diluted with distilled water to a total volume of 50 mL. A volume of 2 mL of diluted Alcalase 2.4 L solution was added to 300 mL of 6 % (by mass per volume) wheat gluten suspension at pH previously adjusted to 8.0 with 1 mol/L of NaOH solution. The final ratio of enzyme activity to protein was $9.1 \cdot 10^{-3}$ AU/g. The whole enzymatic reaction was maintained at 55 °C using water bath. After 1 and 2 h of hydrolysis, the reaction was stopped by heating the suspension in boiling water for 10 min. The suspension was centrifuged in ZOPR-52D refrigerated centrifuge (Hitachi Koki Co., Tokyo, Japan) at 6 °C and 8000×g for 20 min, and the supernatant was lyophilized (ACPA1-4, Christ Co., Osterode, Germany) and kept at 4 °C as hydrolysate. Alcalase 2.4 L hydrolysates of raw wheat gluten taken after 1 and 2 h were named RGH1 and RGH2 respectively, while those of dry-heated wheat gluten were correspondingly named DHGH1 and DHGH2, respectively.

Degree of hydrolysis (DH) was determined before centrifugation using trichloroacetic acid (TCA) method as stated by Drago and González (9). Protein content was determined by Kjeldahl method ($N \times 5.7$).

Size-exclusion high-performance liquid chromatography (SE-HPLC)

Hydrolysates were dissolved in distilled water to form a solution of 1 mg/mL. A volume of 10 µL of the resulting solution was fractionated by a Waters HPLC 600 system (Waters, Milford, MA, USA) fitted with a TSKgel 2000 SWXL column (0.78×30 cm; Tosoh Bioscience, Tokyo, Japan) and diode array detector. The elution rate was 0.5 mL/min and the elution solvent was acetonitrile/water/trifluoroacetic acid (45:55:0.1, by volume). The absorbance was recorded at 220 nm at 30 °C. The optimum separation range of the SE-HPLC column was 100–30 000 Da. All data were collected and analyzed by Breeze software (Waters).

Cytochrome c ($M_r=12\ 500$ Da), bovine pancreatic trypsin inhibitor ($M_r=6500$ Da), bacitracin ($M_r=1450$ Da), tripeptide ($M_r=451$ Da) and dipeptide ($M_r=189$ Da) were used as the comparable standards of molecular mass. The molecular mass of hydrolysates was calculated based on the elution time. The fitted linear equation was calculated and shown below:

$$\log y = 6.97 - 2.37x \quad (R^2 = 0.9974) \quad /1/$$

where y is the relative molecular mass per mol (Da/mol) and x is the elution time (min).

FTIR spectra

The wheat gluten samples were brought to equilibrium with 1 mL of water for 48 h and then mixed vigorously with 2 mL of water to form a paste. Infrared spectra (128 scans) of wheat gluten samples were recorded with a resolution of 2 cm^{-1} on Nicolet® infrared spectrophotometer (Model 'Nexus 470', Nicolet Co., Shanghai, China) using a thunderdome attenuated total reflectance (ATR) accessory. The interpretation of the changes in the overlapping amide I band components was made possible by Gaussian deconvolution of FTIR spectra using Peak-Fit v. 4.11 software (Galaxy Industries Corp., Canton, MI, USA).

To avoid the interference of the OH from water solvent with the amide I band in FTIR spectrum, deuterium oxide (D_2O) was used to disperse the wheat gluten samples instead of distilled water in the experiment to determine the secondary structure of wheat gluten.

Sulphydryl quantification

The quantification of sulphydryl groups was done using the method of Cui *et al.* (1). A volume of 3 mL of wheat gluten solution (0.3 mg/mL) was diluted with 3 mL of phosphate buffer solution (0.1 mol/L, pH=8.0) containing EDTA (1 mM) and SDS (1 %), and then 0.1 mL of DTNB was added. Free sulphydryl groups of wheat gluten reacted with DTNB and released thionitrobenzoate, which was quantified at 412 nm after 60 min of reaction.

To determine the disulphide group level, the wheat gluten protein solution (1 mL) was mixed with 4 mL of Tris-glycine buffer containing 10 M urea and 0.05 mL of mercaptoethanol. After incubation at 25 °C for 1 h, 10 mL of 12 % (by mass per volume) TCA were added and left to rest for 1 h. The sample was then subjected to centrifugation at 5000×g for 10 min. The residues were dissolved in 3 mL of phosphate buffer solution (0.1

mol/L, pH=8.0) containing EDTA (1 mmol/L), SDS (1 %) and 0.1 mL DTNB. The absorbance was measured at 412 nm.

Surface hydrophobicity

Values of surface hydrophobicity (H_0) were determined by the hydrophobicity fluorescence probe using ANS. Wheat gluten dispersions (1 mg/mL) were prepared in 0.01 M phosphate buffer (pH=7.0), stirred for 1 h at 25 °C, and centrifuged for 25 min at 3000×g. Protein concentration in the supernatants was measured by the micro-Kjeldahl procedure ($N \times 5.7$).

Each supernatant was serially diluted with 0.01 mol/L of phosphate buffer to obtain protein concentrations ranging from 0.02 to 0.3 mg/mL. A 3-mL aliquot of each diluted sample was then added to 20 μ L of 8.0 mM ANS solution in 0.1 M phosphate buffer (pH=7.0). Fluorescence intensity (FI) was measured at 405 nm (excitation) and 480 nm (emission). The protein concentrations without ANS were used as blank. The initial slope of the FI *vs.* protein concentration plot (calculated by linear regression analysis) was used as the H_0 index.

Statistical analysis

Each determination was carried out on three separate samples and the results were shown as mean value \pm standard deviation. The significant difference of the results was analyzed by SPSS v. 13.0 software, and PeakFit v. 4.11 software was used to deconvolute the amide I band in FTIR spectra of wheat gluten samples.

Results and Discussion

Enzymatic hydrolysis of dry-heated gluten

The typical curves of the degree of hydrolysis of wheat gluten samples by Alcalase 2.4 L are shown in Fig. 1 and the results are in agreement with Kong *et al.* (10). The degree of hydrolysis of wheat gluten was dramatically improved by dry heat pretreatment. The DH of dry-heated gluten reached 30 % after 1 h of hydrolysis and showed an increase of 10 % compared to the raw wheat gluten. Similar result was reported by Achouri *et al.* (11), who studied the proteolysis of soy protein isolate prior to aqueous heat treatment.

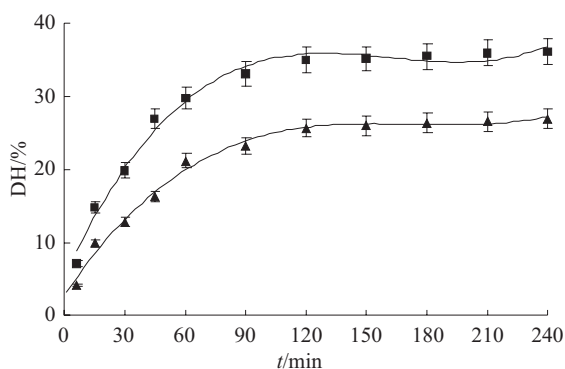


Fig. 1. Curves of enzymatic hydrolysis progress (—■— DH of dry-heated wheat gluten, —▲— DH of raw wheat gluten)

The molecular mass distributions of Alcalase 2.4 L hydrolysates of dry-heated gluten were evaluated by SE-HPLC and compared with those of raw gluten. The peaks on the chromatogram were divided into five groups according to the relative molecular mass, and the percentages of individual groups were calculated by the percentages of the corresponding peak area ratios to the total peak area as listed in Table 1. The M_r of the hydrolysates of raw gluten (RGH) ranged from 285 to 14 209 Da and those of dry-heated gluten (DHGH) varied from 279 to 13 444 Da. The percentage of the fraction with M_r lower than 1000 Da in DHGH1 and RGH1 was 30.7 and 17.6 % respectively. Moreover, the percentage of the fraction with M_r lower than 5000 Da reached 62.7 % in DHGH1 and 43.5 % in RGH1. The percentages of the fraction with M_r lower than 5000 Da in DHGH2 and RGH2 were higher, up to 66.4 and 51.6 %, respectively. There was a significant difference among the various protein hydrolysate fractions.

Table 1. Molecular mass distribution of enzymatic hydrolysates of raw wheat gluten (RGH) and dry-heated wheat gluten (DHGH)

Group (M_r /Da)	Molecular mass distribution/% (M_r /Da)			
	RGH1	RGH2	DHGH1	DHGH2
I (>10000)	32.6 (14209)	25.4 (13718)	21.3 (13444)	18.6 (13247)
II (5001–10000)	24.5 (7764)	23.7 (7700)	16.4 (7869)	15.6 (8032)
III (1001–5000)	25.9 (2476)	29.7 (2435)	32.2 (2330)	31.2 (2284)
IV (501–1000)	7.3 (737)	9.3 (735)	11.5 (740)	13.0 (738)
V (<500)	10.3 (285)	12.6 (297)	19.0 (279)	22.2 (303)

As demonstrated by the data in Fig. 1 and Table 1, Alcalase 2.4 L hydrolysis of wheat gluten can be accelerated by dry heat pretreatment, resulting in larger amount of lower molecular fraction (M_r lower than 5000 Da). This result compelled us to further study the structure of dry-heated wheat gluten. We assume that the structure of wheat gluten could be altered by dry heat in a way to facilitate enzymatic hydrolysis.

Secondary structure analysis of wheat gluten exposed to dry heat

Shifts in frequencies of amide I, II or III band in FTIR spectrum corresponded to the changes in the secondary structure of proteins (12). Fig. 2a shows the original infrared spectra in the amide I and amide II bands of wheat gluten with or without dry heat treatment. Intensities of amide I and amide II bands revealed a decrease in the spectra of dry-heated gluten (Fig. 2a). This decrease showed an important change in the secondary structure of wheat gluten that occurred as a result of dry heating. The decrease of signal intensity was presumably attributed to the thermal dissociation of molecular polymer formed by two typical components in wheat gluten (gliadin and glutenin). This decrease is consistent with the finding by Hargreaves *et al.* (13), who studied the molecular flexibility of wheat gluten proteins subjected to aqueous heat.

The amide I band of proteins consists of many overlapping component bands that represent different struc-

tural elements such as α -helices, β -sheets, turns and unordered or irregular structures (14). In order to quantitatively estimate the content of secondary structure segments (α -helices, β -sheets or β -turns), the amide I band of the spectra in Fig. 2a was deconvoluted as done by Goormaghtigh *et al.* (15) and Elangovan *et al.* (16). The deconvoluted amide I band of raw gluten is shown in Fig. 2b, and 6 typical components centered at wavenumbers of deconvoluted amide I band were in agreement with those summarized by Mejri *et al.* (17). The information about the assignment of deconvoluted peaks and the content of each secondary structure segments are shown in Table 2. Comparison of the secondary struc-

tures of raw gluten and dry-heated gluten revealed a great increase in β -sheet amount from 41.5 to 51.2 % and a slight enhancement in α -helices from 24.5 to 26 % accompanied by the disappearance of extended structures. Extended structures are those uncommon secondary structures like the polyproline helix and α -sheet. The α -helices/ β -sheet ratio became smaller in the dry-heated gluten and expressed the variation of secondary structures observed in it. Structural changes of wheat gluten after dry heat treatment consisted of the disappearance of the proportion of extended structures and a concomitant increase in the proportions of β -sheet and α -helices.

Tertiary structure analysis of wheat gluten under dry heat treatment

Except for the secondary structure, proteins are known to form three-dimensional structures mainly stabilized by the non-bonding interactions and the disulphide bond. Surface hydrophobicity, as a quantitative index of non-bonding interactions, is responsible for the longer wavelength variations that correlate with the protein tertiary structural features (18). In this paper, the effect of dry heat treatment on the tertiary structures of wheat gluten was demonstrated by detecting the surface hydrophobicity, hydration capacity and disulphide-sulphydryl content.

Surface hydrophobicity (H_0) was determined with regression analysis using SPSS v. 13.0 software. H_0 of wheat gluten increased from 56.7 to 74.5 after dry heat treatment (Table 3) and this result was contradictory to that of aqueous solution treatment (19). When protein was heated in aqueous solution, H_0 decreased mainly due to the interaction of the thermally exposed hydrophobic groups in the protein and this interaction was driven by polar media (water) by which heat was delivered, herein the number of ANS binding sites was reduced (20,21). In dry heat processing, the hydrophobic group interaction could be avoided by the lack of water, thus increasing the H_0 of dry-heated gluten. Furthermore, the exposure of more hydrophobic amino acids on the surface of wheat gluten polymer, such as glutamine and proline, which are rich in wheat gluten, might also contribute to the increase in H_0 .

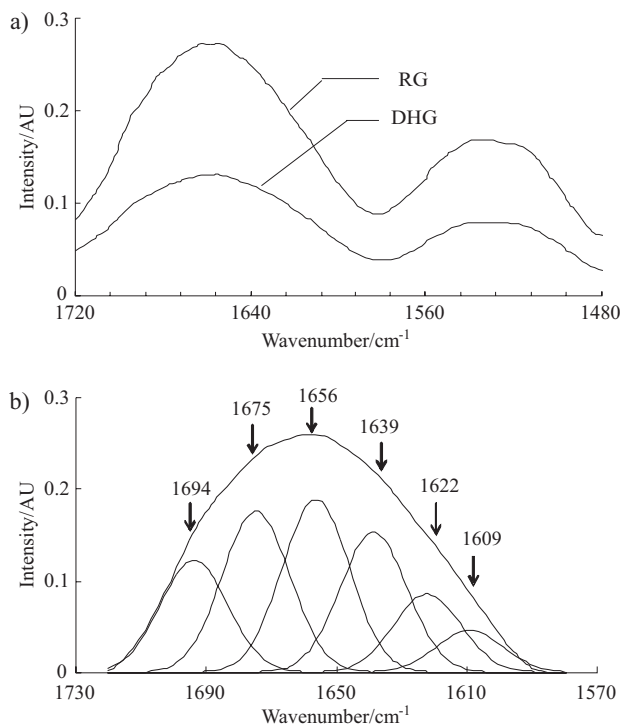


Fig. 2. Secondary structure of wheat gluten determined using FTIR spectra: a) the amide I and amide II regions of raw wheat gluten (RG) and dry-heated wheat gluten (DHG), b) deconvoluted infrared amide I band of raw wheat gluten

Table 2. Determined frequencies of amide I band components and their distributions in raw wheat gluten (RG) and dry-heated wheat gluten (DHG)

Assignment	RG		DHG	
	Frequency	Structure distribution	Frequency shift	Structure distribution
	cm ⁻¹	%	cm ⁻¹	%
β -sheet	1609	41.5	3	51.2
β -sheet	1639		-5	
β -sheet	1694		0	
α -helices	1656	24.5	-2	26.0
β -turn	1675	22.9	-1	22.7
extended structures	1622	11.1		
α H/ β S		0.59		0.50

Table 3. Sulphydryl groups and surface hydrophobicity of raw wheat gluten (RG) and dry-heated wheat gluten (DHG)

Parameter	RG	DHG
(n(sulphydryl)/m)/(μ mol/g)	4.74	16.18
(n(disulphide)/m)/(μ mol/g)	22.48	16.76
H_0	56.68	74.53

Hydroxyl group (OH) stretching region (2800–3800 cm⁻¹) in FTIR spectrum of protein is representative of hydration. The intensity of this band was correlated with the amount of water absorbed by the protein polymer (22). The intensity of OH in wheat gluten decreased after dry heat treatment (Fig. 3) and these results confirmed that dry-heated gluten was less hydrated, which corresponded to its higher H_0 .

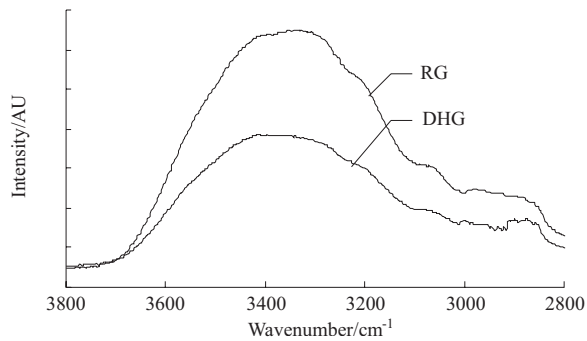


Fig. 3. Infrared spectrum in the OH region of raw wheat gluten (RG) and dry-heated wheat gluten (DHG)

Wang *et al.* (23) reported that the sulphhydryl content of wheat gluten exposed to wet heat decreased as the temperature increased, which indicated cross-linking of gluten proteins through disulphide bonding. Compared with findings of Wang *et al.* (23), the decrease in sulphhydryl content of wheat gluten was not found during dry heat treatment. The differences in sulphhydryl content of wheat gluten exposed to wet heat and our results of dry heat treatment are attributed to the water, which plays an important role in the formation of typical wheat gluten viscoelasticity. Disulphide content of wheat gluten was dramatically decreased by dry heat (Table 3), and this finding was inconsistent with the findings of Kieffer *et al.* (24), who claimed that an increase in disulphide content was due to the cleavage of intrachain disulphide bonds and their rearrangement into interchain bonds in wheat gluten treated with thermal hydrostatic pressure. However, it is suggested by the result of sulphhydryl/disulphide content of dry-heated gluten that the rearrangement did not occur in dry heat process. The reason of alteration mechanism in sulphhydryl/disulphide content of dry-heated gluten will be a subject of further investigation.

Correlation of enzymatic hydrolysis efficiency with structural alteration by dry heat treatment

The large increases in the amount of low molecular fraction (lower than 1000 Da; Table 1) as well as in the degree of hydrolysis (Fig. 1) of wheat gluten were correlated with the structural changes affected by dry heat. Under dry heat treatment, the secondary (Fig. 2 and Table 2) as well as tertiary structure (Fig. 3 and Table 3) of wheat gluten were disturbed. A disappearance of extended structure and a concomitant increase in β -sheet were found in the secondary structure of wheat gluten under dry heat, which indicated the formation of non-compact structure, whereby the enzyme was facilitated to be more easily accessible to the active sites in the protein. Together with the secondary structure alterations, the larger amount of hydrophobic clusters (indicated by higher H_0) was exposed on the surface of wheat gluten protein. Higher H_0 may have special impact on the action of the enzymes such as Alcalase 2.4 L and α -chymotrypsin, which preferably cleave peptide bonds involving hydrophobic amino acids (25). Moreover, the exposure of a large amount of hydrophobic clusters and the breakage of disulphide bonds (Table 3) guaranteed that the Alcalase 2.4 L could reach certain sites that had previ-

ously been inaccessible to enzyme action. Furthermore, the lower hydration of dry-heated gluten shown in Fig. 3 demonstrated that the weakness of hydrogen bond in the intra- or inter-chain of wheat gluten, herein the Alcalase 2.4 L, may have easy access to the peptide bond in the wheat gluten backbone (peptide chains). In favour of the structure alteration by dry heat pretreatment, the hydrolysis of wheat gluten by Alcalase 2.4 L was greatly improved. As a consequence, the higher degree of enzymatic hydrolysis and the larger amount of lower M_r peptide components were obtained in the process of hydrolyzing dry-heated wheat gluten (Fig. 1 and Table 1). Herein, dry heat pretreatment was assumed to be a potential way to improve the enzymatic hydrolysis of wheat gluten.

Conclusion

FTIR investigation of dry-heated wheat gluten showed the alterations of its secondary structure by an increase in β -sheet content and concomitant disappearance of extended structure. Along with the secondary structure alteration affected by dry heat, tertiary structures of wheat gluten were also disrupted, which was demonstrated by the increases in H_0 , sulphhydryl content as well as in the decrease of hydration capacity. This altered structure makes Alcalase 2.4 L more accessible to the active sites in wheat gluten protein. As a result, the degree of hydrolysis of wheat gluten was improved by dry heat pretreatment and a high yield of peptides with M_r lower than 5000 Da was shown by SE-HPLC. The results in this paper suggest that dry heat treatment can shorten the time and save energy consumption in the industrial process of enzymatic hydrolysis of wheat protein.

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