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Effect of the Structural Features of Hydrochloric Acid-Deamidated Wheat Gluten on Its Susceptibility to Enzymatic Hydrolysis

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ABSTRACT: The effect of the structural features of hydrochloric acid-deamidated wheat gluten with different degrees of deamidation (DDs) on the susceptibility to enzymatic hydrolysis by pancreatin was investigated. The wheat gluten deamidated by hydrochloric acid with a DD of 55% revealed the highest susceptibility to enzymatic hydrolysis as evaluated by the hydrolysis degree and nitrogen solubility index of the hydrolysates. An increase of peptides with MW below 3000 Da was observed as the DD increased. Raman spectra in the 1740−1800 cm^{-1} and 521−530 cm^{-1} range suggested that wheat gluten had taken off the deamidation with different DDs and that the disulfide bond had disrupted the sulfhydryl groups with different intensities, respectively. Results from the deconvolution of the amide I region of FTIR spectra in the 1600−1700 cm^{-1} range showed that the content of the α-helix decreased and that the content of the β-turn and β-sheet increased with increasing DDs, which improved the molecular structure and flexibility of wheat gluten. A scanning electron microscope (SEM) revealed that the image of HDG-55% presented the smoothest surface and the least uniform pore, enabling the sample to be more susceptible to enzymatic hydrolysis. The above information will enable us to better understand the effect of structure on the susceptibility of deamidated wheat gluten.

KEYWORDS: *wheat gluten, hydrochloric acid deamidation, susceptibility, Raman spectra, Fourier transform infrared spectroscopy (FTIR), scanning electron microscope (SEM)*

INTRODUCTION

Over the recent decades, with a continuous increase in requirements for value-enhanced food products, there has been considerable research concentrating on various relatively low-cost sources of plant proteins.[1−3] Wheat gluten is a byproduct of wheat starch, which is a widespread and relatively inexpensive source. Nevertheless, as a result of inferior water dispersion properties, the application of wheat gluten to and its efficiency during enzymatic hydrolysis have been strongly restricted and limited.

A lot of trials including chemical deamidation and enzymatic hydrolysis have been done to improve the properties of wheat gluten and have turned out to be the most effective way to modify wheat gluten.[3,5,6] Chemical deamidation including acid-deamidation, alkali-deamidation, and salt-deamidation could modify the proteins from several aspects such as increasing the electrostatic repulsion, stretching proteins, changing charge density, and breaking hydrogen bonds by means of transforming amides of Gln and Asn into carboxyl groups. These treatments could unfold protein conformation and strengthen protein−water interactions.[7] In previous studies, hydrochloric acid has been widely used for protein deamidation[8−11] and proved to be useful in enhancing the efficiency of hydrolysis.[9,12,13] Our previous study also stated that hydrochloric acid-deamidated wheat gluten showed better susceptibility to pancreatin hydrolysis than the native wheat gluten.[4,14] Enzymatic hydrolysis is also widely and frequently used to catalyze deamidation with different reaction mechanisms in comparison to acid-deamidation.

Most investigations of enzymatic hydrolysis of proteins have featured enzymology with such main factors as the composition of the substrate, the extent of protein denaturation, the properties of enzymes, and the kinetics and mechanism of enzymatic conversion.[15,16,19] Actually, proteolysis can also be influenced by the substrate structure; for example, the available binding surface of substrate to enzyme will affect the rate of hydrolysis.[17,18] However, to date, limited concern is focused on the impact of the structrual properties on the susceptilbility of the HCl-deamidated wheat gluten with different degrees of deamidation (DDs) to enzymatic hydrolysis. The objective of the present work was thus to elucidate the changes in the susceptibility of HCl-deamidated wheat gluten with increasing DDs to enzymatic hydrolysis. The effects of substrate structural characteristics on the in vitro digestion of the whole resultant deamidated wheat gluten with different DDs were investigated in detail.

MATERIALS AND METHODS

Materials. Commercial vital wheat gluten with 74.83% (w/w, dry basis) crude protein, 6.68% moisture, 6.18% fat, and 9.51% carbohydrate was obtained from Lianhua Co. Ltd. (Zhoukou, China). Protamex and flavourzyme were obtained from Novozymes (Beijing, China). Alcalase and pancreatin were kindly donated by Haitian Co., Ltd. (Foshan, China). The chemical l-anilino-8-naphthalene sulfonate (ANS^-) was obtained from Sigma Chemical Co. Ltd. (St. Louis, MO,USA). All other chemicals and solvents were of analytical grade or HPLC grade and were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) or Sigma Chemical Co. Ltd. (St. Louis, MO, USA), respectively.

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Preparation for Hydrochloric Acid Deamidation and Enzymatic Hydrolysis. The preparation of acid-deamidated wheat gluten was with HCl (0.30 mol/L) with different deamidation times as revealed in Figure 1, according to a method reported previously.[2]

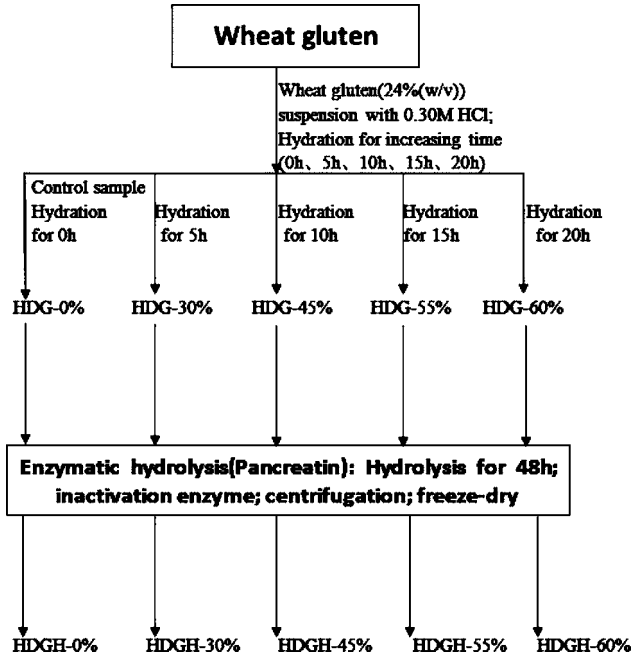

Figure 1. Process diagram of the enzymatic hydrolysis of the deamindated wheat gluten by HCl.

Wheat gluten (24% (w/v)) was first mixed with hydrochloric acid (0.30 mol/L) to form suspensions. The suspensions were hydrated for 0−24 h in a shaking water bath at 65 °C and were referred to as HCl-deamidated wheat gluten at 0−60% DDs (abbreviated, respectively, as HDG-30%, HDG-45%, HDG-55%, and HDG-60%). After that, each sample was divided into two aliquots. The whole deamidated wheat gluten of those four aliquots (HDG-30%, HDG-45%,HDG-55%, and HDG-60%) were collected and dialyzed in deionized water at 4 °C for 24 h to remove ammonium and freeze-dried for the determination of particle size and surface hydrophobicity and evaluation of conformational changes. Wheat gluten without acid treatment under the same conditions was used as the control sample (abbreviated as HDG-0%).

Afterward, as shown in Figure 1, the control sample and the four deamidated wheat gluten suspensions were hydrolyzed under conditions based on optimum hydrolysis conditions for pancreatin. Five aliquots (12% (w/v)) were adjusted to pH 9.0 with 1 M sodium hydroxide and incubated in a water bath until the suspensions reached 50 °C; then, pancreatin was added to the suspensions at an enzyme to substrate ratio of 1:100 (w/w) to initiate the enzymatic reaction and continued up to 48 h. At the reaction time of 0, 12, 24, 36, and 48 h, the mixture was heated at 95 °C for 20 min to inactivate the enzyme and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatants of the hydrolysates from HDG-0%, HDG-30%, HDG-45%, HDG-55%, and HDG-60% were denoted as HDGH-0%, HDGH-30%, HDGH-45%, HDGH-55%, and HDGH-60%, respectively. HDGH-0%, HDGH-30%, HDGH-45%, HDGH-55%, and HDGH-60% at the given hydrolysis times were collected and used to conduct the determination of the degree of hydrolysis and protein recovery and gel permeation chromatography analysis.

Chemical Analysis. The ratio of the amount of ammonia liberated from the deamidated samples to the total ammonia of wheat gluten was used to calculated the degree of deamidation of modified samples. The amount of liberated ammonia from the ammonium generated during deamidation was assayed according to the previous method[4] before dialysis and lyophilization. The nitrogen solubility index (NSI) of samples was regarded as the ratio of the soluble nitrogen content in the supernatant to the total nitrogen content (TN) of suspension in the original reaction; the protein contents of the supernatant and initial suspension were determined by the Kjeldahl method with a nitrogen−protein conversion coefficient of 5.7. The content of a-amino nitrogen (AN) in supernatants (AN, mg/mL) was analyzed by formal titration. The degree of hydrolysis (DH) was defined as a percentage of AN/TN.

Gel Permeation Chromatography (GPC). Molecular weight distribution profiles of the supernatants from the deamidated wheat gluten hydrolysates were determinated by gel permeation chromatography (Waters 1525, USA). Protein purification chromatography (Amersham plc, Buckinghamshire, United Kingdom) with a Superdex Peptide 10/300 GL column was applied to the analysis. The mobile phase, which was applied at isocratic mode, was 0.02 M sodium phosphate buffer containing 0.25 M NaCl (pH 7.2). The flow rate of the mobile phase was 0.5 mL/min. Deionized water was used to dilute the solutions of the unfreeze-dried hydrolysates to gain the dilution samples with 2 mg/mL protein concentration. The absorbance was monitored with UV detection at 214 nm followed by 450 mm membrane filtration. The final dilution was filtered through a micropore film (0.22 μm of pore size). A mixture of six protein standards containing Globin III (2512 Da), Globin II (6214 Da), Globin I (8519 Da), Globins I + III (10700 Da), and Globins I + II (14404 Da) was taken to calibrate the column and make the standard curve. The plot of log *MW* against elution time was constructed, and the molecular weight distribution for each hydrolysate was then calculated according to the plot. UNICORN 5.0 software (Amersham Biosciences Co., Piscataway, NJ,USA) was used to analyze the chromatographic data. The final equation was gained by linear regression of the fitting curve, which was log $MW = -0.0578Rt + 4.6289$, $R^2 = 0.9913$.

Determination of the Particle Size of Deamidated Wheat Gluten. The average particle size of the whole deamidated wheat gluten was determined by Malvern MasterSizer 2000 (Malvern Instruments Co. Ltd., Worcestershire, UK). It is measured by laser light scattering. The refractive index and adsorption of the dispersed phase were set as 1.414 and 0.001, respectively. The surface weighted average diameter ($d_{3,2}$, μm) and volume weighted average diameter ($d_{4,3}$, μm) were calculated as follows:

$$d_{3,2} = \sum n_i d_i^3 / \sum n_i d_i^2 d_{4,3} = \sum n_i d_i^4 / \sum n_i d_i^3$$

where n_i is the number of particles with the same diameter d_i; d_i is the particle size.

Determination of Surface Hydrophobicity. Values of surface hydrophobicity (H_0) of the whole deamidated wheat gluten was determined by a fluorescence probe using the 1-anilino-8 naphthalene sulfonate anion (ANS^-) according to a previously published method[20] with some modifications. The freezing-dried samples were diluted with phosphate buffer (pH 7). The dilutions were centrifuged for 20 min at 8000*g*. The protein content in the supernatants was determinated by the micro-Kjeldahl method. A certain amount of 8.0 mM ANS^- solution was prepared for later interaction with the protein solutions. Four milliliter supernatants of each deamidated wheat gluten solution at protein contents of 0.05, 0.1, 0.2, 0.5, and 1 g/L in 10 mmol/L phosphate buffer (pH 7) were separately added to a 20 μL aliquot of ANS^- stock solution. Each mixed solution was shaken in a vortex mixer for 5 s before its quick analysis. The fluorescence intensity (FI) was measured at wavelengths of 365 nm (excitation) and 484 nm (emission) using an RF-5301 PC spectrofluorometer (Shimadzu Corp., Kyoto, Japan) at 26 ± 0.5 °C with a constant excitation and emission slit of 5 nm. The FI of each sample with probe was then exported by subtracting the FI of protein dilution without adding the fluorescence dye ANS^- solution. The initial slope of the FI versus protein concentration plot, which was calculated by linear regression analysis, was used as the H_0 index.

Raman Spectroscopy. Freeze-dried samples of the supernatants from the acid-deamidated wheat glutens after their centrifugation at 10,000*g* for 15 min at 4 °C were used for Raman spectroscopy. Raman spectra were collected point-by-point in a backscattered configuration

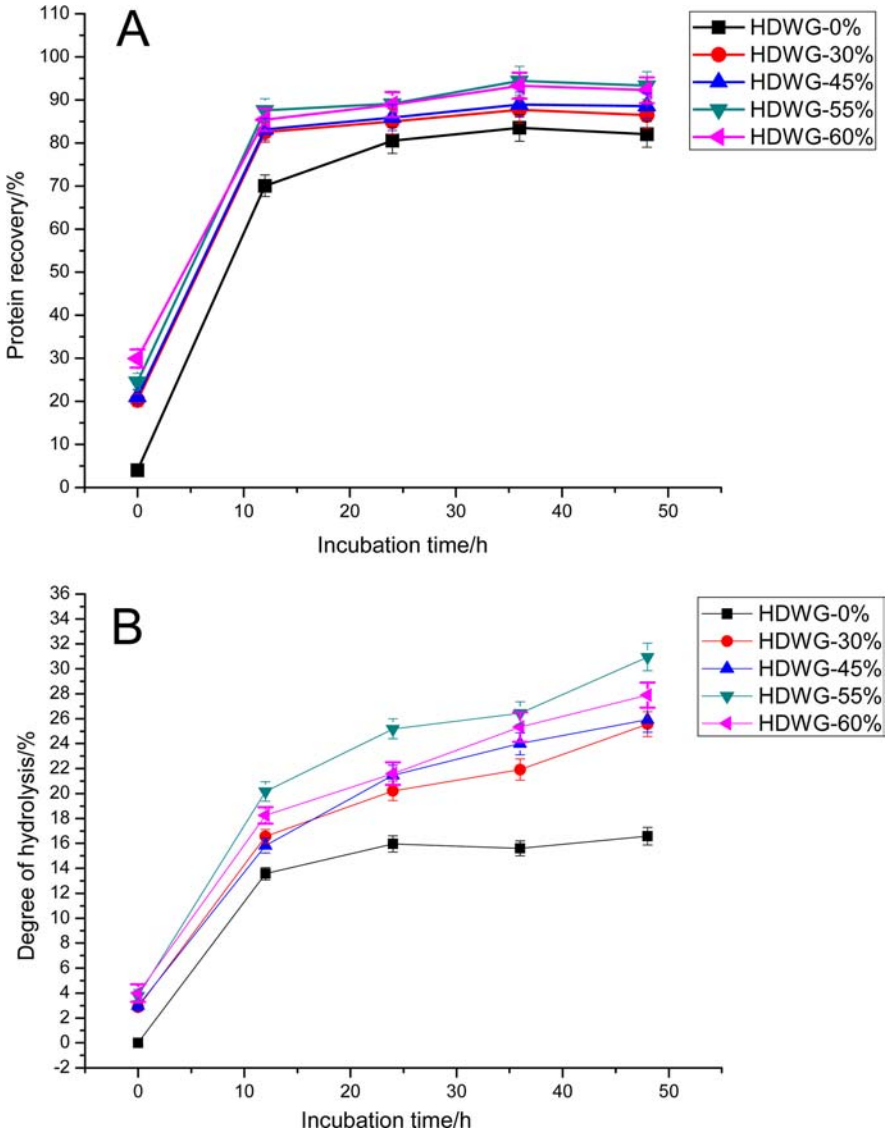

Figure 2. Hydrolysis degree (A) and soluble nitrogen index (B) profiles of the hydrolysates from the control and hydrochloric acid-deamidated wheat gluten.

by a LABRAM-Aramis spectrometer (Horiba Jobin-Yvon, France). The instrument was operated at room temperature with excitation at 632.8 nm (He/Ne laser, less than 10mW on sample). The samples of deamidated wheat gluten were laid onto the microscope slides for Raman measurement.The laser was focused on the lyophilized samples. The conditions of each spectrum conducted were 80 scans, 2 s exposure time, and 2 cm^{-1} resolution. Each numerical calculation of the Raman intensity ratio was based on the averaged spectral data from the scans of samples in the Raman spectrophotometer with at least triplicate measurements. The Raman spectra in this article were smoothed, baseline calibrated, and normalized to minimize intensity variations due to surface roughness.

Fourier Transform Infrared (FTIR) Spectroscopy. The FTIR technique is highly selective and produces a spectral fingerprint of each compound, which provides information on molecular structure based on vibrations at the molecular level. Therefore, it can be well-used for the identification of substances. FTIR spectroscopy is based on an absorption process. FTIR spectra of lyophilized samples were carried out on a Nicolet 8210E FTIR spectrometer (Nicolet, USA) fitted with a deuterated triglycine sulfate detector. The spectrometer was continuously purged with dry air from a Balston dryer (Balston, USA). Two milligrams of each sample was accurately weighed to be added to 198 mg of KB. Then, the samples were prepared and kept at room temperature for subsequent homogenization using an agate mortar and pestle. Finally, the samples were formed into pellets (1−2 mm thick) using a 15-ton hydraulic press to conduct FTIR. FTIR spectra were collected under the following conditions: wavenumber range from 400 to 4000 cm^{-1}, 128 scans, and 2 cm^{-1} resolution. Changes in the components of the overlapping amide I band (1580−1700 cm^{-1}) were interpreted by deconvolution using PeakFit software, version 4.12 (SPSS Inc., Chicago, IL, USA), according to the previous method.[2]

Scanning Electron Microscope (SEM). The SEM is a microscope that uses electrons instead of light to form an image that is largely magnified. At the top of the microscope, an electron gun is equipped to produce a beam of electrons. Special treatments must be done to the sample for the reason that the SEM utilizes vacuum conditions and uses electrons to form an image. As the water would vaporize in the vacuum, all water must be removed from the samples. Therefore, we used freeze-dried samples of the whole deamidated wheat gluten for determination. The samples need to be made conductive by covering the sample with a thin layer of conductive material. This is conducted by a device called a sputter coater. The sputter coater uses an electric field and argon gas. The sample is placed in a small chamber that is under vacuum conditions. Argon gas and an electric field cause an electron to be removed from the argon, making

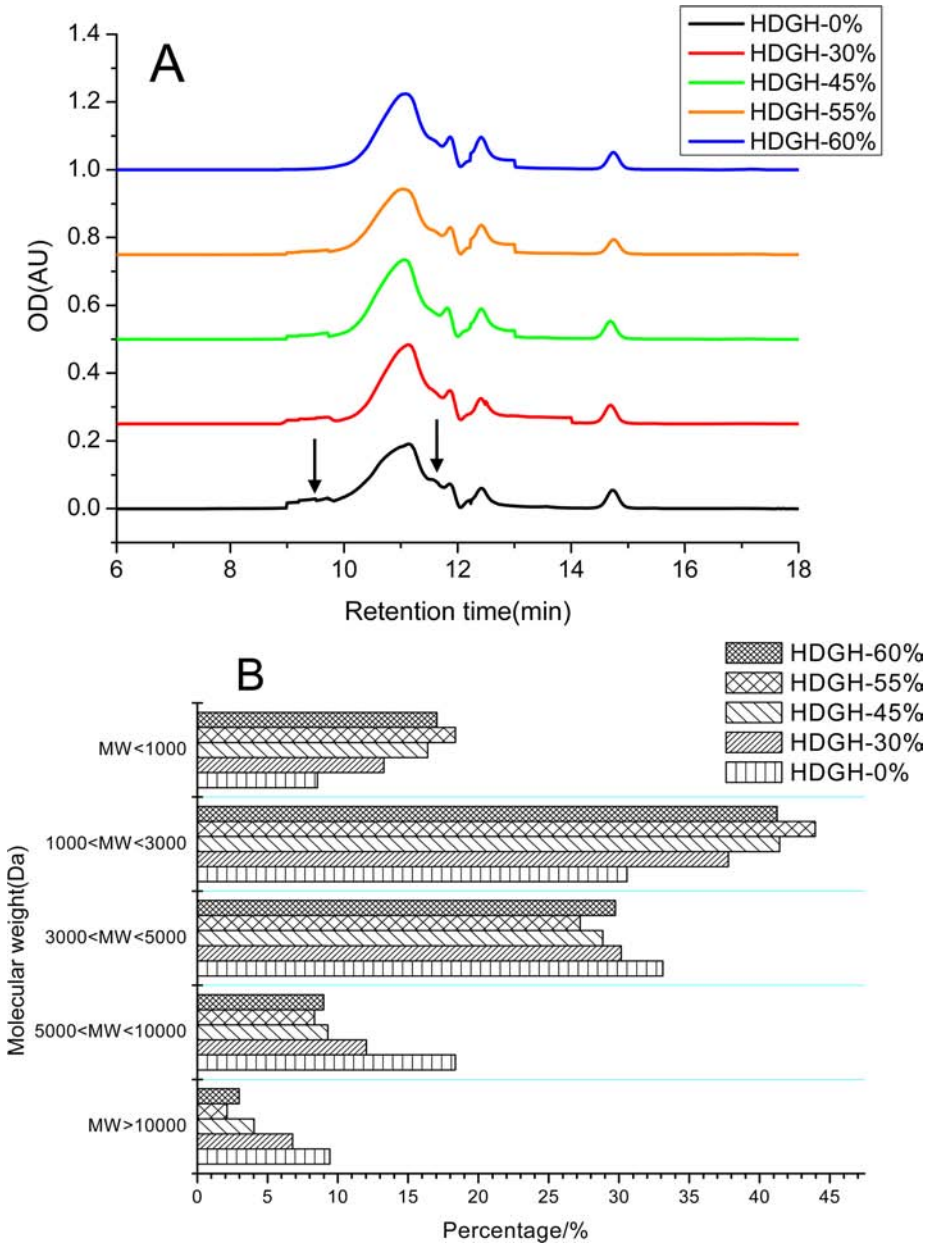

Figure 3. GPC profiles (A) and molecular weight distribution (B) of the hydrolysates from the control and the hydrochloric acid deamidated wheat gluten.

the atoms positively charged. The argon ions then become attracted to a negatively charged gold foil. The argon ions knock off gold atoms from the surface of the gold foil. These gold atoms fall and settle onto the surface of the sample producing a thin gold coating. Finally, we used SEM to observe the samples and take images of them.

Statistical Analysis. All of the assays in this study were conducted in triplicate, and the data were reported as the mean ± standard deviation (SD). Analysis of variance and significant difference tests were performed using SPSS 10.0 statistical software. It was considered that $p < 0.05$ was statistically significant.

RESULTS AND DISCUSSION

Susceptibility of Wheat Gluten to Enzymatic Hydrolysis. First of all, to test and verify whether the susceptibility of the acid-deamidated wheat gluten to enzymatic hydrolysis could be modified by enzymes, we carried out a reaction simultaneously with pancreatin, alcalase, protamex, and flavorzyme. The results showed that the susceptibilities to enzymatic hydrolysis by alcalase or flavorzyme were similar to that by pancreatin, while that by protamex was different (data not shown). Furthermore, the acid-modified wheat gluten hydrolyzed by pancreatin exhibited the greatest improvement in susceptibility to proteolysis. Therefore, pancreatin was selected as the working enzyme in the following research.

Protein recoveries (PR) and degrees of hydrolysis (DH) of HDGHs treated by pancreatin with different reaction times are shown in Figure 2A and B, respectively. As shown in Figure 2, the DH and PR values of all of the hydrolysates increased rapidly during the first 24 h and then reached a plateau, which was in agreement with the report of Liao et al.[4] At each given time, the HDG-55% hydrolysate (HDGH-55%) showed the highest DH and PR values, followed by HDGH-60%, HDGH-45%, HDGH-30%, and the control (HGDH-0%). For example, the DH and PR values of the HDGH-55% hydrolysate reached 30.95% and 93.31% after hydrolysis for 48 h, while those of

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HGDH-0% were only 16.59% and 81.99%, respectively. It suggested that HCl deamidation pretreatment improved the susceptibility of wheat gluten to pancreatin proteolysis, and the susceptibility increased along with increasing of DDs until 55%. However, further increase of DD values decreased the susceptibility. It was probably due to the fact that the exposure of hydrophobic residues increased with increasing of DDs until 60% so that the corresponding conformational changes led to a deamidated substrate more easily hydrolyzed with more available peptidyl residues.[21] What is more, because of the ionic effect of ammonium produced in deamidation or partial hydrolysis of peptides bonds, the HCl deamidation with 55% DD made the substrate more dispersible by increasing negative charge density and decreasing hydrogen bonding, which improved protein−water interactions and prevented the aggregation and gelation of wheat gluten in proteolysis.[2,7] On the contrary, lower susceptibility of the acid-modified wheat gluten with DD ≤ 55% was probably due to the fact that wheat gluten after HCl deamidation pretreatment might bind less to the active sites of pancreatin as a result of the less favorable changes of substrates in molecule size, charge density, and conformation.[22] The somewhat lower susceptibility of HDGH-60% to enzymatic hydrolysis might be due to the formation of large molecule aggregates after the long time treatment. As a result, the active sites lacking deamidation or with excessive deamidation were buried inside the molecules and were resistant to enzymatic hydrolysis by pancreatin.[22]

Molecule Weight Distribution of the Hydrolysates. The molecular weight distribution of the hydrolysates obtained from the deamidated wheat gluten incubated for 48 h was analyzed by gel permeation chromatography. The chromatogram profiles of the hydrolysates are shown in Figure 3A. The corresponding area percentages of peptide fractions with different molecular weight (MW) to the total peptide content in the hydrolysates from the control and the HCl-deamidated wheat gluten are demonstrated in Figure 3B. The area percentages are calculated according to integration of peak areas in GPC profiles and are equal to the ratio of peak area to the whole area. Compared with the chromatogram profile of the control, the first peak of peptides eluted at 9.7 min (with larger MW) gradually disappeared after the acid-deamidation pretreatment (see the arrow on the peak at 9.7 min in Figure 3A), and the peak areas of peptides eluted at 11−13 min (with the smaller MW) significantly increased as the DD increased (see the arrow on the peak at 11−13 min min in Figure 3A). As shown in Figure 3B, the control sample (HDGH-0%) was characterized by the highest percentage of peptides with MW more than 10000 Da, which decreased markedly as the DD of the substrate increased to 55% and then slightly grew when the DD further increased to 60%. The HCl deamidation process improved the susceptibility of wheat gluten to enzymatic hydrolysis, thereby increasing the area percentages of peptides with MW ≤ 3000 Da. After the HCl pretreatment, the area percentage of ≤3000 Da fractions of HDGHs increased with increasing DD, which reached the maximum value when DD increased to 55%. It indicated that HDGH-55% possessed more smaller peptides than the other HDGHs, confirming that HCl pretreatment for 15 h (55% DD) induced a more loose structure of wheat gluten and a higher efficiency of enzymatic hydrolysis, which were consist with the results of Figure 2A. The MW ≥5000 Da fraction of the control sample (HDGH-0%) was observed to become lower as DD increased to 55% and then increased slightly with DD increased up to 60%, indicating that more peptide bonds appeared in wheat gluten with the increase of DD less than 55% after the pretreatment with HCl and then became less when DD further increased to 60%, which was in accordance with results shown in Figure 2A. This phenomenon might be explained by the fact that excessive deamidation together with extended time might induce aggregation of protein molecules.[16,23,24]

Evaluation of Surface Hydrophobicity and Particle Size. Surface hydrophobicity, which was proposed as the property to estimate "average surface hydrophobicity",[23,24,25] was determined by the hydrophobic contribution of amino acids in wheat gluten. Changes in the surface hydrophobicity (H_0) and particle size including the mean suface diameter ($D_{3,2}$) and mean volume diameter ($D_{4,3}$) of the whole deamidated wheat gluten with different DDs are shown in Table 1. It was found that surface hydrophobicity of HCl-deamidated wheat gluten significantly improved compared to that of the control (HDG-0%). This was possibly due to the fact that the strong effect of peptide cleavage by HCl-deamidation treatment led to more exposed hydrophobic residues, which consequently increased the surface hydrophobicity of HDGs. On the basis of this definition of surface hydrophobicity, the current results demonstrated that hydrophobic amino acids exposed in HDGs increased as the DD increased to 55% and then decreased slightly when deamidating for 20 h with 60% DD. Pancreatin is mainly made up of chymotrypsin and trypsin.[26] Hydrophobic or aromatic amino acids including Tyr, Try, Phe, Val, Leu, Ile, Pro, and Ala have been reported to be preferentially cleaved from peptide linkages by chymotrypsin, while basic amino acids such as Ser, Lys, and Arg are from the peptide linkages by trypsin.[26] Therefore, if product inhibition, enzyme inactivation, and substrate recalcitrance were not considered as important factors, we assumed that the susceptibilities of these acid-deamidated wheat gluten to proteolysis would be HDG-55% > HDG-60% > HDG-45% > HDG-30% > HDG-0% (control).

Table 1. Surface Hydrophobicity and Particle Size of Wheat Gluten Deamidated with HCl at Different Degrees of Deamidation with Different Deamidation Times[a]

	surface	particle size (μm)	
assignments	hydrophobicity (H_0)	D3,2	D4,3
0 h deamidation	27.954 ± 3.0 a	50.276 ± 2.5 a	98.276 ± 4.0 a
5 h deamidation	179.49 ± 4.0 b	13.94 ± 1.0 b	40.843 ± 2.2 b
10 h deamidation	195.71 ± 4.5 bc	8.363 ± 1.1 a	32.927 ± 1.5 a
15 h deamidation	210.62 ± 3.8 a	8.038 ± 1.3 bc	32.408 ± 1.9 bc
20 h deamidation	198.84 ± 3.5 a	8.256 ± 1.2 b	33.627 ± 1.4 b

[a]Values are the mean ± SD of triplicate measurements. Means with different letters in a column are significantly different ($P < 0.05$).

The difference in the average particle sizes of the whole deamidated wheat gluten can also influence their susceptibilities to hydrolysis. Table 1 reveals that the particle size decreased with the increase of DD until 55%, then the size increased slightly when the DD value further increased due to particle aggregation. The trend of particle size went just opposite to that of the PR, DH, and H_0. Therefore, we presumed that the smaller particle size of the whole acid-deamidated wheat gluten would result in its better susceptibility to pancreatin hydrolysis.

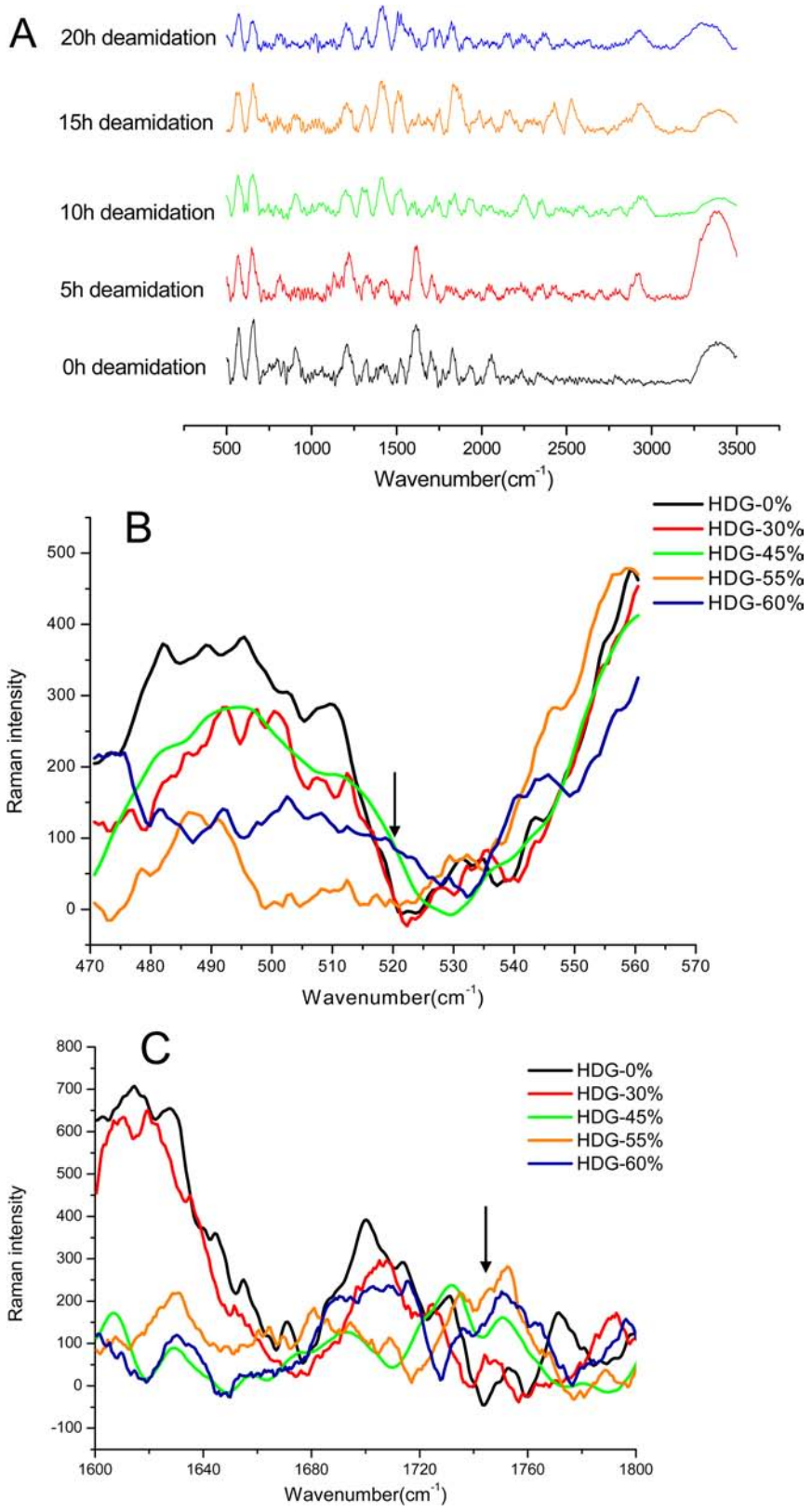

Figure 4. Raman spectra of hydrochloric acid-deamidated wheat gluten at different degrees of deamidation.

Evaluation of Conformational Changes. *Raman Spectroscopy of Substrates.* Previous reports have figured out that differences among structural conformational changes of deamidated wheat gluten may influence their susceptibilities to hydrolysis.[27] Therefore, the conformational changes of wheat gluten after deamidation with HCl at different DDs (0%−60%) were detected by Raman spectroscopy. Raman spectroscopy has been well credited for characterization of protein structure. This technique can be used to investigate the secondary structure of both liquid and solid proteins and to characterize

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protein side groups (e.g., whether they are buried or at the protein surface).[20,28] The full Raman spectrum of the 500−3500 cm^{-1} region is presented in Figure 4A. The assignments of the Raman bands have been carried out according to literature references.[21] The frequency and intensity changes in the Raman bands were the main indicators of changes in the secondary protein structure and of variations in the local environments of deamidated proteins. According to previous reports,[2,13,14] the carboxyl group (the carbonyl stretching frequencies of the carboxylic acid monomer) has obvious stretching vibration in the Raman spectral wavenumber of 1740−1800 cm^{-1}. As shown in Figure 4B, all of the deamidated wheat gluten have different absorption strengths of the C═O absorption peak at 1745 cm^{-1} as compared to the original wheat gluten, which confirmed that Gln and Asn had taken off the reaction of deamidation. The amide groups of protein molecules changed into carboxyl group to form aspartic acid and glutamic acid.[21]

Chen et al. reported that Raman spectroscopy was sensitive enough to be the best selection to detect the conversion between disulfide bonds and sulfhydryl groups in deamidated wheat gluten.[29] The disulfide bond disruption of sulfhydryl groups, which plays an important role in protein folding and assembly, may occur during the HCl deamidation of wheat gluten and contribute to the tertiary conformational change. As revealed in Figure 4C, the presence of Raman stretching at 521 cm^{-1} clearly demonstrated disulfide bonding in HCl-deamidated wheat gluten. The rank of the intensity of Raman peaks in this area (521 cm^{-1}) was HDG-55% < HDG-60% < HDG-45%, showing that HDG-55% had the least number of disulfide bonds. This demonstrated that the tertiary structure of HDH-55% was less compacted than that of HDG-60% and HDG-45%. It may be due to the fact that changes happened in hydrogen bonding and electrostatic repulsion in wheat gluten when deamidated with HCl for 15 h to reach 55% DD, leading to a decrease in protein−protein interaction and an enhancement for water−protein interaction, which could improve the susceptibility of HCl-deamidated wheat gluten to enzymatic hydrolysis. However, the Raman intensity of the control sample (HDG-0%) and HDG-30% was less than 0 because they were strongly overprinted by a fluorescence signal due to impurity of the control sample (HDG-0%) and HDG-30% protein even after handwashing. As such, these two spectra have not been analyzed here.

Secondary Conformation. Since the secondary structure information obtained from Raman spectroscopy is not precise enough to determine the contents of sheet, helix, and random coil in protein, FTIR analysis was conducted to further investigation protein conformation. FTIR spectroscopy is highly sensitive to protein secondary structure; each protein possesses its own secondary structure type, which has specific bands with characteristic wave numbers and intensities.[2] The full FTIR spectra in the region (400−4000 cm^{-1}) is shown in Figure 5. By analyzing the amide I region (1600−1700 cm^{-1}) (see the arrows on the full spectra) that could best reflect the secondary structure in the spectra of samples, the contents of their secondary structure components were calculated (Table 2). Deconvolution of the amide I region of the samples indicated that they were composed of five components located at 1606, 1635, 1652, 1679, and 1697 cm^{-1}, representing intermolecular β-sheets of protein aggregation, extended β-sheet (hydrated), α-helix, β-turn, and extended β-sheet, respectively.[28]

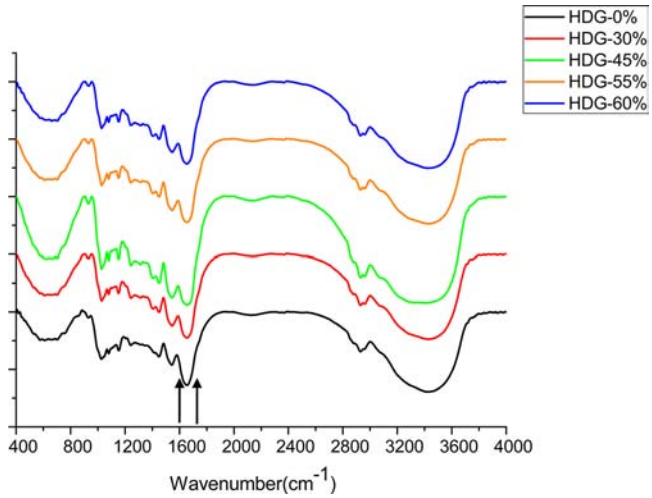

Figure 5. FTIR spectra of hydrochloric acid-deamidated wheat gluten at different degrees of deamidation.

Table 2 showed that native wheat gluten contained 34.54% α-helix, 17.78% β-turn, and 47.84% β-sheet, which was in accordance with the reports of Wang et al.[30] Deamidated wheat gluten at a DD of 60% (HDG-60%) did not change the secondary structure contents significantly compared with that of original wheat gluten (HDG-0%). The reason may be that when the degree of deamidation reached a certain value, the extended structure of the protein would remain stable. That is to say, the helix structure of wheat gluten has a certain stable region, and once it reaches the certain value, the surface hydrophobicity and molecular flexibility will not change any more. With increasing degrees of deamidation (DD < 55%), the modified samples reduced in α-helix (from 34.38% to 32.77%) and increased in β-turn (from 17.78% to 20.11%) and β-sheet (from 47.82% to 50.10%) forms. As Glu and Asp are the main constituents of the β-turn form, the increase of β-turn might be due to the fact that more Glu and Asp were exposed as the degree of deamidation increased. As a result of increased β-turn in modified samples, the charge density and electrostatic repulsion of protein consequently became stronger after deamidation with HCl, facilitating unfolding of the protein conformation and protein−water interactions and improving the susceptibility of wheat gluten to enzymatic hydrolysis.

The ratio of α-helix to β-sheet represented the molecular flexibility of proteins and indicated the susceptibility of proteins to enzymatic hydrolysis. The values of α- helix to β-sheet of HCl-deamidated wheat glutens with increasing degrees of deamidation became smaller and smaller (from 0.712 to 0.654) and were all lower than that of native wheat gluten (0.719), demonstrating that HCl-modified wheat gluten was more flexible compared to that of native wheat gluten. Owing to the high degree of protein hydrolysis by HCl with the increase of deamidation time (see Figure 2A), the peptides produced in the deamidation of wheat gluten with HCl were shorter and shorter with the increase of the deamidation time. This was the main reason why HDG with longer deamidation time showed more molecular flexibility than that with shorter deamidation time.

On the basis of the Raman and FTIR data, it could be concluded that hydrochloric acid deamidation changed the molecular structure of wheat gluten and that the molecular flexibility of HDG was improved compared with that of native

Table 2. Secondary Structure Contents of Wheat Gluten Deamidated with HCl at Different Degrees of Deamidation with Different Deamidation Times

	intermolecular β-sheet of protein aggregation		extended β-sheet (hydrated)		α-helix		β-turn		extended β-sheet			
assignments	peak (cm^{-1})	content (%)[a]	peak (cm^{-1})	content (%)[a]	peak (cm^{-1})	content (%)[a]	peak (cm^{-1})	content (%)[a]	peak (cm^{-1})	content (%)[a]	α-helix/ total β	α-helix/ β-sheet
0 h deamidation	1604.651	13.022	1635.51	33.685	1652.868	34.384	1679.869	17.780	1695.298	1.129	0.528	0.719
5 h deamidation	1608.508	13.241	1637.438	32.242	1654.796	33.247	1681.798	19.053	1695.298	1.215	0.505	0.712
10 h deamidation	1606.579	14.251	1637.438	32.602	1654.796	33.582	1681.798	19.784	1695.298	1.579	0.492	0.693
15 h deamidation	1606.579	14.299	1637.438	34.219	1654.796	32.765	1681.798	20.109	1695.298	1.607	0.466	0.654
20 h deamidation	1606.579	13.137	1637.438	32.956	1654.796	33.894	1681.798	18.301	1695.298	1.210	0.517	0.716

[a]Estimated as (secondary structure area/total amide I band area) × 100%.

wheat gluten, which enhanced the susceptibility of HCl deamidated wheat gluten to enzymatic hydrolysis.

Comparison of Deamidated Wheat Gluten through a Scanning Electron Microscope (SEM). The SEM is a microscope that uses electrons instead of light to form an image. Figure 6 shows the SEM images of the microscopic

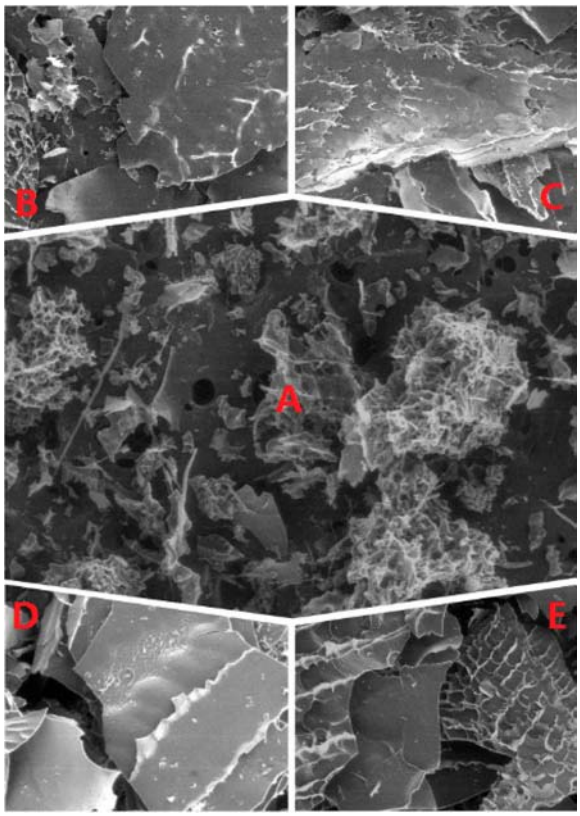

Figure 6. SEM of hydrochloric acid-deamidated wheat gluten at different degrees of deamidation.

structure for whole deamidated wheat glutens at different degrees of deamidation. As observed in Figure 5, image A shows that the original wheat gluten presents a dense block network structure; image B reveals HDG-30% with a less compact structure and some sheet parts; image C manifested that the surface structure of HDG-45% tends to be smooth and that some stomas gradually appear; image D illustrates that the hydrochloric acid-modified wheat gluten at a DD of 55% (HDG-55%) presents a laminated structure with smooth surface and stomas; image E indicates that the structure of HDG-60% is close to that of image C of HDG-45%. In general, the hydrochloric acid-deamidated wheat gluten at a DD of 55% demonstrated the smoothest surface and the least uniform pore, which enabled the sample to be easier to enzymatically hydrolyze. It was probably due to the fact that the deamidation with hydrochloric acid at a DD of 55% had more influence on the molecular weight and molecular size, which was in accordance with the former results of determination in this work. The smaller the molecular weight and size of modified wheat gluten, the greater was the susceptibility of the modified protein to enzymatic hydrolysis.

In conclusion, we found that enzymatic hydrolysis is dependent on the surface area of the deamidated wheat gluten that binds to the enzyme and it also depends on conformational properties of substrates. Deamidated wheat gluten with HCl at a DD of 55% (HDGH-55%) showed the highest susceptibility to enzymatic hydrolysis. HDG-55% could more efficiently enhance the charge density and electrostatic repulsion of proteins compared with other HDGs. Consequently, it facilitated protein unfolding, improved protein−water interaction, and enhanced surface properties to boost the efficiency of enzymatic hydrolysis. Deamidation with different DDs has a distinct influence on the structural characteristics of wheat gluten substrates. HDG-55% showed more significant changes in secondary structure as determined by Raman and FTIR. Their resultant conformational differences significantly influence the exposure level of amino acids in binding to the enzyme and their susceptibility to in vitro enzymatic hydrolysis. Therefore, this study provides a better understanding of the susceptibility of wheat gluten to enzymatic hydrolysis.

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Notes

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REFERENCES

(1) Gorinstein, S.; Pawelzlk, E.; Licon, E. D.; Haruenkit, R.; Weisz, M.; Trakhtenberg, S. Characterisation of pseudo cereal and cereal proteins by protein and amino acid analyses. *J. Sci. Food Agric.* **2002**, *82*, 886−891.

(2) Liao, L.; Zhao, M. M.; Ren, J. Y.; Zhao, H. F.; Cui, C.; Hu, X. Effect of acetic acid deamidation-induced modification in functional and nutritional properties and conformation of wheat gluten. *J. Sci. Food Agric.* **2009**, *90*, 409−417.

(3) Peter, R.; Nigel, G. Cereal seed storage proteins: structures, properties and role in grain utilization. *J. Exp. Bot.* **2002**, *53*, 947−958.

(4) Liao, L.; Qiu, C. Y.; Liu, T. X.; Zhao, M. M.; Ren, J. Y.; Zhao, H. F. Susceptibility of wheat gluten to enzymatic hydrolysis following deamidation with acetic acid and sensory characteristics of the resultant hydrolysates. *J. Cereal Sci.* **2010**, *52*, 395−403.

(5) Wang, J. S.; Wei, Z. Y.; Lu, L.; Bian, K.; Zhao, M. M. Characteristics of enzymatic hydrolysis of thermal-treatedwheat gluten. *J. Cereal Sci.* **2009**, *50*, 205−209.

(6) Hrckova, M.; Rusnakova, M.; Zemanovic, J. Enzymatic hydrolysis of defatted soy flour by three different proteases and their effect on the functional properties of resulting protein hydrolysates. *Czech J. Food Sci.* **2002**, *2*, 7−14.

(7) Riha, W. E.; Izzo, H. V.; Zhang, J.; Ho, C. T. Nonenzymatic deamidation of food proteins. *Crit. Rev. Food Sci. Nutr.* **1996**, *6*, 225−255.

(8) Aranyi, C.; Hawrylewicz, E. J. Preparation and isolation of acid-catalyzed hydrolysates from wheat gluten. *J. Agric. Food Chem.* **1972**, *20*, 670−675.

(9) Chan, W. M.; Ma, C. Y. Acid modification of proteins from soymilk residue (okara). *Food Res. Int.* **1999**, *32*, 119−127.

(10) Matsudomi, N.; Sasaki, T.; Kato, A.; Kobayashi, K. Conformational changes and functional properties of acid-modified soy protein. *J. Agric. Biol. Chem.* **1985**, *49*, 1251−1256.

(11) Wu, C. H.; Nakai, S.; Powrie, W. D. Preparation and properties of acidsolubilized gluten conformation. *J. Agric. Food Chem.* **1976**, *24*, 504−510.

(12) Aaslyng, M. D.; Martens, M.; Poll, L.; Nielsen, P. M.; Flyge, H.; Larsen, L. M. Chemical and sensory characterization of hydrolyzed vegetable protein, a savory flavoring. *J. Agric. Food Chem.* **1998**, *46*, 481−489.

(13) Liao, L.; Liu, T. X; Zhao, M. M.; Cui, C.; Yuan, B. E.; Tang, S. Functional, nutritional and conformational changes from deamidation of wheat gluten with succinic acid and citric acid. *Food Chem.* **2010**, *123*, 123−130.

(14) Liao, L.; Wang, Q.; Zhao, M. M. Investigation of the susceptibility of acid-deamidated wheat gluten to in vitro enzymatic hydrolysis using Raman spectra and free amino acid analysis. *J. Agric. Food Chem.* **2012**, *92*, 1865−1873.

(15) Kong, X.; Zhou, H.; Qian, H. Enzymatic preparation and functional properties of wheat gluten hydrolysates. *Food Chem.* **2007**, *101*, 615−620.

(16) Wang, J. S.; Wei, Z. Y.; Li, L.; Bian, K.; Zhao, M. M. Characteristics of enzymatic hydrolysis of thermal-treated wheat gluten. *J. Cereal Sci.* **2009**, *50*, 205−209.

(17) Lee, D.; Yu, A. H. C.; Wong, K. K. Y.; Saddler, J. N. Evalution of the enzymatic suscepibility of cellulosic substrates using speific hydrolysis rates and enzyme adsorption. *Appl. Biochem. Biotechnol.* **1994**, *45*, 407−415.

(18) Thompson, D. N.; Chen, H. C.; Grethlein, H. E. Comparison of pretreament methods on the basis of available surface area. *Bioresour. Technol.* **1992**, *39*, 155−163.

(19) Sinitsyn, A. P.; Gusakov, A. V.; Vlasenko, E. Y. . Effect of structural and physico-chemical features of cellulosic substrates on the efficiency of enzymatic hydrolysis. *Appl. Biochem. Biotechnol.* **1991**, *1*, 43−59.

(20) Haskard, C. A.; Li-Chan, E. C. Y. Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRODAN) and anionic (ANS^-) fluorescent probes. *J. Agric. Food Chem.* **1998**, *46*, 2671−2677.

(21) Wong, H. W.; Mei, S. C.; Phillips, D. L.; Ma, C. Y. Raman spectroscopic study of deamidated food proteins. *Food Chem.* **2009**, *113*, 363−370.

(22) Palmer, B. A. Monomeric and Oligomeric Enzymes. In *Understanding Enzymes*, 2nd ed.; Ellis Horwood: Chichester. U.K., 1985; Vol. *5*, pp 88−96.

(23) Schofield, J. D.; Bottomley, R. C.; Timms, M. F.; Booth, M. R. The effect of heat on wheat gluten and the involvement of sulfhydryl-disulphide interchange reactions. *J. Cereal Sci.* **1983**, *1*, 241−253.

(24) Liao, L.; Liu, T. X.; Zhao, M. M.; Cui, C.; Zhao, H. F. Aggregation behavior of wheat gluten during carboxylic acid deamidation upon hydrothermal treatment. *J. Cereal Sci.* **2011**, *54*, 129−136.

(25) Mahn, A.; Lienqueo, M. E.; Asenjo, J. A. Effect of surface hydrophobicity distribution on retention of ribonucleases in hydrophobic interaction chromatography. *J. Chromatogr., A* **2004**, *1043*, 47−55.

(26) Jafari, S. M.; Assadpoor, E.; Bhandari, B.; He, Y. Nano-particle encapsulation of fish oilby spray drying. *Food Res. Int.* **2008**, *41*, 172−183.

(27) Kato, A.; Tanaka, A.; Lee, Y.; Matsudomi, N.; Kobayashi, K. Effects of deamidation with Chymotrypsin at pH 10 on the functional properties of proteins. *J. Agric. Food Chem.* **1987**, *35*, 285−288.

(28) Chen, H. L.; Wang, C. H.; Chang, C. T.; Wang, T. C. Effects of Taiwanese yam (Dioscorea japonica Thunb var. pseudojaponica Yamamoto) on upper gut function and lipid metabolism in Balb/c mice. *Nutrition* **2003**, *19*, 646−651.

(29) Chen, W. Nie SMand Kuck JFR. Near-infrared Fourier transform Raman and conventional Raman studies of calf-crystallins in the lyophilized state and in solution. *J. Biophys.* **1991**, *60*, 447−455.

(30) Wang, J. S.; Zhao, M. M.; Yang, X. Q. Improvement on functional properties of wheat gluten by enzymatic hydrolysis and ultrafiltration. *J. Cereal Sci.* **2006**, *44*, 93−100.