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Calcium-Binding Capacity of Wheat Germ Protein Hydrolysate and Characterization of Peptide−Calcium Complex

Feng-Ru Liu, Li Wang, Ren Wang, and Zheng-Xing Chen*

State Key Laboratory of Food Science and Technology, National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, People's Republic of China

ABSTRACT: This study investigates the ability of various wheat germ protein hydrolysates (WGPHs) to bind calcium and characterizes the peptide−calcium complexes. We demonstrate that the amount of Ca bound depended greatly on the type of enzyme, degree of hydrolysis (DH), amino acid composition, and molecular mass distribution of different hydrolysates. The maximum level of Ca bound (67.5 mg·g^{-1}) occurred when Alcalase was used to hydrolyze wheat germ protein at a DH of 21.5%. Peptide fragments exhibiting high calcium-binding capacity had molecular mass <2000 Da. The calcium-binding peptides mainly consisted of Glu, Arg, Asp, and Gly, and the level of Ca bound was related to the hydrophobic amino acid content in WGPHs. UV−visible and Fourier transform infrared spectra demonstrate that amino nitrogen atoms and oxygen atoms on the carboxyl group were involved in complexation. Therefore, wheat germ protein is a promising protein source for the production of calcium-binding peptides and could be utilized as a bioactive ingredient for nutraceutical food production.

KEYWORDS: *wheat germ protein, hydrolysates, calcium binding, peptide−calcium complex*

INTRODUCTION

Calcium is an essential mineral nutrient required for biological functions in the body such as nerve conduction, muscle contraction, mitosis, blood coagulation, and structural support of the skeleton.[1] Insufficient calcium uptake will compromise the density of bones, resulting in rickets and osteoporosis.[2] Organic calcium has been proposed as a superior alternative to inorganic calcium. It is thought to overcome two of the major limitations of inorganic calcium supplements: low bioavailability at low concentrations and biological toxicity at high levels.[3] Calcium ion can form complexes with organic ligands. A molecule possessing affinity for a biomineral, in general, must have a basic structure to provide calcium binding, such as polycarboxylic acid or polyphosphoric acid.[4] Casein phosphopeptides (CPP) and citrate were respectively found to have many phosphoseryl and carboxylic groups that have the capacity of chelating calcium. However, some other proteins or peptides without phosphate groups still contribute to calcium absorption, by binding calcium via the amino group as well as carboxyl groups of amino acid residues. Numerous investigations about binding of amino acids and formation of calcium complexes and chelates have been described in the literature.[5−7] Most of the data reveal that the α-amino and carboxylate of amino acid functional groups were used to chelate the calcium. A small number of amino acid side chains are potential metal ligands. Ligand groups commonly encountered include the thiolate of cysteine (Cys), the imidazole of histidine (His), the carboxylates of glutamic acid (Glu) and aspartic acid (Asp), and the phenolate of tyrosine (Tyr). Less frequently, the thioether group of methionine (Met) and the amino groups of asparagine (Asn) and glutamine (Gln) are involved in metal binding. Metal ions can also bind to peptide bonds, through the carbonyl or the deprotonated amino nitrogen, and to the terminal amino and carboxyl groups of the protein.[8]

The increasing utilization of metal supplements is based on the theory that the absorption of metal could be inhibited by the further reaction of mental with antagonists. Most chelates remain soluble throughout changes in pH during digestion and are electrically neutral. The strength of the bond between ligand and metal upon formation of a metal chelate can prevent dissociation and loss of mineral as it passes through the digestive system.[9] Casein phosphopeptides (CPPs), phosphorylated peptides derived from milk, have proved to be effective in promoting calcium absorption and are now widely used for mineral binding.[10] Meat and fish have also long been considered to promote calcium absorption, possibly due to the release of calcium-binding peptides during muscle tissue digestion.[11,12] However, some consumers cannot use protein from dairy products for calcium supplements because they have lactose intolerance, and the relatively high price of animal protein increases the cost of products. Therefore, production of calcium-binding peptides from plant protein could be a new choice for consumers.

Wheat germ represents about 2.5−3.8% of the total seed weight and is an essential byproduct of the flour milling industry. Defatted wheat germ has a relatively high protein content (30−32%), is rich in albumin (34.5% of total protein) and globulin (15.6%), and thus presents a well-balanced amino acid profile.[13] It has been reported that wheat germ protein has various functional and biologic properties such as antioxidant, angiotensin-converting enzyme (ACE) inhibition, eumelanin inhibition, and immunity adjustment.[14] Thus, many studies have been performed to utilize wheat germ protein and to improve its bioactive peptide properties. However, the study of

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calcium-binding peptides from wheat germ protein has not previously been reported. Moreover, the precious wheat germ source has poor utility for human applications, while the majority is used for animal feeding purposes. Wheat germ protein, which is cheaper than milk protein and many other animal proteins, is thus an attractive potential resource to produce calcium-binding peptides in large quantities. Therefore, it is interesting to know whether wheat germ protein produced high calcium-binding peptides and to attempt to determine the calcium-peptide binding mechanism. The aim of this study was to produce and evaluate wheat germ protein−calcium (WGP−Ca) complex from enzymatic hydrolysis of wheat germ protein, using analytical techniques such as ultraviolet−visible (UV−vis) spectroscopy and Fourier transform infrared (FTIR) spectroscopy. The results provide a better understanding of the WGP−Ca complex and open up the possibility of further investigation.

MATERIALS AND METHODS

Materials. Wheat germ (WG) was obtained from the Anyang Mantianxue Food Co., Ltd. (Henan, China). Alcalase 2.4 L (declared activity of 2.4 AU/g and density of 1.18 g/mL); Neutrase 1.5 MG (declared activity of 1.5 AU/g), Flavourzyme 500 MG (declared activity of 500 LAPU/g), and Protamex (declared activity of 500 LAPU/g) were obtained from Novozymes (Bagsvaerd, Denmark). Papain (a cysteine protease from papaya latex with a declared activity of 800 units/mg) was purchased from Sinopharm Chemical Co. Ltd. (Shanghai, China). Other chemical reagents used in this study were of analytical grade and commercially available.

Preparation of Wheat Germ Protein Hydrolysates. Wheat germ protein isolates produced as previously described[15] were utilized as a substrate for production of wheat germ protein hydrolysate. Raw wheat germ was defatted with *n*-hexane for 8 h and air-dried at room temperature. The defatted wheat germ meal was then dispersed in 1.0 mol/L NaCl solution (1:10 w/v) and stirred for 30 min at ambient temperature. The pH of suspension was adjusted to 9.5 with 1 mol/L NaOH and stirred for 1 h. The protein-rich suspension was centrifuged at 8000 rpm for 20 min at 4 °C to remove the fiber and other suspended solids and was then adjusted pH to 4.0 with 1.0 mol/L HCl to precipitate the proteins and centrifuged again at 8000 rpm for 20 min at 4 °C. The precipitates were washed several times with distilled water, dispersed in a small amount of distilled water, and adjusted to pH 7.0 with 0.1 mol/L NaOH. The dispersed product was freeze-dried.

Wheat germ protein isolates were hydrolyzed sequentially with proteases in a hydrolysis reactor equipped with a stirrer, digital heating circulating water bath, and pH electrode. Hydrolysis parameters were as follows: protein isolates concentration 5% (w/v); enzyme/substrate ratio 1/50 (w/w); Alcalase (50 °C, pH 8.0); Protamex (50 °C, pH 6.5); Flavourzyme (50 °C, pH 7.0); Neutrase (50 °C, pH 7.0); papain (55 °C, pH 7.0); and after 30, 60, 120, 180, 240, and 300 min, hydrolysis was inactivated by heating at 100 °C for 10 min.

A portion of the supernatant containing target peptides was freeze-dried as wheat germ protein hydrolysate, while the remaining portion was passed through ultrafiltration membranes with molecular weight cutoff (MWCO) of 3K, 5K, and 10K by use of an ultrafiltration device. Ultrafiltration was performed sequentially: first through the 10K membrane and retentate was passed through 5K membrane, and then retentate from the 5K membrane was passed through the 3K membrane. The permeate from each MWCO membrane was collected as >10K (P4), 5−10K (P3), 3−5K (P2), and <3K (P1) peptide fractions. All the permeates were freeze-dried and stored at −20 °C until needed for further analysis. The protein contents of the freeze-dried wheat germ peptide (WGP) fractions were determined by the modified Lowry method.[16]

Preparation of Wheat Germ Peptide−Calcium Complex. Solid WGP was dissolved in 20 mM Tris-HCl (pH 7.8) buffer solution at room temperature to give a freshly prepared solution of WGP at a concentration of 20 mg·mL^{-1}. The WGP solution was mixed by vortex to ensure complete protein dissolution. A solution of 0.2 M $CaCl_2$ was added slowly, with the ratio of WGP to Ca^{2+} (weight/millimolar) reaching 1:1. The reaction mixture was maintained for 1 h at 40 °C and then centrifuged sequentially at 8000*g*. Ethanol was added to the precipitating supernatant that eventually made WGP−Ca complex. The yield (percent) of WGP−Ca complex was calculated as follows:

$$\text{yield} = \frac{\text{amount of complex} \times 100}{\text{protein content} + \text{added amount of calcium}} \quad (1)$$

Analysis of Calcium Binding Ability. Calcium binding activity was determined according to the ethylenediaminetetraacetic acid (EDTA) titration method of "GB/T 5009.92-2003 Determination of calcium in foods" (National Standard of the People's Republic of China) and Nielsen[17] with some modifications. Calcium contents are expressed as milligrams of calcium per gram of protein.

Aliquot gram sample was taken to ca. 50 mL with double-distilled water in a 125-mL Erlenmeyer flask, and a blank of distilled water equal to specimen volume was prepared. The pH was adjusted to 10 ± 0.05 with 10% NaOH. Calmagite (1 mL) was added to each flask, and they were then titrated with EDTA standard (*M*, 0.01 M) solution slowly, with continuous stirring, until the last reddish tinge disappears. Color at the end point is blue. The volume (*V*, milliliters) of EDTA solution used for each titration is recorded. Samples were prepared and analyzed in triplicate. The amount of calcium in sample (milligrams per gram) was calculated as follows:

$$\text{calcium} = \frac{40.085MV}{\text{protein in sample}} \quad (2)$$

Determination of Degree of Hydrolysis. The degree of hydrolysis (DH), defined as the ratio of peptide bonds cleaved to total peptide bonds in the protein substrate (h_{tot}), was calculated from the amount of base (NaOH) added to keep the pH constant during hydrolysis:[18]

$$\text{DH} = \frac{BN_b}{\alpha M_p h_{tot}} \times 100\% \quad (3)$$

where *B* is the amount of NaOH consumed (milliliters) to keep the pH constant during the reaction. N_b is the normality of the base, M_p is the mass (grams) of protein ($N \times 6.25$), and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis:

$$\alpha = \frac{10^{\text{pH}-\text{p}K}}{1 + 10^{\text{pH}-\text{p}K}} \quad (4)$$

where pH and p*K* are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in the wheat germ protein substrate was assumed to be 8.3 mequiv·g^{-1}.[18]

Analysis of Amino Acid Composition. The amino acid composition of hydrolysate was determined according to the method of Yang et al.[19] with a slight modification. Amino acid composition was determined by an automatic amino acid analyzer (835-50, Hitachi). The total amino acid composition of hydrolysates was determined after hydrolysis at 110 °C for 22 h with 6 M HCl. External standards were used for quantification.

Determination of Molecular Weight Distribution. The molecular mass distribution profile was determined by gel-permeation chromatography on a TSK gel G2000 SWXL 7.8 × 300 mm column (Tosoh, Tokyo, Japan) with an Agilent 1100 HPLC system (Agilent Technologies) according to the method of Zhuang et al.[20] HPLC was carried out with the mobile phase (acetonitrile/water/trifluoroacetic acid = 30:70:0.1 v/v/v) at a flow rate of 0.5 mL/min and monitored at 220 nm at 30 °C. The standards used were tripeptide GGG (M_r 189), tetrapeptide GGYR (M_r 451), bacitracin (M_r 1450), aprotinin (M_r 6500), and cytochrome *c* (M_r 12500) (Sigma Chemical Co., St. Louis, MO).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. SDS−PAGE was performed on a discontinuous buffer system according to the method of Laemmli [21] with a 12% separating gel and

5% stacking gel. The protein samples were solubilized in 0.125 M Tris-HCl buffer (pH 6.8) containing 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol (2-ME), 5% (v/v) glycerol, and 0.025% (w/v) bromophenol blue and heated for 5 min in boiling water before electrophoresis. For each sample, 10 μL was applied to each lane. After running at a constant current of 20 mA for ≈3 h, the separating gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% trichloroacetic acid and then destained in 7% acetic acid.

Ultraviolet−Visible Spectroscopy. The sample was dissolved in double-distilled water to obtain a concentration of 1 mg·mL^{-1}. The UV−vis absorption measurements of the sample solutions were performed on a Unico spectrophotometer (UV-2008) at room temperature (25 ± 1 °C). Prior to measurement, the baseline was set with double-distilled water. The spectra were recorded by scanning the wavelength from 190 to 800 nm with quartz cuvettes (1 cm). The peak signals in the spectra were analyzed by Omnic 6.0 software (Thermo-Nicolet Co., Madison, WI).

Fourier Transform Infrared Spectroscopy. The FTIR spectra were obtained from discs containing 1 mg of sample in approximately 100 mg of potassium bromide (KBr). An FTIR spectrophotometer (Bruker Equinox-55, Germany) was used in the experiment. All spectra were recorded within a range from 400 to 4000 cm^{-1} with 4 cm^{-1} resolution and 32 scans. All measurements were performed in a dry atmosphere at room temperature (25 ± 1 °C). The results were presented in transmittance units.

Statistical Analysis. The experiments were performed in triplicate, and values are expressed as mean ± standard error (SD). All data were subjected to analysis of variance (ANOVA), and the differences between means were evaluated by Duncan's multiple range test.

RESULTS AND DISCUSSION

Effect of Protease and Hydrolysis Time on Degree of Hydrolysis. The degree of hydrolysis (DH), defined as the percentage of cleaved peptide bonds, is a fundamental parameter for monitoring the protein hydrolysis reaction. The effects of enzymes and treatment periods on DH of wheat germ protein isolate are shown in Figure 1. Wheat germ protein was hydrolyzed by five commercial enzymes (Alcalase, Protamex, Flavourzyme, Neutrase, and papain) at their optimal temperature and pH values, and the process of hydrolysis was monitored for 5 h via pH-stat. A typical hydrolysis curve obtained under experimental conditions shows a high rate of hydrolysis for the first 1 h. The hydrolysis rates of papain, Neutrase, and Flavourzyme gradually slowed down and subsequently became constant, and DH reached respective maximum values of 10.4%, 5.7%, and 4.5% at 5 h of hydrolysis. Hydrolysis by Alcalase and Protamex gradually increased until DH reached maximum values of 22.4% and 21.1%, respectively. In the first step of the overall reaction, the enzyme molecules become associated with and bound to the protein particles. Subsequently, hydrolysis took place, resulting in the release of soluble peptides and amino acids. After addition of the enzyme, there was an initial rapid phase during which a large number of peptide linkages were cleaved per unit time, and a large proportion of soluble material was released into solution. The most compact core proteins were hydrolyzed more slowly.[22] Consequently, the rate of enzyme cleavage of peptide bonds controlled the overall rate of hydrolysis. The results agree with the earlier report of Zhu et al.[15] Based on the degree of hydrolysis, Alcalase 2.4 L was the best protease and revealed a much higher potential to hydrolyze wheat germ protein, which might be attributed to its specific catalytic site. Alcalase contains subtilisin-like activity in addition to a minor glutamyl endopeptidase activity, and therefore, the degradation of wheat germ protein could be an economically feasible way of producing calcium-binding peptides.

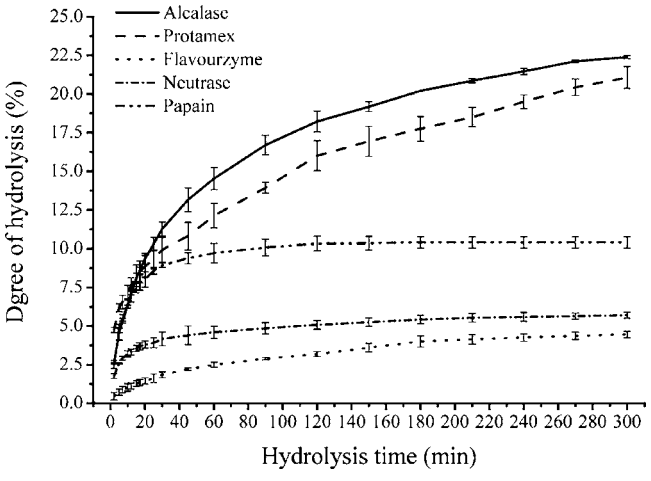

Figure 1. Degree of hydrolysis of protease-digested wheat germ protein hydrolysates (WGPHs) at various time points. Results represent the means of three determinations ± standard deviation. Means are significantly different ($P < 0.05$).

Effect of Protease on Calcium-Binding Capacity. The amount of Ca bound and the yield of WGP−Ca complex at different hydrolysis times are shown in Figure 2. The calcium binding ability of wheat germ protein hydrolysate is shown in Figure 2a. Calcium content of WGP−Ca ranged from 4 to 18 mg·(g of protein)$^{-1}$, and the products from every hydrolysis stage of Alcalase had a higher calcium binding ability than other proteases. The calcium binding capacity of protease hydrolysates all increased with the degree of hydrolysis except that of protein hydrolysate by papain.

The calcium binding ability of enzymatic hydrolysates from strong to weak is as follows: Alcalase > Protamex > papain > Neutrase > Flavourzyme. Figure 2b shows that the yield of all enzymatic hydrolysates was between 15% and 35%, increasing slightly throughout the hydrolysis process. The highest yield of complex was from Alcalase hydrolysate, followed by Protamex, papain, Neutrase, and Flavourzyme. Therefore, Alcalase 2.4 L was considered the most suitable enzyme to make the hydrolysates for the preparation of calcium-binding peptides. Alcalase hydrolysate has a high level of calcium affinity because of its strong proteolytic activity. The maximum level of Ca bound (18 mg·g^{-1}) occurred when Alcalase was used to hydrolyze wheat germ protein at DH of 21.5%. Too high and too low degree of hydrolysis is unfavorable. This indicates that the molecular mass of wheat germ peptides plays an important role in calcium-binding capacity.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. Analysis of Figures 1 and 2 showed that the calcium binding capability of WGP hydrolysates was dependent not only on the type of enzyme but also on enzymolysis time. SDS−PAGE in tricine buffer was selected to account for this situation, and the patterns of WGP hydrolysates (300 min treatment) of various enzymes are shown in Figure 3. The four kinds of protein (albumin, globulin, glutelin, and prolamin)-extracted from wheat germ by the Osborne procedure were compared to wheat germ protein isolates (WGPI), and the results showed that WGPI contains above four kinds of protein, mainly albumin and globulin. The molecular mass distribution was 15−100 kDa, and there were four distinct areas: 50, 32−34, 28, and 14−17 kDa. Three high molecular mass subunits of

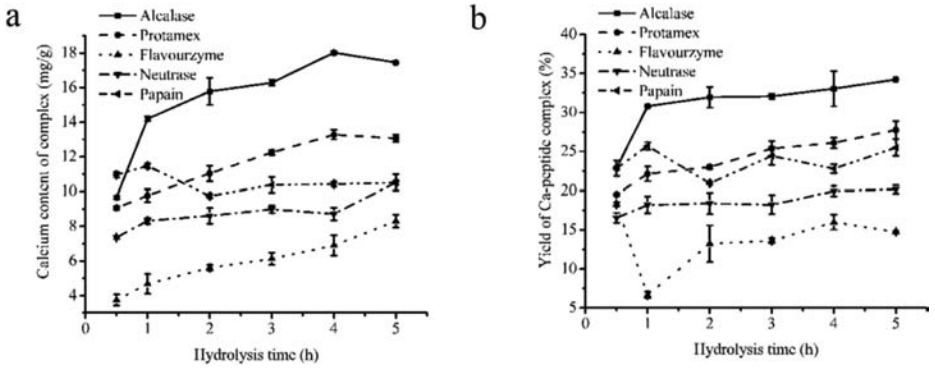

Figure 2. (a) Amount of bound calcium (milligrams per gram of protein) in wheat germ peptide−calcium complex (WGP−Ca). (b) Yield of WGP−Ca from various WGPHs. Results represent the means of three determinations ± standard deviation. Means are significantly different ($P < 0.05$).

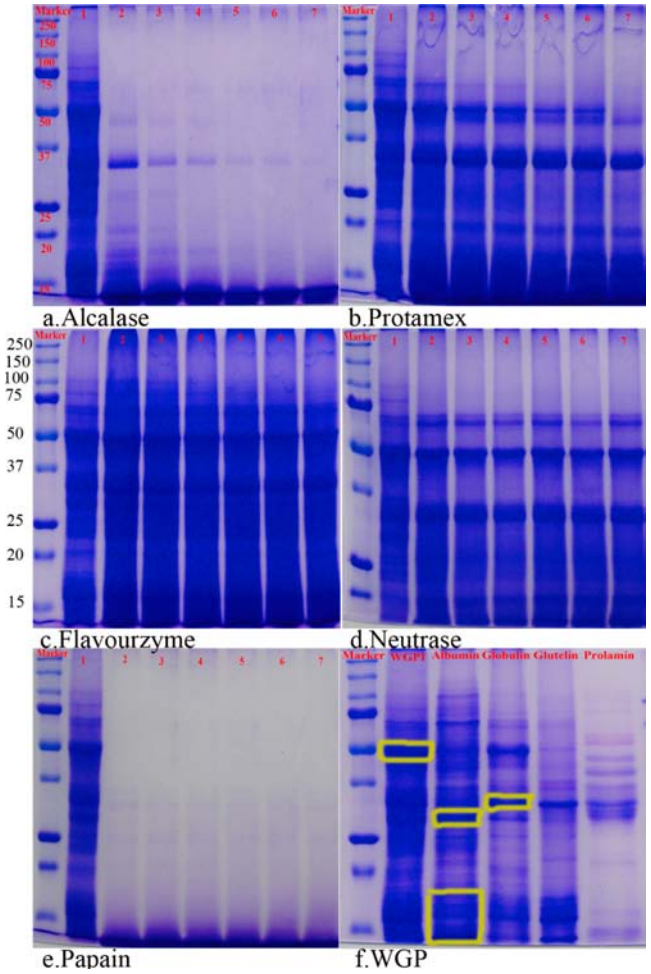

Figure 3. Electrophoresis pattern of WGP and WGPHs at various hydrolysis times. Molecular mass markers are given in kilodaltons. Lane 1, wheat germ protein isolate (WGPI); lanes 2−7, WGPHs at hydrolysis times of 0.5, 1, 2, 3, 4, and 5 h. (a) Alcalase; (b) Protamex; (c) Flavourzyme; (d) Neutrase; (e) papain; (f) wheat germ protein (containing WGPI, albumin, globulin, glutelin, and prolamin).

100, 80, and 70 kDa, as well as a low molecular mass subunit of 20 kDa, were present. The molecular mass distribution of albumin ranged from 14 to 100 kDa with a wealth of subunits. Globulin mainly included four distinct regions of 50, 32, 28, and 17 kDa.

Figure 3 panels a and e respectively show the hydrolysis bands of Alcalase and papain with obvious changes. In Figure 3a, the high molecular mass subunit of WGPI was degraded significantly in the initial 0.5 h, with only two obvious regions at 34 and 17 kDa, as well as small amounts at 47, 20, and 18 kDa. Subsequently, all wheat germ protein subunits were gradually hydrolyzed into small peptides of molecular mass less than 15 kDa. In Figure 3e, the molecular mass of WGPI was dramatically degraded to less than 15 kDa within 0.5 h by papain, but it was disadvantageous for obtaining a peptide with high calcium binding capacity, as confirmed from Figure 2. The high molecular mass subunits (100 and 80 kDa) of WGPI were hydrolyzed first, followed by hydrolysis of 70 and 50 kDa fractions, and two significant bands of 34 and <17 kDa were mainly present in the final product. The changes of bands of hydrolysates by Flavourzyme and Neutrase are shown in Figure 3 panels c and d, respectively. There were no obvious effects of hydrolysis time on the foremost bands of protein hydrolysates. That was similar to the change in degree of hydrolysis in Figure 1.

To sum up, Alcalase (Figure 3a) and papain (Figure 3e) successfully hydrolyzed all the major protein fractions of WGPI, but papain was not suitable for the production of peptides with high calcium affinity. Protamex (Figure 3b) and Neutrase (Figure 3d) were able to partially hydrolyze the 100, 80, 70, and 50 kDa fractions of WGP. Flavourzyme (Figure 3c) was the least effective in hydrolyzing 70, 50 and 32−34 kDa fractions of WGP. In the present study, a higher DH and hydrolysis of 50, 28−34 and 17 kDa from WGP with Alcalase may be attributed to its capacity to break large numbers of peptide bonds. Alcalase was extracted from *Bacillus licheniformis* and contains several different proteinases with broad specificity.[23] This property of Alcalase resulted in a significantly higher DH of wheat germ protein fractions compared with enzymes such as Protamex, Neutrase, or Flavourzyme.

Amino Acid Analysis. The function of any peptide is principally dependent on its amino acid composition. Removal of basic residues (such as Arg) from the peptide leads to elimination of a major protonation site, favoring deprotonation of the peptide and calcium binding.[24] A previous report has indicated that the carboxyl group of acidic amino acids (Glu and Asp) and the imidazole group of His were considered as main calcium binding sites.[25,26] Moreover, calcium binding ability increased linearly with the increment of carboxyl content found in soy protein hydrolysate.[5] The amino acid compositions of various enzymatic hydrolysates revealed that they were rich in Glu (16.01%∼21.58%), Arg (9.91%∼11.40%), Asp (8.68%∼9.06%), and Gly (6.54%∼7.61%) (Table 1). Although there is little difference among different portions,

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Table 1. Analysis of Amino Acid Composition Proportion in Wheat Germ Protein Hydrolysates

amino acid	composition (%)				
	Alcalase	Protamex	Flavourzyme	Neutrase	papain
Asp	8.99	9.22	8.68	8.73	9.06
Glu	16.01	19.99	21.58	20.42	18.20
Ser	4.89	4.95	4.35	4.62	4.62
His	3.06	3.24	2.80	2.93	2.84
Gly	6.54	6.66	7.61	7.22	6.89
Thr	4.12	3.80	3.59	3.67	3.92
Arg	10.96	11.19	11.40	10.84	9.91
Ala	6.07	5.98	6.04	6.02	6.17
Tyr	2.36	2.37	1.98	2.02	2.77
Cys-s	0.33	0.39	0.32	0.33	0.28
Val	7.17	6.01	5.79	5.99	6.40
Met	1.70	1.65	1.33	1.47	1.69
Phe	5.04	4.34	4.08	4.43	4.42
Ile	4.28	3.64	3.28	3.53	3.86
Leu	7.13	6.51	5.67	6.05	6.59
Lys	5.38	5.51	5.40	5.60	6.40
Pro	5.99	4.55	6.10	6.12	5.99
Trp	*a*	*a*	*a*	*a*	*a*
total hydrophobic aa[b]	43.92	39.34	39.90	40.83	42.01

[a]Not detected. [b]Containing Gly, Ala, Val, Leu, Pro, Met, Phe, Trp, and Ile, but statistics do not count Trp because it was not detected.

there are some differences from other types of protein. Jung et al.[27] reported calcium-binding peptide prepared from Alaska pollock backbone was Val-Leu-Ser-Gly-Gly-Thr-Thr-Met-Ala-Met-Tyr-Thr-Leu-Val (1.4 kDa). Hoki frame protein was Val-Leu-Ser-Gly-Gly-Thr-Thr-Met-Tyr-Ala-Ser -Leu-Tyr-Ala-Glu (1.6 kDa).[28] Porcine blood plasma protein was Val-Ser-Gly-Val-Glu-Asp-Val-Asn (1.2 kDa).[12] The amino acid sequence of shrimp byproducts hydrolysate was Thr-Cys-His (<1 kDa).[25] These acidic amino acid residues from the peptide provided a major protonation site, favoring deprotonation of the peptide and calcium binding.[3] Meanwhile, Alcalase hydrolysates had a higher proportion of hydrophobic amino acids, such as Val (7.17%), Phe (5.04%), Leu (7.13%), and Ile (4.28%), than other hydrolysates. In addition, Table 1 shows that Alcalase hydrolysate had the highest overall proportion of hydrophobic amino acids (44%) among the hydrolysates. Therefore, the carboxyl group of acidic amino acids and hydrophobic amino acids are favorable to bind calcium.[5,29]

Molecular Weight of Calcium-Binding Peptides. Wheat germ protein hydrolysate prepared by Alcalase 2.4 L at DH of 21.5% had the highest calcium-binding capacity and was subsequently fractionated through ultrafiltration membranes with molecular weight cutoffs (MWCO) of 3K, 5K, and 10K. The molecular weight distribution of four cutoff portions (P1, P2, P3, and P4) was determined by HPLC and the results are presented in Table 2. Calcium-binding capacity of the hydrolysate derived from ultrafiltration was similarly determined. The highest calcium-binding capacity found at P2 was 67.5 mg·(g of protein)$^{-1}$.

Calcium binding capacity of portions P2 and P3 was significantly higher ($p < 0.05$) than that of P1 and P4. This is mainly due to the component of <500 Da in P1, accounting for 56.65%; most of them are amino acids. P4 contains 10.51% of the protein with a molecular mass >5000 Da. This means that the protein fragments with molecular masses greater than 5000 Da and less than 500 Da were disadvantageous for binding calcium. P2 and P3 component had high calcium affinity peptides of molecular mass 500−2000 Da, the proportion of which was 39.22% for P2 and 37.79% for P3. Therefore, the molecular mass of the main peaks of the high calcium affinity parts was lower than 2000 Da (range from 1000 to 180 Da). This indicates that the molecular mass of wheat germ peptides plays an important role in calcium-binding capacity. The result is similar to previous reports[7,28] and has laid a good foundation for further separation and purification.

UV−Visible Spectra. The UV−vis spectra of WGP and its calcium complex are shown in Figure 4. The formation of complexes of organic ligands with transition metal ions can result in the appearance of new absorbance peaks or shifting/disappearance of pre-existing ones.[30] Wheat germ peptide had a strong absorption peak at 216 nm, which arises from n → π^* transition of C=O in the peptide bond;[31] the strong absorption peak at 260 nm is generally the UV absorbance of phenylalanine.[32] However, the disappearance of characteristic absorption peaks, the detected increase in the absorption, and a bathochromic shift of the absorption maximum compared to the free WGP indicates an interaction between WGP and calcium ions and formation of a new chemical compound.

Fourier Transform Infrared Spectroscopy. Variations in peptide FTIR spectra are observed when calcium ions bind to the amino acid residues in peptides.[33] Infrared spectra of

Table 2. Molecular Weight Distribution of Alcalase Hydrolysates after Ultrafiltration[a]

mol weight range	distribution (%)			
	P1	P2	P3	P4
>5000	0.30	1.02	2.05	10.51
5000−3000	1.73	3.09	3.90	7.06
3000−2000	4.03	5.53	5.85	7.45
2000−1000	14.62	17.10	16.93	17.51
1000−500	22.68	22.12	21.86	18.52
500−180	34.96	31.58	28.85	22.33
<180	21.69	19.56	20.56	16.61
bound Ca,[b] mg·(g of protein)$^{-1}$	24.2 ± 0.9 a	67.5 ± 1.4 c	48.6 ± 1.8 b	34.2 ± 0.4 a

[a]Relative contents of components were determined by the area normalization method. Ultrafiltration cutoff portions are as follows: P1 = M_w < 3K; P2 = 3K < M_w < 5K; P3 = 5K < M_w < 10K; and P4 = M_w > 10K. [b]Results are presented as mean ± standard deviation ($n = 3$). Mean values with different letters are significantly different at $p < 0.05$.

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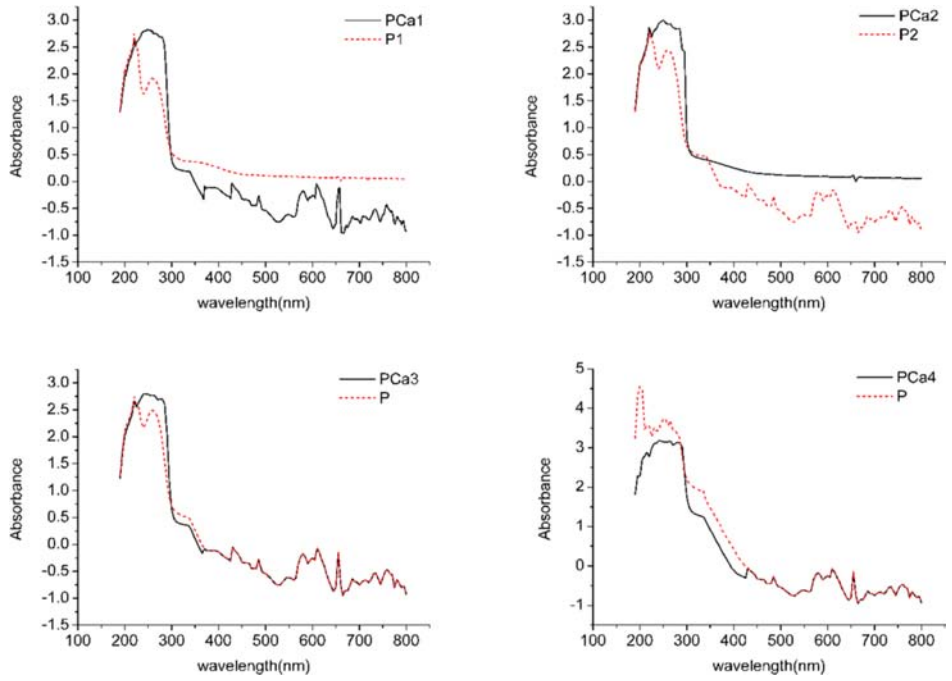

Figure 4. UV−vis spectroscopy of wheat germ peptides (P1−P4) and wheat germ peptide−calcium complexes (PCa1−PCa4). Wheat germ peptides were from Alcalase-digested hydrolysates at DH of 21.5% and separated by an ultrafiltration membranes with cutoffs of 3K, 5K, or 10K.

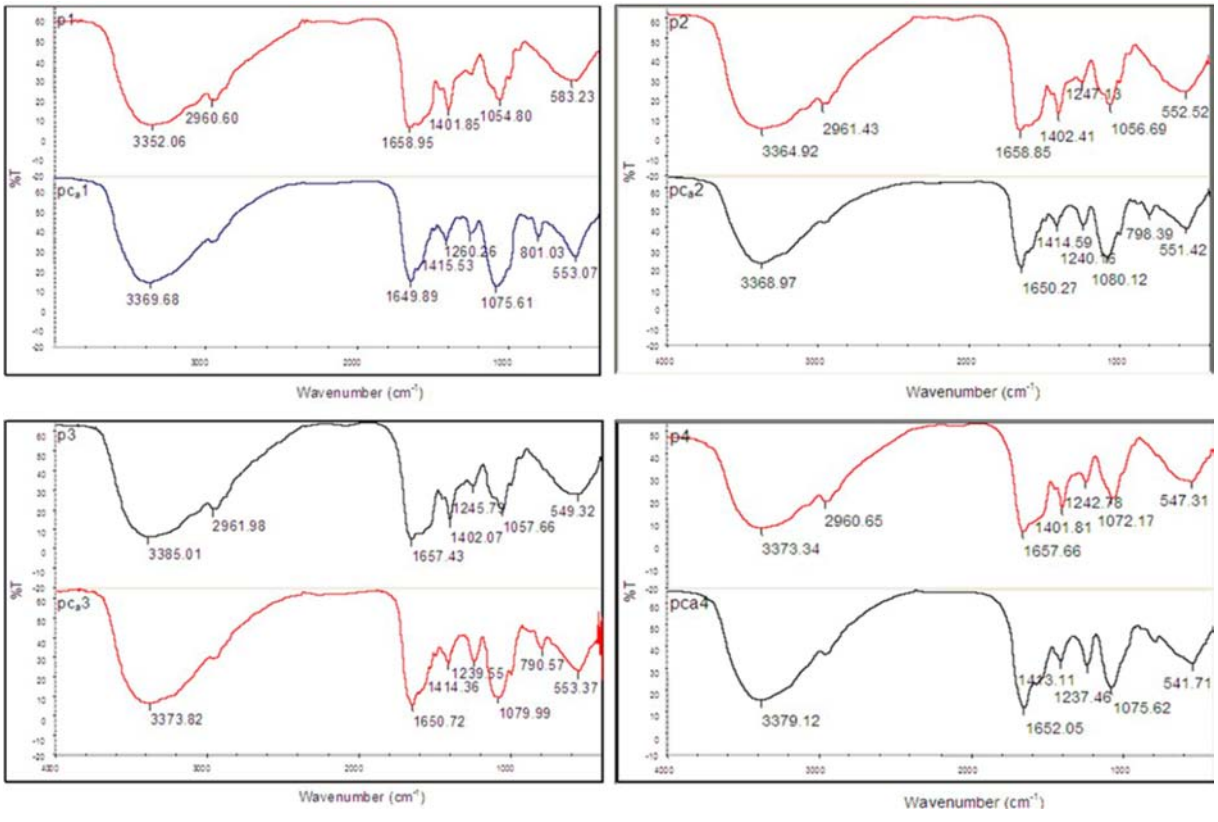

Figure 5. FTIR spectra of wheat germ peptides (P1−P4) and wheat germ peptide−calcium complexes (PCa1−PCa4). The wheat germ peptides were from Alcalase-digested hydrolysates at DH of 21.5% and were separated by ultrafiltration membranes with cutoffs of 3K, 5K, or 10K.

calcium-binding peptides and calcium−peptide complexes are shown in Figure 5. These band shifts, indicative of complex formation, are visible in all the calcium−peptide complexes (Figure 5). After addition of calcium, changes to the N−H stretching vibration were observed; the red shift suggests the involvement of the $-NH_2-$ group in complex formation. As mentioned previously, band shifts in the C=O stretching vibration were observed in the region 1658−1650 cm^{-1} and are indicative of involvement of the carboxylic group in covalent bonding to the metal ion. The peak (1402 cm^{-1}) for the

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$-COO^-$ carboxylate group moved to a higher frequency (1414 cm^{-1}) in the spectrum of the bound peptide and showed that −COOH probably bound calcium ion, turned into −COO−Ca. C−O stretching and −OH deformation vibrations were at 1100−1000 cm^{-1},[34] the wavenumber (1056 cm^{-1}) of which shifted to a higher frequency (1080 cm^{-1}). Additionally, several absorption bands for the peptide−calcium complex in the range 810−790 cm^{-1} arise from the vibration of the C−H and N−H bonds,[35] and these bands were not present in the calcium-binding peptide spectra. These results demonstrate that calcium binds to the wheat germ peptide primarily through interactions with carboxyl oxygen and amino nitrogen atoms.

As described above, we found that type of enzyme, degree of hydrolysis, amino acid content, and molecular mass distribution affect the calcium-binding peptide's capacity. Alcalase 2.4 L was shown to be the most suitable enzyme to produce WGPHs for binding calcium. Alcalase-hydrolyzed peptides exhibited the highest calcium binding capacity at DH of 21.5%. The molecular weight of the obtained calcium-binding peptide determined by ultrafiltration was <2000. Calcium-binding peptide possesses essential residues: Glu, Arg, Asp, Gly, and hydrophobic amino acid content played an important role in calcium-binding capacity. In the IR spectra experiment, after wheat germ peptides were combined with calcium, the amide I and II wavenumbers were displaced, indicating that amino nitrogen atoms and oxygen atoms in the carboxyl group were involved in complexation. In the UV scan spectra, the characteristic absorption peak of the wheat germ peptide's carbonyl and the peptide bond is clearly shifted, indicating that the wheat germ peptides had a reaction with calcium. Therefore, wheat germ protein is a promising protein source for the production of calcium-binding peptides and could be utilized as a bioactive ingredient for nutraceutical food production. Additional studies are warranted to identify the responsible calcium-binding peptide sequences from Alcalase hydrolysate of WGP and to study its bioavailability through in vitro and in vivo assays.

AUTHOR INFORMATION

Corresponding Author

*Telephone +86-510-85197856; fax +86-510-85197856; e-mail zxchen_2008@126.com.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Miller, G. D.; Jarvis, J. K.; McBean, L. D. The importance of meeting calcium needs with foods. *J. Am. Coll. Nutr.* **2001**, *20*, 168S−185S.

(2) Guéguen, L.; Pointillart, A. The bioavailability of dietary calcium. *J. Am. Coll. Nutr.* **2000**, *19*, 119S−136S.

(3) Byrne, L. A. Analytical assessment of peptide−metal interactions and subsequent stability. Doctoral Dissertation, National University of Ireland, 2010.

(4) Chen, H.-t.; Hsieh, K.-c.; Chang, J.-k.; Wang, G.-j.; Fu, Y.-c.; Ho, M.-l.; Tzeng, C.-c. Conformations of divergent peptides with mineral binding affinity. U.S. Patent 20,120,270,811, 2012.

(5) Bao, X. L.; Lv, Y.; Yang, B. C.; Ren, C. G.; Guo, S. T. A study of the soluble complexes formed during calcium binding by soybean protein hydrolysates. *J. Food Sci.* **2008**, *73*, C117−C121.

(6) Charoenphun, N.; Cheirsilp, B.; Sirinupong, N.; Youravong, W. Calcium-binding peptides derived from tilapia (*Oreochromis niloticus*) protein hydrolysate. *Eur. Food Res. Technol.* **2013**, *236*, 57−63.

(7) Rui, X. Calcium binding of peptides derived from enzymatic hydrolysates of whey protein concentrate. *Int. J. Dairy Technol.* **2009**, *62*, 170−173.

(8) Crichton, R. In *Interactions between Iron Metabolism and Oxygen Activation*; Ciba Foundation Symposium 65, Oxygen Free Radicals and Tissue Damage, 2008; Wiley Online Library, 2008; pp 57−76.

(9) Ashmead, H. D. *Amino Acid Chelation in Human and Animal Nutrition*; CRC Press: Boca Raton, FL, 2012.

(10) Mcdonagh, D.; FitzGerald, R. J. Production of casein-ophosphopeptides (CPPs) from sodium caseinate using a range of commercial protease preparations. *Int. Dairy J.* **1998**, *8*, 39−45.

(11) Jeon, S. J.; Kim, W.-G.; Song, K. B. Isolation of a calcium-binding substance from anchovy. *J. Korean Soc. Appl. Biol. Chem.* **2010**, *53*, 516−518.

(12) Lee, S. H.; Song, K. B. Isolation of a calcium-binding peptide from enzymatic hydrolysates of porcine blood plasma protein. *J. Korean Soc. Appl. Biol. Chem.* **2009**, *52*, 290−294.

(13) Brandolini, A.; Hidalgo, A. Wheat germ: not only a by-product. *Int. J. Food Sci. Nutr.* **2012**, *63*, 71−74.

(14) Zhu, K.; Zhou, H. Purification and characterization of a novel glycoprotein from wheat germ water-soluble extracts. *Process Biochem.. (Oxford, U. K.)* **2005**, *40*, 1469−1474.

(15) Zhu, K.; Zhou, H.; Qian, H. Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase. *Process Biochem. (Oxford, U. K.).* **2006**, *41*, 1296−1302.

(16) Markwell, M. A. K.; Haas, S. M.; Bieber, L.; Tolbert, N. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **1978**, *87*, 206−210.

(17) Nielsen, S. S. *Food Analysis Laboratory Manual*; Springer: New York, 2010.

(18) Adler-Nissen, J., *Enzymic Hydrolysis of Food Proteins*. Elsevier Applied Science Publishers: London, 1986.

(19) Yang, B.; Yang, H.; Li, J.; Li, Z.; Jiang, Y. Amino acid composition, molecular weight distribution and antioxidant activity of protein hydrolysates of soy sauce lees. *Food Chem.* **2011**, *124*, 551−555.

(20) Zhuang, Y.; Sun, L.; Zhao, X.; Wang, J.; Hou, H.; Li, B. Antioxidant and melanogenesis-inhibitory activities of collagen peptide from jellyfish (*Rhopilema esculentum*). *J. Sci. Food Agric.* **2009**, *89*, 1722−1727.

(21) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(22) Guerard, F.; Dufosse, L.; De La Broise, D.; Binet, A. Enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacares*) wastes using Alcalase. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 1051−1059.

(23) Doucet, D.; Otter, D. E.; Gauthier, S. F.; Foegeding, E. A. Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. *J. Agric. Food Chem.* **2003**, *51*, 6300−6308.

(24) Nemirovskiy, O.; Gross, M. Determination of calcium binding sites in gas-phase small peptides by tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1020−1028.

(25) Huang, G.; Ren, L.; Jiang, J. Purification of a histidine-containing peptide with calcium binding activity from shrimp processing byproducts hydrolysate. *Eur. Food Res. Technol.* **2011**, *232*, 281−287.

(26) Choi, D.-W.; Lee, J.-H.; Chun, H.-H.; Song, K. B. Isolation of a calcium-binding peptide from bovine serum protein hydrolysates. *Food Sci. Biotechnol.* **2012**, *21*, 1663−1667.
(27) Jung, W.-K.; Karawita, R.; Heo, S.-J.; Lee, B.-J.; Kim, S.-K.; Jeon, Y.-J. Recovery of a novel Ca-binding peptide from Alaska Pollack (*Theragra chalcogramma*) backbone by pepsinolytic hydrolysis. *Process Biochem. (Oxford, U. K.)* **2006**, *41*, 2097−2100.
(28) Jung, W.-K.; Kim, S.-K. Calcium-binding peptide derived from pepsinolytic hydrolysates of hoki (*Johnius belengerii*) frame. *Eur. Food Res. Technol.* **2007**, *224*, 763−767.
(29) Jin, Y.-G.; Fu, W.-W.; Ma, M.-H. Preparation and structure characterization of soluble bone collagen peptide chelating calcium. *Afr. J. Biotechnol.* **2011**, *10*, 10204−10211.
(30) Blat, D.; Weiner, L.; Youdim, M. B.; Fridkin, M. A novel iron-chelating derivative of the neuroprotective peptide NAPVSIPQ shows superior antioxidant and antineurodegenerative capabilities. *J. Med. Chem.* **2007**, *51*, 126−134.
(31) Yu, Y.; Fan, D. Characterization of the complex of human-like collagen with calcium. *Biol. Trace Elem. Res.* **2012**, *145*, 33−38.
(32) Yan, M.; Li, B.; Zhao, X.; Ren, G.; Zhuang, Y.; Hou, H.; Zhang, X.; Chen, L.; Fan, Y. Characterization of acid-soluble collagen from the skin of walleye pollock (*Theragra chalcogramma*). *Food Chem.* **2008**, *107*, 1581−1586.
(33) Nara, M.; Morii, H.; Tanokura, M. Coordination to divalent cations by calcium-binding proteins studied by FTIR spectroscopy. *Biochim. Biophys. Acta, Biomembr.* **2012**, DOI: 10.1016/j.bbamem.2012.11.025.
(34) Ren, Z.-y.; Huang, G.-r.; Jiang, J.-x.; Chen, W.-w. Preparation and characteristic of iron-binding peptides from shrimp processing discards hydrolysates. *Adv. J. Food Sci. Technol.* **2011**, *3*, 348−354.
(35) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectroscopic Identification of Organic Compounds*; Wiley: New York, 1981; pp 249−303.