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Optimization of the Enzymatic Deamidation of Soy Protein by Protein-Glutaminase and Its Effect on the Functional Properties of the Protein

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ABSTRACT: The effects of enzymatic deamidation by protein-glutaminase (PG) on the functional properties of soy protein isolate (SPI) were studied. Conditions for the deamidation were evaluated by means of response surface methodology (RSM). Optimal conditions based on achieving a high degree of deamidation (DD) with a concurrently low degree of hydrolysis (DH) were 44 °C, enzyme:substrate ratio (E/S) of 40 U/g protein and pH 7.0. Under optimal conditions, both DD and DH increased over time. SDS−PAGE results indicated that lower molecular mass subunits were produced with increasing DD. Far-UV circular dichroism spectra revealed that the α-helix structure decreased with higher DD, while the β-sheet structure increased until 15 min of deamidation (32.9% DD), but then decreased at higher DD. The solubility of deamidated SPI was enhanced under both acidic and neutral conditions. SPI with higher DD showed better emulsifying properties and greater foaming capacity than SPI, while foaming stability was decreased. It is possible to modify and potentially improve the functional properties of SPI by enzymatic deamidation using PG.

KEYWORDS: soy protein, protein deamidation, enzyme, protein-glutaminase, response surface methodology, circular dichroism, protein functional properties

INTRODUCTION

Soy protein is widely used in the food industry due to its excellent nutritional and functional properties. It is possible to further improve the solubility and functional properties of soy proteins, especially for specific end uses, by physical, chemical and/or enzymatic modification. Deamidation is one type of modification that can improve solubility and other functional properties of food proteins.[1] Hydrolysis by deamidation can alter secondary and tertiary structure of proteins by removal of amide groups from glutamine and asparagine residues. During deamidation amide groups are converted into acid residues (carboxyl groups) with the subsequent release of ammonia. This leads to a decrease in the isoelectric point (p*I*) of the protein due to the increase in number of negatively charged carboxyl groups. As a consequence, deamidated proteins will be more soluble under weakly acidic conditions.[2] Deamidation can be conducted both enzymatically and nonenzymatically (chemically). Enzymatic deamidation has several advantages over chemical methods, including mild reaction conditions, higher specificity and greater safety.[1] Enzymes that have been used for protein deamidation include transglutaminase, protease, peptide-glutaminase and protein-glutaminase (PG).[1,3]

PG was first isolated in 2000 from the bacterium *Cryseobacterium proteolyticum*.[4] This enzyme catalyzes the deamidaton of proteins at glutamine residues in both short peptide chains and proteins, but does not deamidate asparaginyl residues or free glutamines.[3] The specific activity of PG for various protein substrates, including soy protein isolate (SPI), has been previously reported.[3] With respect to deamidation PG differs from other enzymes in that it does not cause side reactions, such as cross-linking (transglutaminase), peptide hydrolysis (protease) or the deamidation of glutamine residues in short peptide chains (peptidoglutaminase). Studies on the deamidation by PG of some proteins and food materials, including α-lactalbumin, α-zein, wheat gluten and skim milk, have demonstrated that protein solubility and various functional properties can be improved.[5−8]

The most important factors in enzymatic hydrolysis are enzyme concentration, reaction temperature, reaction time, pH and the nature of the protein substrate.[9,10] Since there are several factors affecting enzymatic hydrolysis, the optimization of the process parameters is essential in order to achieve an economical and optimal process. Response surface methodology (RSM) has been used for process optimization. RSM is a mathematical and statistical technique used for modeling and analysis of complex reactions or processes, in which the response of interest (dependent variable) is influenced by several independent variables.[11] Various researchers have used RSM to study enzymatic hydrolysis of various types of protein such as crayfish processing byproduct,[12] fish protein,[13] chicken meat,[14] and mussel meat.[15]

To our knowledge there are no detailed reports on the use of PG for deamidation of soy protein to modify its conformation,

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Table 1. Experimental Design (Coded and Actual Values) for the Degree of Deamidation (DD, %) and Degree of Hydrolysis (DH, %) for the Enzymatic Deamidation of Soy Protein Isolate (SPI) Using Protein-Glutaminase (PG)

	indep variables						dep variables	
	coded			actual (uncoded)				
design point	x_1	x_2	x_3	E/S	T (°C)	pH	DD (%)	DH (%)
1	−1	−1	−1	14	44	5.8	24.9	4.54
2	1	−1	−1	41	44	5.8	28.9	2.45
3	−1	1	−1	14	56	5.8	28.7	3.16
4	1	1	−1	41	56	5.8	39.2	3.39
5	−1	−1	1	14	44	8.2	25.4	3.26
6	1	−1	1	41	44	8.2	33.4	4.10
7	−1	1	1	14	56	8.2	30.7	3.19
8	1	1	1	41	56	8.2	34.9	2.96
9	−1.68	0	0	5	50	7	24.4	3.90
10	1.68	0	0	50	50	7	33.2	4.13
11	0	−1.68	0	22.5	40	7	33.4	3.09
12	0	1.68	0	22.5	60	7	35.2	2.82
13	0	0	−1.68	22.5	50	5	29.2	1.94
14	0	0	1.68	22.5	50	9	19.5	2.28
15	0	0	0	22.5	50	7	33.9	3.49
16	0	0	0	22.5	50	7	34.9	3.76
17	0	0	0	22.5	50	7	33.9	3.90

which in turn can affect its solubility and other functional properties. Hence, the objective of this study was to employ RSM in order to optimize process parameters for the PG deamidation of soy protein isolate (SPI) and to evaluate the effect of deamidation on solubility and functional properties, including emulsifying and foaming properties, compared to untreated SPI.

MATERIALS AND METHODS

Materials. Soy protein isolate (SPI; Profam 974) was obtained from Archer Daniels Midland Company (Decatur, IL). Protein-glutaminase "Amano" 500 (500 U/g) was obtained from Amano Enzyme, Inc. (Elgin, IL).

Enzymatic Deamidation. Deamidation of SPI was performed in 0.01 M citrate−phosphate−borate buffer[16] containing 20 mg/mL SPI and incubated for 90 min. The experiments were conducted in 50 mL test tubes with Teflon lined caps under different conditions with respect to E/S (5−50 U/g protein), temperature (40−60 °C), and pH.[5−9] The enzymatic activity was stopped by increasing temperature to 80 °C for 10 min.[3]

Optimization of PG Deamidation. A central composite design was used to determine the optimum condition for deamidation using three independent variables including E/S, temperature, and pH. The selection of the ranges of these factors was based on available literature[5−8] and on preliminary studies of enzyme properties conducted by the enzyme manufacturer. The experimental plan with the total of 17 combinations is shown in Table 1. The estimated response surface $\hat{y}$ (dependent variable) can be described as a second-order mathematical model:

$$\hat{y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 + \beta_{33} {x_3}^2 \quad (1)$$

where β_0 is the constant term; $\beta_1, \beta_2, \beta_3$ are linear terms; $\beta_{12}, \beta_{13}, \beta_{23}$ are interaction effect terms; $\beta_{11}, \beta_{22}, \beta_{33}$ are quadratic terms of the model, while x_1, x_2, x_3 represent the independent variables in coded values. The predicted regression coefficients of the model were calculated by using SPSS Statistics software version 17.0 (SPSS Inc., Chicago, IL). The surface plots were produced by Statistica software version 8.0 (StatSoft, Inc., Tulsa, OK).

The following deamidation conditions were selected from RSM and used in additional time dependent experiments: reaction temperature of 44 °C, E/S ratio of 40 U/g protein and pH of 7.0. The enzymatic deamidation was performed in triplicate at the indicated conditions for various reaction time periods until 24 h. A control sample of SPI was treated under the same conditions without addition of PG for 24 h. Degree of deamidation (DD) and degree of hydrolysis (DH) were measured at each individual period of time.

Determination of Degree of Deamidation (DD). The DD was determined according to the methods of Yong et al.[7] and Cabra et al.[17] with some modifications. The amount of ammonia released from deamidated glutamine residues was determined by using an ammonia assay kit (Sigma-Aldrich, Inc., St. Louis, MO). The DD was expressed as the ratio (in percentage) of the amount of released ammonia by PG reaction and the total glutamine residues of proteins, which was determined by the released ammonia when protein was treated with 2 N sulfuric acid at 100 °C for 4 h.

Measurement of Degree of Hydrolysis (DH). DH was performed as described by Cabra et al.[17] with some changes. The DH is expressed as the percentage of the dissolved protein in the deamidated soy protein samples after precipitation with 0.2 N trichloroacetic acid (TCA), compared to the total dissolved protein (100%), which was obtained after complete hydrolysis with 2 N sulfuric acid at 100 °C for 4 h.

Determination of Total Soluble Protein (TSP). TSP was determined by using the DC Protein Assay (Bio-Rad Laboratories, Inc.; Hercules, CA) according to the method described by Dia et al.[18] The absorbance was measured at 630 nm. Total soluble protein concentration was quantified using a bovine serum albumin (BSA) standard curve ($r^2 \cong 0.99$).

Sample Preparation for Determination of Functional Properties. Deamidated samples were performed in citrate−phosphate−borate buffer (pH 7.0) containing 60 mg/mL SPI and incubated at various time periods (15 min, 2 h, and 12 h). A control sample of soy protein isolate was treated under the same conditions without PG for 2 h. Five hundred milliliters of SPI in mixed buffer solution with PG (E/S of 40 U/g protein) was incubated in 1000 mL reagent bottles with caps. The temperature was controlled in a water bath at 44 °C. The enzymatic activity was stopped by increasing temperature to 80 °C for 10 min. The resultant solution and precipitate were dialyzed in 0.1 M acetic acid overnight and then freeze-dried. The dried samples were kept at 4 °C for the entire study.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed according to the method described by Laemmli.[19] Precast polyacrylamide gel electrophoresis (4−20% Mini-PROTEAN TGX; catalog number 456-1093, Bio-Rad Laboratories Inc., Hercules, CA) was used in the study. Each protein sample (~2 mg/mL) was diluted (1:1 ratio, volume) with sample loading buffer (950 μL of Laemmli sample buffer, Bio-Rad catalog number 161-0737; and 50 μL of 2-mercaptoethanol for electrophoresis, >98% from Sigma) and then vortexed. Protein samples were boiled for 5 min and then briefly spun (6000 rpm) in a minicentrifuge (Fisher Scientific, Pittsburgh, PA). The samples (30 μL, equivalent to 30 μg of protein) and 5 μL of Precision Plus Protein Dual Color standard (161-0374, Bio-Rad) were loaded into the wells of precast gel, run at 200 V for 30 min (PowerPac 300, Bio-Rad) in Tris/glycine SDS buffer. The gel was placed in fixing buffer (40% methanol and 10% acetic acid) for 15 min and then stained overnight with Coomassie blue. The stained gel was destained using by 10% acetic acid for 20 min and then washed with deionized water. The photo of the gel pattern was taken by

Kodak Image Station 440CF (Eastman Kodak Co., New Haven, CT). Band intensities of protein samples were used for molecular mass calculation.

Circular Dichroism (CD). Soy protein dispersions were prepared at a concentration of 10 μM in phosphate buffer (pH 7.0) at 20 °C. The CD spectrum in the far UV region (190−250 nm) of each sample was determined using a JASCO spectropolarimeter (model J-715, Tokyo, Japan) equipped with a temperature controller and a water bath (Neslab RTE 111; Thermo Neslab, Newington, NH). The samples were analyzed in a 1-cm path length square quartz cuvette with a Teflon cap with a speed of 50 nm/min, resolution 1 nm, sensitivity 50 mdeg, response 0.5 s; 50 scans were averaged. The molar ellipticity values were calculated using the formula given by Kelly et al.[20] as

$$[\theta]_{\text{molar},\lambda}(\text{deg cm}^2\ \text{dmol}^{-1}) = 100 \times \frac{\theta_\lambda}{m} \times d \quad (2)$$

where θ_λ is the observed ellipticity (degrees) at wavelength λ, m is molar concentration of a solute, and d is the path length (cm). Prediction of the percent of protein secondary structure from CD spectra was obtained using software from webserver: http://perry.freeshell.org/raussens.html, which uses the method of Raussens et al.[21]

Determination of Solubility. Solubility was determined in triplicate according to Puppo et al.[22] and Yong et al.[7] with some modifications. The freeze-dried samples (1 mg) were dispersed in acetate−phosphate buffers of various pH values (3.0, 5.0, and 7.0) in 1.5 mL microcentrifuge tubes. All samples were kept at 25 °C overnight and then vortexed. The samples were then centrifuged at 3000 rpm (1000g) at 10 °C by Eppendorf centrifuge model 5417R (Brinkmann Instruments, Westbury, NY) for 10 min. The supernatants (soluble fraction) were collected, and total soluble protein was determined. The solubility was calculated as

$$\text{solubility}\ (\%) = \frac{\text{protein in supernatant (mg/mL)}}{\text{initial protein (mg/mL)}} \times 100 \quad (3)$$

Determination of Emulsifying Properties. Emulsifying activity index (EAI) and emulsion stability index (ESI) of protein samples were determined in triplicate following Pearce and Kinsella[23] and L'Hocine et al.[24] with some changes. Emulsions of protein dispersions were prepared by mixing 10 mL of corn oil (Crisco) with 30 mL of 0.5% (w/v) protein dispersion in 0.1 M acetate−phosphate buffer at pH 7.0. The mixtures were emulsified using homogenizer (Ultra Turrax T18, IKA Works Inc., Wilmington, NC) at 22,000 rpm for 1 min. An aliquot of emulsion was immediately diluted 200 times with 0.1% (w/v) sodium dodecyl sulfate (SDS) solution and also for 15 min after homogenization. The absorbance of diluted emulsion was measured at 500 nm with a UV−visible spectrophotometer (DU-64, Beckman Coulter, USA). EAI and ESI were calculated by the following equation:

$$\text{EAI}\ (\text{m}^2/\text{g}) = \frac{2T \times A_0 \times \text{dilution factor}}{c \times \Phi \times 10000} \quad (4)$$

$$\text{ESI}\ (\text{min}) = \left(\frac{A_0}{A_0 - A_{15}}\right) \times t \quad (5)$$

where T is turbidity (2.303), A_0 and A_{15} are absorbance at time 0 and 15 min, dilution factor is 200, c is the weight of protein per unit volume (g/mL) of protein aqueous phase before forming emulsion, Φ is oil volume fraction of the emulsion (0.23, based on preliminary experiments), and t is time interval (15 min).

Determination of Foaming Properties. Evaluation of foaming capacity (FC) and foaming stability (FS) was performed in triplicate according to the method described by Kanu et al.[25] with some changes. Protein dispersions (0.5%, w/v) were prepared in 0.1 M phosphate buffer at pH 7.0, and then 50 mL of each sample was poured into a 100 mL graduated cylinder. The aqueous sample was mixed using a homogenizer (Ultra Turrax T18, IKA Works Inc., Wilmington, NC) at 18,000 rpm for 1 min inside the cylinder. FC was calculated as the percentage of increasing volume upon mixing. FS was expressed as the percentage of remaining foam after 5, 10, 20, 40, and 60 min without disturbing.

Statistical Analysis. Analysis of variance (ANOVA) and least significant difference (LSD) were used in order to determine the differences among treatments ($p < 0.05$) by SAS (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Optimization of Deamidation of SPI by PG. Experimental data from the central composite design were obtained using 17 combinations of three independent variables: enzyme:substrate ratio (E/S), temperature and pH (Table 1). Two models were fitted with second-order polynomial equations to explain DD and DH using actual values as shown in eqs 6 and 7:

$$\begin{aligned}\text{DD}\ (\%) = {} & -10.218 - 4.132x_1 - 1.555x_2 \\ & + 20.188x_3 - 0.013{x_1}^2 - 2.366{x_3}^2 \\ & + 0.100x_1x_2 + 0.670x_1x_3 \\ & + 0.236x_2x_3 - 0.013x_1x_2x_3 \end{aligned} \quad (6)$$

$$\begin{aligned}\text{DH}\ (\%) = {} & 12.365 - 1.620x_1 - 0.189x_2 \\ & + 0.001{x_1}^2 - 0.005{x_2}^2 - 0.350{x_3}^2 \\ & + 0.029x_1x_2 + 0.211x_1x_3 \\ & + 0.090x_2x_3 - 0.004x_1x_2x_3 \end{aligned} \quad (7)$$

where DD is degree of deamidation (%), DH is degree of hydrolysis (%), and x_1, x_2 and x_3 represent E/S, temperature (°C) and pH, respectively. Both models (eqs 6 and 7) were examined by ANOVA without considering nonsignificant term(s). The coefficients of determination (r^2) of the models (DD and DH) were 0.867 and 0.855, respectively. This means that the models explain 86.7% and 85.5% of the total variation for DD and DH, respectively.

Response surface plots (a) and contour plots (b) generated by the models for DD and DH are shown in Figures 1 and 2, respectively. The plots show the interaction between two independent variables (E/S and pH), while the third variable with the least significance in the fitted model (temperature) was maintained at 44 °C. This was the lowest temperature that gave response values (i.e., DD and DH from the model) that were closest to the experiment data.

Quadratic trends were observed between both independent and dependent variables (Figures 1 and 2). DD increased as a function of E/S until around 36−38 U/g protein, after which it declined with further increases in E/S (Figure 1). In addition, greater DD was obtained at higher pH values, with the highest DD occurring at around pH 7.1−7.4, after which DD declined at the higher pH values. The decreased activity at higher pH values could be explained by a loss of enzyme stability.[26] Furthermore, Yamaguchi et al.[3] showed that PG was most active at a pH range of 5.0−7.0 and then declined slightly at higher pH values. As shown in Figure 2, DH was lowest either at the combination of high E/S and low pH or at low E/S and high pH. The lower deamidation activity at low pH (even at high E/S) was expected since soy protein exhibits a low electrostatic repulsion as it draws closer to its isoelectric point (pI ∼ 4.5), thus reducing the

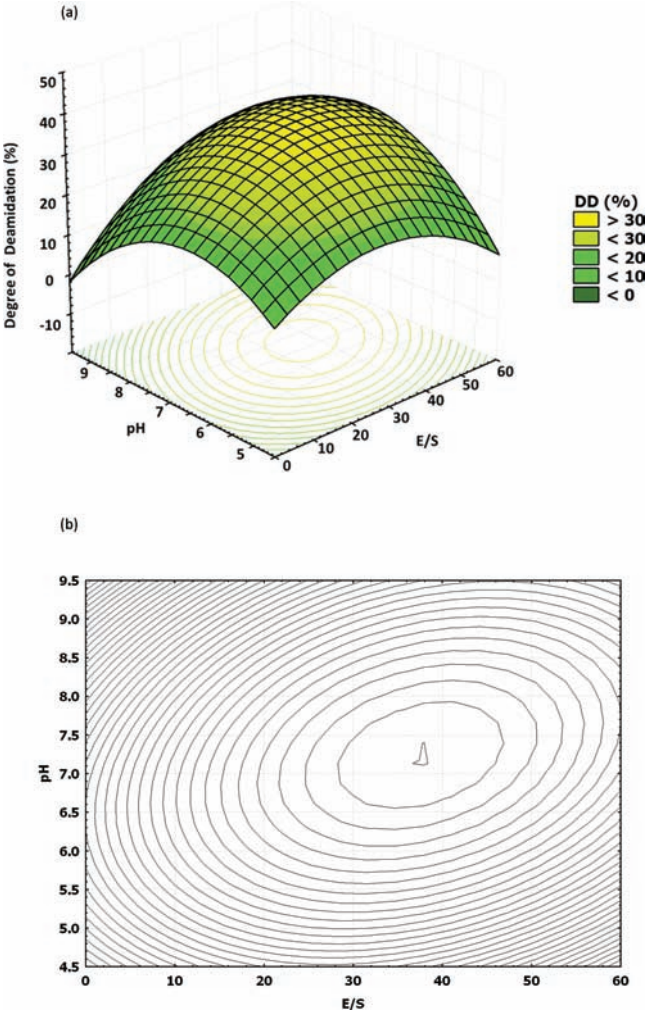

Figure 1. Response surface (a) and contour (b) plots of the effect of enzyme:substrate ratio (E/S) and pH on the degree of deamidation (DD, %) of soy protein isolate (SPI) by protein-glutaminase at 44 °C (from eq 1).

number of sites at which the enzyme can react. In addition, the deamidation activity of PG is lower at higher pH since the p*I* of this enzyme is around 10.0.[3] In contrast, highest DH was observed both at the combination of low E/S and low pH and at high E/S and high pH (Figure 2).

In order to optimize the PG deamidation of SPI using RSM, a high DD and low DH were considered as being the most desirable outcome. Figure 3 shows superimposed contour plots of DD and DH. At the highest value of DD (discussed above), the DH was less than 3%, which was deemed acceptable.

The adequacy of the DD model was confirmed by conducting an additional RSM experiment using same central composite design discussed previously, with 17 combinations of three independent variables (data not shown). The model was also fitted in the second-order polynomial equation as shown in eq 8:

$$\begin{aligned}\mathrm{DD}\ (\%) = {} & -39.656 + 0.548x_1 - 0.153x_2 \\ & + 19.437x_3 - 0.009{x_1}^2 - 0.007{x_2}^2 - 1.829{x_3}^2 \\ & + 0.008x_1x_2 + 0.032x_1x_3 \\ & + 0.114x_2x_3 - 0.001x_1x_2x_3 \end{aligned} \quad (8)$$

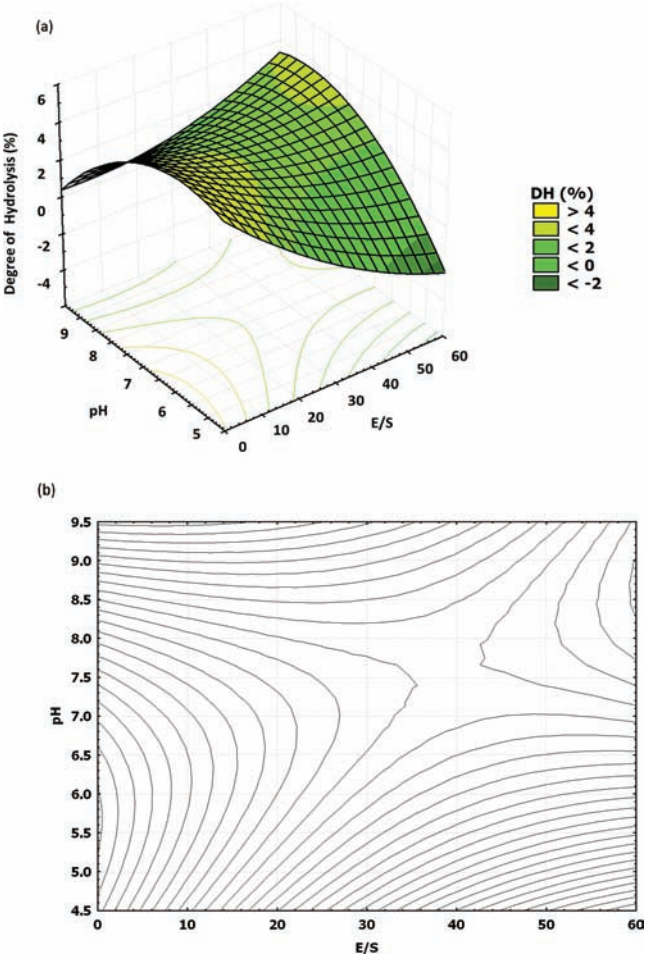

Figure 2. Response surface (a) and contour (b) plots of the effect of enzyme:substrate ratio (E/S) and pH on the degree of hydrolysis (DH, %) of soy protein isolate (SPI) by protein-glutaminase at 44 °C (from eq 2).

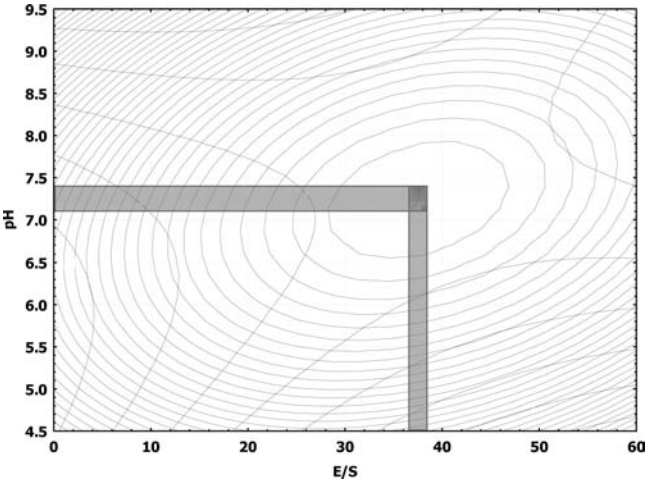

Figure 3. Superimposed contour plots for the response variables: degree of deamidation (DD, %) and degree of hydrolysis (DH, %) showing the optimal region for the deamidation of soy protein isolate (SPI) by protein glutaminase at 44 °C.

The coefficient of determination (r^2) of the model was 0.933, which means the model could explain 93.3% of total variation.

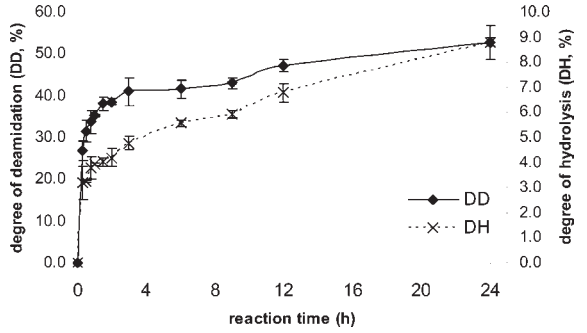

Figure 4. Change in degree of deamidation (DD, %) and degree of hydrolysis (DH, %) as a function of reaction time.

Furthermore, the response surface and contour plots generated for the model from eq 8 with temperature maintained at 44 °C (data not shown) were similar to those shown previously in Figure 1. Based on this model, the optimum conditions for the PG deamidation of SPI for the three variables were (approximately) at a temperature of 44 °C, an E/S of 40 U/g protein and a pH of 7.0. These conditions were used for all additional studies.

Changes in DD and DH as a function of reaction time are shown in Figure 4. DD rapidly increased to about 40% within the first 2 h and then gradually reached a level of about 53% after 24 h. In addition, the reaction might continue after 24 h since it had not yet reached a plateau at 24 h. The appearance of the deamidated SPI solution was more turbid and had lower apparent precipitate and viscosity compared to the control SPI solution (without PG). The rate of PG deamidation of SPI determined in the present study was faster than what was reported for native state α-lactalbumin and α-zein.[5,6] This agrees with results of Yamaguchi et al.,[3] who reported that the specific activity of the PG on SPI (1.170 μmol/min·mg) was higher than for both α-lactalbumin (0.836 μmol/min·mg) and zein (0.655 μmol/min·mg). DH was comparatively lower than DD at all time points, but both showed a parallel pattern. DH increased rapidly within the first 2 h to approximately 4% and then gradually increased to nearly 9% at 24 h.

Previous reports indicated that PG can catalyze the deamidation of protein without proteolysis;[3,7] however, the enzyme used in the present study is a commercial product and might contain some residual protease activity. In addition, it is also possible that some of the increase in DH can be attributed to the effects of deamidation. During deamidation amide groups are converted to carboxyl groups.[2,3] This increases the number of negative charges and causes an increase in the electrostatic repulsion within the protein molecule. As a consequence protein unfolding might occur leading to release of small peptide fragments, thus contributing to the increase in the measured DH.

Reaction times of 15 min, 2 h, and 12 h were used to prepare larger samples under optimal conditions for use in the determination of functional properties. The DD levels for the resulting 15 min, 2 h, and 12 h deamidated protein samples were 32.9, 43.7, and 52.3%, respectively. Meanwhile, the DH levels for these same samples were 3.45, 4.81, and 10.7%, respectively. DD and DH of the large scale samples were higher than those observed for the aforementioned time dependent study, which might be due to the higher substrate concentration used in the large scale reactions (60 mg/mL).[27] In addition, the large scale reactors were stirred throughout the deamidation process, thus providing better mass transfer between the PG and SPI compared with the time dependent experiments.

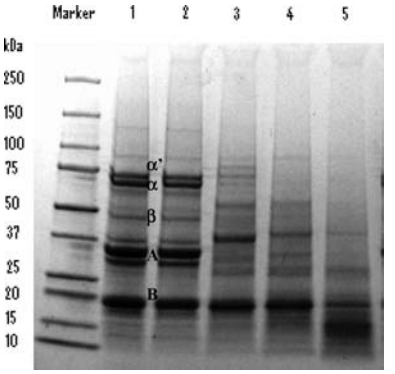

Figure 5. SDS−PAGE patterns for nondeamidated and enzyme deamidated soy protein isolate (SPI): (1) untreated SPI; (2) control SPI (SPI treated without PG); (3) SPI deamidated for 15 min (32.9% DD, 3.45% DH); (4) SPI deamidated for 2 h (43.7% DD, 4.81% DH); (5) SPI deamidated for 12 h (52.3% DD, 10.7% DH). α', α, and β indicate subunits of β-conglycinin (7S); A, B indicate acidic and basic subunits of glycinin (11S), respectively.

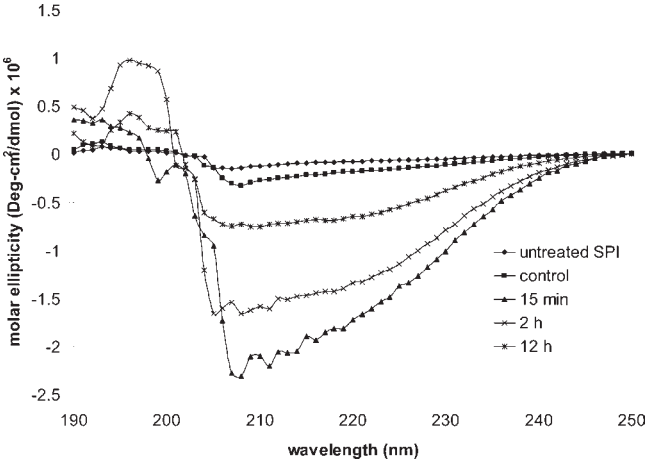

Figure 6. Far UV−CD spectra of nondeamidated and enzyme deamidated soy protein isolate (SPI): untreated SPI; control SPI (SPI treated without PG); SPI deamidated for 15 min (32.9% DD, 3.45% DH); SPI deamidated for 2 h (43.7% DD, 4.81% DH); SPI deamidated for 12 h (52.3% DD, 10.7% DH).

Molecular Mass Distribution of Deamidated SPI. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) profiles of untreated SPI, control SPI, and SPI deamidated for 15 min, 2 h, and 12 h are shown in Figure 5. The average molecular masses of untreated SPI, control SPI, and SPI deamidated for 15 min, 2 h, and 12 h (lanes 1−5, respectively) were approximately 40.2, 42.6, 31.2, 24.5, and 17.4 kDa, respectively. The gel pattern (Figure 5) shows that the deamidated samples (lanes 3−5, respectively) differed from the untreated SPI and control SPI (lanes 1 and 2, respectively). Band patterns for deamidated proteins indicated a downward shift to lower molecular mass units, which is supported by the observed increase in DH as a function of reaction time. However, Yong et al.[6,7] reported that no apparent protein hydrolysis occurred for the PG deamidation of other proteins (α-zein and wheat gluten). The untreated SPI and control had similar SDS−PAGE patterns and were in

Table 2. Secondary Structures[a] of Non-Deamidated and Deamidated Soy Protein Isolate

			deamidation reaction time		
	untreated SPI	control	15 min	2 h	12 h
α-helix (%)	21.3	18.0	10.7	10.2	10.0
β-sheet (%)	22.2	24.9	28.9	25.2	23.4
β-turn (%)	12.5	12.5	12.5	12.5	12.5
random (%)	37.2	37.5	38.5	37.9	38.3
sum (%)	93.2	92.9	90.5	85.8	84.5

[a] Data were derived by analysis of CD spectra.

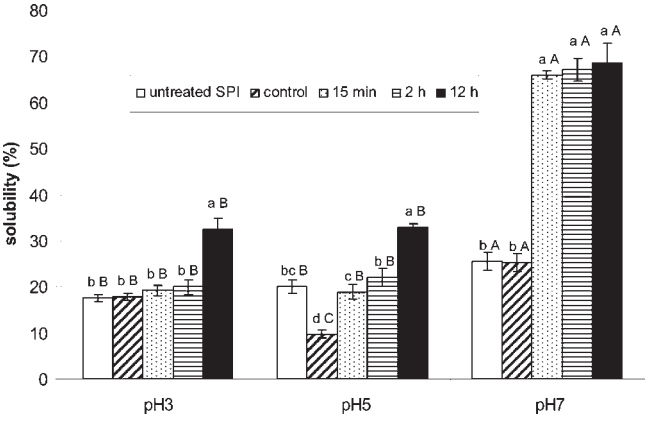

Figure 7. Solubility of nondeamidated and enzyme deamidated soy protein isolate (SPI) under various pH conditions: untreated SPI; control SPI (SPI treated without PG); SPI deamidated for 15 min (32.9% DD, 3.45% DH); SPI deamidated for 2 h (43.7% DD, 4.81% DH); SPI deamidated for 12 h (52.3% DD, 10.7% DH). Same lowercase letters at same pH are not significantly different ($p > 0.05$, $n = 3$); same uppercase letters within the same sample across different pH values are not significantly different ($p > 0.05$, $n = 3$).

agreement with those previously reported in the literature.[28] The 20 kDa band had the highest intensity in the 15 min and 2 h deamidated SPI samples (lanes 3 and 4, respectively), while the SPI deamidated for 12 h produced a high intensity band around 15 kDa. Based on these data, among the various protein fractions in SPI, subunit B of glycinin may be the most resistant to hydrolysis by PG, especially for the shorter reaction times of 15 min and 2 h.

Circular Dichroism (CD). Secondary structure changes caused by deamidation were evaluated by measuring the far-UV CD spectra (190−250 nm) of the above samples. Figure 6 shows CD spectra of the soluble fractions of the control and deamidated SPI samples. The CD spectra of the untreated and control SPI samples had patterns similar to those previously reported for $\alpha + \beta$ proteins,[29] which showed a positive band near 190−195 nm and negative board band at around 210−220 nm. The CD spectra of the deamidated SPI samples indicated that the increase in reaction time resulted in a reduction of the α-helix structure. The α-helix contents of untreated SPI and control SPI samples were around 21.3 and 18.0%, respectively, and then subsequently decreased to 10.7, 10.2, and 10.0% as a result of deamidation for 15 min, 2 h, and 12 h, respectively (Table 2). The 15 min deamidated SPI sample showed the greatest increase in β-sheet structure, which then decreased as a function of further reaction time. The increase in β-sheet formation after deamidation agrees with the results of Yong et al.,[6] who reported that β-sheet structure in deamidated α-zein increased from 24% to 32%. In addition, β-turn structure remained constant, while random coil did not differ among samples. These observations are supported by the SDS−PAGE results (Figure 5), where α' and α fraction band intensities dramatically decreased, indicating that they might have changed their structure to β-sheet form.

Effect of Deamidation on Protein Solubility. The effect of deamidation on the solubility of SPI was evaluated at different pH values (3.0, 5.0, and 7.0) (Figure 7). The solubility of all samples was highest at neutral pH (pH 7.0) and lowest at low pH values (pH 3.0 and 5.0). At pH 3.0, the solubility of the 12 h deamidated SPI sample was the highest (∼32%), while the solubilities of other samples were similarly low (∼18−20%). The solubility data for pH 5.0 was similar to that of pH 3.0 except that the control SPI sample showed the lowest solubility. The lower solubility of the control SPI compared to untreated SPI can be explained by its partial protein denaturation caused by the heating step (80 °C for 10 min). This in turn could have resulted in an increase in the hydrophobicity of protein at the surface after rearrangement, thus reducing its water solubility.[30] Also, after the rearrangement, the p*I* of control SPI might be increased to nearly 5.0 as a result of the increase in positively charged amine groups at the surface of protein molecule in solution. Thus, the solubility of the control SPI was lowest at pH 5.0. The shift in p*I* of soy protein from 4.5 to 5.0 was also reported when the soy protein was hydrothermally cooked (154 °C) for 19 s or longer.[31] At pH 7.0, the solubility of the deamidated samples was much higher (>65%) than that of the untreated SPI and control SPI (∼25%). Therefore PG deamidation for even a short period of time (15 min) can lead to an increase in solubility of SPI at neutral pH. These results are in agreement with those of Yong et al.,[7] who demonstrated that partial deamidation of wheat gluten (1 h, 22% DD) remarkably increased its solubility in a neutral buffer (pH 7.0).

Effect of Deamidation on Emulsifying Properties. Based on the preliminary experiments (data not shown), emulsifying properties could not be reliably measured at pH 3.0 and 5.0 because of the poor solubility of the protein. Yong et al.[6,7] found that deamidated α-zein and wheat gluten displayed excellent emulsifying properties at pH 7.0. Thus, the emulsifying activity index (EAI) and emulsion stability index (ESI) were determined at only pH 7.0 in the present study (Table 3). EAI of all deamidated SPI samples were higher than untreated SPI and control SPI ($p < 0.05$). The 15 min deamidated SPI had the highest EAI, which was slightly higher than the EAI values for the 2 and 12 h deamidated SPI samples. These results indicated that deamidation could improve emulsifying properties by increasing the solubility of SPI, thus enhancing the protein's ability to form a layer around fat globules allowing them to better associate with the aqueous phase of the emulsion.[32] Mirmoghtadaie et al.[33] also stated that the improvement in the emulsifying activity of deamidated oat protein isolate was caused by an increase in solubility and surface hydrophobicity, which resulted in a better balance of the hydrophobic and hydrophilic ratio necessary for emulsification. The slightly lower EAI values for the 2 and 12 h deamidation SPI samples compared to the 15 min deamidation SPI sample can be explained by the higher solubility caused by the increase in deamidation. This could increase the net charge of the protein, which could affect protein−protein interactions of the protein film around the fat droplets.[33] The ESI values for all deamidated SPI samples were higher than for the untreated SPI and control

Table 3. Emulsifying and Foaming Properties of Non-Deamidated and Deamidated Soy Protein Isolate[a]

				foaming stability (%)				
sample	EAI[b] (m^2/g)	ESI[c] (min)	FC[d] (%)	5 min	10 min	20 min	40 min	60 min
untreated SPI	17.5 ± 0.2 c	20.1 ± 0.3 c	26.0 ± 3.5 c	21.1 ± 3.0 cA	15.3 ± 2.1 dB	11.9 ± 1.6 cBC	9.8 ± 2.2 dC	8.5 ± 1.1 dC
control	16.8 ± 0.7 c	20.0 ± 0.3 c	38.7 ± 2.3 b	29.6 ± 1.1 bA	27.9 ± 0.7 bA	25.0 ± 1.3 aB	21.6 ± 1.8 aC	21.6 ± 1.8 aC
15 min	45.0 ± 1.6 a	26.6 ± 0.4 b	54.7 ± 2.3 a	36.6 ± 3.5 aA	30.6 ± 1.3 abB	14.9 ± 0.5 cC	11.4 ± 0.2 cdD	10.8 ± 0.7 cdD
2 h	40.4 ± 1.0 b	31.1 ± 0.9 a	50.0 ± 6.0 a	37.3 ± 2.5 aA	24.0 ± 1.7 cB	18.2 ± 1.4 bC	13.8 ± 0.4 bcD	12.5 ± 0.9 cD
12 h	41.2 ± 0.8 b	29.2 ± 0.9 ab	53.3 ± 6.4 a	39.2 ± 1.7 aA	31.8 ± 2.8 aB	26.1 ± 2.9 aC	17.5 ± 3.8 bD	15.7 ± 3.2 bD

[a] Average ± standard deviation ($n = 3$). Within columns, values with same lowercase letters are not significantly different at $p > 0.05$. Within rows, values with same uppercase letters are not significantly different at $p > 0.05$. [b] Emulsifying activity index. [c] Emulsifying stability index. [d] Foaming capacity.

SPI samples, which also increased as a function of reaction time. The 2 and 12 h deamidated SPI samples had the highest ESI values (~31 min).

Effect of Deamidation on Foaming Properties. Foaming capacity (FC) and foaming stability (FS) were measured in pH 7.0 phosphate buffer (Table 3). FC of the deamidated SPI samples did not differ from one another and were higher than those of the untreated SPI and control SPI samples. This might be due to the increase in solubility caused by deamidation, since foaming is enhanced by soluble proteins.[34] In addition, the FC of the control SPI sample was higher than of that of the untreated SPI sample. Although the increase in % DD enhanced FC, the FS decreased. The FS of the deamidated SPI samples decreased as a function of resting time. This might be due to the reduction of protein−protein interaction, which is affected by the excessive increase in protein charge, which interferes with the formation of a cohesive protein film at the air−liquid interface.[35] These results agree with those of Chan and Ma,[36] who studied the effect of acid deamidation on the functional properties of okara (soymilk residue) protein isolate and found that higher DD leads to greater foaming ability. In that study, the FS decreased over resting time, and also decreased with higher DD. Kanu et al.[25] also reported a similar result, in that the FC of hydrolyzed defatted sesame flour protein was higher than that of nonhydrolyzed sample. Furthermore, the FS also decreased over resting time.

In conclusion, optimization of the enzymatic deamidation of SPI by PG was successfully carried out using RSM. The optimum conditions to obtain a deamidated SPI with high DD and acceptably low DH was a temperature of 44 °C, an E/S of 40 U/g protein and a pH of 7.0. The deamidated SPI had enhanced solubility in both acidic and neutral conditions. The higher DD (longer deamidation time) showed better emulsification properties, including both EAI and ESI. Furthermore, the deamidated SPI had higher FC, but decreased FS over resting time. Deamidation has great potential to produce SPI with modified functional properties that can be used for various purposes in the food industry, especially for use in acidic soy-based beverages. However, studies on the conformational changes and other functional properties, such as impact on the flavor profile and flavor binding properties, are still needed.

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