Effect of Limited Proteolysis on the Enzymatic Phosphorylation of Soy Protein

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The effects of limited proteolysis on the enzymatic phosphorylation of commercial soy protein isolate were evaluated for several proteases. Proteases were chosen on the basis of their ability to hydrolyze peptide bonds between specific amino acids and included trypsin (basic), chymotrypsin (aromatic), endoproteinase Glu-C from *Staphylococcus aureus*, strain V8 (acidic), and Pronase E (nonspecific). Hydrolyzed soy isolate was phosphorylated with the catalytic subunit of bovine cardiac muscle protein kinase, and phosphorylation was followed by incorporation of ^{32}P into the protein. Phosphorylation decreased with increased proteolysis for all proteases. Decreased phosphorylation was most pronounced for Pronase E-hydrolyzed protein and least pronounced for trypsin-hydrolyzed soy isolate. The solubility−pH profile of the trypsin-hydrolyzed, phosphorylated soy isolate was compared to those of both the untreated and the hydrolyzed profiles. There was no significant difference in solubility among the profiles over the pH 3−6 range.

Keywords: *Polypeptide; modification; functionality; hydrolysis; interaction*

INTRODUCTION

Many food proteins, especially of plant origin, require modification to achieve the proper functional properties for use as food ingredients. For example, commercial soy protein isolate (SPI) has limited solubility at acidic pH, which negatively impacts its use in acidic foods (Wolf, 1970; Kinsella, 1979; Kinsella et al., 1985). Solubility is a particularly important functional property since other functional properties in food systems, such as emulsifying activity, are strongly correlated to solubility (Kinsella et al., 1985). Improved solubility of SPI at acidic pH values has been achieved by both chemical and enzymatic modifications. However, chemical modification is less desirable because of the presence of unreacted chemicals, unwanted side reactions, and harsh reaction conditions. Enzymatic modification is generally more specific and generates a uniform product under milder reaction conditions without unwanted side reactions.

Enzymatic modification of soy protein to improve solubility under acidic conditions has been achieved by either limited proteolysis (Adler-Nissen, 1976), deamidation (Hamada and Marshall, 1989; Hamada, 1994), or phosphorylation (Seguro and Motoki, 1990; Campbell et al., 1992). Soy protein solubility can also be enhanced by a combination of the above enzymatic modifications. Hamada and Marshall (1988) observed that limited proteolysis followed by deamidation with peptidoglutaminase increased the degree of deamidation and, as a result, increased solubility in the pH 2−10 range. Casella and Whitaker (1990) reported on the chemical phosphorylation and deamidation of zein hydrolysates generated using trypsin for limited specific hydrolysis. Chemical phosphorylation and deamidation of the hydrolysates increased the water solubility in the pH range from 4 to 10, but the emulsifying and foaming properties were lower as compared to those of the native protein. To the authors' knowledge, no studies have appeared that document the enzymatic phosphorylation of enzymatically hydrolyzed soy protein. The present investigation attempts to determine the effect of prior proteolysis on the extent of enzymatic phosphorylation of soy protein.

MATERIALS AND METHODS

Materials. SPI (Purina protein 620; 91% protein, dwb) was a gift from Ralston Purina Co., St. Louis, MO, and [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from DuPont, Boston, MA. Pronase E (Type XXV), trypsin (Type I), endoproteinase Glu-C (XVII-B), α-chymotrypsin (Type I-S), and the catalytic subunit of bovine cardiac muscle protein kinase (EC 2.7.1.37) were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals used were of reagent grade.

Preparation of Hydrolyzed SPI. A 10 mg/mL suspension of SPI in water was solubilized by heating to 50 °C, and the pH was adjusted to 8.0 with 0.1 N NaOH. Various proteases were added to 90 mL of protein solution and incubated at 50 °C for various time periods. Appropriate amounts of enzyme (α-chymotrypsin, 10−20 mg; trypsin, 1−3 mg; endoproteinase, 1.1 mg; Pronase E, 2 mg) were used to achieve the desired degree of hydrolysis (DH) for SPI. Proteolysis was terminated by heating the protein mixture to 95 °C for 5 min to inactivate the proteases. For the control (DH = 0%), proteases were heat inactivated before they were added to the protein solution. DH was estimated using the equation (Hill et al., 1982)

$$\mathrm{DH} = [(BN)/(\alpha(\mathrm{mp})h_{\mathrm{total}})] \times 100 \qquad (1)$$

where B = mL of NaOH added, N = normality of NaOH, α = temperature and pH correction factor and is 1.13 at 50 °C and pH 8.0, mp = mass of protein in grams, and h_{total} = hydrolysis equivalent and is 7.75 for soy protein. Using eq 1, the amount of NaOH at a specific normality can be determined which will yield a preselected degree of hydrolysis. Therefore, the proteolysis was terminated when a specific amount of NaOH was used to maintain the pH at 8.0 as determined from eq 1. The hydrolyzed SPI was lyophilized, and the degree of hydrolysis was verified by the trinitrobenzenesulfonic acid (TNBS) method for the determination of free amino groups (Alder-Nissen, 1979).

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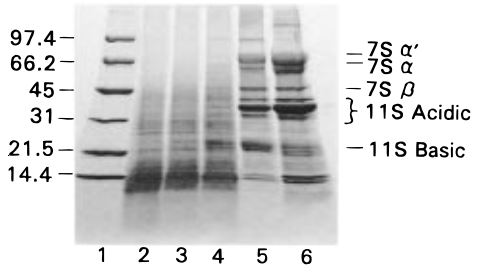

Figure 1. Gradient SDS−PAGE of trypsin-hydrolyzed SPI: lane 1, M_w standard; lanes 2−5, DH of 2.0%, 0.9%, 0.4%, 0.0%, respectively; lane 6, SPI.

Lyophilized SPI hydrolysates were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The hydrolysates (20 μg of protein per gel lane) were mixed with an equal volume of 2× electrophoresis treatment buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol) and heated at 90 °C for 2 min. SDS−PAGE (4−20% gradient) was performed using the method of Laemmli (1970). The Coomassie blue R-250 stained gels were scanned by densitometry at 560 nm with a Shimadzu spectrophotometer.

Phosphorylation of Hydrolyzed SPI. Hydrolyzed SPI was phosphorylated using the catalytic subunit of bovine cardiac muscle protein kinase and ATP for 2 h at 37 °C as previously described (Campbell et al., 1992). The phosphorylated protein was examined by quantitative analysis using scintillation counting and by qualitative analysis using SDS−PAGE (4−20%) followed by autoradiographic analysis. For the large-scale phosphorylation assay (up to 350 mg of SPI), all reactants were scaled-up linearly, nonradioactive ATP was substituted for radioactive ATP, and the protein kinase reaction was performed at 37 °C for 12 h with occasional mixing of reactants. Hydrolyzed SPI was incubated under the same conditions in the absence of protein kinase to obtain a nonphosphorylated control. A small-scale phosphorylation assay using $^1/_{10}$ the amount of material as the larger-scale phosphorylation was performed using radiolabeled ATP to determine the amount of phosphate incorporated by SPI. Nonphosphorylated SPI and phosphorylated SPI were desalted and concentrated by ultrafiltration using an Amicon stirred cell equipped with a PM-10 membrane.

Solubility−pH Profile. Protein solubility was determined using the method of Morr et al. (1985) with modification. SPI (1.0 mg/mL) was suspended in 0.1 M sodium acetate buffer at various pH values between 3 and 6. The suspensions were stirred for 2 h at 2 °C and then passed through a 0.45 μm filter. The filtrate was diluted 1:2 and the absorbance recorded at 280 nm. The percent solubility was calculated using the expression

$$\% \text{ solubility} = (A_{pH}/A_{max}) \times 100 \quad (2)$$

where A_{pH} = absorbance of protein in the filtrate at a specific pH and A_{max} = absorbance of protein at pH 12.

RESULTS

Characterization of Hydrolyzed SPI. The hydrolysates generated by treatment with the various proteases were examined using SDS−PAGE and scanning densitometry (data not shown). The extent of disappearance of 7S subunits and 11S acidic and basic polypeptides due to proteolysis was determined from the gel profiles. Increased trypsin hydrolysis of SPI showed progressive reduction of 7S subunits and 11S polypeptides and a progressive increase in low molecular weight peptides at the bottom of the gel (Figure 1). At the highest DH value of 2.0, all 7S subunits had disappeared along with nearly complete hydrolysis of the 11S basic subunits. Proteolysis of the 11S acidic subunits was not as extensive. Since trypsin preferentially

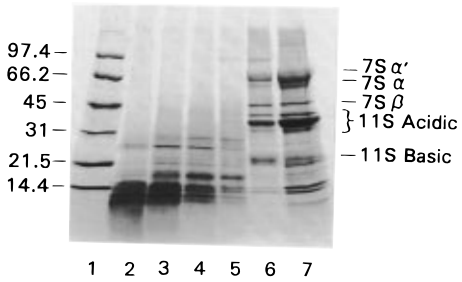

Figure 2. Gradient SDS−PAGE of chymotrypsin-hydrolyzed SPI: lane 1, M_w standard; lanes 2−6, DH of 4.0%, 2.0%, 1.4%, 0.3%, 0.0%, respectively; lane 7, SPI.

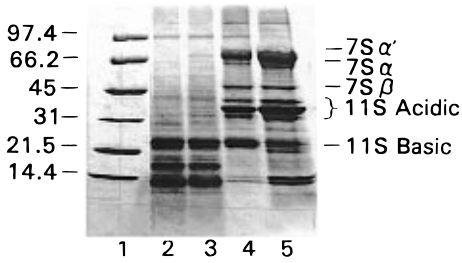

Figure 3. Gradient SDS−PAGE of endoproteinase-hydrolyzed SPI: lane 1, M_w standard; lanes 2−4, endoproteinase DH of 1.4%, 1.0%, 0.0%, respectively; lane 5, SPI.

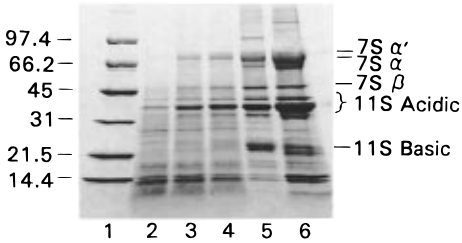

Figure 4. Gradient SDS−PAGE of Pronase E-hydrolyzed SPI: lane 1, M_w standard; lanes 2−5, DH of 3.8%, 2.6%, 1.9%, 0.0%, respectively; lane 6, SPI.

hydrolyzes peptide bonds adjacent to basic amino acids, and since the acidic polypeptides have the smallest percentage of basic amino acids per 20 kDa unit of any of the soy globulins (Moreira et al., 1981; Staswick et al., 1984; Coates et al., 1985; Fukazawa et al., 1985), our results could be anticipated. Incubation of SPI in the presence of α-chymotrypsin resulted in the extensive disappearance of 7S subunits and 11S acidic polypeptides even at the lowest DH of 0.3% (Figure 2). Basic polypeptides were more refractory toward the enzyme. These results suggest that basic polypeptides may have the lowest percentage of aromatic amino acids per 20 kDa unit of the soy globulins. Indeed, this is verified by the amino acid analyses of these subunits and polypeptides (Fukushima, 1985). Endoproteinase hydrolysis of SPI caused degradation of the 7S subunits and the 11S polypeptides (Figure 3). Among the 11S polypeptides, the acidic fraction appeared to be degraded more extensively, probably because of its higher content of acidic amino acids, which provide hydrolysis sites for endoproteinase. Pronase E hydrolyzed soy globulins in a nonpreferential manner, with both 7S and 11S fractions extensively proteolyzed (Figure 4). Since the sites of proteolysis for Pronase E are nonspecific, the SDS−PAGE gel profile observed is not unexpected.

Phosphorylation of Hydrolyzed SPI. All proteases produced hydrolysis products that generally showed a decrease in phosphate incorporation with an increase in DH (Figure 5). The SPI hydrolysates produced by incubation with trypsin showed the highest

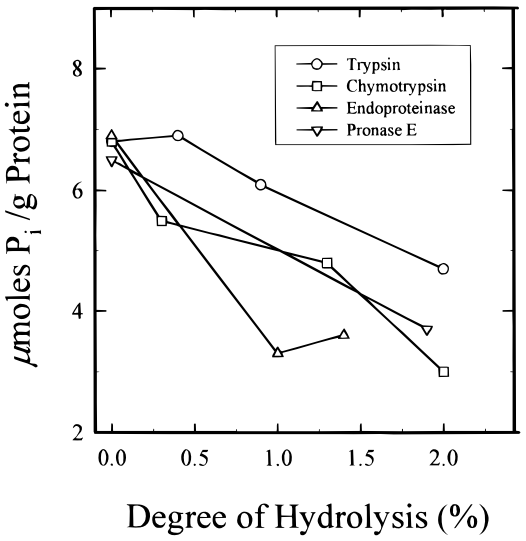

Figure 5. Phosphorus incorporation into SPI hydrolysates as a function of DH.

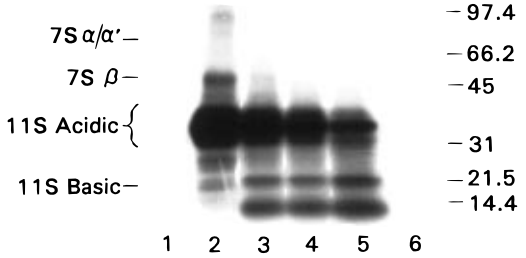

Figure 6. Autoradiogram of SDS−PAGE of phosphorylated SPI (trypsin hydrolyzed): lanes 2−5, trypsin DH of 0.0%, 0.4%, 0.9%, 2.0%, respectively.

level of phosphate incorporation at a specific DH value. Moreover, phosphate incorporation into trypsin-hydrolyzed SPI was slightly higher than the control at a DH of 0.4%.

Since phosphorylated hydrolysates from trypsin proteolysis had the highest level of phosphorylation, these samples were used for further study. Autoradiography of the samples at different degrees of proteolysis showed decreased incorporation of radiolabeled inorganic phosphate in the 7S β subunit upon proteolysis (Figure 6). The SPI fraction most heavily phosphorylated was the 11S acidic polypeptides, both before and after proteolysis. The basic polypeptides were also phosphorylated, but as the samples were further hydrolyzed, a significant portion of the labeled phosphate appeared in the low M_w (<14 400) fraction. A likely source of this low M_w material is the hydrolysis products of acidic polypeptides. This statement is supported by the evaluation of a timed protein kinase assay for a trypsin-generated hydrolysate at DH = 0.9%. Figure 7 shows labeled inorganic phosphate incorporated after 5 min into acidic polypeptides and low M_w material from the hydrolysis of these polypeptides. The low M_w band was more intense after 5 min than the acidic polypeptide band. During the course of the assay, label was incorporated into both the acidic and basic polypeptides along with the low M_w fraction. No label was observed in the 7S subunits, although some α′/α and β subunits were not hydrolyzed at a DH = 0.9% (Figure 1). Campbell et al. (1992) showed that significant phosphorylation of 7S subunits does not occur until about 2 h of exposure to the protein kinase.

Solubility−pH Profile. Nontreated SPI, trypsin-hydrolyzed SPI (DH = 0.9%), and trypsin-hydrolyzed

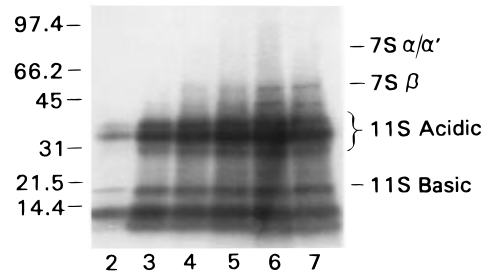

Figure 7. Autoradiogram of SDS−PAGE of timed protein kinase assay to produce phosphorylated SPI (trypsin hydrolyzed; DH of 0.9%): lanes 2−7, 5, 15, 30, 45, 60, 120 min, respectively.

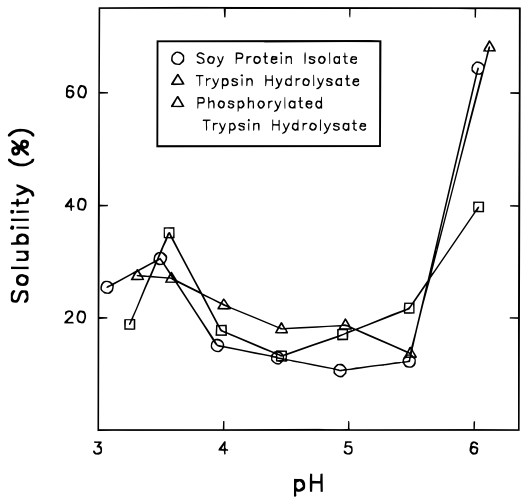

Figure 8. Solubility profile as a function of pH for SPI.

(DH = 0.9%) and phosphorylated SPI were used to evaluate the effect of enzymatic modification on solubility in the pH 3−6 range. Compared with the control (intact SPI), both the trypsin hydrolysate and the phosphorylated trypsin hydrolysate showed slight improvement in solubility in the pH 3−6 range (Figure 8). There was no significant difference in solubility of the phosphorylated SPI over the trypsin-hydrolyzed SPI.

DISCUSSION

Seguro and Motoki (1990) reported that protein kinase selectively phosphorylated the 11S acidic polypeptides of soy protein and also reported that the acidic polypeptides contained at least 18 phosphorylation sites. Campbell et al. (1992) increased the enzyme dosage and reaction time and found phosphorylation of 11S and 7S globulins. After 2 h of exposure to the protein kinase, the amount of labeled phosphate incorporated was in the following order: 11S acidic polypeptides ≫ 11S basic polypeptides ≫ 7S β subunit > 7S α′/α subunits. In this time period, very little label was incorporated into 7S globulin. Therefore, hydrolyzed SPI which contains largely intact acidic polypeptide should be a desirable substrate for phosphorylation with protein kinase. This seems to be the case with trypsin-treated SPI because the acidic polypeptides were more resistant to trypsin hydrolysis (Figure 1) and the highest level of phosphate incorporation was seen at a specific DH value (Figure 5). Chymotrypsin extensively hydrolyzed 7S and 11S globulins (Figure 2), and this resulted in a lower level of phosphate incorporation at all DH values compared to SPI hydrolyzed using trypsin. Lower phosphorylation levels were also obtained at all DH values using endoproteinase and Pronase E hydrolysates. An im-

portant consideration for maximizing proteolysis and phosphorylation of soy protein is the selective hydrolysis of the various subunits and polypeptides.

Protein conformation is another factor that determines the extent of proteolyis and subsequently the extent of phosphorylation of SPI. The less structured segments of polypeptide chains, such as the C terminus of the 11S subunits, are the most susceptible to enzymatic attack. Consequently, they are the ones most likely degraded during the limited hydrolysis. The same segments are, evidently, the most available for protein kinase and have higher degrees of phosphorylation. Their degradation during proteolysis will result in a decrease in the number of bound phosphate groups per gram of protein during subsequent phosphorylation.

Limited proteolysis or phosphorylation increases a protein's negative surface charge and thereby increases solubility and improves other functional properties such as emulsifying activity and emulsion stability. In the present study, limited proteolysis using trypsin followed by phosphorylation had only a small effect on soy protein solubility. There are two possible explanations for the small change in soy protein solubility with enzymatic modification. While both hydrolysis and phosphorylation cause an increase in charge, the effect of hydrolysis dominated, particularly at high DH. As a result, the effect of phosphorylation would be relatively insignificant for the sample with prior hydrolysis. This result would indicate that most of the increased negative charge in SPI was created by proteolysis rather than phosphorylation. Methods to enhance the incorporation of phosphate into hydrolyzed SPI will be required to improve its functional properties. Suggested methods include the application of other protein kinases where phosphorylating activity may be enhanced by proteolysis. The second explanation deals with the method used to desalt the proteins prior to the solubility experiments. The protein samples were desalted by ultrafiltration using an Amicon stirred cell equipped with a PM-10 membrane (10 000 molecular weight cutoff). For the phosphorylated hydrolysates, a significant portion of the labeled phosphate appeared in the low M_w (<14 400) fraction, and this fraction was probably lost during the desalting procedure. Although ultrafiltration is a faster procedure, future studies should use dialysis for desalting of hydrolysates.

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LITERATURE CITED

Adler-Nissen, J. Enzymatic hydrolysis of proteins for increased solubility. *J. Agric. Food Chem*. **1976**, *24*, 1090−1093.

Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J. Agric. Food Chem*. **1979**, *27*, 1256−1262.

Campbell, N. F.; Shih, F. F.; Marshall, W. E. Enzymatic phosphorylation of soy protein isolate for improved functional properties. *J. Agric. Food Chem*. **1992**, *40*, 403−406.

Casella, M. L. A.; Whitaker, J. R. Enzymatically and chemically modified zein for improvement of functional properties. *J. Food Biochem*. **1990**, *14*, 453−475.

Coates, J. B.; Medeiros, J. B.; Thanh, V. H.; Nielsen, N. C. Characterization of the subunits of beta-conglycinin. *Arch. Biochem. Biophys.* **1985**, *243*, 184−194.

Fukazawa, C.; Momma, T.; Hirano, H.; Harada, K.; Udaka, K. Glycinin A_3B_4 mRNA: cloning and sequencing of double-stranded cDNA complementary to a soybean storage protein. *J. Biol. Chem*. **1985**, *260*, 6234−6239.

Fukushima, D. Recent progress of soybean protein foods: chemistry, technology, and nutrition. *Food Rev. Int*. **1991**, *7*, 323−351.

Hamada, J.; Marshall, W. Enhancement of peptidoglutaminase deamidation of soy protein by heat treatment and/or proteolysis. *J. Food Sci.* **1988**, *53*, 1132−1134, 1149.

Hamada, J.; Marshall, W. Preparation and functional properties of enzymatically deamidated soy proteins. *J. Food Sci.* **1989**, *54*, 598−601, 635.

Hamada, J. Deamidation of food proteins to improve functionality. *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 283−292.

Hill, B. S.; Snyder, H. E.; Wiese, K. L. Use of the pH Stat to evaluate trypsin inhibitor and tryptic proteolysis of soy flours. *J. Food Sci*. **1982**, *47*, 2018−2020, 2059.

Kinsella, J. Functional properties of soy proteins. *J. Am. Oil Chem. Soc.* **1979**, *56*, 242−258.

Kinsella, J. E.; Damodaran, S.; German, B. Physicochemical and functional properties of oilseed proteins with emphasis on soy protein. *New Food Proteins* **1985**, *5*, 107−178.

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

Moreira, M. A.; Hermodson, M. A.; Larkins, B. A.; Nielsen, N. C. Comparison of the primary structure of the acidic polypeptides of glycinin. *Arch. Biochem. Biophys.* **1981**, *210*, 633−642.

Morr, C. V.; German, B.; Kinsella, J. E.; Regenstein, J. M.; van Buren, J. P.; Kilara, A.; Lewis, B. A.; Mangino, M. E. A collaborative study to develop a standardized food protein solubility procedure. *J. Food Sci.* **1985**, *50*, 1715−1718.

Seguro, K.; Motoki, M. Functional properties of enzymatically phosphorylated soybean proteins. *Agric. Biol. Chem*. **1990**, *54*, 1271−1274.

Staswick, P. E.; Hermodson, M. A.; Nielsen, N. C. The amino acid sequence of the A2B1a subunit of glycinin. *J. Biol. Chem*. **1984**, *259*, 13424−13430.

Wolf, W. J. Soybean proteins: their functional, chemical, and physical properties. *J. Agric Food Chem*. **1970**, *18*, 969−976.

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