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Enzymatic Phosphorylation of Soy Protein Isolate for Improved Functional Properties

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A commercial soy protein isolate (SPI) was phosphorylated using the catalytic subunit of a commercially available protein kinase from bovine cardiac muscle. On the basis of scintillation counting and autoradiography, incorporation of $^{32}P_i$ into SPI increased with increased incubation time and reached a level of 13 μmol of phosphorus incorporated/g of protein after a 4-h incubation at 37 °C. SDS-PAGE and autoradiography of timed protein kinase assays showed $^{32}P_i$ initially incorporated into glycinin acidic polypeptides and then into glycinin basic polypeptides. Very little $^{32}P_i$ was associated with the β, α/α' subunits of β-conglycinin. Compared with nonphosphorylated SPI, the phosphorylated protein showed significantly improved solubility and emulsifying activity over a pH range of 3-6. Emulsion stability and foam expansion were also significantly improved with phosphorylation, but foam stability was lower using the phosphorylated protein.

INTRODUCTION

Soy protein isolate (SPI) has gained importance as a food ingredient because of its high protein content and good functional properties in some food systems (Wolf, 1970; Kinsella et al., 1985; Peng et al., 1984). Unfortunately, SPI has limited use in acidic foods due to poor solubility and emulsifying ability in the pH 3-6 range (Kinsella et al., 1985). Therefore, SPI cannot expand into high-volume food products such as coffee whitener and pourable and nonpourable dressings without modification to improve its solubility and emulsification properties under the acidic conditions present in these products. The introduction of negatively charged phosphate groups by either chemical or enzymatic methods could improve solubility and emulsification properties of SPI in mildly acidic foods. Chemical modification has been used to phosphorylate soy protein for this purpose (Matheis, 1991; Matheis and Whitaker, 1984; Hirotsuka et al., 1984). However, chemical modification is not very desirable for food applications because of harsh reaction conditions, nonspecific chemical reagents, and the difficulty of removing unreacted reagents from the final product. Enzymatic phosphorylation of soy protein could prove to be a more acceptable method of modification. Enzyme reactions are generally more specific and generate uniform product under milder conditions (Cheetham, 1986; Feeney and Whitaker, 1985).

Protein kinase (EC 2.7.1.37) has been used to phosphorylate food proteins. Ross (1989) used a commercially available protein kinase to successfully phosphorylate SPI. In this study we have examined the kinetics of the enzymatic phosphorylation of SPI and investigated the effects of phosphorylation of SPI on its functional properties in the acidic pH range.

MATERIALS AND METHODS

Materials. Soy protein isolate (Purina protein 620) was a gift from Ralston Purina Co., St. Louis, MO, [γ-^{32}P]-ATP (3000 Ci/mmol) was purchased from Du Pont, Boston, MA, BCA (bicinchoninic acid) protein assay reagent was procured from Pierce Chemical Co., Rockford, IL, and the catalytic subunit of bovine cardiac muscle protein kinase (EC 2.7.1.37) was purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were of reagent grade.

Sample Preparation. SPI was solubilized in 0.1 M sodium phosphate buffer (pH 8.0) at a concentration of 5 mg/mL and heated to 90 °C for 5 min in a water bath. The protein solution was cooled and filtered through two layers of miracloth. The protein concentration was determined using the bicinchoninic acid method (BCA) of Smith et al. (1985), and the final concentration was adjusted to 0.1% using 0.1 M sodium phosphate buffer (pH 8.0). Bovine serum albumin, diluted in 0.1 M sodium phosphate buffer (pH 8.0), was used as the protein standard.

Phosphorylation of SPI. SPI was phosphorylated in a reaction mixture containing 2 mM $MgCl_2$, 5 mM DTT, 0.07% SPI, 60 μM ATP (100-250 cpm/pmol), and 2.5 units of protein kinase in a 100-μL reaction volume (Ross and Bhatnagar, 1989). The specific activity of the kinase used was 36-56 pmol of ^{32}P transferred min^{-1} (mg of casein)$^{-1}$ at pH 6.5 at 30 °C. The mixture was incubated at 37 °C in triplicate for various time periods. The enzymatic reaction was stopped by removing aliquots (30-50 μL), transferring to phosphocellulose paper, and washing the paper with 10% trichloroacetic acid/10 mM pyrophosphate. The amount of $^{32}P_i$ incorporation was determined by liquid scintillation counting as described previously (Ross and Bhatnagar, 1989). For polyacrylamide gel electrophoresis analysis, the protein kinase reaction was performed as stated previously and the enzymatic reaction was stopped by adding an equal volume of 2× electrophoresis treatment buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol) and heating at 90 °C for 1.5-2 min. SDS-polyacrylamide gel electrophoresis (4-20% gradient) was performed using the method of Laemmli (1970). Autoradiographic analysis was performed on gels stained with Coomassie Brilliant Blue (R-250), dried, and stored at -70 °C with a sheet of X-ray film (Kodak X-OMAT RP) in direct contact with the gel.

When larger amounts (up to 350 mg) of phosphorylated SPI were required for functionality tests, all reactants were increased by a linear scale-up, nonradioactive ATP was substituted for radioactive ATP, and the protein kinase reaction was performed at 37 °C for 4 h with occasional mixing of reactants. SPI was incubated under the same conditions in the absence of protein kinase to obtain a nonphosphorylated control sample. The phosphorylated soy protein product was desalted by ultrafiltration using an Amicon stirred cell equipped with a PM-10 membrane.

Solubility-pH Profile. Protein solubility was measured using the procedure of Morr et al. (1985) with slight modification. The protein solution (0.5 mg/mL in 0.1 M sodium phosphate buffer) at each pH was filtered using a 0.45-μm filter, and the protein concentration of the filtrate for each pH was determined using the BCA protein assay. Unmodified protein similarly treated at pH 12 was used for maximum solubility. The percent solubility was calculated using the formula

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

where A_{pH} is the absorbance of protein in the filtrate at the indicated pH values and A_{max} is the absorbance of SPI at pH 12.

Solubility–Calcium Profile. Protein solubility was measured as stated above at pH 7 using varying concentrations of $CaCl_2$ in 0.1 M Hepes (pH 7.0). Protein solution containing no $CaCl_2$ was used for maximum solubility.

Emulsifying Activity–pH Profile. The emulsifying activity was determined using the method of Pearce and Kinsella (1978). The emulsions were prepared by homogenizing protein samples with a IKA Ultra-Turrax T25 mixer at 20 500 rpm for 30 s at room temperature. The samples consist of 9 mL of 0.1% protein solution in 0.3 M sodium phosphate buffer at various pH values and 3 mL of peanut oil. The emulsions were diluted to 1/1000 using 0.1% SDS and 0.1 M NaCl, and the absorbance at 500 nm was determined. The emulsifying activity index (EAI) was expressed as interfacial area per unit weight of protein (m^2/g).

Emulsion Stability. The stability of heated emulsions was determined using the method of Huang and Kinsella (1987). The emulsions were heated at 80 °C for various times up to 20 min and diluted 1/1000 with 0.1% SDS and 0.1 M NaCl, the absorbance was determined at 500 nm, and the EAI was calculated.

Foaming Properties. The foaming properties were measured using the method of Yasumatsu et al. (1972) with slight modification. Ten milliliters of protein solution (10 mg/mL in water) was homogenized at 24 000 rpm for 30 s at room temperature in a 3 cm diameter glass container. The resultant foam height (centimeters) was used for foam expansion. The foam height was measured after the solution had stood for 30 min at room temperature and used to calculate percent foam stability by the formula

$$\% \text{ FS} = (FH_2/FH_1) \times 100$$

where FH_2 is the foam height at 30 min and FH_1 is the foam height at 0 min.

Isoionic Point. The isoionic point was determined using the procedure of Ho and Waugh (1965). The protein solution was desalted by ultrafiltration and diluted using deionized water for a final concentration of 1 mg/mL. The protein solution (25 mL) was mixed with 3.0 g of AG 501-X8D (Bio-Rad; mixed bed ion-exchange resin) and stirred at 750 rpm for 1 h. The solution was allowed to settle for 1 h and the pH determined.

Moisture Sorption. Moisture sorption was estimated using the method of Lang et al. (1981). Lyophilized protein (50 mg) was placed on Whatman filter paper (5.5 cm; weighted) and incubated in a desiccator containing a saturated potassium chloride solution (a_w = 0.8295; Greenspan, 1976) at 34 °C for 7 days. The sample and filter paper were removed from the desiccator, weighed, and incubated in a vacuum oven at 60 °C and 760 mmHg for 5 h. The sample and filter paper were weighed after they were removed from the oven. Moisture sorption was calculated from the weight of moisture (milligrams) divided by the weight of sample (milligrams).

Statistical Methods. Functional properties of phosphorylated and nonphosphorylated soy protein isolate were compared using *t*-tests. All curve comparisons were made using analysis of covariance (Steel and Torrie, 1980; *SAS/STAT*, 1989).

RESULTS

SPI Phosphorylation. The amount of phosphorus incorporated into SPI increased with increased incubation time, and 13 μmol of phosphorus was incorporated/g of protein after 4 h at 37 °C (Figure 1). Autoradiography of the gels showed $^{32}P_i$ incorporated into the glycinin acidic polypeptides within the first minute of the phosphorylation reaction. After 2 min, there was incorporation of $^{32}P_i$ into the glycinin basic polypeptides, and after 10 min, $^{32}P_i$ incorporation was also observed to occur within the β and α/α′ subunits of β-conglycinin (Figure 2).

Functionality Tests. SPI showed significantly improved solubility (P = 0.0001) over the pH range 3–6 when compared with SPI (Figure 3). SPI-P was 19% more soluble at pH 3, 25% more soluble at pH 4, and 43% more soluble at pH 5 than SPI. There was no significant difference in the solubilities of SPI and SPI-P using $CaCl_2$ in a concentration range of 0–20 mM (Figure 4). The emulsifying activity of SPI increased significantly (P < 0.01) over the pH range 3–6 after phosphorylation (Figure 5). The EAI for SPI-P increased 27% at pH 4 when

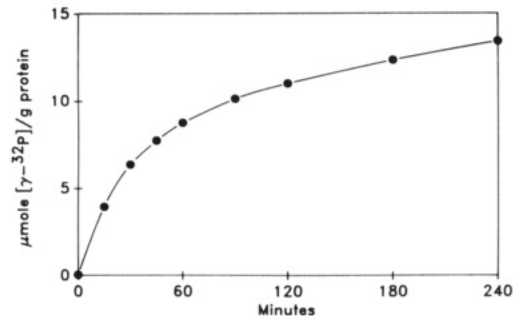

Figure 1. Effect of reaction time on the phosphorylation of denatured SPI. Each point represents the mean of 10 determinations with a standard error of less than 0.8.

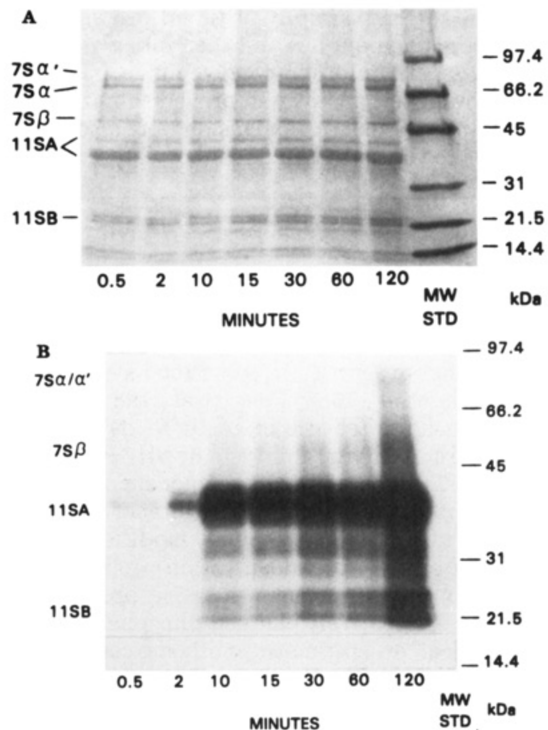

Figure 2. (A) Gradient SDS–PAGE of timed protein kinase assay. 11SA, 11S acidic subunit; 11SB, 11S basic subunit. (B) Autoradiogram of SDS–PAGE in (A).

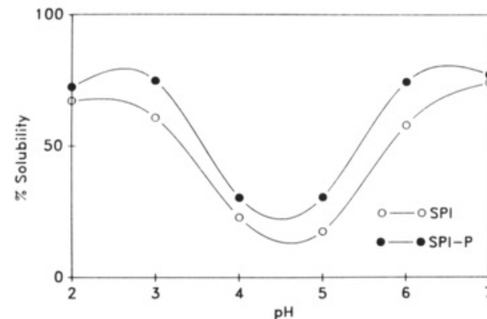

Figure 3. Effect of pH on percent solubility of native and modified soy protein isolate. Each point represents the mean of 15 determinations. Using analysis of covariance with a quadratic curve fit between pH 3 and pH 6, the two curves were significantly different (P = 0.0001), showing similar trends with respect to pH.

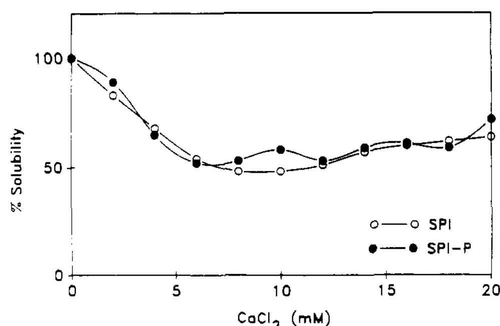

Figure 4. Solubility of native and modified soy protein isolate using $CaCl_2$ concentrations of 0–20 mM. Each point represents the mean of nine determinations. Using analysis of covariance with a quadratic curve fit, there was no significant difference ($P > 0.05$) between the two curves.

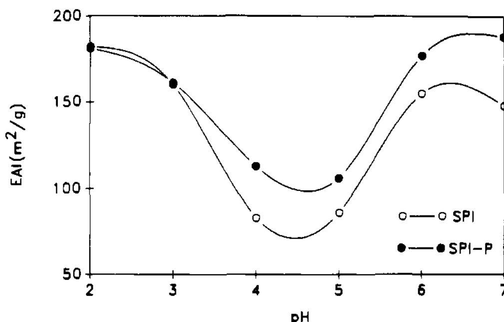

Figure 5. Emulsifying activity of native and modified soy protein isolate at pH values 2–7. Each point represents the mean of 17 determinations. Using analysis of covariance with a quadratic curve fit between pH 3 and pH 6, the two curves were significantly different ($P < 0.01$), showing similar trends with respect to pH.

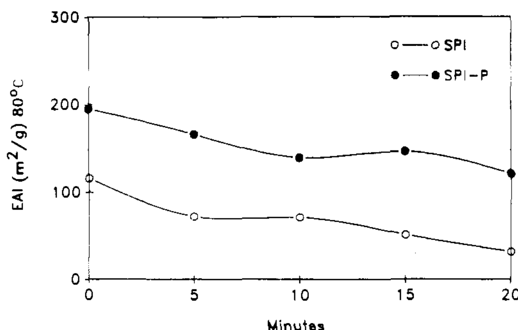

Figure 6. Emulsion stability of native and modified soy protein isolate at pH 7.0. Each point represents the mean of nine determinations. Using analysis of covariance with a linear curve fit, the two curves were significantly different ($P = 0.0001$), showing similar trends with respect to time.

compared with that of SPI. There was also significant improvement ($P = 0.0001$) in emulsion stability at pH 7 for the phosphorylated soy protein (Figure 6). The heat stabilities of emulsions formed with SPI-P were twice as great as those of emulsions formed with SPI. There was a significant increase ($P = 0.01$) in foam expansion and a significant decrease ($P < 0.001$) in percent foam stability after phosphorylation (Table I). There was no significant difference ($P > 0.05$) in the isoionic point or moisture sorption after phosphorylation (Table I).

DISCUSSION

The major soybean storage proteins, glycinin and β-conglycinin, were phosphorylated using the catalytic subunit of a commercial protein kinase. Seguro and Motoki (1989) reported that protein kinase phosphorylated glycinin acidic polypeptides but did not phosphorylate glycinin basic polypeptides or any of the β-conglycinin subunits. We found more extensive phosphorylation by using more units of protein kinase in our assay and monitoring the reaction for various time intervals up to 4 h. Our results show sequential and selective phosphorylation of the major soybean globulins. This information could be used to selectively phosphorylate specific globulins. Also, the order in which the polypeptides and subunits are phosphorylated corresponds to the number of potential phosphorylation sites in the known primary structures for these molecules (Moreira et al., 1981; Staswick et al., 1984; Coates et al., 1985; Fukazawa et al., 1985; Utsumi et al., 1987).

Table I. Functional Properties of Phosphorylated and Nonphosphorylated Soy Protein Isolate[a]

property	SPI	SPI-P
isoionic pH	4.91 ± 0.19 (9)[b]	4.83 ± 0.14 (9)[b]
moisture absorption, mg of H_2O/mg of protein	0.65 ± 0.10 (4)[b]	0.57 ± 0.01 (3)[b]
foam expansion, cm	3.1 ± 0.1 (3)[c]	3.8 ± 0.1 (3)[c]
foam stability, %	74 ± 1 (3)[d]	53 ± 3 (3)[d]

[a] Mean ± standard error with the number of observances in parentheses. [b] $P > 0.05$. [c] $P < 0.05$. [d] $P < 0.001$.

The solubility of SPI at pH 3–6 increased after phosphorylation because of negative charges introduced into the soy protein. Our solubility results are comparable to the findings of Seguro and Motoki (1986). Phosphorylation of SPI had a negligible effect on the protein sensitivity toward calcium chloride with no observable difference in solubility using solutions containing up to 20 mM calcium chloride.

The emulsifying activity increased in the pH range 3–6 with increased emulsion stability at pH 7. This was expected since there is a strong correlation between solubility and emulsifying activity at a given pH (Kinsella et al., 1985). However, mixed results were obtained for the foaming properties with an increase in foam expansion and a decrease in percent foam stability after phosphorylation. Phosphorylation of SPI did not affect its water sorption ability. This is in agreement with Ochiai-Yanagi et al. (1978), who reported that phosphorylation of soybean protein did not increase its water holding capacity.

There was no significant difference in the isoionic pH after phosphorylation. The isoionic pH is comparable to the isoelectric pH and more suitable to our application because SPI is a mixture of different subunits and polypeptides. Isoelectric pH is more applicable to situations using a highly purified protein.

In conclusion, enzymatic phosphorylation improved the emulsifying activity and solubility of soy protein isolate in the acidic pH range. We would anticipate SPI-P would exhibit improved functional properties compared to those of unmodified SPI in acidic food products. We are currently evaluating less costly sources of protein kinase as a prelude to developing a cost effective scale-up process for the enzymatic phosphorylation of soy protein.

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