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Enzymatic Phosphorylation of Soybean Proteins

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Soybean proteins were enzymatically phosphorylated with the catalytic subunit of cAMP-dependent protein kinase isolated from bovine cardiac muscle. Both of the major storage proteins, β-conglycinin and glycinin, were phosphorylated. Heat denaturation of the soy proteins increased the amount of γ-^{32}P incorporation. Soy isolates incorporated up to 0.88 mol of phosphate/mol of β-conglycinin and over 1.0 mol of phosphate/mol of glycinin. Enzymatic phosphorylation could provide a means of increasing the solubility of soy proteins at mildly acidic pH and thus extend the availability of soy proteins for use in the food system.

The utilization of soybeans is gaining importance in the food system because of their high protein content. They are used not only in the form of flour or grits but also in the form of concentrates and isolates and hydrolysates of the soy proteins known as peptones. The concentrates and isolates are made by removing nonprotein components. Soy isolates are over 90% protein (Smith and Circle, 1972). Although native soy proteins have good functional properties, namely high solubility and emulsifying ability, these properties do not function in the acidic range (pH 3.0–6.0) since solubility decreases in this range due to the isoelectric point of the proteins. This prohibits their use in acidic foods such as beverages, coffee whiteners, or mayonnaise.

Several attempts have been made to modify soybean proteins by phosphorylation in order to improve their functional properties. The degree of chemical phosphorylation with phosphorus oxychloride of soy protein isolates is 452 pmol of phosphate/mg of soy protein (Hirotsuka et al., 1984). Chemically phosphorylated soybean proteins exhibit increased water solubility and emulsifying activity, particularly in the acidic range (Sung et al., 1983; Hirotsuka et al., 1984). Chemical phosphorylation of soybean proteins has been achieved with sodium trimetaphosphate (Sung et al., 1983) and phosphorus oxychloride (Hirotsuka et al., 1984). When sodium trimetaphosphate was used, the phosphorylated amino acids were lysine and serine (Sung et al., 1983), while lysine and histidine were phosphorylated when phosphorus oxychloride was used (Hirotsuka et al., 1984). Nitrogen-bound phosphate, such as on histidine or lysine residues, is very acid labile; the phosphorus may dissociate in a neutral aqueous solution (Matheis et al., 1983). Proteins containing oxygen-bound phosphate are more desirable for incorporation into food products because of their stability under acid conditions (Matheis and Whitaker, 1984).

Chemical modification of food proteins has its drawbacks. The presence of unreacted chemicals may not allow the modified proteins to be used in the food system. Side reactions could also cause deleterious effects in the final food product. A more specific and milder way of modifying vegetable proteins for use in the food industry would be with the use of enzymes. Enzymes have been used to hydrolyze and to cross-link soy proteins for use in food products (Feeney and Whitaker, 1985). There has been one report of an enzymatic phosphorylation of glycinin, one of the major soy storage proteins (Seguro et al., 1986).

Protein kinase (E.C. 2.7.1.37) has been found to phosphorylate proteins, both in vivo and in vitro. They are usually subclassified based on the amino acid acceptor of the phosphoryl residue (Krebs, 1986). One group phosphorylates the alcohol group of serine and threonine, another the nitrogen group of histidine and lysine, and a third the phenolic group of tyrosine. Since the aim of this investigation was to extend the use of soy proteins in acidic foods such as beverages, we choose to work with adenosine cyclic 3′,5′-monophosphate dependent protein kinase (cAMPdPK), which is known to phosphorylate serine and threonine (oxygen-bound phosphate) because the phosphoprotein product would be acid stable (Matheis et al., 1983).

cAMPdPK exists as an inactive tetrameric holoenzyme (R_2C_2) made of two regulatory (R) and two catalytic subunits (C) (Krebs and Beavo, 1979). In general, each R subunit binds two molecules of cAMP, which leads to the dissociation of the holoenzyme and activation of the

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phosphotransferase activity (C subunit) (Smith et al., 1981). This kinase is most active toward serines that are located several peptides away from basic amino acids such as arginine and lysine, the highest specificity being toward the amino acid sequences Arg-Arg-x-Ser and Lys-Arg-x-x-Ser (Krebs and Beavo, 1979). The subunits of some of the major soybean storage proteins that have been sequenced do contain these amino acid sequences. Potential serine receptors for phosphorylations in the soy proteins are the sixth residue in the α-subunit of β-conglycinin (Coates et al., 1985), residue 62 of the A_2 subunit (Staswick et al., 1984), and residue 4 of the A_4 subunit of glycinin (Moreira et al., 1981).

In this study, the extent of phosphorylation of several soybean proteins by the catalytic subunit of the cAMPdPK from bovine cardiac muscle was determined.

MATERIALS

Defatted soybean flour, Nutrisoy 7B, was obtained from Archer Daniels Midland Co., Decatur, IL 62525. Soy protein isolate, Purina Protein 620, was obtained from Ralston Purina Co., St. Louis, MO. Soy isolate made by ultrafiltration was obtained from the Food Protein Research and Development Center, Texas A&M University. Soybean storage proteins that had been purified were also used as substrates. Purified β-conglycinin (7S globulin) was a gift from W. Wolf (NRRC, USDA—ARS, Peoria, IL), and purified glycinin (11S globulin) was a gift from H. Marshall, Jr. (SRRC, USDA—ARS, New Orleans, LA). [τ-^{32}P]ATP was obtained from Du Pont, Boston, MA. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Sample Preparation. The soy protein was extracted from the flour and isolates in a 5–10% aqueous solution under slightly alkaline conditions (pH 7.8) with constant stirring for 1 h at room temperature. Sodium hydroxide (1 N) was used to adjust the pH. The samples were filtered through six layers of cheesecloth and then centrifuged at 4000g to remove particulate matter. The total protein concentrations of the flours and isolates in the resulting supernatant ranged from 2.5 to 6% as determined according to Bradford (1976) using bovine serum albumin as a standard. This low protein concentration may be due to incomplete dispersion of the flour and isolate in solution at the slightly alkaline pH (7.8). Conglycinin and glycinin were solubilized in 0.1 M phosphate buffer containing 0.4 M sodium chloride at a protein concentration of 1%. Half of the soy protein extracts was used without further treatment, and the remaining half of each sample was denatured. Samples were denatured by heating in a heating block at 100 °C for 5 min. The whole denatured sample was used. The soy protein extracts and denatured soy protein extracts were prepared within 16 h of use. Hydrolyzed soy protein (Peptone Types III and IV, Sigma) used in this study were 1% aqueous solutions.

Phosphorylation Procedure. The catalytic subunit from Type II cAMPdPK holoenzyme was purified from bovine cardiac muscle as described by Zoller et al. (1979). The specific activity of the C subunit measured by the method of Roskoski (1983) using 100 μM Ser peptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) and 200 μM [τ-^{32}P]ATP (Cook et al., 1982) was 12.6 μmol of τ-^{32}P transferred/min per mg of protein. The soy proteins were phosphorylated by incubating 10 μL of the proteins with 2.28 ng of C subunit in a reaction volume of 100 μL consisting of 50 mM 3-N-morpholinopropanesulfonic acid (MOPS) (pH 7.0), 10 mM $MgCl_2$, 2.5 mg/mL bovine serum albumin, and 100 μm [τ-^{32}P]ATP (1500 cpm/pmol) for 3 h at 30 °C in triplicate.

At the end of the reaction period, 25-μL aliquots were removed and spotted on phosphocellulose paper strips. The τ-^{32}P incorporation into the protein was determined by liquid scintillation counting after the unreacted [τ-^{32}P]ATP was washed from the phosphocellulose paper. The degree of phosphorylation was measured by the method of Roskoski (1983).

Electrophoresis sample reducing buffer was added to the remaining reaction mixture and the resultant mixture heated at 100 °C for 5 min. Polyacrylamide gel electrophoresis (PAGE) using 15% gels (20 cm × 20 cm × 1.5 mm) of the phosphorylated protein mixture was run under denaturing conditions using the method of Laemmli (1970) with lithium dodecyl sulfate being used in place of sodium dodecyl sulfate. The radioactivity contained in the protein bands on the gels was counted and integrated on a Model RS thin-layer radiochromatograph (RTLC; Radiomatic Instruments & Chemical Co., Inc. Tampa, FL).

For calculations, molecular weights of 360 000 and 175 000 were used for glycinin and β-conglycinin, respectively, and subunit molecular weights of 37 000 and 20 000 for the acidic and basic subunits of glycinin and 72 000, 68 000, and 52 000 for α'-, α-, and β-subunits of β-conglycinin.

Table I. τ-^{32}P Incorporation into Soybean Proteins (cpm × 10^{-3}/mg Total Protein)

	native	denatured
flour	58	40
ultrafiltrate isolate	4343	1156
Purina Protein 620 isolate	8683	9876
β-conglycinin	2409	1045
glycinin	2634	16790

Table II. Nanomoles of Phosphate Incorporated per Milligram of Total Soy Protein[a]

	native	denatured
flour	0.05	0.03
ultrafiltrate isolate	2.85	7.58
Purina Protein 620 isolate	5.69	6.49
β-conglycinin	1.58	6.83
glycinin	1.75	11.00

[a] Protein concentrations determined according to Bradford (1976) using bovine serum albumin as a standard.

Table III. Moles of Phosphorus Incorporated per Mole of Soy Protein[a]

	β-conglycinin	glycinin
ultrafiltrate		
native	0.16	0.70
denatured	0.83	1.01
Purina Protein 620		
native	0.45	1.14
denatured	0.88	0.53
β-conglycinin		
native	0.28	
denatured	1.20	
glycinin		
native		0.63
denatured		3.96

[a] Calculations described in Results.

RESULTS

The extract of Nutrisoy 7B soybean flour was phosphorylated with the cAMPdPK, but the extent of incorporation was very low for both the native and heat-denatured samples (Table I). Peptone Type III and Peptone Type IV did not incorporate any measurable phosphate. As shown in Table II phosphate incorporation of the soy isolates ranged from 2.85 to 7.58 nanomol of phosphate/mg of total soy protein. The degree of phosphorylation for the two isolates, the Purina Protein 620 and the ultrafiltrate, was 50–150 times higher than that of the flour. Both the β-conglycinin and glycinin were phosphorylated. For most soy protein substrates the heat-denatured proteins were phosphorylated to a greater degree than the native proteins.

Scans of the LDS-PAGE for τ-^{32}P-labeled proteins are shown in Figure 1. The upper scan in each of the panels represents the heat-denatured sample. The scans are normalized to the denatured Purina Protein 620 isolate, which had the highest degree of τ-^{32}P incorporation. The moles of τ-^{32}P incorporated per mole of storage protein are given in Table III. Calculations were based on the total amount of τ-^{32}P incorporated per milligram of total protein and percent incorporation in the individual subunits of

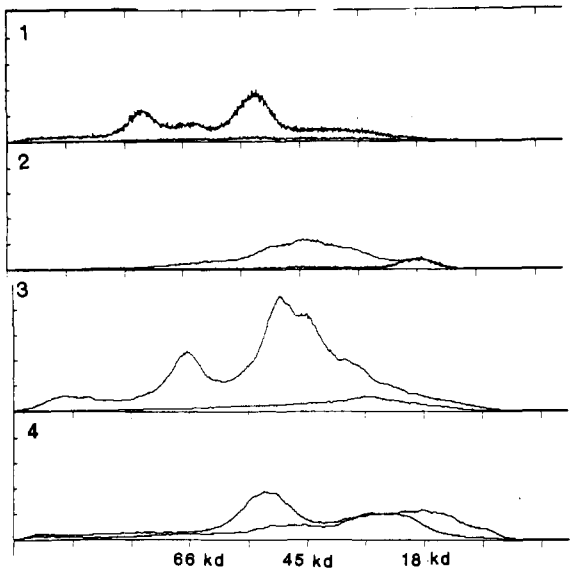

Figure 1. Scans of LDS-PAGE for τ-^{32}P-labeled proteins: 1, β-conglycinin; 2, glycinin; 3, Purina Protein 620 soy isolate; 4, ultrafiltrate soy isolate. Lower scan in each panel is of native protein; upper scan in each panel is of denatured protein. Scans normalized to denatured Purina Protein 620 soy isolate (upper scan, panel 3).

glycinin and β-conglycinin as determined by RTLC integrations of the gels and the molecular weights of β-conglycinin and glycinin. There was a linear relationship between the τ-^{32}P incorporation as determined by scintillation counting and τ-^{32}P incorporation as determined by the RTLC scans.

For the β-conglycinin and the glycinin preparations, percents of the total serine and threonine residues phosphorylated were calculated (Kinsella et al., 1985). Of the 153 mol of serine and threonine in β-conglycinin, 0.2% was phosphorylated in the native protein while 0.78% was phosphorylated in the denatured protein. Of the 348 mol of serine and threonine in glycinin, 0.18% was phosphorylated in the native protein and 1.13% was phosphorylated in the denatured protein.

DISCUSSION

The major soybean storage proteins, glycinin and β-conglycinin, could be enzymatically phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. As shown in Figure 1, both the acidic and basic subunits of glycinin were phosphorylated as well as the α'-, α-, and β-subunits of β-conglycinin. Since this kinase is known to phosphorylate serine and threonine residues (Krebs and Beavo, 1979), we feel that these residues are being phosphorylated on the soy proteins. From amino acid sequences of the soy proteins, we know there are potential phosphorylatable sites in the α-subunit of β-conglycinin and acidic subunits of glycinin. On the basis of the number of serine and threonine residues in glycinin and β-conglycinin, it was determined that a maximum of 1.13% of these residues was phosphorylated in glycinin and 0.78% in β-conglycinin during the incubation of the proteins with the cAMPdPK. Since very few of the serine residues in these proteins are preceded by the basic amino acids lysine and arginine, we would not expect to find a large number of the serine residues to be enzymatically phosphorylated. However, even this degree of phosphorylation could shift the isoelectric points of the soy storage proteins and enable them to be more soluble under mildly acidic conditions. The degree of phosphorylation of soy protein isolates treated with phosphorus oxychloride was 452 pmol of phosphorus/mg of soy protein (Hirotsuka et al., 1984). As shown in Table II the degree of phosphorylation of soy protein isolates reacted with the kinase is 2.85–7.58 nanomol of phosphate/mg of soy protein. This is a 6–17-fold increase over phosphorus oxychloride in the amount of phosphate attached to the soy protein by the kinase. Chemical phosphorylation does improve the solubility of soy proteins, but additional studies will be necessary to confirm whether enzymatically phosphorylated soy proteins exhibit an increased solubility at mildly acidic pHs.

Heat denaturation caused an increase in the phosphorylation of β-conglycinin, glycinin, and the two isolates. Heat denaturation for short periods of time may allow changes in the tertiary structure of the soy proteins such that a greater number of the amino acid residues in the proteins become accessible to the enzyme for phosphorylation (Kinsella et al., 1985).

Hydrolyzed soy proteins are usually more easily modified than intact soy proteins. However, when hydrolyzed soy proteins (peptones) were used as substrates in this study, they were not phosphorylated by cAMPdPK. This could be due to lack of an appropriate amino acid sequence in these hydrolyzed proteins for the phosphorylation of the serine by this enzyme or to the dissociation of the required sequence involving basic amino acids preceding the serine residue when the soy proteins are hydrolyzed or modification (i.e., oxidation) of serine and threonine residues during hydrolysis.

The effects of enzymatic phosphorylation of soy proteins by protein kinase on alterations in the isoelectric points of these proteins is under investigation. Initial studies to determine whether the soy proteins and storage proteins like glycinin and β-conglycinin could be enzymatically phosphorylated have proven successful. A significant degree of phosphorylation of soy proteins was achieved under both denaturing and nondenaturing conditions. The use of this particular enzyme offers several additional advantages. It can be obtained commercially (Sigma). The amino acid sequence of the 40 580-Da enzyme has been determined (Shoji et al., 1981, 1983). The DNA that encodes this enzyme has been sequenced, cloned, and expressed (Uhler et al., 1986a,b; Uhler and McKnight, 1987). Thus, this enzyme could be made economically available in large quantities if it proved useful in the food processing industry. We are currently investigating the use of commercial preparations of the cAMPdPK (Sigma) as well as attempting to determine the conditions for further increases in the amount of phosphate incorporated into the soy storage proteins.

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Oxygen Uptake by Gallic Acid as a Model for Similar Reactions in Wines

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Autoxidation in highly alkaline solution consumed 4.9 atoms of oxygen/molecule of gallic acid oxidized. Hydrogen peroxide was proven (for the first time at least in a wine context) to be produced during the oxidation and was quantitated at about 2 mol of hydrogen peroxide/mol of gallic acid. Production of carbon dioxide was not detected. After acidification, the oxidized products of gallic acid and ellagic acid (gallic acid dimer) were studied by paper chromatography and HPLC. Two major acidic products having polarity higher than gallic acid were produced, and they appeared to be the same for both gallic and ellagic acids. Analysis of the results indicates a gallic acid oxidation mechanism primarily via a dimer equivalent to ellagic acid resulting finally in opened-ring acidic products. The numerical integrations of the rate equations for the proposed scheme are in good agreement with the actual measurements of oxygen uptake. The results are discussed in relation to oxidations during wine aging and related food processing.

Oxidation of constituents other than lipids is an important cause of modification, sometimes spoilage and sometimes improvement, in foods and beverages. Sherries, raisins, black tea, and chocolate are examples in which such oxidation is part of desirable changes essential to the product. White table wine and many other fruit and vegetable products are examples in which oxidative browning and associated flavor modification are considered undesirable. Nutritional value can be decreased by reactions associated with or following oxidation of food phenols to quinones. In still other cases, notably red wines, optimum quality is associated with a shift to a somewhat tawny color and complexing flavor changes that result from limited oxidation and partial consumption of oxidizable substrates (Singleton and Esau, 1969). Important as it is, relatively little research beyond visible spectral studies has been devoted to such nonenzymic autoxidation in aqueous systems.

In a number of these foods and beverages, notably grape juices and wines and probably all such vegetable products without appreciable ascorbic acid content, naturally present phenolic substances are considered the major

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