citronellal, 106-23-0; isoborneol, 124-76-5; borneol, 507-70-0; terpinen-4-ol, 562-74-3; cryptone, 500-02-7; β-terpineol, 138-87-4; α-terpineol, 10482-56-1; verbenone, 18309-32-5; cuminal, 122-03-2; carvone, 99-49-0; neral, 106-26-3; terpinyl acetate, 8007-35-0; β-caryophyllene, 87-44-5; aromadendrene, 72747-25-2; eremophilene, 10219-75-7; β-sesquiphellandrene, 20307-83-9; globulol, 489-41-8; *d*-limonene, 5989-27-5; terpin-4-ol, 80-53-5; isomenthol, 490-99-3; *trans*-caryophyllene, 87-44-5; alloaromadendrene, 25246-27-9; β-eudesmol, 473-15-4.

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Optimization of Enzymatic Phosphorylation of Soybean Storage Proteins: Glycinin and β-Conglycinin

Lynne F. Ross

Enzymatic phosphorylation could provide a means of increasing the solubility of soy proteins at mildly acidic pH and thus extending the availability of soy proteins for use in food systems. Soybean storage proteins, glycinin and β-conglycinin, were enzymatically phosphorylated with a commercial preparation of the catalytic subunit of cAMP-dependent protein kinase isolated from bovine cardiac muscle. The method was optimized for soybean protein substrates. The degree of phosphorylation was increased 150-fold, from 50 pmol of phosphate/mg of soy protein to 8.9 nmol of phosphate/mg of total soy protein. Soy flours and isolates incorporated up to 1.24 mol of phosphate/mol of β-conglycinin and over 2.0 mol of phosphorus/mol of glycinin.

Due to their high protein content, soybean flours, concentrates, and isolates are gaining importance in food systems. These proteins have good functional properties, such as high solubility and emulsifying ability, except in the acidic range since solubility decreases in this range due to the isoelectric point of most soy proteins. Therefore, these proteins cannot be used in acid foods such as beverages, coffee whiteners, or mayonnaise.

Phosphorylation increases solubility and emulsifying ability of soybean proteins, particularly in the acidic range (Sung et al., 1983; Hirotsuka et al., 1984). In previous experiments (Ross and Bhatnagar, 1989) a homogeneous preparation of the catalytic subunit of cAMP-dependent protein kinase (cAMPdPK) with high specific activity (12.6 μmol of γ-^{32}P transferred/min per mg of Ser peptide) was found to phosphorylate soybean storage proteins, glycinin and β-conglycinin. With the homogeneous enzyme preparation, protein from soy flour incorporated 50 pmol of phosphate/mg of soy protein and protein from soy isolates incorporated from 2.85 to 7.58 nmol of phosphate/mg of protein. Since the ultimate aim of this research is to use this method of phosphorylation in the food processing industry, the commercially available enzyme preparation

SRRC, USDA, New Orleans, Louisiana 70179.

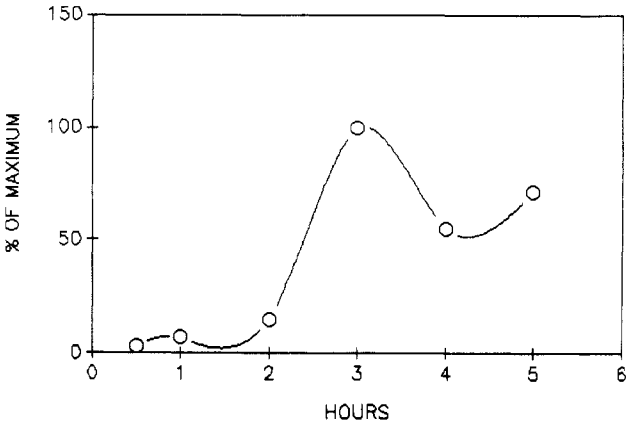

Figure 1. Effect of reaction time on phosphorylation of denatured soybean flour.

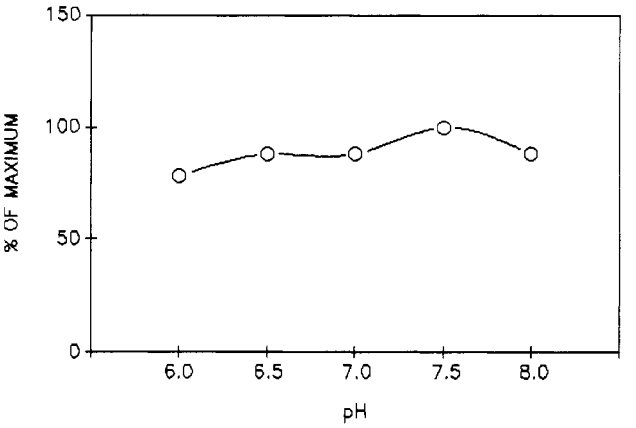

Figure 2. Effect of pH on phosphorylation of denatured soybean flour.

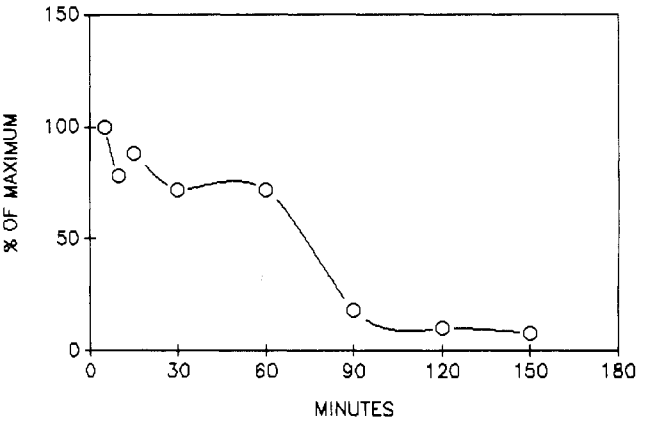

Figure 3. Effect of length of heat denaturation of soy proteins on phosphate incorporation of denatured soybean flour.

with lower specific activity was used in this study. The goal of this study was to optimize enzymatic reaction parameters and reactant concentration to increase the degree of phosphorylation of the soybean storage proteins.

METHODS AND 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 63164. Soy isolate made by ultrafiltration was obtained from the Food Protein Research and Development Center, Texas A&M University. Soybean storage proteins 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 DuPont, Boston, MA 02118. Acrylaide cross-linker was obtained from FMC BioProducts, Rockland, ME. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Samples were prepared as previously described (Ross and Bhatnager, 1989).

The catalytic subunit of the cAMPdPK (Sigma) holoenzyme used in this study was measured to have a specific activity of 36 pmol of [τ-^{32}P]ATP transferred/min per mg of casein. One kinase unit is equal to the transfer of 1 pmol of phosphate/min per mg of casein. The soy proteins were phosphorylated by incubating the proteins with 1 unit of this enzyme in a reaction volume of 100 μL consisting of 30 mM phosphate buffer (pH 7.5), 10 mM $MgCl_2$, 100 μM [τ-^{32}P]ATP (1500 cpm/pmol), unless noted otherwise. The degree of phosphorylation of soy protein was measured by the method of Roskoski (1983) in triplicate. Denatured soy flour was used as the substrate in a series of experiments designed to optimize the extent of phosphorylation by varying one parameter (i.e., reaction time or temperature) or reactant concentration at a time.

In the final experiment, the two soy isolates as well as the soy flour and glycinin and β-conglycinin preparations in both native and denatured forms were used. Polyacrylamide gel electrophoresis (PAGE) was performed as previously described (Ross and Bhatnagar, 1989) except acrylaide cross-linker was used in place of bisacrylamide.

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 33611).

RESULTS

A denatured extract of Nutrisoy 7B was used as the substrate in the experiments to optimize the phosphorylation of the soy storage proteins with the commercially available protein kinase. Each optimization step represents the overall results from a number of seperate experiments with each assay done in triplicate. For each series of experiments one reaction parameter or reactant concentration was changed. The soybean proteins required longer incubations with the enzyme than casein for optimum phosphorylation. A time course study for soy protein phosphorylation is shown in Figure 1. The maximum amount of phosphorylation occurred in a 3-h incubation. Preincubation of the soy protein with all the reactants except the kinase did not decrease the time required for maximum phosphorylation. The recommended pH for optimum activity of the commercial preparation was 6.5 (Sigma), but as shown in Figure 2, the kinase exhibited a broad pH range for optimum activity between pH 6.0 and 8.0.

Heat denaturation of the soy proteins increased the phosphorylation 7-fold in the flour substrate from 60 to 450 pmol of phosphate/mg of total soy protein. A study was conducted to see whether increasing the duration of heat denaturation of the proteins would increase the degree of phosphorylation. Although heating the soy proteins for 5 min increased phosphate incorporation, extending the duration of heating caused a decrease in phosphate incorporation (Figure 3). The recommended temperature for the kinase reaction was 30 °C (Sigma), but with soy protein as substrate a higher degree of phosphate incorporation was observed at 35 °C (Figure 4).

The optimum concentration for ATP was determined to be 40 μM (Figure 5), and for $MgCl_2$ it was 10 mM (data not shown) for maximal phosphorylation of soy protein. Dithiothreitol is required for kinase activity (Sigma); the optimum concentration of dithiothreitol in the reaction mixture, as shown in Figure 6, was determined to be 1.28 mmol.

The soy protein concentration in the reaction mixture was also found to affect the amount of phosphate incorporation (Figure 7). Initially 1–2% soybean protein was used in the reaction mixture. Decreasing the amount of protein in the reaction mixture leads to an increase in the

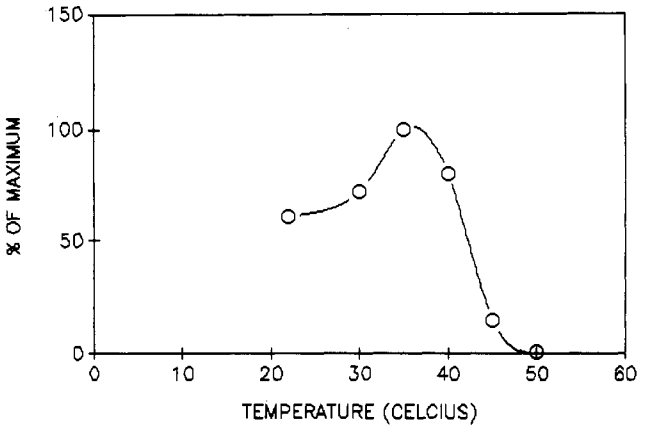

Figure 4. Effect of reaction temperature on phosphate incorporation of denatured soybean flour.

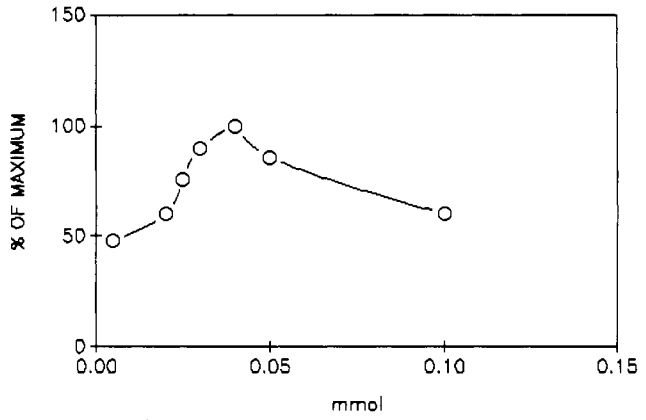

Figure 5. Effect of ATP concentration on phosphate incorporation of denatured soybean flour.

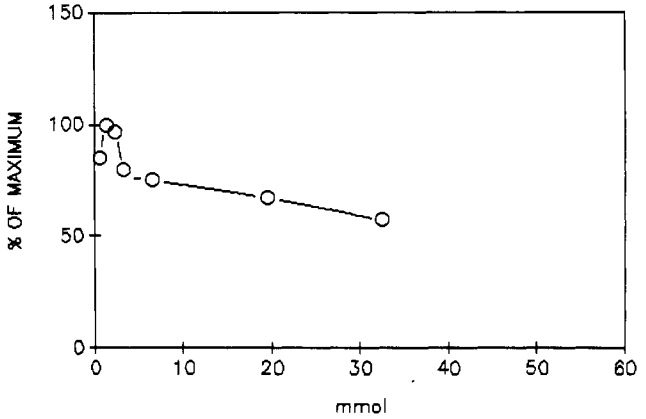

Figure 6. Effect of dithiothreitol on phosphate incorporation of denatured soybean flour.

phosphate incorporated in the soybean proteins. The optimum protein concentration for the reaction was 0.1%.

For the initial studies, 1 unit of enzyme was used in a 100-μL reaction mixture. As shown in Figure 8, increasing the enzyme concentration up to 3 units increased the degree of phosphorylation, the incorporation leveling off at the higher concentrations.

These optimized conditions for maximum phosphorylation of soy proteins was then used to measure the phosphorylation of five soybean protein extracts, Nutrisoy 7B flour, Purina Protein 620 isolate, ultrafiltrate isolate, glycinin, and β-conglycinin, in their native and denatured states. As shown in Table I, phosphate incorporation ranged from 0.2 to 15.5 nmol of phosphate/mg of protein. Scans of the polyacrylamide gels for γ-^{32}P-labeled proteins are shown in Figure 9. These scans were integrated to

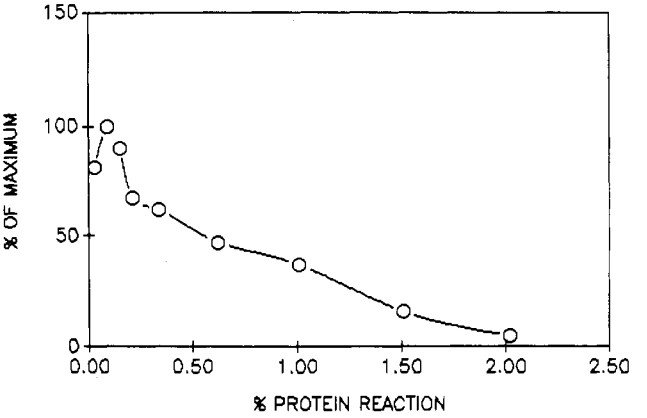

Figure 7. Effect of protein concentration on phosphate incorporation of denatured soybean flour.

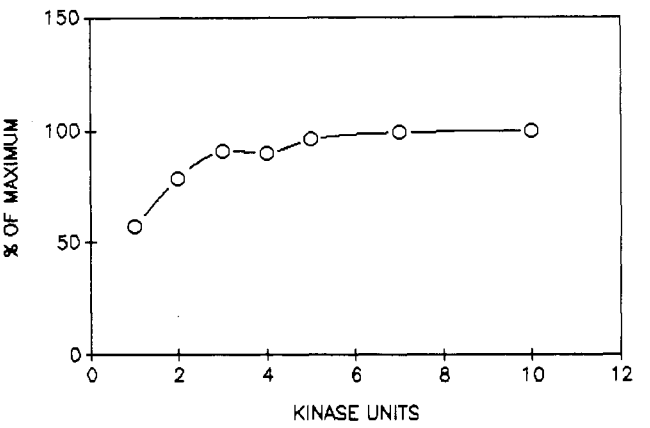

Figure 8. Effect of enzyme concentration on phosphate incorporation of denatured soybean flour.

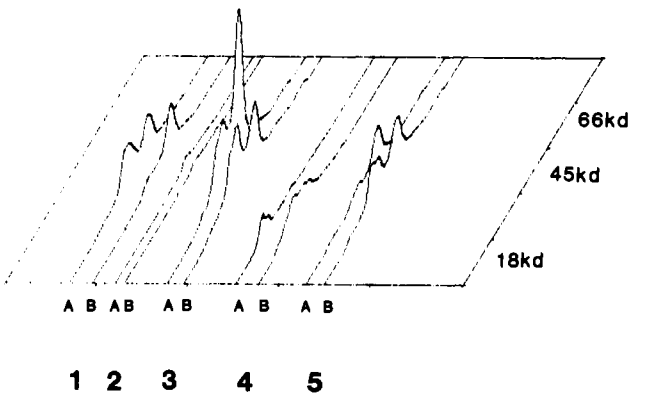

Figure 9. Scans of LDS-PAGE for γ-^{32}P incorporation into soybean storage proteins: 1, Nutrisoy 7B flour; 2, β-conglycinin; 3, Purina Protein 620 isolate; 4, glycinin; 5, ultrafiltrate isolate; A, native protein; B, denatured protein.

Table I. Nanomoles of Phosphate Incorporated per Milligram of Soy Protein

	native[a]	denatured[a]
flour	2.3	8.9
ultrafiltrate isolate	10.0	7.6
Purina Protein 620	10.4	10.6
β-conglycinin	0.2	1.2
glycinin	5.8	15.5

[a] Native substrates are aqueous extracts and denatured substrates are aqueous extracts that have been heated for 5 min at 100 °C. Reaction conditions were 3 h at 35 °C. Reaction mixture contained 30 mM phosphate buffer, pH 7.5, 10 mM $MgCl_2$, 40 mM ATP, 1.28 mM dithiothreitol, 0.01% protein, and 2 units of protein kinase.

Table II. Moles of Phosphorus Incorporated per Mole of Soy Protein

	β-conglycinin	glycinin
flour		
native	0.23	0.37
denatured	1.24	0.64
ultrafiltrate isolate		
native	1.15	1.27
denatured	0.66	1.38
Purina Protein 620		
native	1.16	1.36
denatured	0.83	2.09
β-conglycinin		
native	0.04	
denatured	0.21	
glycinin		
native		2.08
denatured		5.57

[a] Native substrates are aqueous extracts and denatured substrates are aqueous extracts that have been heated for 5 min at 100 °C. Reaction conditions were 3 h at 35 °C. Reaction mixture contained 30 mM phosphate buffer, pH 7.5, 10 mM $MgCl_2$, 40 mM ATP, 1.28 mM dithiothreitol, 0.01% protein, and 2 units of protein kinase.

determine the percent τ-^{32}P incorporated into the individual subunits of the β-conglycinin and glycinin. Subunit molecular weights for β-conglycinin of 72 000, 68 000, and 52 000 for the α′-, α-, and β-subunits (Sykes and Gayler, 1981) were used in the calculations, and for the acidic and basic subunits of glycinin 37 000 and 20 000 (Kinsella et al., 1985) were used. The percentages of the total serine and threonine residues phosphorylated were calculated. Of the 153 mol of serine and threonine in β-conglycinin, 0.02% was phosphorylated in the native protein while 0.14% was phosphorylated in the denatured protein. Of the 348 mol of serine and threonine in glycinin, 0.60% was phosphorylated in the native protein and 1.60% was phosphorylated in the denatured protein.

The moles of τ-^{32}P incorporated per mole of storage protein based on molecular weights of 360 000 and 175 000 for glycinin and β-conglycinin, respectively (Kinsella et al., 1985), are given in Table II. Calculations were based on total amount of τ-^{32}P incorporated into the total protein and percent incorporation into individual subunits as determined by RTLC integration of the gels.

DISCUSSION

By optimizing the reactants and parameters for the protein kinase reaction, the degree of phosphorylation of the soybean proteins was increased from 60 pmol of phosphorus (Ross and Bhatnager, 1989) to 8.8 nmol of phosphate per milligram of total soy protein from soy flours. As shown in Table I the degree of phosphorylation of soy protein isolates reacted with the commercially available kinase is 7.6–10.6 nmol of phosphorus/mg of soy protein as compared to 2.9–7.6 nmol of phosphate/mg of soy protein, with the homogeneous protein with a higher specific activity (Ross and Bhatnager, 1989). Chemical phosphorylation improves the solubility of soy proteins (Hirotsuka et al., 1984) but additional studies will be necessary to confirm whether enzymatically phosphorylated soy proteins exhibit an increased solubility at mildly acidic pH.

The moles of phosphorus incorporated per mole of β-conglycinin ranged from 0.04 to 1.24 and 0.37 to 5.57 for glycinin (Table II). This is higher than was achieved with the homogeneous protein kinase enzyme preparation with a higher specific activity than the commercial preparation used in this study in which soy isolates incorporated up to 0.88 mol of phosphate/mol of β-conglycinin and 1.14 mol of phosphate/per mol of glycinin (Ross and Bhatnagar, 1989). The method used in the earlier report was not optimized for soy proteins. Both of the major storage proteins, glycinin and β-conglycinin, were phosphorylated significantly. These storage proteins are large and globular in their native state. The catalytic subunit of cAMPdPK phosphorylated serine and threonine residues but preferentially phosphorylates serines, which are several residues away from basic amino acids such as arginine and lysine (Krebs and Beavo, 1979). There are only a few amino acid sequences in glycinin and β-conglycinin in the right configuration for preferential phosphorylation by the protein kinase (Coates et al., 1985; Moreira et al., 1981; Staswick et al., 1984). Thus, the enzyme has to search along these large globular proteins to find sequences in the required arrangement, which may be internal sequences in the tertiary structure of these globular proteins. This may be the reason for the 3 h it takes to achieve a significant degree of phosphorylation.

There was no significant difference in phosphate incorporation between pH 6.0 and 8.0; the slight increase at pH 7.5 is probably due to the fact that the soybean proteins are more soluble at an alkaline pH. Heat denaturation may also increase the solubility of the soy proteins, resulting in higher phosphorylation. When soy proteins are heated for a short time, the subunits are known to dissociate and unfold and the protein becomes more soluble (Kinsella et al., 1985), and as they are heated for longer periods, they start to aggregate and form gels. As the proteins form gels and become less soluble, and appropriate serine sequences become less accessible and, as is seen in Figure 3, the extent of phosphorylation decreases under similar circumstances.

Heat denaturation increases phosphorylation of soy flours almost 4-fold, but it seems to have little effect on either of the soy isolates (Table I). Thus, the short period of heating may be destroying enzymes or inactivators that could be present in the soy flour. Processing, including heat treatment, used to make soy isolates from soy flour could already have destroyed enzymes or inactivators in the soy isolates or already denatured the soy protein. Of the commercially available soy protein preparations, the soy isolates, ultrafiltrate, and Purina Protein 620 were found to have the highest degree of phosphate incorporation.

The optimum concentration of ATP was 40 μM and of dithiothreitol was 1.28 mM. Initially, 100 μM ATP was used as suggested in the method of Roskoski (1983). As shown in Figure 5 the amount of phosphate incorporated by the soy proteins nearly doubled when ATP was decreased to 40 μM. The recommended level of dithiothreitol was higher for the casein substrate (Sigma). The reducing agent affects the solubility of the soy protein substrates as well as the kinase. Dithiothreitol depolymerizes the disulfide bridges of the soy storage proteins (Kinsella et al., 1985). Glycinin is affected to a greater degree due to the extensive number of disulfide bridges within subunits and between acidic and basic pairs (Hoshi and Yamauchi, 1983). If the concentration of dithiothreitol is too high, the degree of phosphorus incorporation into the soy protein is decreased (Figure 6).

As the concentration of protein in the reaction mixture decreases, the degree of phosphate incorporation is increased to a maximum at 0.1% protein (Figure 7). The aqueous extract of soy flours and isolates used in these experiments contains a mixture of proteins, mainly the storage proteins glycinin and β-conglycinin. This phenomena of increasing degree of phosphorylation by a

protein kinase with decreasing substrate concentration has been reported by Lin and Key (1976) and was attributed to a heterogeneous substrate with one of the proteins in the mixture inhibiting the kinase.

Using more enzyme will increase phosphate incorporation for up to 3 units of the enzyme. As seen in Figure 8 the phosphate incorporation levels off with additional levels of the enzyme. As mentioned previously, there are only so many sites on the soy proteins with the appropriate sequence that can be phosphorylated with the enzyme so the presence of more enzyme will not increase the degree of phosphorylation after all available sites are phosphorylated and a steady state is attained.

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 carried out enzymatically have proven successful. This study represents the optimization of methodology and reaction conditions for the phosphorylation of soy protein substrates with a commercial preparation of cAMPdPK. Future studies will focus on scaling up the reaction and characterizing the phosphorylated proteins in relation to their solubility under acidic conditions with a view to utilization of the economical soy proteins in various food products such as beverages, coffee whiteners, or mayonnaise.

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Identification by Two-Dimensional NMR of *myo*-Inositol Tris- and Tetrakis(phosphates) Formed from Phytic Acid by Wheat Phytase

Brian Q. Phillippy

The pathway of hydrolysis of phytic acid (*myo*-inositol hexakis(phosphate)) by wheat phytase from the tetrakis(phosphate) to the tris(phosphate) level was determined. Individual tetrakis(phosphate) isomers were hydrolyzed to tris(phosphates), which were purified by ion-exchange chromatography. Chemical shifts were determined by one-dimensional ^1H NMR, and structural assignments were confirmed by two-dimensional ^1H–^1H NMR. Hydrolysis of D-*myo*-inositol 1,2,5,6-tetrakis(phosphate) produced a mixture of D-*myo*-inositol 1,2,6-tris(phosphate) and D-*myo*-inositol 1,5,6-tris(phosphate). Hydrolysis of L-*myo*-inositol 1,2,3,4-tetrakis(phosphate) gave only *myo*-inositol 1,2,3-tris(phosphate). The role of phytic acid in inositol phosphate mediated calcium metabolism may depend on whatever functions its hydrolysis products are found to perform.

The recent discoveries of IP_6 (*myo*-inositol hexakis(phosphate), phytic acid) in a variety of animal cells (Heslop et al., 1985; Morgan et al., 1987; Jackson et al., 1987; Tilly et al., 1987) bring the possibility that it plays a role in the inositol phosphate pathway regulating calcium metabolism in plant (Schumaker and Sze, 1987; Rincon and Boss, 1987) and animal (Streb et al., 1983) cells. D-$I(1,4,5)P_3$ has shown more potency than any other inositol phosphate in mobilizing calcium from intracellular stores (Irvine et al., 1984, 1986), specifically identified as endoplasmic reticulum (Delfert et al., 1986). D-$I(1,3,4,5)P_4$ can control entry of calcium into cells (Irvine and Moor, 1986) and can be dephosphorylated to D-$I(1,3,4)P_3$, which has

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