Enzymatic Phosphorylation of Soybean Proteins by Protein Kinase

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Soybean proteins were subjected to phosphorylation with cyclic adenosine monophosphate-dependent protein kinase (A-kinase). As a result, acidic subunits of the 11S fraction were found to be phosphorylated by A-kinase. To estimate the effect of the phosphorylation, 11S acidic subunits were isolated and subjected to A-kinase phosphorylation. The optimal enzyme amount and Mg\(^{2+}\) concentration for the phosphorylation of 11S acidic subunits were determined to be 1.5 U/ml and 1.6 mM, respectively. The rate of phosphorylation was 2 mol/mol acidic subunits (MW 38,000) under the above conditions. The protein structures of 11S acidic subunits, as determined from UV and CD spectra, were slightly affected by the enzymatic phosphorylation.

The physicochemical properties and functionalities of proteins largely depend on their amino acid compositions or, in other words, the side chain structures of the amino acid residues in proteins. Thus, intentional changes in the side chains of amino acid residues by means of chemical or enzymatic modification are believed to alter the functionality.\(^1\)\(^-\)\(^2\) Among such modifications, phosphorylation results in a higher solubility, greater water-holding capacity or greater calcium-binding capacity of a protein.\(^3\)\(^-\)\(^8\) These changes are mainly dependent on the introduction of hydrophilic and negatively charged phosphoryl groups, though some influence due to subsequent conformational changes can’t be neglected. Chemical phosphorylation of proteins has been widely performed so far. However, when it is used for edible proteins, there are troublesome drawbacks, such as the formation of unknown products by highly reactive reagents, phosphorus oxychloride, trichloraceticimidoyl phosphate, phosphorus pentoxide, etc. Also a high pH in the chemical reaction can result in the formation of lysinoalanine in proteins.\(^9\) Furthermore, the removal of remaining reagents and side-reaction products is difficult.

Protein kinases, on the other hand, catalyze phosphorylation of protein-bound serine and/or threonine residues with adenosine triphosphate as a source of phosphoryl groups.\(^10\)\(^-\)\(^13\) This enzymatic phosphorylation is highly specific and the products, such as caseins, phosvitin, etc., are found in milk and eggs, which are edible and nutritious.\(^14\) Therefore, we attempted to phosphorylate soybean proteins, commonly known food proteins, with cyclic adenosine monophosphate-dependent protein kinase. Here, we report evidence that 11S acidic subunits were phosphorylated and the phosphorylation conditions for 11S acidic subunits. Also we investigated the effect of the enzymatic phosphorylation on the structures of 11S acidic subunits using the spectroscopic techniques.

Materials and Methods

Protein kinase and other chemicals. The catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase (EC 2.7.1.37, A-kinase) was purchased from Sigma Chemical Co., Ltd. (U.S.A.). Most of the other chemicals were purchased from Koso Chemical Co., Ltd. (Japan). Radioactive adenosine 5'-[\(^\gamma\)-\(^{32}\)P]triphosphate (ATP, 3000 Ci/mmol) was obtained from Amersham International plc (England).

Soybean proteins and their acidic subunits. Soybean
proteins from defatted soybean flakes were fractionated into whey, 7S and 11S fractions by the method of Thanh and Shibasaki. Further, 11S was fractionated into acidic subunits by the method of Kitamura et al. with stepwise elution with 0.4 M NaCl. A commercially available soybean protein isolate, Ajipron S2 (Ajinomoto Co.), was also used.

**Phosphorylation of substrate proteins.** Phosphorylation of substrate proteins by A-kinase was carried out by the method described previously. In brief, 1 vol of a substrate protein solution (10 mg/ml), 4 vol of sodium phosphate buffer (0.1 M, appropriate pH) and 5 vol of magnesium chloride (appropriate concentration) were mixed in a test tube. To the mixture, 0.01 vol of ATP (0.8 mM) and 0.2 vol of A-kinase (appropriate amount) were added to start the enzymatic reaction. After a certain period, the reaction was stopped by the addition of trichloroacetic acid (TCA, 19%) to the reaction mixture. The precipitated protein was collected by centrifugation at 4000 rpm and then washed repeatedly with the TCA. Finally, the precipitate was dissolved in deionized water and lyophilized. When the optimal conditions and phosphorylation sites were determined, radioactive ATP was added to 1% of nonradioactive ATP and the volumes of the reaction mixture were 1/60 to 1/120 of that of nonradioactive labeling.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.** SDS-PAGE was performed as described by Laemmli. The gel was either stained with Coomassie Brilliant Blue or autoradiographed to visualize the protein bands. Autoradiography was carried out as follows: the SDS-PAGE gel was dried and stored with a sheet of X-ray film (Fuji, X-RAY Medical RXO-H) at -80°C overnight. Then, the film was developed.

**Rate of phosphorylation.** When nonradioactive ATP was used as the source of phosphate groups, the amount of phosphate groups introduced into a substrate protein was estimated by the method of Fiske-Subbarow. In brief, protein was hydrolyzed by heating with 10 N sulfuric acid and a few drops of 30% hydrogen peroxide. After the hydrolysis, inorganic phosphate liberated from the protein was spectrophotometrically detected with 10 mM 1-amino-2-naphthol-4-sulfonic acid and 0.2 M ammonium molybdate. The rate was estimated with the following equation:

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\text{Phosphorylation rate (mol/mol)} = \frac{(N \times F \times 1.5 \times 10^{-19})}{\text{protein (mol)}}
\]

where \(N\) is the count per min (cpm), \(F\) the dilution factor of radioactive ATP, and \(1.5 \times 10^{-19}\) the activity of radioactive ATP (mol min per count).

**Circular dichroism (CD).** Protein solution (1.5 mg/ml) was prepared with 0.16 M potassium phosphate buffer (pH 7.0) and then placed in a 0.1 mm path length quartz cell. CD spectrum, ranging from 200 to 250 nm, was recorded with a polarimeter (JASCO J-20A).

**Ultracentrifugation analysis.** Ultracentrifugal sedimentation constant for 11S acidic subunits was obtained for protein solution (2.3 mg/ml) in McIlvaine buffer (pH 7.6) with an ultracentrifuge system (HITACHI 70P-72) equipped with an optical detector (HITACHI ASD). The centrifugation conditions were 55,000 rpm and 20°C.

**Protein concentration.** Protein concentrations were determined by the method of Lowry et al.

### Results

**Phosphorylation of soybean proteins**

Soybean proteins were fractionated into three fractions. They were subjected to enzymatic phosphorylation (Fig. 1). Acidic subunits of 11S and some low molecular-weight fractions were phosphorylated with A-kinase. In contrast, 11S basic subunits, and the 7S and whey fractions were not phosphorylated.

**Optimal phosphorylation conditions for 11S acidic subunits**

The optimal conditions for the enzymatic...
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Fig. 1. Phosphorylation of Soybean Protein Fractions.

a) Fractionation scheme for soybean proteins. b) SDS-PAGE pattern of each fraction after A-kinase treatment. b-1) Autoradiogram. b-2) Coomassie brilliant blue staining. Fraction A, whole soybean proteins; B, whey; C, commercial soybean protein isolate (SPI); D, 7S, E, 11S. The letters in b) correspond to the fractionated proteins in a).

Phosphorylation of 11S acidic subunits were determined using radioactive ATP. The concentrations of 11S acidic subunits and ATP were fixed at 1.2 mg/ml and 0.8 mM, respectively (Fig. 2). First, the effect of pH was investigated (Fig. 2a). As the pH increased, the amount of radioactive phosphate incorporated into 11S acidic subunits increased. However, the reaction pH was set at 8, to avoid alkaline denaturation and the formation of lysinoalanine in substrate proteins. The optimal enzyme amount and Mg\(^{2+}\) concentration were determined to be 1.5 U/ml and 1.6 mM, respectively (Figs. 2b and 2c).

Physicochemical properties of phosphorylated acidic subunits

Acidic subunits of 11S were phosphorylated on a large scale using nonradioactive ATP under the conditions determined above. The rate of phosphorylation, as determined by the method of Fiske–Subbarow, was 2.0 mol/mol acidic subunits. The molecular weight of 11S acidic subunits, as determined by SDS-PAGE, was about 38,000 and not altered by the phosphorylation (Fig. 3). This shows that no
Fig. 2. Optimal Conditions for Phosphorylation of 11S Acidic Subunits.
Protein and ATP concentrations: fixed at 1.5 mg/ml and 0.8 mM, respectively. Radioactive ATP was used as the source of phosphoryl groups, and counting was done directly using the reaction mixtures under various conditions, as described in Materials and Methods. a) pH profile, b) A-kinase concentration profile, c) magnesium chloride concentration profile.

Fig. 3. SDS-PAGE Pattern of 11S Acidic Subunits.
Lane 1, standard molecular weight proteins; 2, native acidic subunits; 3, phosphorylated acidic subunits. The numerals at the sides are molecular weights (10^3 scale).

Fig. 4. Autoradiography after Tlc of a Hydrolysate of 11S Acidic Subunits Phosphorylated with Radioactive ATP.
Solvent, n-butanol-acetic acid-water=2:1:1. Lane 1, phosphothreonine; 2, phosphoserine; 3, hydrolysate of phosphorylated 11S acidic subunits; 4, phosphotyrosine. The fast-moving radioactive spot in lane 3 corresponded to a fast-moving radioactive spot of acid-treated radioactive ATP. Crosslinking reaction occurred. The sedimentation constants determined by ultracentrifuge analysis for both the native and phosphorylated acidic subunits were 3.0S. This also
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Fig. 5. UV and CD Spectra of Phosphorylated 11S Acidic Subunits.

(a) UV spectra, (b) CD spectra. (-----), native acidic subunits; (-----), phosphorylated acidic subunits.

implies that in the dissolved state no intermolecular interactions occur.

The sites phosphorylated were determined after 6N hydrochloric acid hydrolysis of radioactively labeled 11S acidic subunits by the tlc technique. As a result, one spot was observed on the two-dimension tlc autoradiogram (1st solvent, n-BuOH–AcOH–H₂O = 2:1:1, 2nd solvent, HCl–iso-PrOH–H₂O = 16.6:65:18.4). Further, as shown in Fig. 4, the radioactive spot was found to represent serine residues, as judged on comparison with three phosphoamino acids. CD spectra suggested that the random coil structure was a little increased (Fig. 5a). UV spectra suggested that aromatic side chains were partially exposed to the solvent (Fig. 5b). The above facts imply that the native structures of 11S acidic subunits were changed by the introduction of the phosphate groups, but not to great extent.

Discussion

Acidic subunits of soybean 11S were phosphorylated by the catalytic subunit of A-kinase. The optimal reaction conditions determined were pH 8, 1.6 mM MgCl₂, 1.5 U/ml A-kinase, 1.2 mg/ml acidic subunits and 0.8 mM ATP. The phosphorylation rate attained under the above conditions was 2.0 mol/mol acidic subunits, the average molecular weight being 38,000. The only residues phosphorylated were found to be serine. This implies that the A-kinase used in this experiment was the type I isozyme.11-13)

For serine residues (Ser) to be phosphorylated by the A-kinase, the amino terminal side of Ser-containing sequences must contain basic amino acid residue(s), such as arginine (Arg) and lysine (Lys) residues. In fact, such sequences as -Lys-Arg-X-X-Ser- and -Arg-Arg-X-Ser- have been identified as phosphorylation sites, where X can be any amino acid residue.10,11) Among six acidic subunits of soybean 11S (A₁₁₀, A₁₁₅, A₂, A₃, A₄ and A₅)21), the complete amino acid sequences of four subunits and the partial amino acid sequence of the rest have been determined.22-25) In these sequences, there are at least 18 potential phosphorylation sites. Each subunit contains at least two potential phosphorylation sites, though the molecular weights of each acidic subunit were assumed to be 38,000.

In the series of experiments involving radioactive ATP for the enzymatic phosphorylation, the highest rate obtained, with the method of Corbin and Reimann, was 14 mol/mol acidic subunits. This increase coincides with the fact that lysozyme, bovine serum albumin and creatine kinase were phosphorylated by A-kinase only in the denatured state, i.e., in the native state they were not phosphorylated.26) As an explanation for the above phenomenon, it is thought that the denaturation exposes sequestered serine and/or threonine residues, which satisfy the sequence requirement as substrates, to the surface of protein molecules. And as a result, they become easily accessible to A-kinase.10,11,26) The degree and state of the denaturation may be correlated with the rate of phosphorylation. In the case of 11S acidic subunits, the same argument would be valid, since soybeans must suffer from denaturation during the defatting process or protein fractionation procedure, or because of the rather high pH of the enzymatic reaction medium. As to potential phosphorylation sites, the increase in the phosphorylation rate is likely to be due to 1) further phosphorylation of remaining potential phosphorylation sites, which would be
exposed due to higher degree of the denaturation, 2) the higher content of subunits rich in potential phosphorylation sites among a group of acidic subunits and/or 3) phosphorylation of \( A_{11} \), whose amino acid sequence is unknown. Therefore, regardless of the denaturation and formation of lysinoalanine, a greater change in the functionality can be expected under rather severe conditions, since a greater extent of phosphorylation may be possible.

The protein structures of 11S acidic subunits were slightly affected by the phosphorylation, in a way that the random structure increased and the aromatic side chains were exposed to the solvent. The sedimentation constant and molecular weight of the native acidic subunits were 3.0S and 38,000. On the phosphorylation, both values did not change. These results suggest that the conformational changes in the acidic subunits were small and that crosslinking, which easily occurs on chemical phosphorylation or on the formation of intermolecular lysinoalanine, was not observed in this enzymatic phosphorylation.

As a whole, this enzymatic phosphorylation can be regarded as a mild phosphorylating method, causing small conformational changes only due to the introduction of the phosphoryl groups to serine residues. It is, thus, expected that this phosphorylation may alter the functionalities of 11S acidic subunits in ways different from those reported previously. The functionalities of the phosphorylated 11S acidic subunits are under investigation and the results will be reported soon.

References