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Enhancement of Calcium-Binding Quality of Proglycinin Peptides by Chemical Phosphorylation

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Abstract Glycinin, one of the predominant storage proteins in soybeans, was examined as to whether it could be used as a calcium-binding mediator after chemical phosphorylation and enzymatic hydrolysis. Glycinin is composed of six subunits. One of the proglycinin subunits ($A_{1a}B_{1b}$) was overexpressed in *E. coli* to obtain nonphosphorylated proteins with homogeneity. To investigate the enhanced calcium-binding properties of the phosphopeptides, the proglycinin was purified, phosphorylated, and hydrolyzed with trypsin. The proglycinin expressed in *E. coli* was purified by ammonium sulfate precipitation, ion-exchange chromatography, and cryoprecipitation. Chemical phosphorylation by sodium trimetaphosphate was performed to obtain phosphorylated proglycinin. After the phosphorylation, one-dimensional isoelectric focusing gel electroanalysis confirmed the phosphorylation of the proglycinin. The phosphorylated peptides were then hydrolyzed with trypsin, followed by a binding reaction with calcium chloride. The calcium-bound phosphopeptides were finally separated using immobilized metal (Ca^{2+}) chromatography. Consequently, a limited tryptic hydrolysate of the isolated phosphopeptides exhibited an enhanced calcium-binding ability, suggesting the potential of glycinin phosphopeptides as a calcium-binding mediator with greater availability.

Key words: Proglycinin, calcium, peptides, phosphorylation, 1-D IEF gel

Calcium is the fifth most abundant element in humans and animals, and plays an important role in directing cell functions, nerve impulses, and the blood clotting mechanism. Calcium absorption varies with age from early adulthood onward. As such, the high incidence of osteoporosis among the aged has resulted in an increased focus on dietary sources of calcium and useful substances that improve calcium availability [20]. It is already known that the enhanced calcium binding of milk casein is essentially due to the presence of phosphopeptides, which are formed during luminal proteolysis. Casein phosphopeptides (CPPs), casein tryptic hydrolysates, have also been shown to form soluble complexes with calcium and inhibit the precipitation of calcium phosphate, resulting in an increase of soluble calcium in the lower small intestine [3]. Also, it has been reported that the effect of dietary casein on the enhancement of calcium absorption is due to the phosphates in CPPs [10, 12, 17].

Soybeans are an excellent source of protein, and it has long been recognized in Asia. Recently, many researchers have also reported on the importance of soybeans as a health beneficial component [5, 8]. The various peptides that have been derived from soybean proteins include functional food factors as an angiotensin-converting enzyme inhibitor [11, 19], immunomodulators, cholesterol reducers, and other peptides [20]. Soybean proteins are composed of two major components, glycinin and beta-conglycinin. Of these two proteins, glycinin is known as the predominant storage protein in soybeans, thus it is an important target for improving their functional properties. Glycinin consists of six subunits, each of which consists of an acidic and basic polypeptide that are linked by a disulfide linkage [1, 14, 15]. These six acidic and five basic polypeptides have highly homologous yet distinct sequences [14]. As such, this complexity of glycinin proteins is one of the major obstacles in its application. Thus, to avoid this complexity in the current study, a highly purified proglycinin subunit was prepared by the overexpression of the cDNAs encoding $A_{1a}B_{1b}$ in *E. coli* [7], which facilitated the phosphorylation level of proglycinin to be detected, while its binding to calcium was monitored. In addition, the difference in the calcium binding quality was also compared between nonphosphorylated and phosphorylated glycinin peptides.

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MATERIALS AND METHODS

Bacterial Strains, Medium, and Plasmids

Escherichia coli strain JM105 was used as the host cell. LB medium (pH 7.5) consisted of 1% bactotryptone (Difco), 0.5% yeast extract (Difco), and 1% NaCl. The expression plasmid was pKGA$_{1a}$B$_{1b}$-3, which lacks the mature N-terminal 9 bp sequences from the cDNA encoding the glycinin A$_{1a}$B$_{1b}$ subunit precursor and has 30 bp of its 3'-noncoding region. The plasmid and cDNA were kindly donated by Dr. C.-S. Kim, Cheju National University, Korea.

Overproduction of Proglycinins in *E. coli*

The *E. coli* JM105 strain harboring the expression plasmid was grown at 37°C for 18 h after induction with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested by centrifugation and suspended in a cold 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 1.5 mM phenylmethysulfonyl fluoride (PMSF). The total cell lysates were analyzed by SDS-PAGE.

Purification of Soybean Proglycinin

The *E. coli* cell lysates were suspended 6 times in a 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 1.5 mM PMSF, plus lysozyme was added to a final concentration of 1 mg/ml. The suspension was incubated on ice for 30 min, and the cells were disrupted by sonication. The supernatant was then collected after removing the cell debris and unbroken cells by centrifugation at 10,000 ×*g* for 20 min. After the crude extract was adjusted to 40% saturation with solid $(NH_4)_2SO_4$ and stirred for 30 min, the precipitate was removed by centrifugation at 10,000 ×*g* for 20 min, and the supernatant adjusted to 65% saturation and stirred for 30 min. The precipitated protein was then collected by centrifugation at 10,000 ×*g* for 20 min and resuspended in a 35 mM Tris-HCl buffer (pH 7.6) containing 0.05 M NaCl, 10 mM 2-mercaptoethanol, and 0.02% NaN_3. Next, the suspension was dialyzed overnight with exchanges of the same buffer (2-l). The dialyzed material was applied onto a Mono Q 5/5 column that was washed until the A_{280} of the eluant stabilized. The bound material was eluted using a linear gradient of 0.05–0.5 M NaCl, then the fractions (1 ml/fraction) were monitored based on their absorbance at 280 nm and SDS-PAGE, and pooled. Cryoprecipitation was carried out for the final purification. The pooled fractions were then dialyzed against the dialysis buffer containing a 6 mM Tris-HCl buffer (pH 6.3), 10 mM 2-mercaptoethanol, and 0.02% NaN_3 for 2 days with several changes. Thereafter, the dialysate was centrifuged at 10,000 ×*g* for 20 min, and the precipitate was suspended in a 3.5 mM potassium phosphate buffer (pH 7.6) and dialyzed overnight against the dialysis buffer. Total protein of purified proglycinin was measured using the dye binding method of Bradford or a densitometric analysis of a gel stained with Coomassie Brilliant Blue.

Phosphorylation of Proglycinin by Sodium Trimetaphosphate

The chemical phosphorylation of the proglycinin was performed according to the method of Sung *et al.* [16]. The purified proglycinin was adjusted to pH 11.5 with 0.1 N NaOH. After the addition of powder STMP up to 1%, the mixture was continuously stirred at 35°C for 3 h. During the reaction, a constant pH was maintained by the addition of 0.1 N NaOH. To terminate the reaction, 10% Trichloroacetic acid (TCA) was added and the mixture stored at 4°C for 12 h. The supernatant was then discarded after centrifugation at 10,000 ×*g* for 10 min. One-dimensional gel electrophoresis was performed according to the method of Robertson *et al.* [18] to determine the level of phosphorylation. The electrophoresis was conducted at 250 V for 2 h and the sample volume for loading was 10 ml. Thereafter, the protein bands were stained with 0.1% Coomassie brilliant blue R-250 and destained with 10% methanol and 10% glacial acetic acid.

Phosphorylation of Proglycinin Tryptic Hydrolysates

The proglycinin was digested with porcine pancreas trypsin (E.C 3.4.21.4, Sigma St. Louis, MO, U.S.A.) at E/S ratios of 1:250, 1:500, and 1:1000 (w/w), and the enzyme reaction continued for 3 h at 30°C. The reaction mixtures were then adjusted to pH 11.5 with 0.1 N NaOH for 30 min at 4°C and phosphorylated with STMP according to a previous method [16]. After the phosphorylation, the mixtures were adjusted to pH 5 and centrifuged at 10,000 ×*g* for 20 min. The calcium binding activity was determined according to the degree of hydrolysis, as described below. After $CaCl_2$ was added to the samples, the reaction was incubated at pH 6.0, 37°C for 1 h, and terminated by reacting with 10% TCA at 4°C for 12 h, followed by centrifugation at 10,000 ×*g* for 20 min. The final calcium concentration in the supernatant was estimated using an Atomic Absorption Spectrophotometer (Shimadzu, Kyoto, Japan).

Immobilized Metal Affinity Chromatography

HiTrap chelating column was purchased from Pharmacia (Uppsala, Sweden) and used as the matrix for the immobilized metal in the FPLC system (Pharmacia). To immobilize the Ca^{2+} ions on the gel, the column was applied with a 0.1 M $CaCl_2$ solution after washing with distilled water [2, 13]. After the unbound metal ions were washed off with water, the column was equilibrated with several volumes of a 20 mM sodium phosphate binding buffer (pH 5.0), to accelerate the binding of the calcium-binding peptides to the matrix. A 100 µl of tryptic hydrolysates was then added to the column and the unbound

peptides washed out. The calcium-binding peptides bound to the matrix were then eluted with calcium ions using a buffer containing 200 mM sodium dihydrogen phosphate. For regeneration, the column was washed using several volumes of a 200 mM sodium dihydrogen phosphate buffer supplemented with 0.5 M EDTA.

Calcium-Binding Activity of Phosphopeptides

To remove the free phosphates in the bound fraction, the unbound and bound fraction were both dialyzed through a membrane (MW cutoff 1,000 Da) overnight against 2-l of a dialysis buffer containing 5 mM PIPES (pH 6.0), 25 mM KCl, and 0.5 M PMSF. The calcium-binding activity was then assayed as described above.

RESULTS AND DISCUSSION

Purification of Soybean Proglycinin

The proglycinin gene in the $pKGA_{1a}B_{1b}$-3 was expressed as a soluble protein in *E. coli* JM105 and purified by ammonium sulfate precipitation, Mono Q column chromatography, and cryoprecipitation. The crude cell lysates prepared by 40–65% saturation with ammonium sulfate were fractionated by Mono Q 5/5 column chromatography. The fractions eluted between 0.3 and 0.35 M NaCl were then pooled and subjected to cryoprecipitation, and the proteins obtained from each purification step were analyzed by SDS-PAGE. As shown in Fig. 1, cryoprecipitation as a final purification step produced a portion of proteins with a molecular weight of 55 kDa, which was identical with the estimated molecular weight of proglycinin, rather than a partially degraded one. The yield of proteins following the various purification steps is shown in Table 1. The yield of purified proglycinin was 6.1%, and the purity was higher than 96% according to the image analysis.

Phosphorylation of Proglycinin

Since bacteria-expressing proteins are not easily covalently modified, the proteins isolated from the *E. coli* lysates were not dephosphorylated. To detect more subtle differences in the covalent structure, the isolated proglycinin was chemically phosphorylated. The reaction of chemical phosphorylation in this study was carried out at pH 11.5 and 35°C in the presence of 1% STMP, and the phosphorylation of proglycinin was confirmed by an

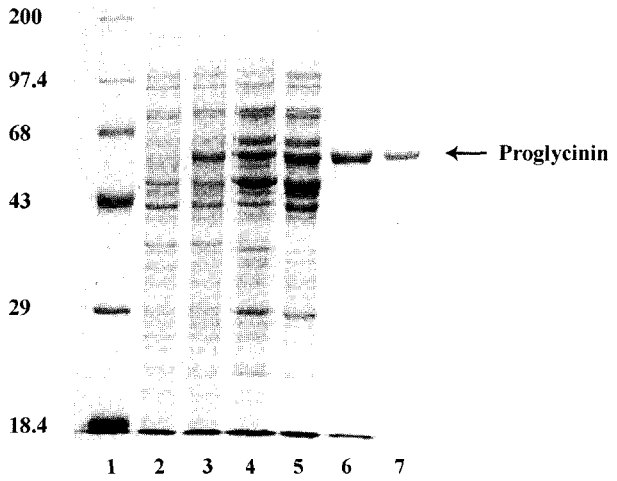

Fig. 1. SDS-PAGE analysis of proglycinin samples at different stages of purification.
Lane 1, molecular markers; Lane 2, non-induced JM105 [$pKGA_{1a}B_{1b}$]; Lane 3, induced [$pKGA_{1a}B_{1b}$]; Lane 4, crude extract; Lane 5, fraction with 40–65% saturation of ammonium sulfate; Lane 6, Mono Q pool; Lane 7, Cryoprecipitation. The arrow indicates the proglycinin band.

isoelectric focusing analysis (Fig. 2). In one-dimensional isoelectric focusing gel electrophoresis, the isoelectric points for the majority of the proglycinin proteins were shifted to the more acidic region when compared to those for the unmodified proglycinin, indicating that the proglycinin proteins were efficiently phosphorylated. Under this condition, a maximum of about 30% of the seryl residues may be phosphorylated in a soy protein isolate. The primary hydroxyl group of serine residues in a soy protein is preferred to have threonine residues as a secondary hydroxyl group to react irreversibly with STMP in alkali condition, resulting in the formation of a stable O-phosphoserine and an equivalent amount of pyrophosphate [16].

Calcium-Binding Activity of Tryptic Hydrolysates

The relationship between the molecular structure of peptides and their calcium-binding property is not yet fully understood. It has been reported that a smaller fragment of less than 1 kDa is unable to bind calcium to a significant extent among the phosphopeptides derived from hen yolk phosvitin [6]. However, the peptides derived from casein by proteolytic digestion are well known to exert various physiological effects, including preventing the precipitation of calcium ions as calcium phosphates [3, 4]. In the present

Table 1. Purification of proglycinin from *E. coli* JM105 ($A_{1a}B_{1b}$).

	Total protein [mg]	Total proglycinin [mg]	Purification [%]	Yield [%]
Crude extract	714.6	87.46	12.24	100
Ammonium sulfate fractionation	409.33	76.59	18.71	76.59
Mono-Q pool	25.65	19.65	76.6	22.47
Cryoprecipitation	5.54	5.34	96.4	6.11

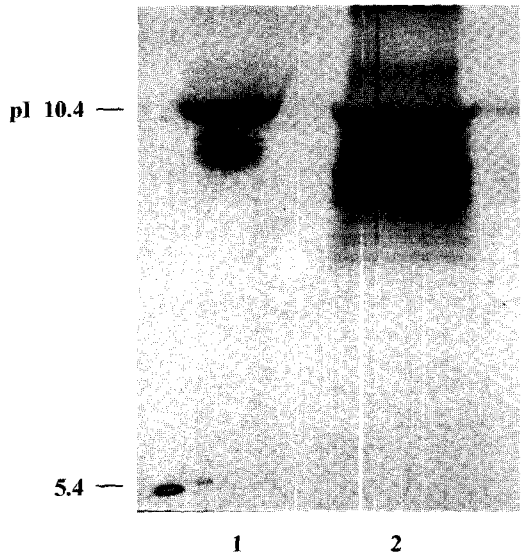

Fig. 2. Determination of isoelectric point of proglycinin.
The protein samples were analyzed using one-dimensional isoelectricfocusing gel, and stained with Coomassie brilliant blue as described in Materials and Methods. Lane 1, purified proglycinin; Lane 2, phosphorylated proglycinin.

study, the effect of the molecular size of the phosphopeptides made from the phosphorylated proglycinin and its subsequent tryptic digestion were studied. After hydrolysis at different E/S ratios, the calcium-binding activity of the non-phosphorylated and phosphorylated samples was analyzed. Figure 3 demonstrates the effect of phosphorylation on the calcium-binding ability at different E/S ratios. The phosphorylated hydrolysates exhibited a higher binding activity than the nonphosphorylated ones for every E/S ratio tested in this study, and the E/S ratio of 1:1,000 showed the highest binding activity. The increase in the binding degree of the hydrolysates after phosphorylation

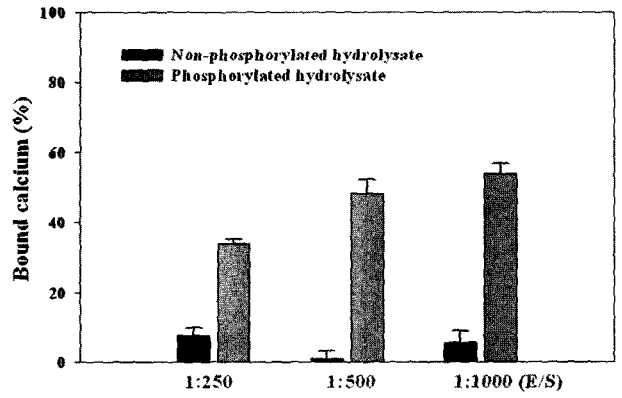

Fig. 3. Calcium-binding activity of nonphosphorylated and phosphorylated tryptic hydrolysate.
After TCA precipitation, the calcium concentration of the supernatant was measured (Bound calcium (%) = Added calcium concentration - calcium concentration of supernatant/added calcium concentration).

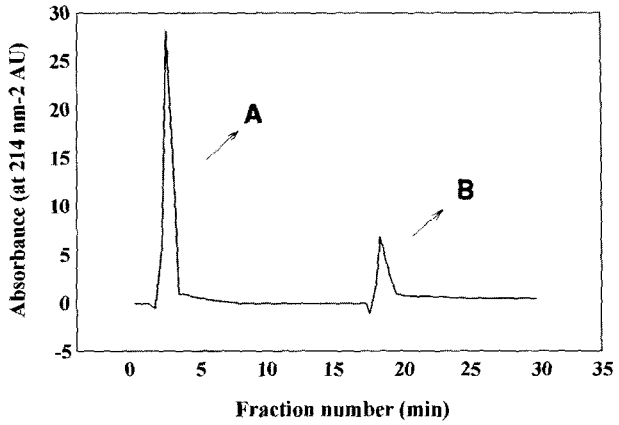

Fig. 4. Fractionation of phosphorylated proglycinin hydrolysate on HiTrap chelating column in FPLC system (E/S = 1:1000).
HiTrap chelating column was used as the matrix for the immobilized calcium in the FPLC system. The elution was carried out with a sodium acetate buffer as described in Materials and Methods.

indicates that the serine residues were converted to phosphoserine by chemical phosphorylation. However, it is still possible that a certain portion of phosphate directly bound to a charged form of amino acid residues, and some of the peptides with continual phosphoserine, aspartic acid, and glutamic acid were chelated to calcium and formed complexes similar to the behavior of CPPs [10].

Figure 4 shows the fractionation pattern of the plasma proteins on the immobilized Ca^{2+} ion affinity column, where peak A represents the proteins that were eluted by the sodium acetate buffer (pH 5.0). The proteins in the fraction were considered to have a relatively low affinity to calcium ions. The peptides in peak B were eluted from the gel with calcium ions by a sodium acetate buffer containing 200 mM of sodium dihydrogen phosphate and had high affinity for calcium ions. The smaller area of peak B compared to peak A indicates that the IMAC chromatogram was able to separate a peptide fraction with a relatively high level of calcium binding. The calcium-binding activities of the unbound and bound fractions, which were prepared at E/S = 1:1,000, were determined after dialysis. To avoid the phosphates in the elution buffer on the IMAC forming insoluble complexes and influencing the calcium-binding activity, the samples were dialyzed overnight against distilled water through a membrane (MW cutoff 1,000 Da). To facilitate the precipitation in a 10% TCA solution, conalbumin, which has a low calcium-binding activity, was also added and co-precipitated. After isolation of the fractions, it was confirmed that the bound fraction had at least 40% higher calcium-binding activity than the unbound fraction (Fig. 5). Further studies are now underway to determine the significance of the primary structure of the proglycinin peptides relative to their calcium-binding activity. Scanff *et al.* [13] purified the casein phosphopeptides

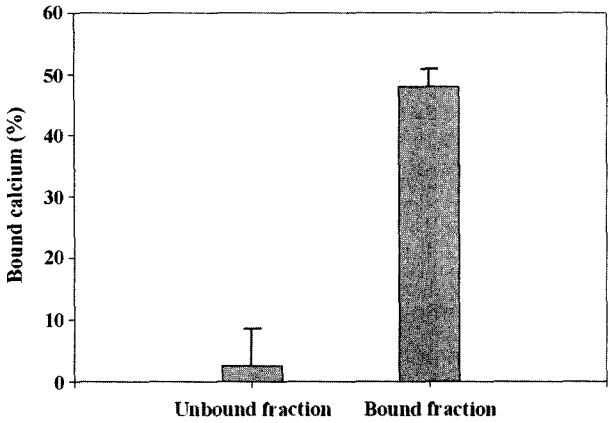

Fig. 5. Calcium-binding activity of IMAC fraction (E/S = 1:1,000).
After TCA precipitation, the calcium of the supernatant was measured (Bound calcium (%) = Added calcium concentration - calcium concentration of supernatant/added calcium concentration).

by IMAC and identified their amino acid composition, and they also demonstrated that the phosphopeptides contained phosphoserine residues. However, the present investigation provided evidence that the phosphopeptides from soybean proglycinin have a calcium-binding activity. Accordingly, these results suggest that the peptides from soy proteins after appropriate phosphorylation could be utilized as promising calcium-absorption enhancers and bioactive agents in future functional food components.

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