

Improvement of functional properties of β -lactoglobulin by conjugation with glucose-6-phosphate through the Maillard reaction

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β -Lactoglobulin (β -Lg) was conjugated with glucose-6-phosphate (G6P) through the Maillard reaction by incubation at 50°C and 65% relative humidity. It was considered that the preferable incubation time was less than 1 day because modification of amino group proceeded quickly through 1 day of incubation and then slowed down; the brown color development and polymerization of β -Lg proceeded steadily from 1 day on. The β -Lg treated with G6P for 0.5 days, of which phosphorus content was 1.06%, was estimated to conjugate 6.9 mol G6P per 1 mol β -Lg. β -Lg-G6P conjugate was completely soluble after heating its solution at 95°C for 10 min whereas more than 90% of intact β -Lg was precipitated at 90°C. Emulsifying properties of β -Lg were improved by conjugation of G6P. No calcium and phosphate were sedimented at 1000 g in the solution containing 30 mM calcium, 22 mM phosphate, and 10 mM citrate at pH 6.7 in the presence of 2% β -Lg-G6P conjugate, but 14.5 mM calcium and 9.1 mM phosphate were sedimented at 100 000 g. The particules of the complex of the β -Lg-G6P conjugate and calcium phosphate with 30–200 nm of diameter were observed in electron micrograph. It was concluded from the above results that modification of β -Lg with G6P was useful in improving its functional properties. © 1998 Canadian Institute of Food Science and Technology. Published by Elsevier Science Ltd. All rights reserved

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INTRODUCTION

Phosphorylation of food proteins improves functional properties such as water solubility, emulsifying activity, foaming properties, and gel-forming properties (Mathies, 1991). Phosphoproteins interact with calcium phosphate. For example, milk caseins interact with calcium phosphate through their phosphate groups, and calcium

and phosphate which are present in excess of their solubilities are solubilized (Holt, 1985). Egg white riboflavin-binding protein (RfBP), which is a globular glycoprophosphoprotein comprising 219 amino acids residues containing eight phosphate groups (Hamazume *et al.*, 1984), also interact with calcium phosphate through its phosphate groups (Aoki *et al.*, 1993). Caseins have been shown to yield phosphopeptides upon digestion in the small intestine. These phosphopeptides also solubilize calcium phosphate, and enhance calcium absorption in the intestine lumen, increasing the calcium concentration by preventing precipitation of calcium phosphate. Solubilization of calcium phosphate is needed for the

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enhancement of calcium absorption in the intestine. Accordingly, it is expected that food proteins with no phosphate groups can be altered to solubilize calcium phosphate and to enhance calcium absorption by phosphorylation.

Chemically phosphorylated proteins are not easily accepted as foods by consumers, although chemical phosphorylation with phosphorus oxychloride is considered to be one of the useful methods for changing the functional properties (Matheis, 1991). Enzymatic phosphorylation is the most preferable method for food proteins with respect to food safety, but gives only a low phosphate group content to proteins because protein kinase has a specificity for the amino acid sequences (Seguro and Motoki, 1990). Recently, some attempts have been made to improve the functional properties of enzyme and food proteins by conjugation of glucose (Kato *et al.*, 1993) or dextran (Kato and Kobayashi, 1991) through the Maillard reaction. Phosphate groups can be introduced to food proteins by conjugation with glucose-6-phosphate (G6P) through the Maillard reaction. Since G6P is conjugated with proteins through mainly amino groups, more phosphate groups can be introduced by G6P conjugation than by enzymatic phosphorylation. We have reported that the heat stability and emulsifying activity of ovalbumin were improved by G6P conjugation (Kato *et al.*, 1995).

Whey proteins represent a valuable and nutritious resource. Recently whey protein concentrate and whey protein isolate have been used as one of materials in food processing. However, improvement of the functional properties of whey proteins is needed for further utilization. β -Lactoglobulin (β -Lg) is a major component in whey proteins, and plays an important role in functional properties of whey proteins, such as heat-induced gelation and emulsifying properties. In this paper we report the improvement of functional properties of β -Lg by modification with G6P through the Maillard reaction.

MATERIALS AND METHODS

Preparation of β -Lg-G6P conjugate and RfBP

β -Lg was prepared from raw skim milk using the method of Armstrong *et al.* (1967) and G6P of which purity was 99.9% was purchased from Oriental Yeast Co. Ltd (Osaka, Japan). β -Lg was dissolved in distilled water in a concentration of 0.5% w/v for measurement of brown color and protein polymerization and 5% for sample preparation with glucose (Glu, 50% of the protein dry wt) or G6P-2Na-6H₂O (94% of the protein dry wt, equivalent to 50% glucose). The β -Lg-G6P solutions were adjusted to pH 7.5 with 1 N NaOH and then lyophilized. The dried samples were incubated at 50°C and 65% relative humidity using a saturated KI solution

for 0.5 to 3 days. For preparation of β -Lg-G6P conjugates, the incubated samples were dialyzed, and lyophilized. RfBP was isolated from egg white from White Leghorn hens by the method of Hidaka and Matsuoka (1977) with a minor modification (Aoki *et al.*, 1993).

Measurement of browning, free amino group content, and polymerized protein content

Browning of protein-Glu or protein-G6P mixture by the Maillard reaction was measured by absorbance at 420 nm of the water solution (5 mg ml⁻¹).

The free amino groups were measured by the trinitrobenzenesulfonic acid (TNBS) method (Haynes *et al.*, 1967).

Polymerized protein content was determined by high performance gel chromatography, at 25°C using Shimadzu LC-5A (Shimadzu Ltd, Kyoto, Japan) or a Toso CCPE chromatograph (Toso Ltd, Tokyo, Japan) equipped, using a TSK-GEL G3000SW column (7.5 mm $\times$ 60 cm, Toso Ltd, Tokyo, Japan) fitted to a TSK guard column (7.5 mm $\times$ 7.5 cm). Elution buffers used were 0.1 M sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl and 25 μ l of sample solution (1 mg protein ml⁻¹) was injected. Polymerized protein content was estimated from the peak area of the chromatogram.

Heat stability

Protein samples were dissolved at concentration of 1 mg ml⁻¹ of 50 mM tris-HCl buffer (pH 7.0) and then heated at 60–95°C for 10 min. The heated sample was diluted to 5 times volume with the buffer and then aggregates were precipitated by centrifugation at 15000 g for 20 min. The UV absorbance at 280 nm of the supernatant was measured to estimate protein concentration of the solution.

Emulsifying property

Emulsifying property was done by the method of Pearce and Kinsella (1978). One ml of corn oil was added to 3 ml of 0.02% protein sample solution (0.2 mg ml⁻¹ of 0.1 M phosphate buffer (pH 7.4)) after which the mixture was homogenized at 12000 rpm at 20°C for 1 min with a PT10-35 Polytron homogenizer (Kinematic Ag, Switzerland). One hundred microliters of the emulsion was taken out from the bottom of a test tube at different times and diluted with 5 ml of 0.1% sodium dodecyl sulfate solution. The absorbance of the diluted emulsion was then determined at 500 nm.

Preparation of test solutions for solubilization of calcium phosphate by proteins

The preparation of test solutions was done according to the procedure for artificial casein micelles (Aoki, 1989).

To 2 ml of 4% protein solution, 200 μ l of 0.2 M potassium citrate, 200 μ l of 0.2 M CaCl_2 , and 240 μ l of 0.2 M K_2HPO_4 were added, followed by 100 μ l of 0.2 M CaCl_2 and 50 μ l of 0.2 M K_2HPO_4 . The addition of 100 μ l of 0.2 M CaCl_2 and 50 μ l of 0.2 M K_2HPO_4 was repeated to give the prescribed concentrations of calcium and inorganic phosphate (Pi). The intervals sets of additions were 15 min, and all additions were accompanied by stirring at pH 6.7. The volume was adjusted to 4 ml by measuring the weight of the solutions. The prepared solutions were allowed to stand for 20 h at 25°C and then centrifuged at 1000 g for 15 min or 100 000 g for 1 h. The precipitated calcium and Pi concentrations were estimated from their differences between total and supernatant fractions.

Electron microscopy

Samples were pre-fixed by mixing 0.5 ml of sample solution, 0.5 ml of cacodylate buffer (pH 6.7) containing 4.9% glutaraldehyde and 0.5 ml of simulated milk ultrafiltrate (SMUF), which was prepared using the method of Jenness and Koops (1962), and then post-fixed by mixing 0.5 ml of pre-fixed sample solution, 0.5 ml of cacodylate buffer (pH 6.7) containing 2% osmium tetroxide and 0.5 ml of SMUF. The fixed samples were diluted with 75% glycerol three times, put on a copper grid coated with carbon supporting film for electron microscopic examination and air dried. The specimen were shadowed with platinum carbon at a 10° angle. A JEM-2000EX II electron microscope (JEOL Ltd, Tokyo, Japan) operating at 160 kV was used. Micrographs were made at the original magnification of 20 000.

Mineral analysis

Phosphorus contents of the β -Lg-G6P conjugates were measured by the method of Allen after digestion in perchloric acid. Calcium and Pi contents were measured in the 12% trichloroacetic acid filtrate by a Hitachi 208 atomic absorption spectrophotometer and the method of Allen (1940).

RESULTS AND DISCUSSION

The decrease in free amino groups of β -Lg incubated with G6P or Glu at 50°C and 65% relative humidity for 3 days was determined by TNBS method because the main groups which participate in the Maillard reaction are amino groups. The free amino groups of β -Lg incubated with G6P decreased to 65 and 51% after 0.5 and 1 days of incubation, respectively (Fig. 1). The numbers of modified amino groups per one β -Lg molecule incubated for 0.5 and 1 day were estimated to be 5.6 and 7.8 since β -Lg contains 15 amino groups of lysine

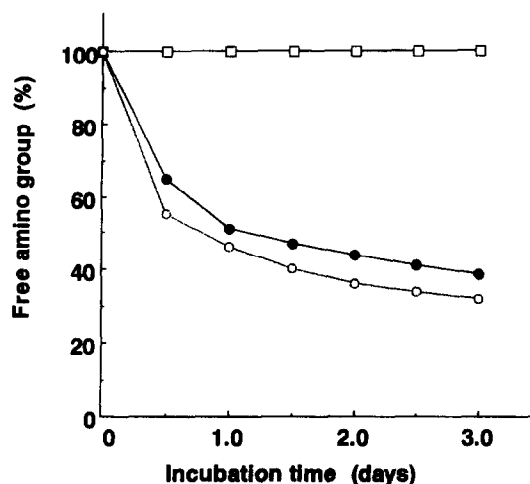


Fig. 1. Decrease in free amino groups of β -Lg during incubation with Glu (○) or G6P (●) at 50°C and 65% relative humidity. β -Lg (□) was incubated in the absence of sugar.

residues and one terminal amino group. The decrease of free amino groups was somewhat smaller in β -Lg incubated with G6P than in incubated with Glu. This tendency was similar to that of ovalbumin incubated with G6P or Glu reported previously (Kato *et al.*, 1995). There were almost no changes in amino groups of β -Lg incubated without sugar.

The brown color development and polymerization of proteins were examined because they occur at the advanced stage of the Maillard reaction. Figure 2 shows the color formation and polymerization of β -Lg incubated with G6P for 3 days at 50°C and 65% relative humidity. The brown color of β -Lg with G6P developed markedly, especially at the later stage, and this intensity was stronger than that of ovalbumin with G6P reported in our previous paper (Kato *et al.*, 1995). The

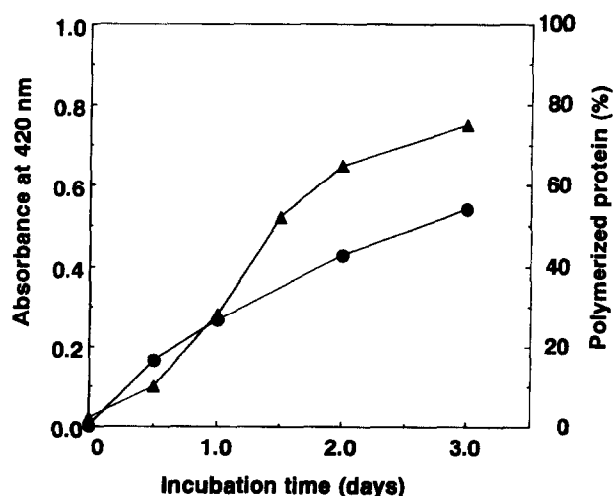


Fig. 2. Brown color development and polymerization of β -Lg incubated with G6P at 50°C and 65% relative humidity. Polymerized protein content was estimated from the peak area of the high-performance gel chromatogram. ●, polymerized protein; ▲, absorbance at 420 nm. Each line shows mean value of the two determinations.

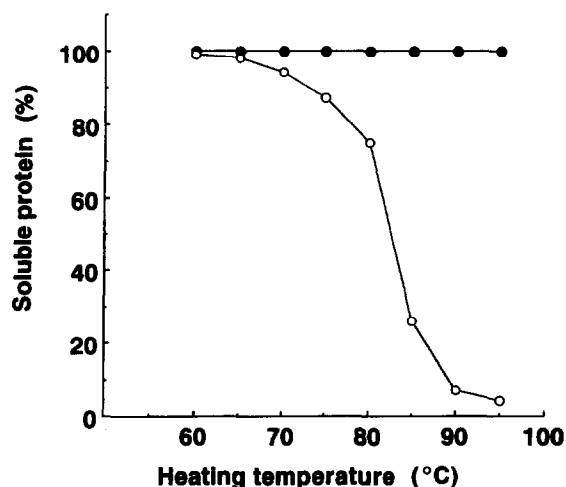


Fig. 3. Heat stability of β -Lg and β -Lg-G6P conjugate. The protein concentration was 0.1% in 50 mM tris-HCl buffer (pH 7.0). $\circ$, intact β -Lg; $\bullet$, β -Lg-G6P conjugate. Each line shows mean value of the two determinations.

polymerized portion contents of β -Lg incubated with G6P for 0.5 and 1 day were 16.5 and 26.4% respectively, and these values were also higher than those of ovalbumin with G6P reported previously (Kato *et al.*, 1995). Accordingly, the preferable incubation time was considered to be less than 1 day because modification of amino group proceeded quickly through 1 day of incubation and then slowed down; the brown color development and polymerization of β -Lg proceeded steadily from 1 day on.

The phosphorus contents of β -Lg-G6P conjugates treated for 0.5 and 1 day were 1.06 and 1.25%, respectively. The number of G6P conjugated with β -Lg was estimated from the phosphorus content of the conjugates and molecular weights of β -Lg and G6P. In this calculation, a molecular weight of 18 362 was used for the β -Lg-A variant. In the β -Lg-G6P conjugate containing 1.06% of phosphorus, 6.9 G6P residues were estimated to be bound to one molecule of β -Lg. This value was higher than that estimated from the determination of free amino group content described above. The residues other than lysine may participate in the Maillard reaction (Kato *et al.*, 1989). The phosphorus content of β -Lg-G6P conjugate incubated for 0.5 and 1 day were higher than that of bovine whole casein, which has a phosphorus content of 0.85%. The β -Lg-G6P conjugate incubated for 0.5 day was used for subsequent experiments.

Woo and Richardson (1983) reported that chemical phosphorylation of β -Lg with phosphorous oxychloride improved functional properties such as emulsifying properties. The heat stability of β -Lg-G6P conjugate was measured. For this purpose, the 0.1% solutions of intact β -Lg and β -Lg-G6P conjugate were heated at 60–95°C for 10 min, and soluble protein was determined. As shown in Fig. 3, β -Lg-G6P conjugate was quite heat-stable when heated at 95°C for 10 min whereas

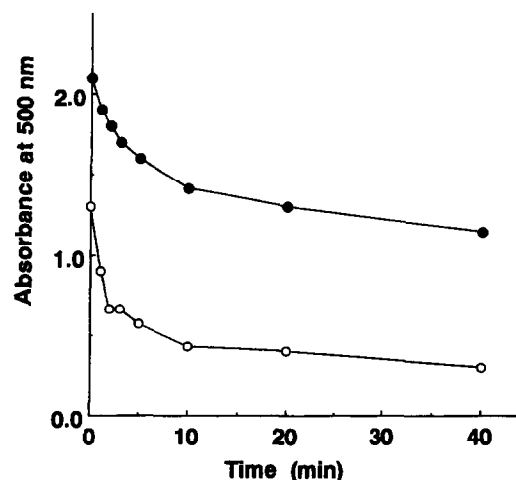


Fig. 4. Emulsifying properties of β -Lg and β -Lg-G6P conjugate. $\circ$, intact β -Lg; $\bullet$, β -Lg-G6P conjugate. Each line shows mean value of the two determinations.

most intact β -Lg was insolubilized at higher temperatures than 90°C.

Figure 4 shows the emulsifying properties of intact β -Lg and β -Lg-G6P conjugate. Emulsion turbidity (ordinate) was plotted against the standing time after emulsion formation (abscissa). The emulsifying activity, which was determined from the absorbance immediately after emulsion formation, of β -Lg-G6P conjugate was 1.6 times that of the intact β -Lg. The emulsion stability of β -Lg-G6P conjugate was also higher than intact β -Lg since the rate of decline in the turbidity was smaller in β -Lg-G6P conjugate than in intact β -Lg.

The solubilization of calcium phosphate by the β -Lg-G6P conjugate was examined and compared to RfBP. Figure 5(A) shows the concentration of calcium and Pi sedimented at 1000g in the solution containing 14–35 mM calcium, 14–24.5 mM Pi, and 10 mM citrate. Calcium and phosphate, of course, were precipitated in the absence of proteins. In the presence of 2% β -Lg-G6P conjugate, no calcium and Pi were sedimented at 30 mM calcium and 22 mM Pi, but small amounts of calcium and Pi precipitated at 35 mM calcium and 24.5 mM Pi. No calcium and Pi were sedimented at all calcium and Pi concentrations examined in the presence of 2% RfBP. The calcium phosphate-solubilizing ability of β -Lg-G6P conjugate was inferior to that of RfBP although the phosphorus content of β -Lg-G6P was higher than that of RfBP, which contains eight phosphate groups per molecule and 0.85% phosphorus (Hamazume *et al.*, 1984).

Although the β -Lg-G6P conjugate solubilized calcium phosphate at 30 mM calcium and 22 mM Pi, the solution was turbid. Subsequently, calcium and Pi sedimented at 100 000g were determined, and the results are shown in Fig. 5B. At 30 mM calcium and 22 mM Pi, 14.5 mM calcium and 9.1 mM phosphate were

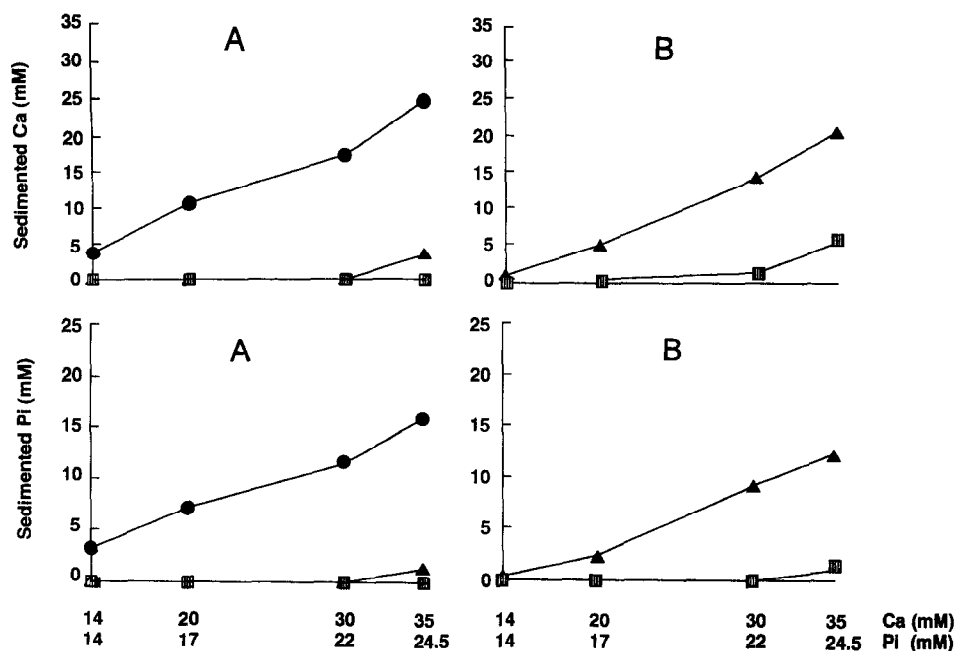


Fig. 5. The amounts of calcium (Ca) and inorganic phosphate (Pi) sedimented at 1000 or 100 000 g from a solution containing 14–35 mM calcium, 14–24.5 mM Pi, and 10 mM citrate at pH 6.7. (A) sedimented at 1000 g; (B) sedimented at 100 000 g; ●, in the absence of protein; ▲, in the presence of 2% β -Lg-G6P conjugate; □, in the presence of 2% RfBP. Each line shows mean value of the two determinations.

sedimented in the presence of 2% β -Lg-G6P conjugate. The sedimented β -Lg-G6P conjugate was also determined. At 30 mM calcium and 22 mM Pi, 12% of β -Lg-G6P conjugate was sedimented at 100 000 g. This means that calcium phosphate are dispersed as fine particles of the complex of calcium phosphate and β -Lg-G6P conjugate. No calcium phosphate was sedimented in the presence of RfBP at 100 000 g at 30 mM calcium and 22 mM Pi although sedimentation of some calcium phosphate was observed at 35 mM calcium and 24.5 mM Pi.

To observe the size and shape of the particles of the complex of calcium phosphate and β -Lg-G6P conjugate, electron microscopy was used. Figure 6 shows the electron micrograph of the sample containing 30 mM calcium, 22 mM Pi, 10 mM citrate, and 2% β -Lg-G6P conjugate. Roughly spherical particles having 30–200 nm diameter were observed and their surface was not smooth. In the Maillard reaction, G6P conjugates with β -Lg mainly through the amino groups. RfBP has eight phosphate groups, which are located between residues Ser (187) and Ser (197) and more localized than in bovine α_{s1} -casein (Hamazume *et al.*, 1984). The phosphate groups of β -Lg-G6P conjugate are not as localized as those of caseins and RfBP. The difference of calcium phosphate-solubilizing ability between β -Lg-G6P conjugate and RfBP may be due to that of localization of phosphate groups. It is also probable that the interaction between β -Lg-G6P conjugate and calcium phosphate is weaker than that between RfBP and calcium phosphate. Solubilization of calcium phosphate is needed for the increase of calcium

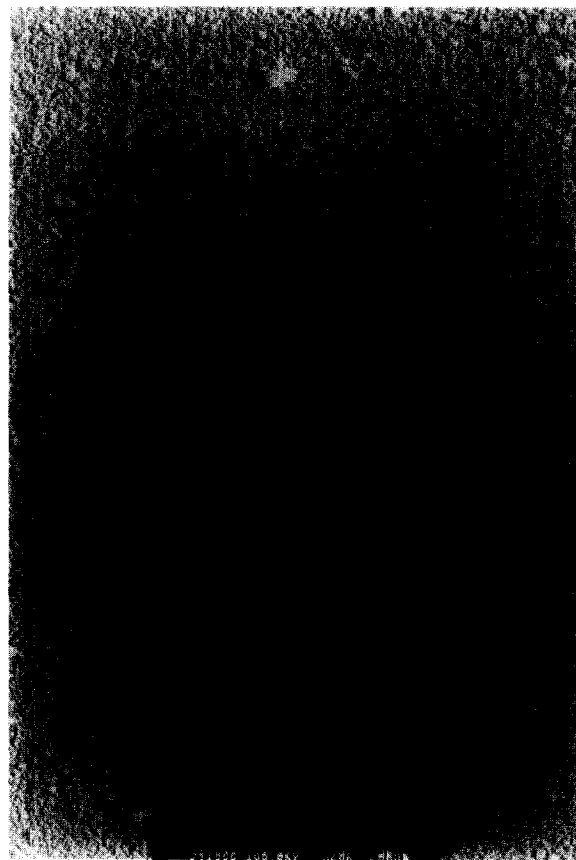


Fig. 6. Electron micrograph of the complex β -Lg-G6P conjugate and calcium phosphate. The solution containing 2% β -Lg-G6P conjugate, 30 mM calcium, 22 mM Pi, and 10 mM citrate was fixed with glutaraldehyde and osmium tetroxide, and then shadowed with platinum. The white bar in the electron micrograph shows 200 nm.

absorption because calcium tends to precipitate as calcium phosphate in small intestine, especially in the distal portion where the pH is slightly alkaline. Although phosphoproteins or phosphopeptides solubilize calcium phosphate, the calcium absorption may not be increased if their interaction with calcium phosphate is too strong. Egg yolk phosvitin, which is highly phosphorylated and contained about 10% phosphorus, does not increase calcium absorption (Naito, 1986). It may be better for calcium absorption-stimulating ability that the interaction of β -Lg-G6P conjugate with calcium phosphate is not strong. The evaluation of the calcium absorption-stimulating ability of β -Lg-G6P conjugate by using animals is needed.

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