Functional improvements in β-conglycinin by preparing edible bioconjugates with ε-polylysine and dextran

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Abstract

β-Conglycinin was conjugated with ε-polylysine (PL) by means of microbial transglutaminase (MTGase) to improve its function. The β-conglycinin-PL conjugate was purified by dialysis. Composition of the β-conglycinin-PL was β-conglycinin:PL = 1:18 (molar ratio) which was confirmed by amino acid analysis. The β-conglycinin-PL was further conjugated with dextran (Dex) by the Maillard reaction. The β-conglycinin-PL-Dex conjugate was purified by dialysis. Conjugation was confirmed by SDS-PAGE and PAS staining. Composition of the β-conglycinin-PL-Dex was β-conglycinin-PL:Dex = 1:41 (molar ratio) which was confirmed by UV spectra measurement and phenol sulfuric acid method. Solubility of β-conglycinin in the acidic range was much improved by conjugation with PL and further improved by further conjugation with Dex. Emulsifying property of β-conglycinin in acidic pH range was much improved by conjugation with PL and Dex. Immunogenicity of β-conglycinin was decreased by conjugation with PL and Dex.

Introduction

β-Conglycinin is a major soy protein, which represents about 30% of total protein in soybean seeds. β-conglycinin has high nutritional value and its various functional properties such as emulsifying (Stone and Campbell 1980; Yamauchi et al. 1982; Tang 2017), foaming (Sirison et al. 2021) and gelling properties (Renkema et al. 2001) are well known. Because β-conglycinin contains saccharide chains, β-conglycinin has high affinity to water and contributes to high emulsion stability. In addition to functional properties, it was clarified that β-conglycinin has serum lipid-lowering (Ma et al. 2013) and anti-obesity effects (Wanezaki et al. 2020). Although β-conglycinin is a useful protein, it is also known that β-conglycinin is a major antigen of soybean allergy (Cordle 2004; Shan et al. 2021). Soybean is recognized as one of 8 major food allergens in Japan. In addition, solubility and emulsifying properties of β-conglycinin decrease in the acidic pH range. Hence, it is strongly desirable to develop a new method that would lower the allergenicity of β-conglycinin and improve functional properties. We have been studying the neoglycoconjugates of protein to achieve this (Hattori 2002). Protein conjugation can simultaneously achieve reduced allergenicity and improved functional properties (such as thermal stability, solubility, and emulsifying ability) while maintaining the physiological functions of proteins.

In the present study, we conjugated ε-polylysine (PL) to β-conglycinin so as to cover the epitopes of β-conglycinin which would lead to reduced immunogenicity and improved solubility and emulsifying property. Then we further conjugated dextran (Dex) to the β-conglycinin-PL conjugate so as to reduce immunogenicity of β-conglycinin more effectively by covering broader area of the epitopes in β-conglycinin. The concept underlying this study is to reduce allergenicity by shielding the epitope with a low allergenic substance and to improve functional properties such as emulsification by binding hydrophilic substances. PL is a cationic, naturally occurring homopolyamide composed of L-lysine with amide linkages between ε-amino and α-carboxyl groups (Shima and Sakai 1981). It was accidentally discovered as an extracellular material produced by *Streptomyces albulus* ssp. *Lysinopolymerus* strain 346 (Shima and Sakai 1977). We used microbial transglutaminase (MTGase) (EC2.3.1.13) to bind PL to

β-conglycinin. This enzyme catalyzes the acyl-transfer mechanism which the γ-carboxamide group act as an acyl donor and primary amines act as acyl-acceptors. γ-carboxamide group of Gln residue in β-conglycinin and ε-amino residue of PL reacts. MTGase is an enzyme used to improve texture, and products catalyzed by this enzyme would be edible, as well as β-conglycinin-PL produced in this manner.

Dextran (Dex) is a polysaccharide consists of D-glucoses. It has low antigenicity and immunogenicity, and few branch structures. Conjugation between the β-conglycinin-PL conjugate and Dex was carried out by the Maillard reaction. The Maillard reaction is a natural occurring reaction which binds between the amino groups and the reducing-end carbonyl groups in saccharide (Kato 2002). By the conjugation with saccharide by the Maillard reaction, functional improvements in a protein would be expected. In the present report, we will describe on the preparation of the β-conglycinin-PL and β-conglycinin-PL-Dex conjugates and improvements in solubility, emulsifying property and immunogenicity of β-conglycinin.

Materials And Methods

Materials

Epsilon-polylysine was a gift from Chisso Corporation (Tokyo Japan) (average polymerization degree; 30). Activa TG-K (E.C.2.3.2.13) was a gift from Ajinomoto Co. (Tokyo, Japan). Dextran (Dex) originate of *Leusonostoc mesenteriodes* was purchased from Sigma Aldrich Co. (St. Luis, USA). Molecular weight of Dex was ~ 10 kDa.

Purification of the β-conglycinin

β-Conglycinin was isolated from defatted soybean seeds which was a gift from Nisshin OilliO Corporation (Tokyo Japan). Crude β-conglycinin was obtained according to the method of Nagano et. al. (1992) and purified by ion-exchange chromatography using a Q Sepharose Fast Flow column (2.5 ID x50 cm, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The column was equilibrated with 35 mM sodium phosphate buffer (pH 7.6) in advance. Crude β-conglycinin was applied to the column and eluted by a 0.1−0.5 M NaCl linear gradient in a 35 mM sodium phosphate buffer (pH 7.6), and eluted by a 0.5 M NaCl in a 35 mM sodium phosphate buffer (pH 7.6) at a flow rate of 5.0 ml/ min. Eluted protein was detected by the absorbance at 280 nm. After dialysis against distilled water with cellulose membrane (MWCO: 14,000) (Viskase Companies, Inc., IL, USA) and lyophilization with ALPHA 1−2 LDplus lyophilizer (Martin Christ, Osterode, Germany), purified β-conglycinin was obtained. As for dialysis, the ratio of the internal fluid to the external fluid was 1:10, and the external fluid for dialysis was changed 5 times every 3 hours. Purity of β-conglycinin was confirmed by SDS-PAGE.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the method of Leammli (1970) with a 15% separating gel and 4% stacking gel. Electrophoresis was carried out at 20 mA constant current, and the gels were stained with CBB and Schiff reagent. Periodic acid Schiff (PAS) staining was carried out under the following conditions: 12.5% trichloroacetic acid was used for fixing proteins in the gel, and 1% orthoperiodic acid was used for oxidation of 1, 2-glycolic groups of carbohydrates. The gel was stained in Schiff reagent in the dark and then washed with 0.5% potassium pyrosulfite.

Preparation of the β-conglycinin-PL conjugate

Activa TG-K (Ajinomoto) was used as MTGase source. Enzyme titer was 100 units/g. 0.2% solution of MTGase was used as MTGase for the enzymatic reaction. Activa TG-K (2.5 g) was dissolved in 10 mL of 0.5 M NaCl/0.1 M imidazole buffer (pH 7.6) and centrifuged at 18,000 rpm for 20 min at 4°C. The supernatant was collected, filtrated and dialyzed overnight against the same buffer to remove calcium lactate and dextrin. MTGase-catalyzed reaction was carried out under the condition of Gln (in β-conglycinin): PL = 1:1. β-conglycinin (60.0 mg) was dissolved in 9 mL of 6 M GdnHCl and dialyzed against 0.1 M imidazole buffer (pH 7.6) containing 0.5 M NaCl. PL (160.0 mg) was dissolved in 9 mL of 0.1 M imidazole buffer (pH 7.6) containing 0.5 M NaCl. β-conglycinin solution (9 mL) and PL solution (9 mL) were mixed, and enzymatic reaction was started by adding 18 mL of MTGase solution to the solution containing β-conglycinin and PL. The reaction mixture was incubated at 40°C for 1 h.

Purification of the β-conglycinin-PL conjugate

After the enzyme reaction, 10 mM 2-mercaptethanol was added to the sample to stop the enzyme reaction, and dialyzed against the 0.5 M NaCl/0.1 M imidazole buffer (pH 7.6) including 10 mM 2-mercaptethanol to remove MTGase and PL using the dialysis membrane which molecular weight cutoff is 100 kDa. Buffer was changed 5 times (1 time/day). Purified β-conglycinin-PL conjugate was dialyzed against distilled water and lyophilized.

Preparation of the β-conglycinin-PL-Dex conjugate

The β-conglycinin-PL-Dex conjugate was prepared by the Maillard reaction. At First, the mixture of β-conglycinin-PL and dextran was made in the following manner. β-conglycinin-PL was dissolved in 0.5 M NaCl/0.1 M imidazole buffer (pH 7.0, 10 mM 2-mercaptethanol) and dialyzed against distilled water, then dextran was dissolved in that solution at a mixing ratio of 1:1, 1:2, 1:3, 1:5 (w/w) and lyophilized. Subsequently this mixture was incubated at 60°C at a relative humidity of 79% for 0, 6, 24, 48 or 72 h. Relative humidity at 79% was kept by saturated KBr in a desiccator.

Purification of the β-conglycinin-PL-Dex conjugate

After the Maillard reaction, the reaction mixture was dissolved in 0.1 M imidazole buffer (pH 7.0) containing 10 mM 2-mercaptethanol and 0.5 M NaCl and dialyzed against distilled water to remove Dex using a dialysis membrane whose molecular weight cutoff is 100 kDa. Buffer was changed every 3 h 10 times and centrifuged at 2,000 rpm at 20°C for 5 min. The supernatant was recovered and lyophilized.

Chemical analysis of the β-conglycinin-PL and β-conglycinin-PL-Dex conjugate *s*

The amount of PL in the β-conglycinin-PL conjugate was measured by determining the amount of Glu by an amino acid analysis with L-8800 amino acid analyzer (Hitachi, Tokyo, Japan). Protein content in the β-conglycinin-PL-Dex conjugate was quantitated by detecting the absorbance at 230 nm by using the β-conglycinin-PL conjugate as standard. Dex content in the β-conglycinin-PL-Dex conjugate was quantitated by phenol sulfuric acid method (Dubois et al. 1956) by using Dex as standard.

Evaluation of solubility

Samples (β-conglycinin, the β-conglycinin-PL and β-conglycinin-PL-Dex conjugates) were stirred in 0.1 M imidazole buffer (pH 2.0–8.0) for 1 h and centrifuged at 18,000 rpm at 4°C for 20 min. Absorbance of the supernatant was measured at 280 nm.

Evaluation of the emulsifying property

Emulsifying property of the β-conglycinin-PL and β-conglycinin-PL-Dex conjugates was evaluated by the turbidimetric method (Pearce and Kinsella 1978). Samples were dissolved in a McIlvaine buffer at pH 3.0, 5.0, 7.0 or in the buffer containing 0.2 M NaCl. Concentration of β-conglycinin was adjusted to 0.5 mg/mL.

To prepare an oil-in-water emulsion, 2 ml of a protein solution and 0.5 ml of corn oil were homogenized by a Polytron PTA-7 (Kinematica, Switzerland) homogenizer at 24,000 rpm at room temperature for 1 min. A 100 μl aliquot was immediately taken (0 min) and after 10, 30, 60 and 120 min from the bottom of the homogenized emulsion and 50-fold diluted with 0.1% SDS solution, the absorbance was measured at 500 nm by a spectrophotometer. The emulsion stability was evaluated by the absorbance at 500 nm of the diluted emulsion at 30 min after emulsification. The emulsifying activity was calculated as follows.

$$EAI \ (m^2/g) = 2T/\varphi C$$

$$T = 2.3 \ A/L$$

$(A = A_{500}, L = 10^{-2} \ m \ (\text{light path}), \varphi = 0.2 \ (\text{oil-phase volume fraction}))$

Immunization

Female BALB/c mice at 6 weeks of age were immunized intraperitoneally with β-conglycinin, β-conglycinin-PL or β-conglycinin-PL-Dex (100 μg as protein) emulsified in Freund's complete adjuvant (Difco Laboratory, MI, USA). 2 weeks after the primary immunization, the mice were boostered with 100 μg of protein emulsified with Freund's incomplete adjuvant (Difco Laboratory). Blood samples were collected from mice seven days after the secondary immunization and stored at 4°C for 24 h to form a clot (Yoshida et al. 2022). Antisera were collected from each blood sample after clot formation. Mice were

sacrificed by cervical dislocation. This study was performed in conformance with the guidelines for the care and use of experimental animals established by the ethics committee of Tokyo University of Agriculture and Technology (R03-186, July 29th, 2021).

Enzyme-Linked Immunosorbent Assay (ELISA)

β-conglycinin, the β-conglycinin-PL or β-conglycinin-PL-Dex conjugates dissolved in PBS at a protein concentration of 0.01% (100 µL) was added to wells of a polystyrene microtitration plate (Maxisorp, Nunc, Roskilde, Denmark), and the plate was incubated at 4°C overnight to coat the well with each antigen. After the removal of the solution, each well was washed three times with 125 µl of PBS-Tween (PBS containing 0.05% Tween 20). 125 µL of 1% OVA/PBS solution was added to each well, and the plate was incubated at 25°C for 2 h, and then the plate was washed three times with 200 µL of PBS-Tween. A 100 µL of antiserum was added and incubated at 25°C for 2 h, and then the plate was washed three times with 200 µL of PBS-Tween. A 100 µL of alkaline phosphate-labeled rabbit anti-mouse immunoglobulin (Dako A/S Denmark) diluted with PBS-Tween was added to each well, and the plate was incubated at 25°C for 2 h. After three washings, 100 µL of 0.1% sodium p-nitrophenyl phosphate disodium/diethanolamine hydrochloride buffer (pH 9.8) was added to each well, and the plate was incubated at 25°C for 30 min. After the addition of 5 M sodium hydroxide solution (20 µl) to each well to stop the reaction, the absorbance at 405 nm was measured with a microplate reader (iMark microplate reader, Bio Rad Laboratories,Inc., California, USA). Statistical analysis was performed on the obtained results by Tukey-Kramer multiple range test.

Results And Discussion

Preparation and characterization of the β-conglycinin-PL and β-conglycinin-PL conjugates

β-conglycinin and PL were conjugated by means of MTGase. Conjugation between β-conglycinin and PL was confirmed by SDS-PAGE. New bands appeared on the border of separation gel and stacking gel and on the top of stacking gel (Fig. 1). Purification of the β-conglycinin-PL conjugate was carried out by dialysis using 100 kDa cutoff membrane. 91.1 mg of the β-conglycinin-PL conjugate was obtained from the enzymatic reaction using 60.0 mg of β-conglycinin and 160.0 mg of PL which was 41.4% yield. Amino acid analysis indicated the molar ratio of β-conglycinin to PL was about 1:18.

The β-conglycinin-PL conjugate was further conjugated with Dex by the Maillard reaction. Formation of the β-conglycinin-PL-Dex conjugate was evaluated by SDS-PAGE (Fig. 2). The results indicated that CBB stained bands of β-conglycinin-PL disappeared by 6 h of the Maillard reaction. As the Maillard reaction advanced, insoluble aggregates seemed to be produced. The results of PAS staining showed that the bands at the upper end of the concentrated gel was observed after 24 h or more at all mixing ratios which indicates glycation by the Maillard reaction.

By visual observation, a relatively large amount of insoluble matter was observed at mixing ratios (β-conglycinin-PL:Dex) of 1:1 and 1:2. On the other hand, since the results of mixing ratios (β-conglycinin-PL:Dex) of 1:3 and 1:5 did not show much differences, we adopted mixing ratio of 1:3 for preparation of the β-conglycinin-PL-Dex conjugates so as to minimize unreacted saccharides. We adopted 48 h as a reaction time, because the Maillard reaction actually occurred. We also observed browning in the Maillard reaction product at that time.

Improvement in solubility of β-conglycinin by conjugation with PL and Dex

Influence of pH on the solubility of the β-conglycinin-PL and β-conglycinin-PL-Dex conjugates was evaluated (Fig. 3). The result shows that improvements in solubility of β-conglycinin-PL in the range of pH 2.0-7.0 and β-conglycinin-PL-Dex in the range of pH 2.0-8.0. These results indicate that the solubility of β-conglycinin was improved by the increase in cationic polar group by conjugation with PL. In addition, it is considered that the change in solubility of β-conglycinin-PL-Dex conjugate was achieved by the influence of the hydrophilic group by Dex.

Improvement in emulsifying property of β-conglycinin by conjugation with PL and Dex

Emulsifying property of the β-conglycinin-PL and β-conglycinin-PL-Dex conjugates was evaluated by turbidity method at various pHs and in the presence of salt. The effect of pH on the emulsifying ability of the conjugates compared to β-conglycinin was evaluated on the basis of the emulsifying activity index (EAI) and emulsion stability.   EAI value and emulsion stability value of the conjugates were higher than these of β-conglycinin at each pH value (Fig. 4). The addition of hydrophilicity and increase in the net charge by conjugation with PL are considered to have enhanced the emulsifying property of β-conglycinin. Nagasawa et al. (1996) revealed that increase in polysaccharide content by conjugation with acidic polysaccharides was more effective to improve the emulsifying property of bovine β-lactoglobulin. Their findings indicate that addition of hydrophilicity is important for the emulsifying property of β-lactoglobulin bioconjugates. In the case of this study, addition of hydrophilicity by conjugation with PL and Dex is considered to be important for improved emulsifying property of β-conglycinin.

Emulsifying property of β-conglycinin-PL and β-conglycinin-PL-Dex in the presence of 0.2 M NaCl was also evaluated (Fig. 5). Emulsifying property of the conjugates in the presence of 0.2 M NaCl was higher than that of β-conglycinin and as high as that in the absence of NaCl. Improvement in emulsifying property of β-conglycinin after conjugation with PL and Dex was considered to be brought about by addition of ion-exchanging ability of PL. Nagasawa et al. (1996) also revealed that addition of net charge by conjugation with acidic polysaccharides was more effective to improve the emulsifying property of β-lactoglobulin in the presence of salt. In the case of this study, addition of net charge by conjugation with PL is considered to be important for improved emulsifying property of BLG in the presence of salt. Since 0.2 M NaCl is similar to salt concentration in food (Hayabuchi et al. 2020), the conjugates obtained in this study are valuable for food application.

Reduced immunogenicity of β-conglycinin by conjugation with PL and Dex

Immunogenicity of the β-conglycinin-PL and β-conglycinin-PL-Dex conjugates was evaluated by noncompetitive ELISA in BABL/c mice after immunization with Freund's adjuvant (Fig. 6a). Results were expressed as relative IgG amount as compared with mixture of anti-β-conglycinin antisera. Immunogenicity of β-conglycinin was reduced by conjugation with PL and further reduced by additional conjugation with Dex. Shielding of epitopes of β-conglycinin was achieved by conjugation with PL and effective shielding was considered to be achieved by further conjugation with Dex. In addition, the emergence of novel immunogenicity was not observed after conjugation with PL and Dex (Fig. 6b, c). Conjugation with PL and Dex was considered to be an effective method to reduce immunogenicity of β-conglycinin without inducing novel immunogenicity.

Concluding remarks

In this study, we prepared the β-conglycinin-PL and β-conglycinin-PL-Dex conjugates with modified functionality by using MTGase and the Maillard reaction. By conjugation, solubility of β-conglycinin was improved and the emulsifying properties of β-conglycinin in the acidic pH region and in the presence of NaCl were much improved. Immunogenicity of β-conglycinin was reduced by this conjugation. Especially, further conjugation with Dex was very effective to reduce the immunogenicity of β-conglycinin. Since the conjugation method used in this study is a safe method, this method is very valuable in that it would be applicable for food processing. We believe that the conjugates prepared in this study can be used as novel food additives in food application with improved functional properties and reduced allergenicity.

Abbreviations

CBB, Coomassie brilliant blue; Dex, dextran; MTGase, microbial transglutaminase; MWCO, molecular weight cut off; PAS, periodic acid Schiff; PL, epsilon polylysine; SDS-PAGE, Sodium dodecyl sulfate poly acrylamide gel electrophoresis.

Declarations

Acknowledgments

Funding information

Data availability

The data underlying this article are available in the article and also from the corresponding author upon request.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Author contributions

TY: Conceptualization, Data curation, Writing manuscript, IH, TH, TM: Data curation, Writing manuscript, MH: Conceptualization, Writing manuscript, Funding acquisition.

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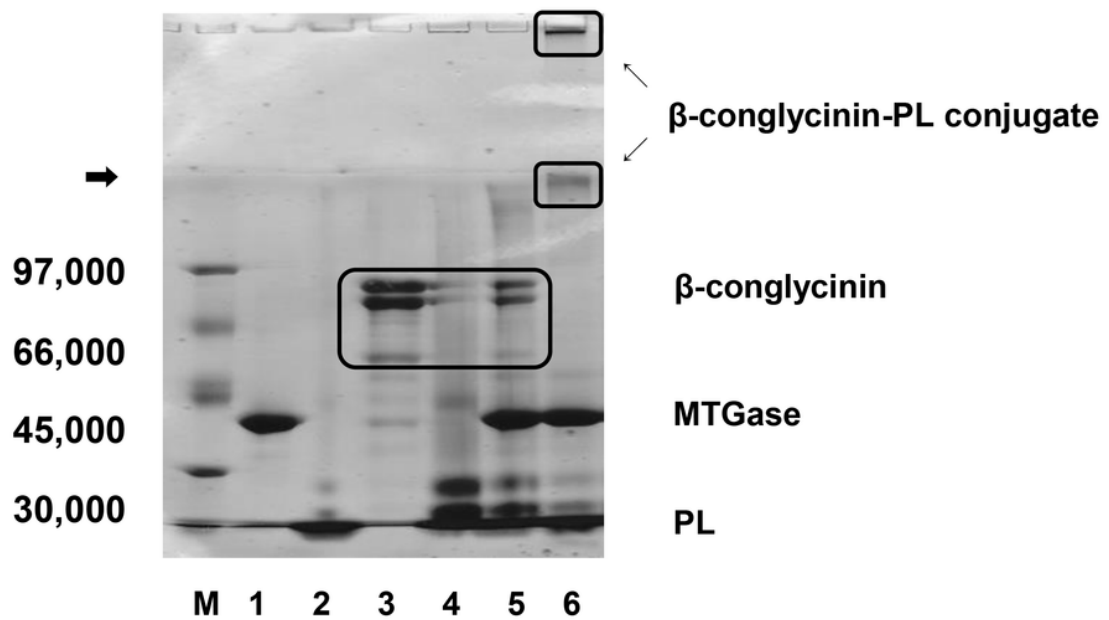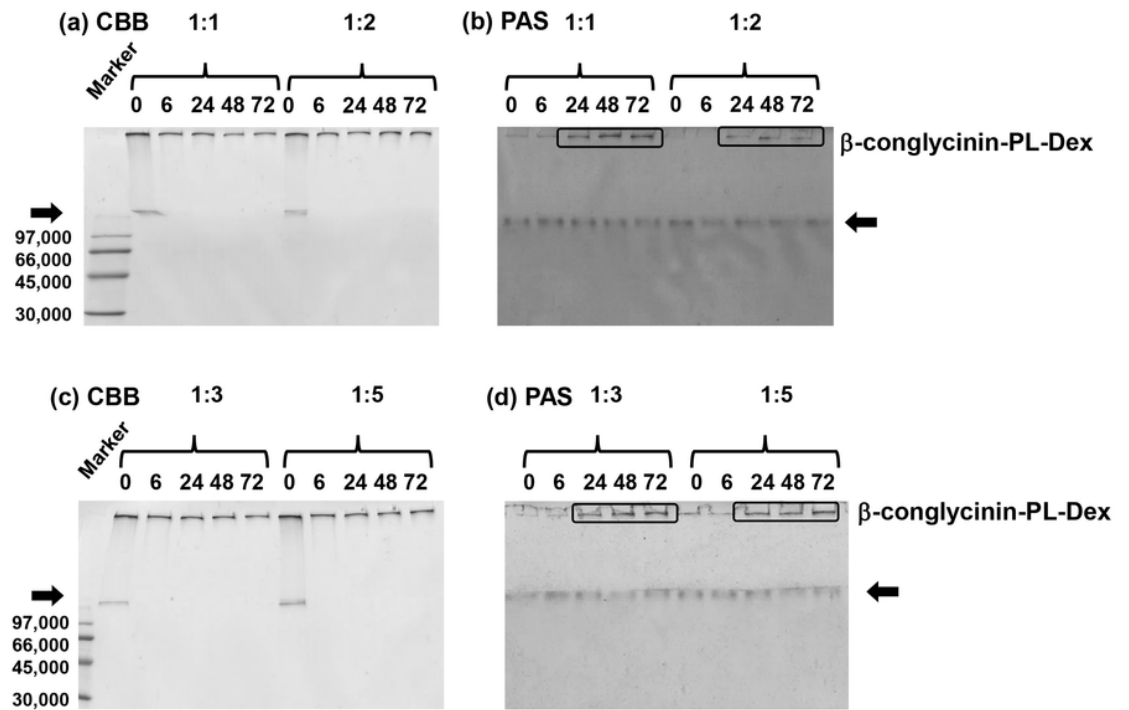
Figures

Fig. 1

Figure 1

Confirmation of conjugation between b-conglycinin and PL.

M: molecular weight marker, 1: MTGase, 2: PL, 3: b-conglycinin, 4: protein solution (b -conglycinin and PL), 5: 0h, 6 = 1h.

A thick arrow indicates the boundary between the stacking (upper) and separating (lower) gels.

Figure 2

Relation between reaction time and mixing ratio of Dex in the Maillard reaction and composition of the b-conglycinin-PL-Dex conjugate.

SDS-PAGE patterns of b-conglycinin-PL-Dex conjugates whose mixing weight ratio is 1:0, 1:1, 1:2, 1:3, 1:5. It was prepared by the Maillard reaction (60°C, relative humidity 79%, 0, 6, 24, 48, 72 h). (a, c) CBB staining, (b, d) PAS staining. Thick arrows indicate the boundary between the stacking (upper) and separating (lower) gels.
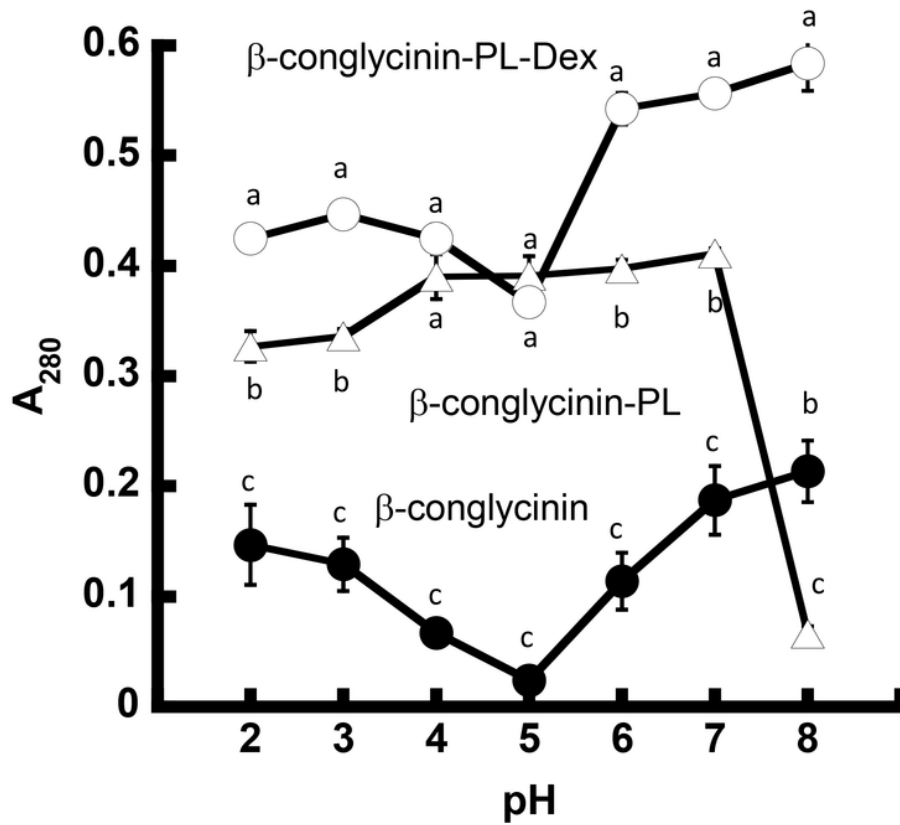
Fig. 3

Figure 3

Solubility of the b-conglycinin-PL and b-conglycinin-PL-Dex conjugates.

●, b-conglycinin; △ , b-conglycinin-PL; , b-conglycinin-PL-Dex.

For each sample, means not followed by the same letter are significant different at p <0.01 level of significance, according to Tukey-Kramer multiple range test.
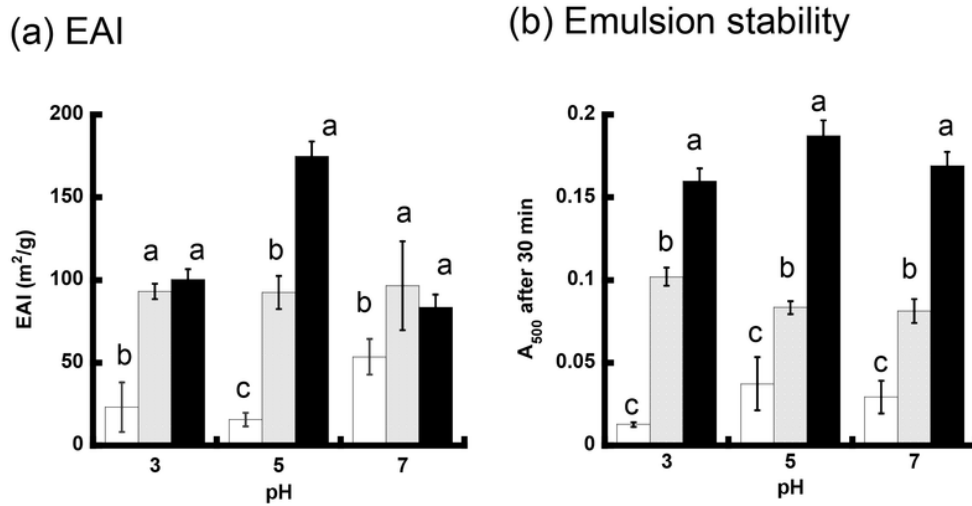
(a) EAI

(b) Emulsion stability

Fig. 4

Figure 4

Emulsifying property of the b-conglycinin-PL and b-conglycinin-PL-Dex conjugates at various pHs.

(a) EAI; (b) Emulsion stability 30 minutes after emulsification.

White bar, b-conglycinin; hatched bar, b-conglycinin-PL; black bar, b-conglycinin-PL-Dex. For each sample, means not followed by the same letter are significant different at $p<0.01$ level of significance, according to Tukey-Kramer multiple range test.
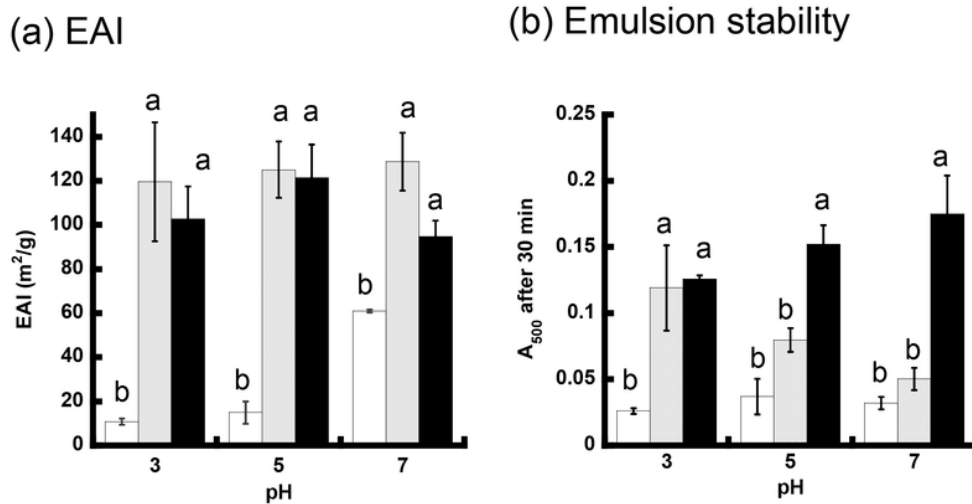
(a) EAI

(b) Emulsion stability

Fig. 5

Figure 5

Emulsifying property of the b-conglycinin-PL and b-conglycinin-PL-Dex conjugates in the presence of 0.2 M NaCl.

(a) EAI; (b) Emulsion stability 30 minutes after emulsification.

White bar, b-conglycinin; hatched bar, b-conglycinin-PL; black bar, b-conglycinin-PL-Dex. For each sample, means not followed by the same letter are significant different at $p < 0.01$ level of significance, according to Tukey-Kramer multiple range test.
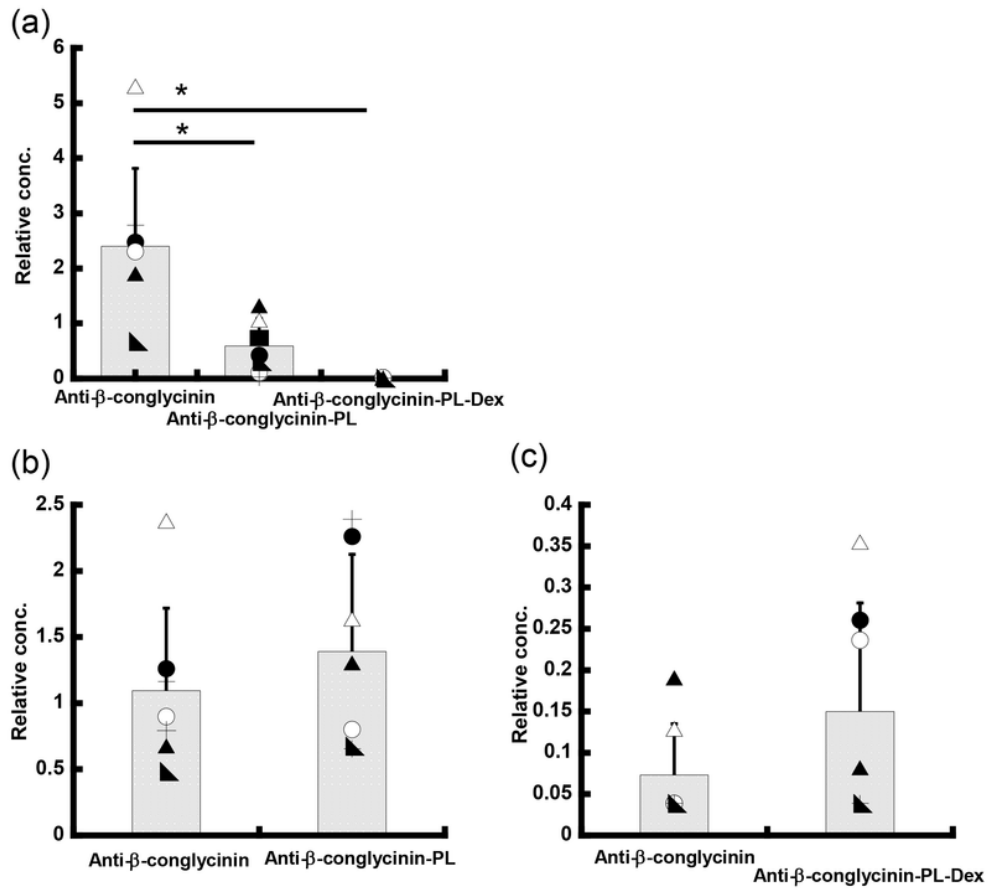
Fig. 6

Figure 6

Immunogenicity of the b-conglycinin-PL and b-conglycinin-PL-Dex conjugates in BALB/c mice.

Anti- b-conglycinin (a), anti- b-conglycinin-PL (b) and anti- b-conglycinin-PL-Dex responses (c) were evaluated by non-competitive ELISA. Markers indicate individual data and bar graphs indicate average values. A significant difference (*:p<0.01) was determined by Tukey-Kramer multiple range test.