Separation of growth-stimulating peptides for *Bifidobacterium* from soybean conglycinin

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**AIM:** To isolate and identify the soybean conglycinin peptides that selectively stimulate the growth of *Bifidobacterium* *in vitro*, and to investigate the effect of soybean conglycinin peptides on intestinal ecosystem *in vivo*.

**METHODS:** Soybean conglycinin was purified from soybean seeds by gel filtration (Sepharose-CL-6B). These proteins were submitted to hydrolysis by pepsin. Several growth-stimulating peptides for *Bifidobacterium* were isolated chromatographically from pepsin hydrolysis of soybean conglycinin and identified by means of matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). Parallel to *in vitro* study, *in vivo* experiments with soybean conglycinin peptides were performed in mice. Ninety male KM mice were randomly assigned into five groups of 16 mice each, and each group was administered for 21d intragastrically with pure protein or various control proteins. The results showed that the peptides which were isolated from soybean conglycinin could stimulate the growth of *Bifidobacterium* *in vitro*, and the molecular mass of purified peptides with MALDI-TOF-MS ranged from 693.32 to 1829.55. Compared with control group, *in vivo* experiments showed that P2-PTC group decreased cecal pH (7.08±0.08 vs 7.21±0.09, *P*<0.05) and *enterococci* counts (5.38±0.26 log10 CFU/g vs 5.78±0.19 log10 CFU/g, *P*<0.05), significantly increased sIgA level (172.08±35.40 ng/g vs 118.27±33.93 ng/g, *P*<0.01) and β-galactosidase activity (1.28±0.23 U/g vs 1.82±0.58 U/g, *P*<0.05).

**CONCLUSION:** The results have shown that conglycinin is good source for enzyne-mediated production of peptides which stimulate the growth of *Bifidobacterium*. These peptides are inactive within the sequence of the parent protein but can be released during enzymatic hydrolysis, and *in vivo* experiments demonstrate that conglycinin peptides may be beneficial for improving gastrointestinal health.

**Key words:** Conglycinin pepsin peptides bifidobacteria

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also evaluated the in vivo effects of soybean conglycinin peptides on gut ecosystem in mice.

MATERIALS AND METHODS

Separation of conglycinin from soybean

The conglycinin was prepared from soybean seeds by the procedure of Iwabuchi[9] and Lovati[10], as reported briefly below. Soybeans were finely ground and defatted with hexane at room temperature. Ground meals, extracted with 30 mmol/L Tris-HCl buffer (pH 8.0) containing 0.01 mol/L β-mercaptoethanol for 1 h at room temperature, were spun by centrifugation (20 min at 4000 r/min, 20 °C). The supernatant (adjusted to pH 6.4) was spun by centrifugation (15000 r/min, 20 min, 4 °C), the precipitates were discarded, and the supernatant was adjusted to pH4.8. The crude conglycinin was collected by the same centrifugation procedure as described above. The precipitated conglycinin was dissolved in 30 mmol/L Tris-HCl buffer (pH 8.0) containing 0.01 mol/L β-mercaptoethanol. The supernatant proteins were fractionated by ammonium sulphate precipitation. The 51-100% saturation fraction was dialyzed against water and applied to a Sepharose-CL-6B column (Pharmacia). Elution was performed with the phosphate buffer (2.6 mmol/L KH2PO4, 32.5 mmol/L K2HPO4, 0.4 mol/L NaCl, 10 mmol/L β-mercaptoethanol, pH 7.6). Finally the purified conglycinin was dialyzed against water. After readjusted to pH7.0 and analyzed for protein concentration by the method of micro-Kjeldahl, the protein solutions were freeze-dried and stored at 4 °C. These materials were used as protein samples.

Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli[11], using 10% polyacrylamide gels. The protein was stained with Coomassie brilliant blue (R-250), scanned, and the profiles of each lane were analyzed by densitometry in the Kodak Digital Science Software 1DTM.

Hydrolysis of conglycinin by pepsin

Conglycinin was hydrolyzed by pepsin (Amersico) using a 1:30 enzyme: substrate ratio. Enzymatic hydrolysis was performed after acidification to pH1.4 with 1 mol/L HCl, and mixture of pepsin and conglycinin was incubated for 2 h at 37 °C. The reaction was terminated by heating at 70 °C for 30 min, and the solution was adjusted to pH 7 with 1 mol/L NaOH. After centrifugation (20 min, 3000 r/min, 4 °C), the supernatant was collected and lyophilized. The nitrogen concentrations of the conglycinin digestion were evaluated by the method of micro-Kjeldahl.

Full hydrolysis of conglycinin by hydrochloric acid

The conglycinin was fully hydrolyzed with 6 mol/L HCl at 110 °C for 24 h. The solution was composed of amino acid composition of conglycinin. The hydrolysates were neutralized and lyophilized.

Separation of peptides

Components of pepsin-treated conglycinin were separated using Sephadex-G15 (Pharmacia) gel filtration chromatography, tracking the maximum growth stimulatory activity of the resulting fractions on Bifidobacterium longum (FCM1192) which was endorsed by Professor Ming-Sheng Dong (Nanjing Agricultural University). The elution was carried out with 0.05 mol/L acetate, and the absorbance of the column effluent was monitored at 280 nm. The molecular mass of the most active fraction was identified by means of MALDI-TOF-MS (BIFLEXTM III, BRUKER). In this study, α-cyano-4-hydroxy-cinnamic acid solution was used as matrix. Peptide concentrations in a sample throughout the purification were determined with the method of micro-Kjeldahl.

Bacterial growth assays in vitro

The basal medium used for a bioassay in vitro was a fully synthetic medium as described by Hassinen[12]. For growth assays, the assay medium (5 mL) was mixed with 0.15 mg/mL (nitrogen concentration) samples and inoculated with 100 μL of bacteria culture. The control contained ammonium acetate which has equal nitrogen concentration with samples. Culture was done under anaerobic conditions at 37 °C. The extent of growth was measured by the absorbance at 460 nm after 48 h of cultivation. The growth experiments were done in triplicates.

Animals and diets

Ninety male KM mice (21 d old, body weight 10-13 g, Shanghai SLAC Experimental Animals Co., Ltd, China) were housed in a room with controlled lighting (12 h/d), and constant temperature. During the first week, all mice had a commercially available basal diet and then randomly assigned into five groups of 16 mice each. Each group was administered for 21 d intragastrically with physiological saline (control), conglycinin, pepsin-treated conglycinin (PTC), the most active fraction which was isolated from pepsin-treated conglycinin (P2-PTC), and HCl-full hydrolysis of conglycinin (HCl-FHC), respectively. The volume of every treatment for the latter four groups was 0.5 mL with equal amount of nitrogen (0.3 mg/mL).

Preparation of sample and enumeration of bacteria

At the end of the experimental period, mice were killed by decapitation. Blood samples were collected and spun by centrifugation at 3000 r/min for 10 min to obtain sera. Serum level of IL-2 was assayed by radioimmunoassay (Beijing North Institute of Biotechnology) using RIA methods. Cecal samples were collected and immediately frozen and stored at -30 °C until analysis and subsequent testing for microbial composition. The following media were used: MacConkey agar for E.coli, bile esculine azide agar for enterococci. Cecal contents were cultured with triplicate plates for the microbial colony count. After incubation at 37 °C for 24 h, results were expressed as colony-forming units per gram of cecal contents. The pH of the cecal contents was measured using a pH meter. Contents of small intestine were also collected and diluted by water a ratio of 1:10. After centrifugation at 3000 r/min for 10 min, IgA level of supernatant was assayed by radioimmunoassay (Beijing North Institute of Biotechnology) using RIA methods.
Growth-stimulating activity of conglycinin hydrolysates in vitro
The results of the in vitro experiments of the effect of soybean conglycinin hydrolysates on the regulation of growth activity for Bifidobacterium longum is shown in Table 1. Compared with control, pepsin-treated conglycinin (PTC) could significantly promote the growth of Bifidobacterium longum \((P<0.01)\), while HCl-full hydrolysis of conglycinin (HCl-FHC) had no significant effect on the growth of Bifidobacterium longum after 48 h.

| Group   | Control Conglycinin         | PTC  | HCl-FHC |
|---------|-----------------------------|------|---------|
| A       | 460 0.11±0.04               | 0.31±0.11  | 0.66±0.07bd  | 0.12±0.02   |

Values are expressed as the mean±SD. \(bP<0.01\) vs control group, \(dP<0.01\) vs HCl-FHC group.

Separation of peptides
Five fractions (P1–P5) were separated from pepsin-treated conglycinin using Sephadex-G15 gel filtration chromatography based on absorbance at 280 nm (Figure 3). Results of bacterial growth assays showed that P2 had the maximum growth stimulatory activity (Table 2). MALDI-TOF-MS analysis of P2 is shown in Figure 4. The molecular mass ranged from 693.32 to 1829.55. It demonstrated that the fraction P2 was comprised of peptides with short lengths.

Enzyme assays
A protocol for detecting fructose-6-phosphate phosphoketolase (F-6-PPK) activity in the extracts from cecum samples was employed based on the assay as previously described by Orban et al.\(^{[13]}\). A positive reaction was recorded if an immediate red-violet color change was visible, and color formation was recorded spectrophotometrically at 505 nm. Quantitative determinations of \(\beta\)-galactosidase enzyme activities were performed in cecal contents suspensions. The method was used for measuring \(\beta\)-galactosidase activity described by Brigidi et al.\(^{[14]}\). One unit of \(\beta\)-galactosidase activity was defined as the amount which released 1 \(\mu\)mol. of \(p\)-nitrophenol per minute and results were expressed as Units/g of wet sample.

Statistical analysis
Data were expressed as mean±SD. Statistical significance was determined by applying one-way analysis of variance (ANOVA). \(P<0.05\) was considered statistically significant.

RESULTS

Soybean conglycinin separation
The procedure for fractionation of conglycinin was based on the isoelectric precipitation and size exclusion chromatography. The elution profile of crude conglycinin on sepharose-CL-6B is shown in Figure 1. The conglycinin was mainly detected in peak II. The gel electrophoretic pattern of purified conglycinin is illustrated in Figure 2. The major bands in the conglycinin were the \(\alpha\), \(\alpha\)' and \(\beta\) subunits, the purification rate of conglycinin was 90.13%.

Figure 1 Chromatography of soybean conglycinin on sepharose-CL-6B. Bed size, 1 cmx100 cm, flow rate 30 mL/h, absorbance at 280 nm.

Figure 2 SDS-PAGE pattern of purified conglycinin. \(\alpha\), \(\alpha\)' and \(\beta\) indicate subunits of conglycinin. M: standard molecular weight proteins.

Figure 3 Chromatography of soybean conglycinin hydrolysates on sephadex-G15. Bed size, 1.0 cmx100 cm, flow rate 15 mL/h, absorbance at 280 nm.

Figure 4 MALDI-TOF-MS analysis of fraction P2.
Effect of conglycinin peptides on serum IL-2 levels and sIgA concentrations of small intestine in mice

Serum IL-2 levels and small intestine sIgA concentrations are presented in Table 3. Level of sIgA in P2-PTC group was higher than that in control group \((P<0.05)\) and HCl-FHC group \((P<0.01)\). The level of serum IL-2 showed no significant difference among the five treated groups.

F-6-PPK activities in cecal contents

The detection of F-6-PPK was considered to be the most reliable indicator that the bacteria belong to the genus \textit{Bifidobacterium}[15]. The results in Table 4 showed that the enzyme F-6-PPK was detected in all samples. Activities of F-6-PPK in the PTC and P2-PTC groups were significantly higher than that in the control group \((P<0.01)\) and HCl-FHC group.

β-galactosidase activities in cecal contents

Compared with control group, β-galactosidase activities in cecal contents of P2-PTC group were significantly increased by 42.2% \((1.82±0.58 \text{ U/g ceecal contents})\). The β-galactosidase activities of conglycinin group were significantly lower than that in P2-PTC group \((1.28±0.23 \text{ U/g ceecal contents})\). But no significant difference was found between P2-PTC and PTC groups, and between P2-PTC and HCl-FHC group \((1.82±0.58 \text{ U/g ceecal contents})\).

Bacterial concentrations and cecal pH

As shown in Table 5, compared with control, cecal pH level and the population of \textit{Enterococci} in P2-PTC group were significantly decreased \((P<0.05)\).

DISCUSSION

In recent years, modification of the human intestinal microbiota has become an important objective of dietetics[20]. This goal can be achieved in two ways: (1) administration of beneficial bacteria with the expectation that they will be able to colonize the intestinal tract; (2) providing prebiotics, which have shown an ability to promote the growth of desirable bacteria[7-15]. Bifidobacteria are part of the beneficial microbiota of the human intestine[20], and they are considered to be important in maintaining intestinal microbiota balance[21]. Therefore, many attempts have been made to increase the number of bifidobacteria in the intestine.

Soybean proteins and their derived foods have been consumed for a long time in the diets of the people in China and other Asian countries. The health promoting properties of soybean proteins have been accepted worldwide. Peptides derived from soybean proteins with short lengths, produced during \textit{in vitro} digestion, are considered to have pharmacological and physiological activities[22]. However, the effect of conglycinin peptides on growth of bifidobacteria had not been reported yet. In this study, we used pepsin to hydrolyze the conglycinin \textit{in vitro} and results demonstrated that conglycinin was a good raw material for enzyme-mediated production of growth-stimulating peptides for bifidobacteria.

The peptides, which were obtained as a consequence of screening for highly active growth stimulators during the separation, were highly hydrophilic and comprised of 6-10 amino acid residues, and these peptides were inactive within the sequence of the parent protein, but could be released during enzymatic hydrolysis. Compared with the HCl-full hydrolysis of conglycinin, the results also indicated that the function of conglycinin peptides was not nutritional but it had physiological properties. Different enzymes in the gastrointestinal tract have different effects on various kinds of soybean proteins.
of foods, which contain numerous sorts of protein. The process above may release prolific bioactive peptides, which have physiological and metabolic programming function on the body.

In conjunction with in vitro studies, pepsin-treated conglycinin was administered to mice in an effort to determine the effects of these peptides on intestinal ecosystem. The results showed that administering these peptides could significantly increase β-galactosidase enzyme activity. It had been reported that the increase in galactosidase was presumably a consequence of elevated numbers of bifidobacteria, which have high levels of this enzyme[21]. This suggests that conglycinin peptides could induce the change of bifidobacteria in the intestine. From our results, conglycinin peptides could lower cecal pH markedly and decrease the population of E. coli. We also observed that conglycinin peptides decreased the population of enterococci, which not only appears to share the main characteristics of lactic acid bacteria (LAB), but also comprises pathogenic species. Enterococci show an extensive range of resistance to various antibiotics, and the evolution of its virulence has been documented[25]. In the present study, the result indicated that conglycinin peptides did not promote the growth of all species of LAB, especially pathogenic species. It had been reported that bifidobacteria could prevent colonization of pathogens in gastrointestinal tract by lowering gut pH, and have health-promoting effects such as enhancement of immune system[24,25]. Gibson et al.[26] suggested that the higher bifidobacteria population, detrimental to other anaerobic bacteria species, may lead to changes in the microbial ecology of the colon. The accepted mechanism that bifidobacteria are inhibitory is related to the higher production of acetic and lactic acids. Increased acid production resulted in a lower pH which prevented enteric colonization of potentially pathogenic microorganisms and growth of putrefactive bacteria[27]. Furthermore, bifidobacteria stimulate immune function. There is considerable evidence from animal studies that probiotic organisms could modulate the mucosal and systemic immune systems[28]. This stimulation of host immunity is thought to relate to the ability of microorganisms to adhere to intestinal cells and interact with the gut-associated lymphoid tissue (GALT)[29,30]. This ability can increase immunoglobulin output into the intestinal lumen[31-33]. sIgA is one of the principal factors preventing bacterial translocation, which can result in sepsis and death of the host. The classic view is that sIgA exerts its effect by aggregating bacteria, thereby mediating clearance of those bacteria from the gut and preventing invasion of bacteria to the body[34]. Some bifidobacteria strains have recently shown to stimulate sIgA production in intestine[35]. Therefore, bifidobacteria may stimulate active IgA production, thereby reducing infections. Our study also showed that local production of sIgA in the small intestine increased significantly, suggesting that the higher amount of bifidobacteria in the cecum of mice caused by ingestion of conglycinin peptides might produce beneficial effects within the gastrointestinal tract. Thus, the health-promoting effect of conglycinin peptides in human beings may have relationship with bifidobacteria of gastrointestinal tract.

In conclusion, our work placed emphasis on the activity (in vitro and in vivo), physiological and biochemical properties of the mixture peptides. The proteolysis of soybean conglycinin with the gastrointestinal protease pepsin results in the generation of growth-promoting peptides for bifidobacteria. Using the MALDI-TOF-MASS strategy, we were able to identify several low-molecular-mass peptides responsible for this activity. The digestion of conglycinin represents an important mechanism to obtain peptides which exhibit significant physiological roles in addition to their nutritional importance. Pepsin is an endopeptidase responsible for the hydrolysis of a wide range of proteins, we may infer that the mixture is a group of bioactive peptides that have identical C-terminus or N-terminus. Further characterization of the composition of the conglycinin hydrolysates, elucidation of the relationship between peptide structure and activity awaits future study.

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