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Isolation and characterization of immunostimulative peptides from soybean

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Immunostimulative peptides were isolated from the pepsin digest of soybean by ion exchange, gel filtration, and reversed-phase high-performance chromatography. The peptides formed blastoids against the splenocytes of C3H/HeN mice. The amino acid sequences of these peptides were: Ala-Glu-Ile-Asn-Met-Pro-Asp-Tyr, Ile-Gln-Gln-Gly-Asn, and Ser-Gly-Phe-Ala-Pro, respectively. (J. Nutr. Biochem. 6:310–313, 1995.)

Keywords: peptide from soybean; blastoid formation; splenocyte

Introduction

Dietary protein should be considered not only as a nutrient but also as an influential factor of the immune system after enzymatic digestion in a living body.[1–4] The peptides derived from the digests of human and cow casein have been shown to regulate the immune system.[6,7] Casein peptides promote the multiplication of lymphocytes that provide the index of mitogenicity.[8] The mitogenicity index has been used to develop the immunostimulators by many investigators.[8–10]

Recently, Yamauchi and Suetsuna reported the effects of soybean peptides and proteins on the immune responses of rats.[18] In this study, we isolated and characterized the peptides, which formed blastoids against mice splenocytes, from the pepsin digest of soybean.

Materials and methods

Materials

Soybeans (*Glycine max* var. Miyagishirome) were purchased from the market. The C3H/HeN mice (6-week-old males) were obtained from CLEA Japan Inc. (Japan). The pepsin (from porcine gastric mucosa, EC.3.4.23.1) was obtained from Merck (Darmstadt, Germany). Mice were housed in a specific pathogen-free condition before use.

Purification of peptides from soybeans

Three hundred grams of soybeans were immersed in deionized water overnight and homogenized in 2 L of deionized water. The pH value of the homogenate was adjusted to 2.0 with 2 N HCl and 3 g of pepsin was added. After 20 hr of digestion at 37°C, the hydrolysate was filtered to remove the residue and centrifuged at 20,000*g* for 20 min at 4°C. The supernatant (200 mL) was applied to a Dowex 50W column (50 to 100 mesh, H^+ from, 4.5 × 20 cm). The column was washed thoroughly with deionized water, and the retained peptides were then eluted with 500 mL of 2 N NH_4OH. The peptide fraction was concentrated to 5 mL under vacuum. The concentrate was applied to a Sephadex G-25 (2.6 × 140 cm; Pharmacia, Uppsald, Sweden) column equilibrated with deionized water, and gel filtrated at a flow rate of 30 mL/hr. Each fraction of 7.8 mL was collected. The peptide fraction (MW range 300 to 5,000) was lyophilized to give a soybean peptide powder. Three grams of the peptide powder was dissolved in deionized water and applied to an SP Sephadex C-25 column (2 × 50 cm, H^+ form; Pharmacia) equilibrated with deionized water.

The column was chromatographed using the linear gradient method with 1 L of deionized water to 1 L of 3% NaCl solution at a flow rate of 30 mL/hr, and fractions of 7.8 mL were collected. The active fractions were collected and freeze-dried. Ten milligrams of the peptide powder dissolved in 500 µL of deionized water was purified by reversed-phase HPLC on a column (4.6 × 150 mm) of Develosil ODS-5 (Nomura Chemicals, Seto City, Aichi, Japan) using a linear gradient method with acetonitrile from

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Received February 23, 1994; accepted December 20, 1994.

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655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(95)00022-R

0 to 50% in 0.1% trifluoroacetic acid for 180 min at a flow rate of 1.0 mL/min. The eluate was monitored at 220 nm.

Biological assay

The blastoid formation activities of peptide fractions were measured by testing the multiplication of the splenocytes from C3H/HeN mice (with no treatment). Splenocytes were isolated from mice spleens, centrifuged (160*g* for 10 min), and adjusted to 1 × 10^9 cells/L in RPMI-1640 medium (Nacalai, Kyoto City, Japan), supplemented with 10% heat-inactivated fetal calf serum (Nacalai, Japan), glutamine (0.3 g/L), and kanamycin (0.05 g/L). The splenocytes suspension (1 mL) and the diluents of peptide fractions were added to 16-mm flat-bottom wells (24 wells/plate; Corning, Corning, NY) and incubated for 48 hr at 37°C in humidified air with 5% CO_2. The splenocytes then were removed by three washings with phosphate-buffered saline, mixed with 2 mL of cell-solubilized solution (sodium lauryl sulfate, 0.125 g/L) and 2 mL ethidium bromide (0.01 g/L). The fluorescent intensity of blank, positive (with concanavalin A (ConA, 0.01 g/L, or peptide sample) and negative (without ConA) were expressed by I_0, I_1, and I_2, respectively. The stimulation index (SI) of the splenocytes was calculated as follows:

$$SI = (I_2 - I_0)/(I_1 - I_0) \times 100$$

Analysis of peptide

Amino acid analysis was carried out for the hydrolysate with 6 N HCl at 110°C for 24 hr using an amino acid analysis system (amino-chrome™, Ciba-Corning, Diagnostics Ltd., Halstead, Essex, England). Sequence analysis was performed by stepwise Edman degradation using a gas-phase automated sequencer Model 477A (Applied Biosystem, Inc., California, USA) coupled with HPLC identification of the resulting PTH-amino acid.

Results

Isolation of peptides from soybean

In this study, immune activating peptides were isolated from the peptic hydrolysate of soybean using an ion-exchange and gel filtration column chromatography as described previously. The Sephadex G-25 chromatogram of the enzymic digest of soybean eluted from Dowex 50W (H^+ form) column is shown in *Figure 1*. The fractions No. 25 to 75 (molecular weight 300 to 5,000) were collected and concentrated to dryness to give a peptide powder. The yield of the peptide powder from 300 g (dry weight) of soybean was 19.6 g. The peptides were fractionated by ion-exchange chromatography on SP Sephadex C-25 (H^+) to give SP-1 (fraction number 34 to 54), SP-2 (55 to 72), SP-3 (73 to 91) and SP-4 (92 to 110) as shown in *Figure 2*.

Blastoid formation activity of peptide fractions

Table 1 shows the blastoid formation activity of soybean peptides from Sephadex G-25 chromatography at various concentrations. The optimum concentration was 0.1 g/L. *Figure 3* shows the blastoid formation activity of soybean peptide fractions from SP Sephadex C-25 chromatography. The stimulation indexes of peptide fractions were SP-1, 108; SP-2, 125; SP-3, 97; and SP-4, 101.

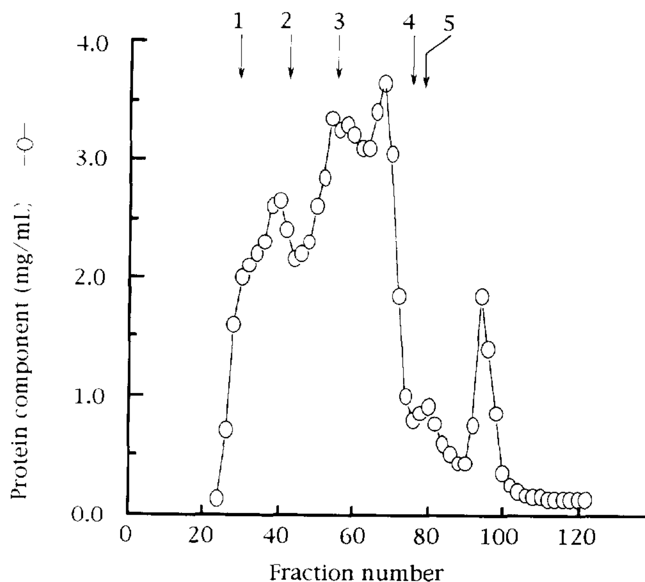


Figure 1 Column chromatogram of soybean peptide on Sephadex G-25. The polypeptides studied (and their molecular weights) were: 1 = insulin (6,000); 2 = insulin B chain (3,500); 3 = insulin A chain (2,550); 4 = bradykinin (1,052); and 5 = glycine (75). —○— The protein component was determined using the Lowry method, and the bovine serum albumin was used as a standard.

Amino acid sequences of active peptides

The active fractions were purified further by reversed-phase HPLC (*Figure 4*). From the SP-1 fraction, an active peptide was obtained (127 min). Two peptides obtained from SP-2, eluting at 109 min and 118 min, were biologically active in vitro. The amino acid sequences of these peptides were determined to be: Ala-Glu-Ile-Asn-Met-Pro-Asp-Tyr, Ile-Gln-Gln-Gly-Asn, and Ser-Gly-Phe-Ala-Pro, respectively (*Table 2*).

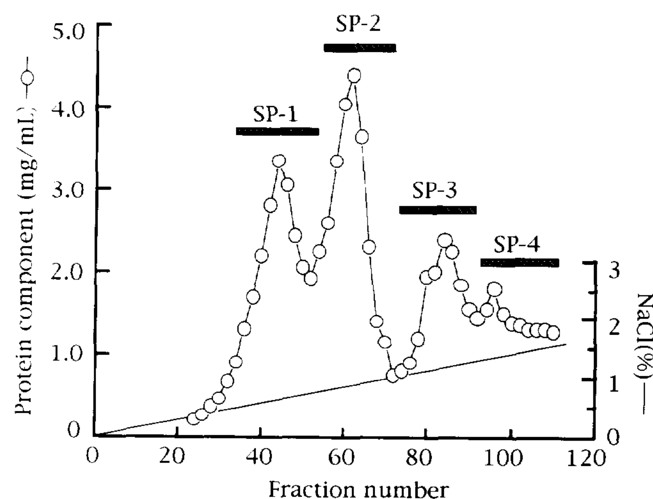


Figure 2 Column chromatogram of soybean peptide on SP Sephadex C-25. —○— The protein component was determined using the Lowry method, and the bovine serum albumin was used as the standard.

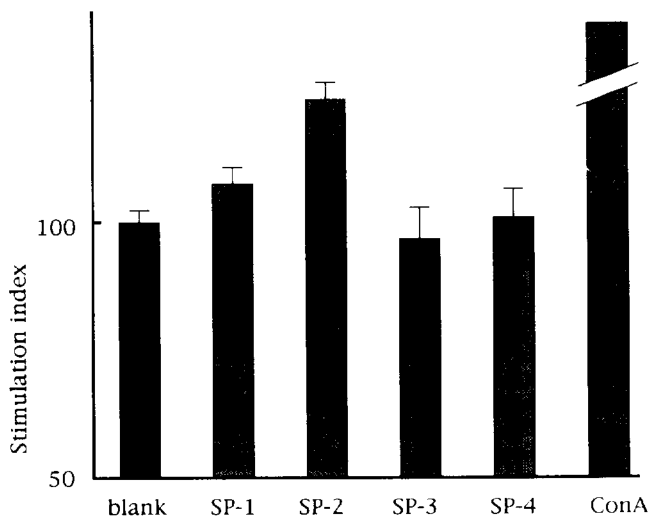


Figure 3 Blastoid formation activity of soybean peptide fraction from SP Sephadex C-25 column chromatographed. ■ blank (SI = 100); ▩ peptide fractions; ▨ positive control (SI = 263).

Discussion

Much has been written about the effects of dietary proteins on host defense mechanisms.[11–13] In order to research the food components that own the function of physiological activities, the immunostimulative peptide fractions were isolated from the enzymatic digest of soybean.

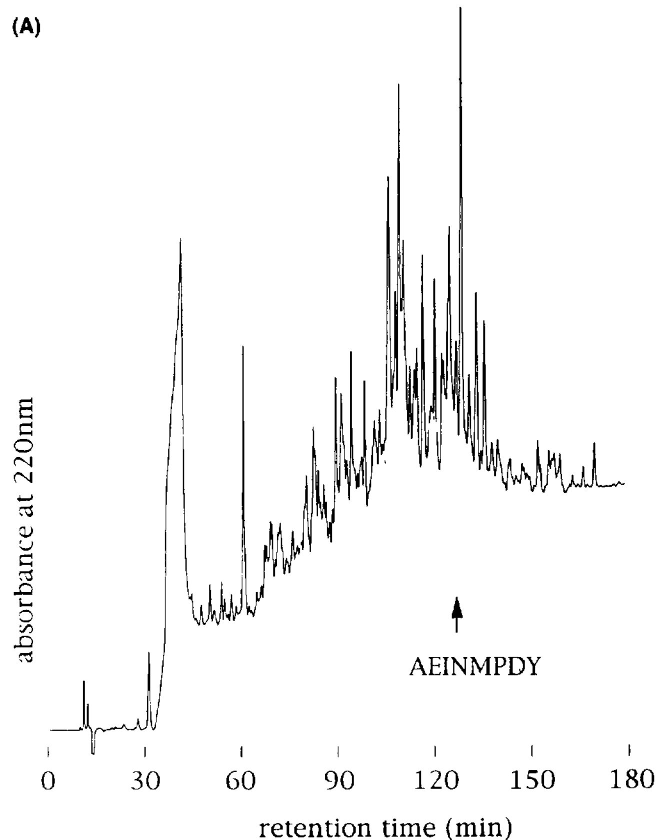
In this study, the soybean was digested using pepsin, and the hydrolysate was fractionated by ion-exchange and gel filtration column chromatography. Peptide fractions had MWs ranging from 300 to 5,000. Blastoid formation activity was determined by the fluorimetry method using the splenocytes of C3H/HeN mice. We could purify the following active peptides from active soybean peptide fraction: Ala-Glu-Ile-Asn-Met-Pro-Asp-Tyr, Ile-Gln-Gln-Gly-Asn, and Ser-Gly-Phe-Ala-Pro.

Jolles et al.[14] suggested the possibility of enzymatic release of immunomodulating peptides from milk casein during the digestive process. Also, Gattegno et al.[15] isolated the peptides that contained immunostimulative activity from human casein hydrolysate. Spleinic T- and B-lymphocyte responses to mitogens such as phytohemagglutinin, concanavalin A, and lipopolysaccharide from *Escherichia coli* were determined.[16] These peptides turned out to be hexapeptide (Val-Glu-Pro-Ile-Pro-Tyr) and tripeptide (Gly-Leu-

Table 1 The blastoid formation activity of soybean peptide from Sephadex G-25 chromatography

	Final concentration (μg/mL)								
	500	250	100	50	25	12.5	6.25	3.1	1.7
Stimulation index*	112	115	127	110	107	102	104	102	102

*The fluorescent intensity of blank, positive (with ConA or peptide sample) and negative (without ConA) were expressed by I_0, I_1, and I_2, respectively. The stimulation index (SI) of the splenocytes was calculated as follows: $SI = (I_2 - I_0)/(I_1 - I_0) \times 100$. The ConA (0.01 g/L) as positive substances were used. The SI of ConA was 263.



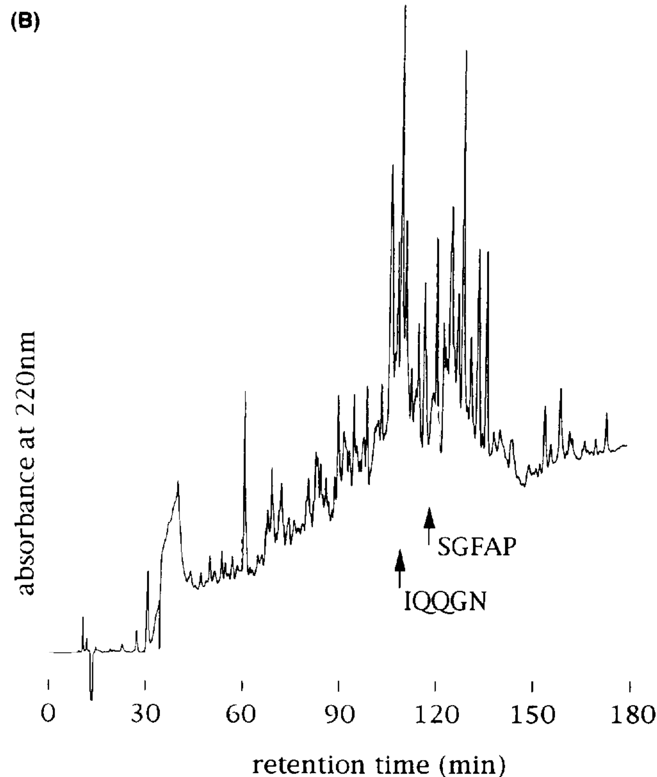


Figure 4 Reversed-phase HPLC on a Develosil ODS-5 column (4.6 × 150 mm) of SP-1 (A) and SP-2 (B) fractions from SP Sephadex C-25. The column was developed with a linear gradient of acetonitrile (0–50% in 180 min) in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. The eluate was monitored at 220 nm. The amino acid sequence of the peptide fragments from SP-1 and SP-2 were AEINMPDY, IQQGN, and SGFAP. These peptides showing biological activity in vitro.

Table 2 Analytical data for purified peptides from SP-1 and SP-2 fractions

Retention time (min)	Amino acid sequence	Amino acid compositions*
127	Ala-Glu-Ile-Asn-Met-Pro-Asp-Tyr†	Ala, 0.91; Glu, 0.98; Ile, 1.01; Asp, 1.97; Met, 0.92; Pro, 1.00; Tyr, 1.01
109	Ile-Gln-Gln-Gly-Asn‡	Ile, 1.00; Glu, 2.01; Gly, 1.21; Asp, 0.85
118	Ser-Gly-Phe-Ala-Pro‡	Ser, 1.00; Gly, 1.08; Phe, 1.11; Ala, 1.08; Pro, 1.08

*Each peptide was hydrolyzed with 6 N hydrochloric acid at 110°C for 24 hr.
†Separated from SP-1 fraction.
‡Separated from SP-2 fraction.

Phe). It is known that these peptides activate in vitro phagocytosis of macrophages and increase the production of antibodies, and the tripeptide enhances the survival rate of mice infected with *Klebsiella pneumoniae*. Suetsuna et al.[17] also found that the oligopeptides derived from sardine muscle had mitogenic activity in vitro, and they supposed that an immune activating peptide requires favorable active sites, which are the proline and branch-chain amino acids (leucine, isoleuicine, and valine). Soybean peptides purified in this study had favorable active sites. It is our future aim to clarify the physiological activities and the mechanisms of the immunological function of the immunostimulative soybean peptides.

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