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SOYBEAN TRYPSIN INHIBITOR AND β-AMYLASE STIMULATE MACROPHAGES

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Mature soybean seeds were found to contain macrophage stimulation activity. The activity existed in a water-soluble fraction of soybean whey. Gel filtration, DEAE-cellulose column chromatography and high-performance liquid chromatography of the water-soluble fraction gave two active proteins. By N-terminal and total amino acid analyses, these were identified as trypsin inhibitor (Kunitz) and β-amylase. Macrophages were effectively stimulated by each of the two proteins to produce nitrite.

Macrophages, when stimulated with lipopolysaccharide, produce a copious amount of intermediate nitric oxide from L-arginine through an enzymatic process (1-3), and the oxide then reacts spontaneously with molecular oxygen and water to yield nitrite and nitrate as final products (1,4). When reactive amino compounds co-exist in the system, N-nitroso-compounds are also formed as final products (5,6). It has been proposed that nitric oxide is one of the most important effectors for macrophage tumoricidal activity (7,8). For this reason we have used nitrite formation as an index of macrophage stimulation, and reported that a wide variety of foods stimulated macrophages (9). Among these foods, soybeans exhibit great activity for macrophage stimulation (9). Here we report details of the purification and identification of the principal stimulants occurring in soybeans.

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Abbreviations: PBS, 10 mM phosphate buffered saline (pH 7.2); V_0, void volume; DEAE, diethylaminoethyl; HPLC, high performance liquid chromatography; SDS, sodium dodecylsulfate; MEM, minimum essential medium; DNS, dansyl.

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Methods

Soybeans. Mature seeds of soybean (Glycine max, var. Hourei) harvested in Ibaraki prefecture, Japan, in 1988, were kindly donated by Dr. K. Kitamura, National Agriculture Center.

Fractionation of components. The soybeans (100 g) were freeze-dried and separated into hull and hulled seeds. The hull was pulverized, mixed with hot water (50 ml), and centrifuged at 5000×g for 20 min to obtain a supernatant. The supernatant was then freeze-dried. Hulled seeds were pulverized and defatted twice with n-hexane (500 ml). The defatted powder was fractionated into whey, a 7S protein-rich fraction, an 11S protein-rich fraction and residue according to the method of Koshiyama (10). Each fraction was heated at 60°C for 5 min to inactivate enzymatic activities. The whey fraction was centrifuged and separated into water-soluble (supernatant) and salt-soluble (precipitate) fractions, each of which was freeze-dried.

Gel filtration. The water-soluble fraction (100 mg) from the whey was loaded on a Sephadex G-75 column (2×80 cm, V_0=92 ml) and eluted with PBS. The eluate was measured for absorbance at 235 nm. Fractions obtained were dialyzed separately and then freeze-dried.

DEAE-cellulose column chromatography. Each (15 mg) of the fractions obtained by gel filtration was chromatographed using a DE-50 (Whatman BioSystems) column (2×70 cm). Non-adsorbed species were washed out with PBS (60 ml) and then a gradient elution was carried out with PBS-2 M NaCl in PBS. The eluate was measured for absorbance at 235 nm and the fraction eluted just around the peak top was dialyzed prior to being freeze-dried.

HPLC. Each fraction obtained by gel filtration and by DEAE-cellulose column chromatography was subjected to HPLC under the following conditions: apparatus, chromatograph type Trirotar VI (Japan Spectroscopic Co.); column, polymethacrylate (Japan Spectroscopic Co.); sample loaded, 2 mg in 0.1% trifluoroacetic acid (100 µl); solvent, 0.1% trifluoroacetic acid in water-acetonitrile (gradient); flow rate, 1 ml/min; temperature, 25°C; detection, UV light at 235 nm. The eluate around each peak top was dried.

Gel electrophoresis. Electrophoresis on SDS-polyacrylamide gel (10-20% gradient, Daiichi Pure Chemicals) was carried out according to the procedure of Weber and Osborn (11).

Cells and medium. A macrophage cell line (RAW 264.7) was obtained from The American Type Culture Collection. The cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Bocknek Laboratories).

Nitrite formation assay. The test samples were each dissolved in the supplemented MEM (5) and then sterilized by passage through a 0.22-µm filter. The cultured macrophages were removed from the dishes by vigorous pipetting and resuspended at a concentration of 1×10^6 cells/ml. Cells were plated at 200 µl/well, allowed to adhere for 30 min, and then the medium was changed to the sample in the supplemented MEM. After 48 h of incubation, nitrite concentration in the culture supernatant was determined by a colorimetric method (5). The isolated compounds were assayed similarly in the presence and absence of polymyxin B (2.5 µg/ml) (12).

Amino acid analysis. The isolated proteins with macrophage-stimulating activities were investigated for their amino acid compositions. Each (50 µg) of the proteins in 6 N HCl (0.5 ml) was degassed and then hydrolyzed at 110°C for 24, 48 and 72 h. The hydrolysate was analyzed for constituent amino acids using an amino acid analyzer (Hitachi type 835) and the amino acid composition was determined by interpolation or extrapolation. For half-cystine determination, performic acid oxidation (13) was carried out before hydrolysis with 6 N HCl. For tryptophan determination, the sample was hydrolyzed with 3 N mercaptoethanesulfonic acid (14). The amino acid composition was expressed on a molar basis.

N-terminal analysis. The stimulants (10 µg) were derivatized by dansylation (15) and then hydrolyzed with 6 N HCl at 110°C for 10 hr. The hydrolysate was subjected to two-dimensional polyamine thin-layer (Schleicher & Schuell) chromatography (16).

LPS analysis. The contaminative LPS was determined with the endotoxin test D (Seikagaku Kogyo, Tokyo) (17).

Results and Discussion

Our previous study (9) revealed that an aqueous extract from soybean had macrophage-stimulating activity. The stimulants responsible were non-diffusible, and therefore, they were apparently high-molecular-weight substances. In order to identify them, we first separated soybean seeds into six fractions. Table 1 shows that two fractions (water-soluble and salt-soluble) from whey were active. The whey fraction has been reported to contain agglutinin, lipoxygenase, β-amylase and two types of trypsin inhibitors (18). We subjected the most active fraction (water-soluble fraction from whey) for further fractionation. The electrophoretic pattern showed that the fraction contained four main components of about 120 kd, 100 kd, 58 kd, and 21 kd respectively. The gel filtration pattern of the water-soluble fraction from whey is shown in Fig. 1. The first fraction (designated G1) was composed of 120 kd, 100 kd and 58 kd components, the second (G2) of 100 kd, 58 kd and 21 kd components, the third (G3) of 100 kd, 58 kd and 21 kd components, and the fourth (G4) of a 21 kd component. All the four fractions were active. We directly submitted the four fractions to HPLC. The chromatograms of the fractions, shown in Fig. 2, suggested that the components in G2 and G3 are separable. The separated components with asterisks (G2-H1, G2-H3, G3-H1 and G3-H3) were active (Fig. 2). G1 and G4 were subjected to DEAE-cellulose column chromatography (Fig. 3a and b). Both of the two peaks (G1-D1 and G1-D2) from G1 were inactive, whereas the two peaks (G4-D1 and G4-D2) from G4 were active. The main active peak, G4-D1, was further purified by HPLC (Fig. 3c) to obtain only one active peak (G4-D1-H1).

Table 1. Macrophage-stimulating Activities of the Fractions from Soybean

Fraction	Concentration (μg/ml medium)	Activity (NO_2^- formed, nmol/10^6 cells)
Hull extract	200 & 20	ND*
Whey water-soluble	200	42.4 ± 3.5**
	20	11.9 ± 1.2
Whey salt-soluble	200	33.8 ± 1.7
	20	ND
7S protein-rich	200 & 20	ND
11S protein-rich	200 & 20	ND
Residue	200 & 20	ND

*Not detected.
**Mean ± standard deviation.

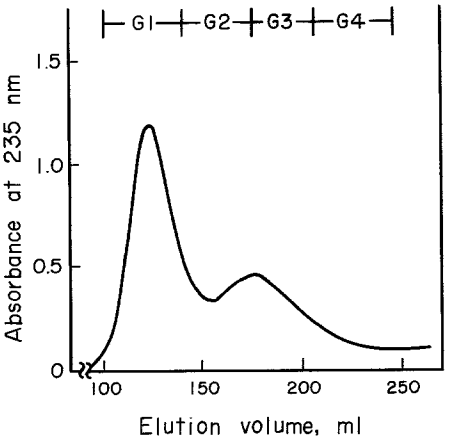
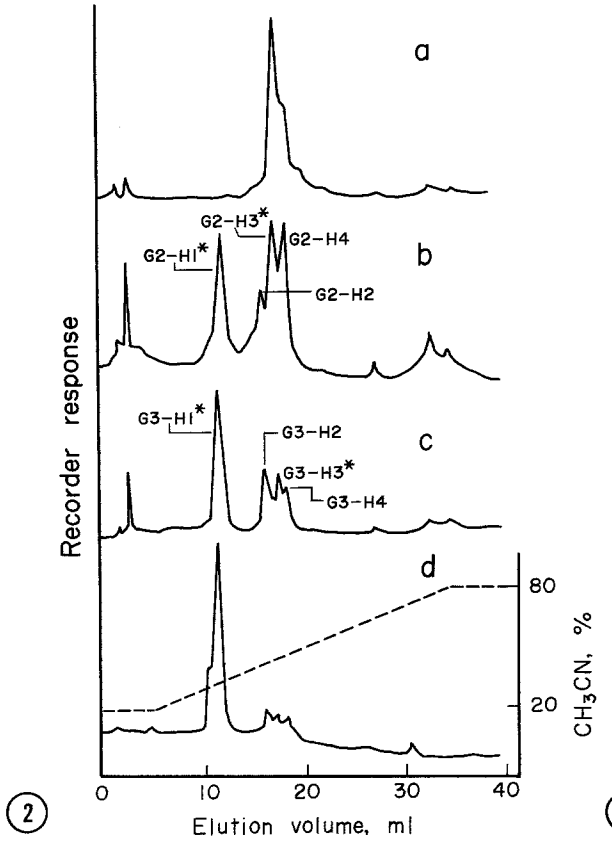


Figure 1. Sephadex G-75 gel filtration pattern of whey water-soluble fraction.



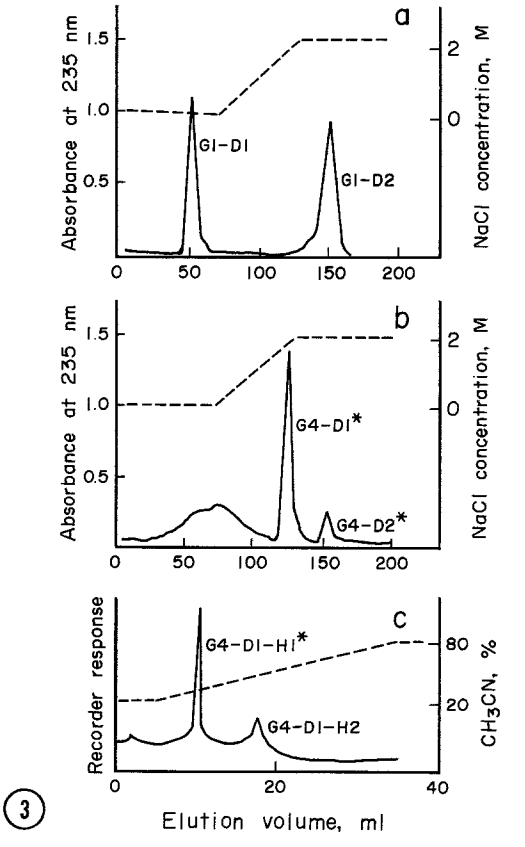


Figure 2. HPLC patterns of G1 (a), G2 (b), G3 (c) and G4 (d). Acetonitrile concentration in the elution solvent is shown with a dotted line in d. The same gradient system as that in d was applied in a, b and c. Active fractions are shown with asterisks.

Figure 3. DEAE-cellulose column chromatograms of G1 (a) and G4 (b), and the HPLC pattern of G4-D1 (c). NaCl concentration in the elution solvent is shown with dotted lines in a and b, and acetonitrile concentration with a dotted line in c. Active fractions are shown with asterisks.

Table 2. Amino Acid Compositions and N-Termini of the Active Components in Comparison with Those in Literature

Amino acid	Component 1*	Kunitz trypsin inhibitor**	Component 2***	ß-Amylase****
Asx	25.3	26	61.7	63
Thr	7.1	7	20.3	18
Ser	10.4	11	25.2	25
Glx	17.4	18	49.7	50
Gly	16.1	16	36.8	38
Ala	8.1	8	31.3	32
Val	13.3	14	32.7	32
1/2 Cys	3.8	4	6.3	6
Met	2.1	2	11.3	11
Ile	13.5	14	29.3	27
Leu	15.6	15	47.2	47
Tyr	4.4	4	21.3	24
Phe	9.2	9	17.6	21
Lys	10.4	10	30.5	30
His	2.3	2	10.2	11
Arg	9.9	9	21.3	19
Trp	2.3	2	31.3	30
Pro	9.9	10	10.7	11
N-terminal	Asp	Asp	Masked	Acetyl

*Calculated as molecular weight of 21 k.
**Quoted from reference 17.
***Calculated as molecular weight of 58 k.
****Quoted from reference 18.

Gel electrophoresis of the active components purified by HPLC was carried out. According to their migration distances in the gel and elution positions (Figs. 2 and 3c), G2-H1, G3-H1 and G4-D1-H1 were the same compound with a molecular weight of 21 k. This was designated compound 1. G2-H3 and G3-H3 were also the same compound with a molecular weight of 58 K, designated compound 2. These two compounds were examined for their N-termini. Compound 1 had aspartic acid at the N-terminal position, and the N-terminal amino group of compound 2 was masked (Table 2). The amino acid compositions of the two compounds are shown in Table 2 in comparison with those of Kunitz-type trypsin inhibitor (19) and ß-amylase (E.C. 3.2.1.2) (20). Putting together the molecular weight data, N-termini and amino acid compositions, we identified compound 1 as trypsin inhibitor (Kunitz) and compound 2 as ß-amylase. Compounds 1 and 2, when dissolved in the medium at a concentration of more than 20 µg/ml and 2 µg/ml, respectively, stimulated macrophages. The macrophage stimulating activities of these compounds were not inhibited by polymyxin B. Each preparation of these compounds contained LPS at less than 100 pg/g (insufficient to activate macrophages to produce nitrite). In conclusion, it is noted that these two proteins are novel macrophage stimulants. The mechanisms of macrophage stimulation by these compounds remain to be studied.

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