A New Growth Effecting Peptide of *Bacillus* Species from Soybean Cake

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A new peptide, which has a marked effect on the growth of *Bacillus* species, was purified from soybean cake by a procedure employing ammonium sulfate fractionation, and active charcoal-, DEAE-Sephadex A-25- and Sephadex gel-column chromatographies. The purified peptide gave a single band on paper electrophoresis. The peptide possessed the following properties: specific extinction ($E_{1\%}^{1\text{cm}}$ at 280 nm), 11.9; N-terminal amino acid, glutamate; molecular weight, 8058; total number of amino acid, 70; chemical composition (%), C, 49.12; H, 6.26; N, 16.44; and S, 2.34; isoelectric point, pH 4.3. The hydrolyzed peptide had no stimulative effect on the growth of *Bacillus* species. Especially, those microorganisms belonging to the genera of *Bacillus*, *Arthrobacter* and *Xanthomonas* were remarkably sensitive to the peptide.

Recent investigations have shown that substances contained in soybean have a nutritional effect on the growth of bacteria; namely, an aqueous extract of soybean seeds,¹⁾ soy proteins²⁾ and a peptide in soybean Miso³⁾ stimulate the growth of *Lactobacillus* species, *Clostridium perfringens*, and *Pediococcus halophilus*, respectively. In the course of our investigation on the growth of aerobic bacteria, we also observed that the addition of soybean cake into a complete synthetic medium markedly stimulated the growth of *Bacillus* species.

The present paper deals with the purification and chemical properties of the purified peptide. The sensitivity of various microorganisms to the purified peptide is also presented in this paper.

MATERIALS AND METHODS

Chemicals. All of the chemicals used in this work were commercial products. Soybean cake was kindly supplied from Marukin Shōyu Co., Ltd., Japan.

Microorganisms. The microbial strains used in this work were supplied from the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University (AKU) and Institute for Fermentation, Osaka (IFO). All of the strains used are listed in Table IV.

Culture and inoculum. Microorganisms were cultured at 30°C under continuous shaking. After testing commonly used nutrients of bacterial growth (e.g. vitamins, minerals, amino acids and bases), the composition of the basal medium used in this work was determined as follows: Glucose 4.0 g, Na-glutamate 3.0 g, $(\text{NH}_4)_2\text{SO}_4$ 3.0 g, KH_2PO_4 250 mg, K_2HPO_4 750 mg, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 300 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 30 mg, NaCl 10 mg, thiamine-HCl 400 μg , nicotinic acid 400 μg , Ca-pantothenate 400 μg , and *p*-aminobenzoate 100 μg in 1 liter of distilled water. The pH was adjusted to 7.2. Samples containing active fractions were added to 2.5 ml of the basal medium of double strength in a test tube, and the total volume was made up to 5 ml with distilled water. After autoclaving at 115°C for 15 min, each tube was inoculated with one drop of the inoculum suspension the optical density of which was prepared to be 0.5 at 660 nm.

Measurement of microbial growth. The growth of microorganisms was determined turbidimetrically by reading the turbidity at 660 nm using a spectrophotometer of Hitachi model 124 or a Klett-Summerson model photoelectric colorimeter.

Bioautography. Growth-effecting substances on the paper chromatogram were bioautographically determined. To 250 ml of the hot basal medium containing 1.5 per cent of agar, 10 ml of an inoculum suspension (*B. cereus*) was added. The mixture was immediately poured on glass plates and cooled. The samples containing growth-effecting substances were paper-chromatographed (Toyo Roshi No. 51) in a mixture of phenol and water (4:1). The developed papers were dried, cut off as paper ribbons and placed

on inoculated agar plates prepared as mentioned above. The plates were incubated at 30°C for about 20 hr until turbid spots owing to growth-affecting substances appeared on the plates.

Other measurements. Methods of molecular weight-determination and others will be referred to in each corresponding section.

RESULTS

Purification of growth-affecting substance

All operations were carried out at 5°C throughout the purification procedures.

Step I. Preparation of extracts from soybean cake

Extracts from soybean cake were prepared by the following procedures. Soybean cake (2 kg) was extracted with 10 liters of boiling water for 30 min. Then the extract was filtered or centrifuged to separate insoluble materials. The clear extract was adjusted to pH 4 with 6 N HCl to remove the globulin fraction, and the precipitate was discarded by continuous-flow-centrifuge at $9000\times g$. The supernatant containing growth-affecting substances was treated as follows.

Step II. Charcoal column chromatography

The absorbent equilibrated with distilled water was packed into a column (5×50 cm). The supernatant solution was adjusted to pH 7 with 6 N NaOH and placed on the top of the column. The column was thoroughly washed

with 5 liters of distilled water. The absorbed active fractions were eluted with 50% ethanol containing 0.2% NH₃ and then the eluates were combined together to give 600 ml of the solution. The ammonia and ethanol in the active fractions were completely evaporated off *in vacuo* at 50°C and the dried sample was dissolved in 100 ml of distilled water.

Step III. First DEAE-Sephadex A-25 column chromatography

The absorbent equilibrated with 0.01 M ammonium carbonate, pH 8.0, was packed into a column (5×50 cm). The dissolved sample was placed on the top of the column, and then the column was thoroughly washed with 2 liters of 0.01 M ammonium carbonate. The absorbed sample was stepwisely eluted with 0.1, 0.2 and 0.5 M ammonium carbonate solutions. As shown in Fig. 1, the active fractions were eluted with 0.2 M ammonium carbonate and the eluates were combined to give 400 ml. The ammonium carbonate in the active fractions was completely evaporated off *in vacuo* at 50°C.

Step IV. Ammonium sulfate fractionation

The dried sample was dissolved in 100 ml of distilled water. To the dissolved solution was added 17.6 g of solid ammonium sulfate up to 30% saturation under mechanical stirring. The pH adjusted to 7.0 with 10% ammonium hydroxide solution. After standing overnight, the precipitate was removed by

FIG. 1. Chromatography of Growth Affecting Substance on DEAE-Sephadex A-25.

Chromatography was performed as stated in the text, and the activity was bioassayed under the standard assay conditions.

centrifugation at $9000\times g$ for 20 min. The ammonium sulfate concentration of the supernatant solution was then increased to 60% saturation by addition of solid ammonium sulfate. The precipitate was collected by centrifugation at $9000\times g$ for 10 min after standing overnight and dissolved in a small volume of distilled water.

Step V. Sephadex G-25 gel filtration

The dissolved solution was subjected to Sephadex gel filtration. The absorbent equilibrated with distilled water was packed into a column (3.5×180 cm). The sample was eluted with distilled water and the active fractions were combined to give 100 ml of the solution.

Step VI. Second DEAE-Sephadex A-25 column chromatography

The eluted active fractions were rechromatographed using a column (2×30 cm) by means of the same procedure as described in the first DEAE-Sephadex A-25 column chromatography. A linear gradient elution was performed by a two chambers system with 200 ml of 0.01 M ammonium carbonate in the first chamber and 200 ml of 0.3 M ammonium carbonate in the second one. The active fractions were eluted with 0.2 M ammonium carbonate and the eluates were combined to give 60 ml of the solution. The ammonium carbonate in the active fractions was completely evaporated off *in vacuo* at 50°C , and then the dried sample was dissolved in a small volume of distilled water.

Step VII. Sephadex G-50 gel filtration

The dissolved solution was subjected to Sephadex G-50 gel filtration. The absorbent equilibrated with distilled water was packed into a column (1.0×180 cm). A part of the active solution was placed on the top of the column and was eluted with distilled water. Figure 2 shows a typical elution pattern of the active fraction. These active fractions were collected and excess water was completely evaporated off *in vacuo* at 50°C , and then the dried sample (about 100 mg) was stored in

FIG. 2. Chromatography of Growth Effecting Substance on Sephadex G-50.

Chromatography was performed as stated in the text, and the activity was bioassayed under the standard assay conditions.

a desiccator.

Properties of purified peptide

Paper electrophoresis and bioautography.

The dried powder was dissolved in a small volume of the same buffers used in electrophoresis. A sample solution was spread on paper (Toyo Roshi No. 51), 4.0 cm in width. Electrophoresis was carried out in each buffer at 5°C with 0.25 mA/cm for 5 hr. After the

FIG. 3. Paper Electrophoresis and Bioautography of Purified Peptide.

Electrophoresis was carried out on paper at 5°C with 0.25 mA/cm for 5 hr. Peptide distribution was checked by staining with ninhydrin reagent. A, Veronal buffer ($\mu=0.05$, pH 8.3); B, phosphate buffer ($\mu=0.05$, pH 6.8); C, pyridine: acetate: water = 1: 10: 89 (pH 3.6); D, 80% formate: acetate: water = 5: 15: 80 (pH 1.9).

run, the distribution of the peptide on the paper was shown by staining with ninhydrin and BPB reagent. As shown in Fig. 3, the peptide gave only one band in paper electrophoresis in each buffer, and *B. cereus* showed a good response to the single band on the bioautogram.

Absorption spectrum. The ultra violet absorption of the peptide was measured under two different conditions. From a spectrum, which had a maximum absorption at 275 nm in 0.1 N HCl, the specific extinction of 1.0% (w/w) solution at 280 nm ($E_{1\text{cm}}^{1\%}$ at 280 nm) was calculated to be 11.9. From another spectrum, which was measured in 0.1 N NaOH, the tryptophan content was estimated to be one residue per a single molecule.⁴⁾

Molecular weight. The molecular weight of the peptide was estimated as approximately 8000 by Andrew's gel filtration method⁵⁾ using pepsin, trypsin, cytochrome C, coenzyme A (dimer) and vitamin B₁₂ as reference samples.

Isoelectric point. The isoelectric point was determined on the basis of the electrophoretic mobility of the peptide as a function of pH. Paper electrophoresis was performed on Toyo Roshi No. 51 in 0.1 M-pyridine-acetate buffer, pH 3.5~5.5, in 0.25 mA/cm for 10 hr at 5°C. It was calculated to be pH 4.3 from the plot of electrophoretic mobility in 0.1 M-pyridine-acetate buffer against pH in the range of 3.5~5.5.

Color reactions. Table I shows the color

reactions of the peptide. The peptide was positive in such protein color reactions as Biuret, Xanthoprotein, Ninhydrin,⁶⁾ and Folin⁸⁾ reactions. And the partially hydrolyzed peptide was negative in such sugar reactions as Elson-Morgan⁹⁾ and Anthrone⁹⁾ reactions.

Chemical composition. The peptide (5 mg each) was hydrolyzed with 6 N HCl at 110±1°C for 24 and 48 hr. Amino acids in the hydrolysate were measured by a Hitachi Amino Acid Analyzer KLA-3B by the method of Moore

TABLE II. AMINO ACID COMPOSITION OF PURIFIED PEPTIDE

Amino acid	Numbers of amino acid per mole of purified peptide ^{a)}	
	Calculated	Integral
Asp	9.6	10
Thr	2.0	2
Ser	2.7	3
Glu	15.4	15
Pro	5.4	5
Gly	4.1	4
Ala	2.5	3
1/2 Cys	4.9	5 ^{b)}
Val	1.9	2
Met	0.9	1
Ileu	2.0	2
Leu	2.9	3
Tyr	0.9	1
Phe	1.0	1
Try	—	1 ^{c)}
Lys	5.5	6
His	1.5	2
Arg	3.6	4

^{a)} Based on a molecular weight of 8000.

^{b)} Estimated from the result of elementary analysis and Ellman reaction.⁷⁾

^{c)} Determined spectrophotometrically.

TABLE I. COLOR REACTIONS OF PURIFIED PEPTIDE

The experiments were carried out according to ordinary methods.

Tested reaction	Result ^{a)}	Tested reaction	Result
Molisch	—	Ellman	+
Anthrone	—	Ninhydrin	+
Orcinol	—	Biuret	+
Nelson-Somogyi	—	Xanthoprotein	+
Elson-Morgan	—	Folin	+
Fiske-Subbarow ¹⁰⁾	—	Nessler	+

^{a)} +, positive; —, negative.

and Stein.¹¹⁾ The results are shown in Table II. Based on the above data that the molecular weight of the peptide is approximately 8000, the total number of amino acids and the molecular weight of the peptide are calculated to be 70 and 8058 respectively. The result of elemental analysis indicated that the peptide consists of C 49.12%, H 6.26%, N 16.44% and S 2.34%.

N-Terminal amino acid. The N-terminal amino acid of the purified peptide was estimated by the DNP method.¹²⁾ Solvent A (toluene: pyridine: 2-chloroethanol: 0.8 N NH_4OH = 50: 15: 30: 30) was used for the first dimension thin layer chromatography and solvent B (chloroform: benzylalcohol: acetic acid = 70: 30: 3) for the second one. The result strongly suggests that the N-terminal amino acid of the peptide is glutamate.

Effect of purified peptide concentration on bacterial growth

The effect of purified peptide-concentration on the growth of *B. cereus* was tested. The result obtained is shown in Fig. 4. The growth of *B. cereus* was greater with increasing concentrations of the added purified peptide.

Effect of purified peptide on the growth of B. cereus at different growth stages

The peptide at a concentration of 0.15 mg/

FIG. 4. Effect of Purified Peptide Concentration on Bacterial Growth.

Incubation was carried out under the standard bioassay conditions for 16 hr.

0.1 ml was added to the culture medium at different growth stages of *B. cereus*. A marked growth was immediately observed after the peptide was added at the lag phase or the initial stage of the exponential phase (Fig. 5). The addition of the peptide at the middle and last stages of the exponential phase had no influence on the growth.

FIG. 5. Effect of Purified Peptide on the Growth of *B. cereus* at Different Growth Stages.

Purified peptide (0.15 mg/0.1 ml) was added at the different growing stages as indicated by arrows: ○—○, 0 hr; ●—○, 12 hr; ●—○, 24 hr; ●—○, 48 hr, 84 hr, none. The microorganisms were cultured for the specified period under the standard culture conditions.

FIG. 6. Paper Chromatography and Bioautography of Hydrolyzed Peptide.

Paper chromatography and bioautography were performed as stated in the text.

Effects of purified peptide and its hydrolysate on growth of B. cereus

The purified peptide (5 mg) was hydrolyzed with 6 N HCl at 110°C for 24 hr. The peptide and its hydrolysate were paper-chromatographed, and their biological activity was bioautographically tested. As shown in Fig. 6, the unhydrolyzed peptide gave only one spot which gave a marked growth owing to a growth-affecting substance, but the hydrolyzed peptide gave many invalid spots.

Stimulation of the microbial growth was also observed when the peptide (equivalent to 30 µg peptide/ml) was added to the basal medium.

TABLE III. EFFECT OF HYDROLYZED PEPTIDE ON GROWTH

Cultured for the specified period under the standard culture condition.

Culture condition	Growth (klett units)	
	24 hr	36 hr
Basal medium	18	215
" +Peptide	134	247
" +Hydrolysate	46	226

As shown in Table III, a stimulative effect on the growth of *B. cereus* after 24 hr-culture at 30°C was obtained only with the unhydrolyzed peptide, although no effect on the total growth after 36 hr.

Response of microorganisms to purified peptide

The response of various genera of microorganisms to the purified peptide was tested. The peptide (30 µg/ml) was added to the basal medium, and microorganisms were cultured at 30°C for the periods as indicated in Table IV. The extent of the growth of each strain was measured turbidimetrically. Almost all of the microorganisms tested showed a considerable growth in response to the added peptide. Especially, those belonging to the genera of *Bacillus*, *Arthrobacter*, and *Xanthomonas* showed a remarkable growth response.

DISCUSSION

Physiological activities of soybean proteins

TABLE IV. RESPONSE OF MICROORGANISMS TO PURIFIED PEPTIDE

Strain	Growth (klett units)			
	24 hr		36 hr	
	Added	Not added	Added	Not added
<i>Escherichia coli</i> 26-A-1	151	120	151	125
<i>Serratia marcescens</i> IFO 3046	137	90	320	274
<i>Proteus vulgaris</i> IFO 3167	94	56	109	101
<i>Bacillus cereus</i> IFO 3001	148	4	223	151
<i>B. aneurinolyticus</i>	90	35	108	89
<i>B. megaterium</i> NI 8100	23	4	164	76
<i>B. natto</i> Sawamura	157	8	198	23
<i>B. natto</i> U. U.	58	4	67	10
<i>B. mesentericus</i> var. <i>flavus</i> IFO 3028	57	40	145	124
<i>B. subtilis</i> IFO 3007	205	29	180	195
<i>B. subtilis</i> IFO 3037	87	20	126	20
<i>B. subtilis</i> IAM 1193	190	166	274	241
<i>B. subtilis</i> var. <i>niger</i> IFO 3214	121	57	120	71
<i>B. brevis</i> IFO 3331	9	1	38	2
<i>B. pumilus</i> IFO 3030	105	80	150	103
<i>B. circulans</i> IFO 3329	21	0	66	0
<i>Micrococcus glutamicus</i> No. 613 ATCC 13059	4	3	10	4
<i>Arthrobacter oxydans</i> IFO 12138	207	30	223	74
<i>Brevibacterium divaricatum</i> NRRL 2311	5	3	77	15
<i>Xanthomonas malvecearum</i> IFO 3383	204	86	182	181
<i>Acetobacter aceti</i> IFO 3284	2	1	2	1

have been investigated and some biological active substances such as trypsin inhibitors,^{13~16)} hemagglutinins,^{17~19)} β -amylase,²⁰⁾ lipoxigenase,^{21,22)} protease²³⁾ and chalconflavanone isomerase²⁴⁾ have been isolated. It was also reported by Thompson *et al.*^{25,26)} that two dipeptides of glutamic acid, γ -glutamyltyrosin and γ -glutamylphenylalanine was found in the non-protein nitrogen fraction of soybeans. Bergey²⁷⁾ reported that the growth of *Bacillus* species and other bacteria was marked on a soybean agar slant. However, the chemical properties and the physiological roles of the active substances in soybean have little been investigated. Nakagaki and Hirano¹⁾ recently reported that an aqueous extract of soybean seeds showed a growth promoting activity for *L. acidophilus* and *L. bulgaricus* and that the activity was mainly due to adenine, guanosine and L-tryptophan. It was also reported by Busta and Schroder²⁾ that some soybean proteins had stimulative effects on the growth of *Clostridium perfringens*.

In this paper, therefore, the purification of the effective substances on the growth of *Bacillus* species was undertaken and the properties of the purified peptide were investigated. This purified peptide was found to differ from the peptidic substance from soybean Miso which shows a remarkable growth effect especially on *Pediococcus halophilus*.³⁾ The present result that the purified peptide was negative in sugar reactions as shown in Table I suggests that the peptide is composed of amino acids. When the peptide was completely hydrolyzed in 6 N HCl for 24 hr the activity on *B. cereus* almost disappeared (Fig. 6). When the peptide or its hydrolysate was added to the basal medium the peptide showed more stimulative effect than the hydrolysate (Table III). These findings suggest that there is an active center in the molecule of this peptide for the activity on *B. cereus*. Hence, it is necessary to partially hydrolyze the peptide using such proteolytic enzymes as pepsin, trypsin, *etc.*

When the purified peptide was added to the culture medium at the first stage, the lag phase

or the initial stage of the exponential phase, the stimulating effect on microbial growth was observed (Fig. 5). As shown in Fig. 5, it was observed by the addition of the peptide that the lag period of the growth of *B. cereus* was shortened. It seems obvious that the peptide plays a significant role during the initial stage of microbial growth. Investigations on the physiological roles of the peptide during the growth are under progress in our laboratory. It is also interesting that a peptide-like substance having biological activities on the growth of microorganisms was observed in various naturally occurring materials such as koji extracts, carrot extracts, corn gluten meal and cotton seed flour.

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