Isolation from Soy Milk of a Growth-stimulating Substance for Lactic Acid Bacteria

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Abstract A growth-stimulating substance for specific lactic cultures such as Lactobacillus casei and Lactobacillus acidophilus was isolated from a precipitate fraction of soybean whey which was followed by ethanol fractionation, and some properties of the substance were investigated. Crude preparation fractionated with ethanol was passed through sulphopropyl-Sephadex C-25 and was resolved into at least three active growth stimulatory fractions of Lactobacillus casei subsp. alactosus 34143 used as the test organism of microbiological assays. One of the fractions, which showed the highest activity, was homogenous and ninhydin positive. From the results of UV absorption spectrum and amino acid analysis, the purified growth-stimulating substance was considered to be peptide and its molecular weight 1,150 daltons.

Key words: lactic acid bacteria, growth stimulation, soy milk, peptide, isolation,

With the increased popularity of fermented milk products, their production in various forms has been recognized on a worldwide basis. Foodstuffs such as vegetable proteins and fruits are now being used as ingredients of cultured dairy foods. Hence, fermentation of bean milk by lactic acid bacteria was tested and an attempt to make yogurt-like bean milk was done using a bean material. In the same study, acid production by Lactobacillus casei subsp. alactosus used was practically nil in skim milk. On the other hand, it produced a substantial amount of acid in bean milk. This suggested that some substances such as carbohydrates and nitrogenous compounds in bean milk were greatly influencing the growth and acid production of L. casei subsp. alactosus. It is well known that the growth of lactic acid bacteria is stimulated by yeast extract, fish soluble factor, enzymatic digests of protein, fruits and vegetable juices. The compounds responsible for the stimulation have been identified mainly as amino acids, peptides, purines, pyrimidines and nucleotides.

The present study was undertaken to isolate stimulatory compound(s) present in soy milk and to gain further information about the nature of such a stimulation in the growth of lactic acid bacteria.
Materials and Methods

1) Cultures

*Lactobacillus casei* subsp. *alactosus* 34143 and its lac*+* mutant designated as N-2515 used have been maintained in this laboratory. Other lactic cultures were kindly given by the National Institute of Animal Industry, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan. They were incubated at 35°C and transferred daily in peptone yeast extract broth (PY broth) consisting of peptone 1%, yeast extract 0.5%, glucose 0.5%, lactose 0.5%, Tween 80 0.1% and L-cysteine 0.01% with a broth pH of 6.8.

2) Preparation of soy milk

Soy milk was prepared from soybeans. Dry, mature, whole beans were thoroughly washed and then soaked in distilled water for 20 hrs at room temperature. The soaked beans were blended for 30 secs in a kitchen mixer with some distilled water, and the final volume was brought up to 4 times the weight of the soaked beans. After heat treatment at 95°C for 10 mins in a water bath, the mixture was filtered through a 120 mesh sieve.

3) Purification procedure

Soy milk was adjusted to pH 4.5 with 2 N HCl and the supernatant (soybean whey) containing the growth-stimulating substance was clarified by centrifugation. Ten parts of cold ethanol were added dropwise to one part of the soybean whey with constant stirring, and the soybean whey was fractionated. After removing the solvent by vacuum evaporation at 40°C, both supernatant and precipitate fractions were dissolved in appropriate volumes of 0.01 M phosphate–citric acid buffer (pH 4.5) to give a total protein concentration of 1 to 10 mg per ml and assayed for the bacterial growth as described below. The precipitate contained the crude growth-stimulating substance. Residual lipids in the precipitate were extracted with cold ethanol twice. This crude preparation was further purified by using sulphopropyl (SP)–Sephadex C-25 column (Pharmacia Fine Chemicals), 2.6 × 30 cm, and eluted with 0.01 M phosphate–citric acid buffer pH 4.5. Two gram eluents were collected at the rate of 18 ml/hr and assayed for bacterial growth.

4) Assay of bacterial growth

Stimulatory effect on the bacterial growth was evaluated by the determination of titratable acidity and microbiological assay.

One ml of the solution containing the ethanol precipitate was filter–sterilized and added to 9 ml of skim milk (reconstituted 10% non–fat dry milk) which was supplemented with 0.5% glucose. This was inoculated with 1% of active lactic culture and incubated at 35°C for 24 hrs. Acid production was determined by titration with 0.1 N NaOH.

Microbiological assay was conducted via the Tokita and Nakaniishi5) method. The composition of the medium is presented in Table 1. The basal medium was equally distributed into several test tubes of uniform diameter, and autoclaved at
Table 1. Composition of basal medium used for microbiological assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free acid hydrolyzed casein (Difco)</td>
<td>0.06 N%</td>
</tr>
<tr>
<td>L-cystine</td>
<td>100 mg</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>100 mg</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Pyridoxal hydrochloride</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 g</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>12 g</td>
</tr>
<tr>
<td>Adenine, guanine, uracil and xanthine</td>
<td>10 mg of each</td>
</tr>
<tr>
<td>K₂HPO₄ and KH₂PO₄</td>
<td>1 g of each</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>400 mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>20 mg</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>20 mg</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>20 mg</td>
</tr>
</tbody>
</table>

a) The basal medium was adjusted at pH 6.8.

110°C for 15 min. *Lactobacillus casei* subsp. *alactosus* 34143 was used as the test organism for all assays. The exponential cells from PY broth were harvested by centrifugation, and washed twice with sterile saline (0.85%). The washed cells were then diluted with the saline to give 10⁷ to 10⁸ cells/ml. Test samples such as soybean whey, ethanol precipitate and column fractions were filter-sterilized and then 0.5 ml of the sample was added to 4.5 ml of the sterile basal medium. In a control 0.5 ml of 0.01 M phosphate-citric acid buffer (pH 4.5) was used instead of 0.5 ml of test sample. Each tube was inoculated with 0.02 ml of the saline cell suspension and incubated at 35°C for 24 hrs. The incubation time was sufficient to give approximately half-maximal growth in the control. Growth was measured turbidometrically at 660 nm with a Hitachi Model 101 spectrophotometer. Increased Optical Density (O.D.) value in contrast to that of the control was read and relative activity of the growth-stimulating substance was expressed as increased O.D. value/g protein.

5) Homogeneity of the purified growth-stimulating substance

Relative activity of different fractions obtained after passing through ion exchange column was measured and fractions of a peak which showed the maximum activity were collected. This pooled fraction was concentrated by vacuum evaporation at 40°C, and used for the estimation of the purification achieved and the homogeneity of the growth-stimulating substance. Purity of the growth-stimulating substance was determined by thin layer chromatography (TLC) and fast protein liquid chromatography (FPLC).
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graphy (FPLC).

TLC plate silica gel 60 F254 (0.5 mm thickness, Merck) was used as the absorbant. The chromatogram was developed in n-butanol : acetic acid : water (8 : 2 : 1 V/V) solvent system. After developing, the dried plate was examined under UV light and also sprayed with ninhydrin. The Rf value of the ninhydrin positive spot was calculated. A Mono S column (HR 5/5) was eluted at the rate of 1 ml/min with 50 mM succinate-NaOH buffer (pH 4.5) using FPLC system (Pharmacia Fine Chemicals). Further elution was carried out by linearly ascending the concentration gradient of NaCl at the flow rate of 1 ml/min. The concentration of NaCl was increased from 0 M to 0.5 M.

6) Amino acid analysis

The purified growth-stimulating substance from the ion exchange column was hydrolyzed in 6 N HCl at 110°C for 24 hrs and analyzed on a Jeol model JLC-6 AH automated amino acid analyzer with a computing integrator.

7) Determination of molecular weight

The mol. wt. of the purified growth-stimulating substance from the ion exchange column was determined by Sephadex G-15 (Pharmacia Fine Chemicals) gel filtration. The column (1.6 by 70 cm) was eluted with 0.01 M phosphate-citric acid buffer (pH 4.5) at the rate of 24 ml/hr. Standard peptides (Serva Feinbiochemica) of known mol. wt. such as phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (mol. wt. 777), actinomycin C (mol. wt. 1,280) and bacitracin (mol. wt. 1,450) were used. The void volume was calculated using blue dextran (mol. wt. 2 × 10⁶).

8) Determination of protein and sugar

Ninhydrin and Molisch tests were carried out to detect primary amines and carbohydrates in test samples, respectively. The protein content of every sample used for microbiological assay was estimated by the method of Lowry et al. using BSA as a standard.

Results and Discussion

The precipitate fraction obtained from solvent fractionation with ethanol contained the majority of the growth-stimulating activity in soybean whey.

The stimulatory effect of this crude preparation on the acid production of lactic acid bacteria is shown in Table 2. This fraction showed a considerable growth-stimulating effect for L. casei subsp. alactosus 34143, L. casei subsp. casei N-25, L. casei subsp. casei L-14 and L. acidophilus L-54, but did not show any significant effect for other lactic cultures. Acid production of L. helveticus B-1 was rather inhibited. These results indicate that growth-stimulating substance(s) present in ethanol precipitate fraction might stimulate acid production in specific lactic cultures. Moreover, since both L. casei subsp. alactosus and its lac+ mutant designated as N-25 produced high amounts of acid, the substantial growth in skim milk of these strains seemed to be caused by stimulant(s) other than carbohydrates. Figure 1 shows a typical dose response curve showing the stimulation for L. casei subsp. alactosus.
Table 2. Effect of growth-stimulating fraction added on acid production of some lactic cultures in skim milk\(^a\)

<table>
<thead>
<tr>
<th>Lactic culture(^b)</th>
<th>Net acid produced (ml of 0.1 N NaOH)(^c)</th>
<th>Medium(A)(^d)</th>
<th>Medium(B)(^e)</th>
<th>(B) − (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. casei</em> subsp. <em>alactosus</em> 34143</td>
<td>1.40</td>
<td>5.60</td>
<td>4.20</td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em> subsp. <em>casei</em> N-25</td>
<td>2.43</td>
<td>7.25</td>
<td>4.82</td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em> subsp. <em>casei</em> L-14</td>
<td>2.90</td>
<td>5.70</td>
<td>2.80</td>
<td></td>
</tr>
<tr>
<td><em>L. helveticus</em> B-1</td>
<td>11.30</td>
<td>9.40</td>
<td>−1.90</td>
<td></td>
</tr>
<tr>
<td><em>L. bulgaricus</em> B-5b</td>
<td>11.10</td>
<td>11.30</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td><em>L. acidophilus</em> L-54</td>
<td>5.85</td>
<td>9.95</td>
<td>4.10</td>
<td></td>
</tr>
<tr>
<td><em>Str. thermophilus</em> 510</td>
<td>6.95</td>
<td>7.05</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td><em>Str. cremoris</em> H-61</td>
<td>4.25</td>
<td>3.60</td>
<td>−0.65</td>
<td></td>
</tr>
<tr>
<td><em>Str. lactis</em> 527</td>
<td>6.15</td>
<td>6.75</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td><em>Str. lactis</em> subsp. <em>diacetylactis</em> N-7</td>
<td>5.40</td>
<td>5.95</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The solution containing precipitate obtained from solvent fractionation with ethanol (total protein in amounts of 7.30 mg per ml) was used as growth-stimulating fraction.

\(^b\) Incubated for 24 hrs at 35°C.

\(^c\) Net acid produced=acid produced in the presence of sample−acid produced in the blank.

\(^d\) Medium(A) indicates skim milk containing 0.5% glucose.

\(^e\) One ml of growth-stimulating fraction was added to 9ml of Medium(A).

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**Fig. 1.** Stimulation of the growth of *L. casei* subsp. *alactosus* 34143 in semi-synthetic medium by the solution containing precipitate obtained from solvent fractionation with ethanol (total protein in amounts of 3.84 mg per ml).
afforded by ethanol precipitate fraction in the semi-synthetic medium. Stimulatory
effects on the growth of lactic acid bacteria in skim milk supplemented with glucose
are not always the same as those in the semi-synthetic medium. However, when L.
casei subsp. alactosus was used as a test organism for microbiological assay, ethanol
precipitate fraction stimulated its growth as well. Therefore, the further purification
of growth stimulatory compounds in this crude preparation was observed by the assay
procedure using the semi-synthetic medium based on that of Tokita and Nakani5).

Figure 2 presents a typical elution profile of an ethanol precipitate fraction which
has passed through an ion exchange column. Three ml of this fraction were applied to
SP-Sephadex C-25 column equilibrated with 0.01 M phosphate-citric acid buffer
(pH 4.5), which later served as the eluent. The column was eluted with the same
buffer. The growth stimulatory fraction recovered between fraction numbers of 26
and 40 and it was resolved into at least three different fractions. The second and
third fractions contained the majority of the total stimulatory activity compared to
the first fraction. Also, the relative activity of Peak 3 was approximately 1.7 times
that of Peak 2. A thin layer chromatogram of Peak 2 on silica gel in an n-butanol-
acetic acid-water solvent system gave three nynhydrin positive spots, while Peak 3
showed only one compound with an Rf value of 0.31 on TLC plates. The third fraction
was pooled and tested for further investigation.

The results of microbiological assays of various fractions from soy milk are
summarized in Table 3. The relative activity of the third fraction recovered from SP-

![Figure 2](https://example.com/fig2.png)

**Fig. 2.** Elution profile of the solution containing precipitate obtained from solvent fractionation with ethanol on a sulphopropyl-Sephadex C-25 column. ○-○ Absorbance at 280 nm; ●-● Relative activity. The elution was carried out as described in the text.
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Sephadex C-25 column was 184.18 and the purification achieved was approximately 160-fold. Its fraction was also negative with respect to the Molisch test. The FPLC chromatogram gave a single peak, although it was not absorbed on cation exchange (Mono S) column (Fig. 3). The UV spectrum of the purified growth-stimulating substance from Peak 3 showed that the maximum absorption occurred at 277 nm with another peak at 210 nm (Fig. 4). The substance was then hydrolyzed with hydrochloric acid at 110°C for 24 hrs in a vacuum-sealed tube. The hydrolysate was analyzed by an amino acid analyzer. Equimolar amounts of amino acids were calculated by dividing each micromolar value by the micromolar value of the amino acid present in least abundance. The molar content of amino acids were lysine (1.14), aspartic acid (1.29), threonine (1.00), serine (3.25), glutamic acid (1.13), glycine (2.86) and alanine (1.27). Therefore, the third fraction was considered to be peptide. The molecular weight

Table 3. Comparison of growth-stimulating activity of various fraction from soy milk by using microbiological assay

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Increased O.D. value$^a$</th>
<th>Protein added (mg/ml)</th>
<th>Relative activity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy milk (Original)</td>
<td>0.011</td>
<td>9.62</td>
<td>1.14</td>
</tr>
<tr>
<td>Soybean whey</td>
<td>0.206</td>
<td>7.77</td>
<td>26.51</td>
</tr>
<tr>
<td>Ethanol precipitate</td>
<td>0.323</td>
<td>3.84</td>
<td>84.11</td>
</tr>
<tr>
<td>SP-Sephadex C-25</td>
<td>0.326</td>
<td>1.77</td>
<td>184.18</td>
</tr>
</tbody>
</table>

$^a$ Subtracted O.D. value in a control from O.D. value in the presence of sample.

$^b$ Expressed as increased O.D. value per gram protein.

Fig. 3. Elution profile of growth-stimulating substance on a Mono S column using fast protein liquid chromatography. —— UV absorption at 280 nm; ----- Molarity of NaCl. 200 μl of growth-stimulating fraction from sulphopropyl-Sephadex C-25 column were injected on a Mono S column. Gradient elution was carried out as described in the text.
of the purified fraction was estimated via the molecular sieving method\textsuperscript{18}). The elution volume of the standard peptides like phenylazobenzyloxy carbonyl-Pro-Leu-Gly-Pro-D-Arg, actinomycin C and bacitracin were calculated as 158, 96 and 76 ml, respectively. The elution volume of the purified growth-stimulating substance was 112 ml. From the molecular weights of standard peptides, the mol. wt. of the purified substance was estimated to be 1,150 daltons (Fig. 5).

It has been shown that a variety of peptides unrelated in their amino acid composition have a stimulatory effect on the growth of \textit{L. casei}. The term strepogenin

![UV absorption spectrum of growth-stimulating substance obtained from sulphopropyl-Sephadex C-25 column.](image)

\textbf{Fig. 4.} UV absorption spectrum of growth-stimulating substance obtained from sulphopropyl-Sephadex C-25 column.

![Standard curve for determination of molecular weight of growth-stimulating substance from sulphopropyl-Sephadex C-25 column by Sephadex G-15 gel filtration. Ve/Vo of bacitracin, actinomycin C, growth-stimulating substance and phenylazobenzyloxy carbonyl-Pro-Leu-Gly-Pro-D-Arg was 1.213, 1.548, 1.787 and 2.542, respectively.](image)

\textbf{Fig. 5.} The standard curve for determination of molecular weight of growth-stimulating substance from sulphopropyl-Sephadex C-25 column by Sephadex G-15 gel filtration. \( \text{Ve/\text{Vo}} \) of bacitracin, actinomycin C, growth-stimulating substance and phenylazobenzyloxy carbonyl-Pro-Leu-Gly-Pro-D-Arg was 1.213, 1.548, 1.787 and 2.542, respectively.
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has been given to peptides showing this effect\(^{19}\). They have, to date, been isolated from tryptic digests of certain proteins, pancreas tissue and ripe Edam cheese\(^{5,19,20}\). The strepogenin activity unit of the peptide isolated in the present study was not determined; however, it seems to be similar to peptides with strepogenin activity in that the peptide showed the property as a growth factor for \(L.\) casei. Since the TLC plate from Peak 2 shown in Fig. 2 gave different ninhydrin positive spots, a variety of nitrogenous compounds like peptide which are responsible for the stimulatory effect would appear to be contained in soy milk. Nucleosides, amino acid and manganese have been also reported as growth-stimulating substances for \(lactobacilli^{10,12,14,21}\). Although their effects were not examined in the present study, many factors present in soy milk might contribute to the growth–stimulating activity. Thus, it appears that the substantial growth of \(L.\) casei subsp. \(a\)lactosus in soy milk depends upon stimulants like the peptide isolated in addition to the fermenting ability of sucrose, the major fermentable sugar in soybeans, as described in an earlier report\(^1\).

Also, the ethanol precipitate fraction from winged bean milk, which was prepared in the same way as soy milk, stimulated acid production of \(L.\) casei and \(L.\) acidophilus in a preliminary study. Therefore, the use of these organisms appears to have comparatively greater potential for the production of fermented foods using bean milk.

Acknowledgement

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References

豆乳から乳酸菌の発育促進性物質の分離

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Lactobacillus casei subsp. alactosus はヨーグルト様豆乳の製造にその利用性が認められた。これは豆乳に含まれる糖や窒素化合物などの組成が、本菌の生育に有効に働いているものと推論された。この点を明らかにする目的で豆乳から乳酸菌の発育促進性物質を分離し、その性状について検討した。

大豆の加熱抽出によって調製した豆乳を、2 N 塩酸で pH 4.5 とし、得られた豆乳ホエーに 10 倍量のエタノールを加えて溶剂分別した。発育促進性物質を含むエタノール沈殿画分の各種乳酸菌に対する酸生成の促進効果は、Lactobacillus casei および Lactobacillus acidophilus において顕著であった。このエタノール沈殿画分は、スルフォプロピルセファデックス C-25 カラムでさらに精製した。溶出液の発育促進効果を、Lactobacillus casei subsp. alactosus を試験菌とした微生物学的検定法により調べたところ、活性を示す 3 つの画分が得られた。最も高い活性を示す画分の薄層クロマトグラムはニンヒドリン反応陽性の単一スポットであった。UV 吸収スペクトルやアミノ酸分析の結果から、単離された発育促進性物質はペプチドと思われ、その分子量は約 1,150 ダルトンと推定された。

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