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# Identification of Growth Stimulatory Compound in the Mixed Culture of *Lactobacillus helveticus* YM-1 and *Streptococcus thermophilus* CH-1 in Milk

**Sung-Sik Yoon and Ju-Hyun Yu***

*Department of Food & Nutrition, Bucheon Technical College, Bucheon, Kyunggi-Do*
**Department of Food Engineering, Yonsei University, Seoul*

# *Lactobacillus helveticus* YM-1과 *Streptococcus thermophilus* CH-1의 혼합배양액 중에 함유된 생육촉진물질의 확인

윤성식 · 유주현*

부천공업전문대학 식품영양과, *연세대학교 공과대학 식품공학과

**Abstract**

A compound stimulatory to the growth of *S. thermophilus* CH-1 was isolated from the cell-free filtrate of *L. helveticus* YM-1 in milk medium. The stimulant was identified as a peptide with a molecular weight of approximately 5000 and exhibited positive ninhydrin reaction. Some kinds of amino acids confirmed as aspartic acid, alanine, valine, glutamic acid, lysine, proline, leucine were rich in the stimulatory peptide hydrolysate. Among them, glutamic acid was most abundant. Judging from bioautographic results, glutamic acid and phenylalanine were expected to exert an important role for the stimulation.

## Introduction

Yoghurt is a traditional food and beverage in Balkans and the Middle East. However its popularity has recentely spread to Europe and to many other parts of the world.[1] The main flora of Yoghurt which has been reported is consisting *L. bulgaricus* and *S. thermophilus*. Therefore their associative growth was much studied by other workers[2-4] who maintained that growth and acid productions of both organisms were stimulated when they were cultivated in mixed. In search of the new yoghurt starter, we selected, *L. helveticus* YM-1 among several lactobacilli strains except L. bulgaricus in the previous paper[5] and also found that the mixed culture of the two strains showed a typical symbiotic relationship. The aim of this study was to examine further the symbiosis existing in two strains and to identify the factors in cell-free filtrate of *L. helveticus* YM-1 responsible for the stimulatory effect.

## Materials and Methods

### Bacterial strains

All the bacterial strains used in this study were kept and maintained the same as the methods mentioned elsewhere.[5]

### Preparation of cell-free filtrate

Cell-free filtrate of *L. helveticus* YM-1 was prepared as previously described.[5]

### Ion exchange chromatography

500*ml* of cell-free filtrate from cultured broth of *L. helveticus* YM-1 was applied on cation exchange column, 2.4 × 60 *cm*, which was packed with Amberite IR-120 (Rohm and Haas Co).[2] Effluent fraction was first obtained by passing the filtrate through the column. The column was washed with decarbonated water until lactose was not detected by adding newly prepared Molish reagent. (Washing fraction) And then eluated by $\frac{N}{10}-NH_4$ $\frac{N}{10}-NH_4OH$ with constant flow rate, 40 *ml*/min. (Eluate fraction). All three fractions obtained were concentrated to dryness with reduced pressure in a rotary vacuum evaporator (Tokyo Rikakikai Co) at 45°C. Each concentrate was dissolved in a small quantity of distilled water and adjusted pH to 6.8 with dilute NaOH solution.

**Sephadex G-25 column chromatography**

The stimulatory fraction, 10*ml*, separated by ion exchange column was applied on 2.5 × 40 *cm* column packed with fine Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) and equilibrated with N/10-$NH_4OH$ solution for 24 hours at room temperature. This column was subsequently eluted by N/10-$NH_4OH$ solution with constant flow rate of 1*ml*/min. The eluate from this column was to pass through Single Path UV-Monitor (Pharmacia Co, Model UV-1 214). Each fraction was collected on automatic fraction collector (Toyo, Model SF-100).

**Paper chromatography and bioautography**

Concentrated active fraction was mixed with 6 volumes of 6N-HCl and hydrolyzed completely at 105°C for 12 hours in a steam bath. After removing residual HCl, the hydrolisate was spotted on Whatman No 1 paper strip for 1-dimensional (5.0×20 *cm*) and 2-dimensional chromatography (20×20 *cm*) and then developed by ascending technique with following solvent systems; n-butanol-acetic acid-water (5:1:4 *v/v*), phenol-water (8:2, *v/v*), chloroform-methanol -17% ammonium hydroxide (4:4:2, *v/v*).[6] $R_f$-values on paper chromatogram were compared with authentic amino acids whose colors were developed by 0.02% ninhydrin solution. Bioautographic test was carried out by modified method of Speck et al.[7] Sterile litmus milk agar was poured to solidify in a large glass plate which can accommodate a paper strip for 2-dimensional chromatography. Overnight culture of *S. thermophilus* CH-1 in lactic broth was harvested by centrifugation and washed once with physiological saline. The washed cell was uniformly spreased over the agar medium and developed paper strip was gently placed on it followed by incubation at 42°C.

**Determination of amino acid contents**

Amino acids contained in th stimulatory fraction were analysed by amino acid analysis system (Waters Associates, U.S.A.).[8-9] The conditions for this analysis was as follows; Injection volume: 20*ml*, Chart speed: 20*mm*/min, Detector; fluorescence detector model 420 AC, Post column reagent: o-phthalaldehyde, Temperature: 62°C, Mobile phase: 0.2N Na-Citrate buffer (pH 3.10), 0.2N Na-Borate buffer (pH 9.60), Stationary phase: CAT EX Resin, Flow rate: 0.4 *ml/min*. Contents of amino acids were calculated by external standard method.

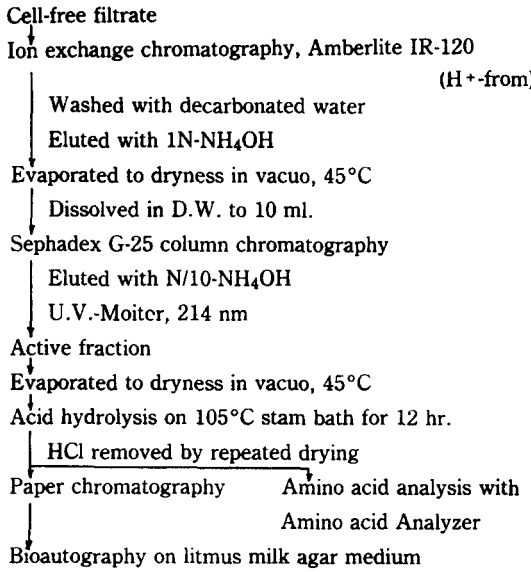


**Fig. 1. Identification procedure of growth stimulatory compound in cell free filtrate**

**Measurement of growth stimulatory activity**

10% reconstituted non-fat milk solid was distributed into 1.5 × 12 *cm* screw capped test tubes in 9 *ml* quantities and then added 1 *ml* of test fractions to make 10 *ml* of final volume. For control, distilled water was added instead. After active *S. thermophilus* CH-1 grown in 10% non-fat milk solid was inoculated and mixed thoroughly, the mixture was incubated without shaking for 6 hours at 42°C and developed acidity was measured.

## Results

**Fractionation by ion exchange chromatography**

As described in previous paper, we found that the stimulatory compound was contained in the culture filtrate of *L. helveticus* YM-1 in milk. Each fraction of effluent, washing and eluate obtained by the ion exchange column was tested for their ability to stimulate the acid production by *S. thermophilus* CH-1, respectively. The active compound was found to be present mainly in the fraction eluted with $\frac{N}{10}$-$NH_4OH$ solution. (Table 1) So it seemed that lactose did not exert the stimulatory effect.

**Effect of heat treatment**

The eluate fraction was subjected to heat treatment

**Table 1. Effect of each fractions on the growth of *S. thermophilus* CH-1 in milk.**

| Fractions | Titratable acidity | | | Growth(%), 6 hr |
|---|---|---|---|---|
| | 0 hr | 3 hr | 6 hr | |
| Control | 1.60 | 3.0 | 5.0 | 100 |
| Effluent fraction | 1.60 | 3.1 | 5.0 | 100 |
| Washing fraction | 1.60 | 3.5 | 5.4 | 108 |
| Eluate fraction | 1.60 | 4.0 | 6.5 | 130 |

Effluent, Washing and Eluate fractions were prepared by passing through Amberlite IR-120 Ion-exchange column.

at 100°C for 15 min and its stimulatory activity was also tested by using *S. thermophilus* CH-1 in milk. As shown in Table 2, eluate fraction was proved to be relatively resistant to heat.

**Gel filtration of active fraction**

When eluate fraction from the ion exchange column was concentrated and submitted to gel filtration on Sephadex G-25 column. As Fig. 2 shows, there appeared three distinct peaks which had a strong absorbance at 214 *nm*. Peptides, in general, absorb light of short wavelength at the proximity of 200*nm*. So three compounds corresponding to the peaks were assumed to be peptides. As the result shows in Fig. 3, both fraction 2 and 3 had stimulatory activities strongly and the rest slight. Judging from the elution profiles of the compound in the active fractions on the gel filtration column, its molecular weight might be estimated to be over several thousands. In the other hand, ninhydrin reactions for the two fractions proved positive.

**Paper chromatography and bioautography**

After fraction 2 and 3 were mixed and then subjected to acid hydrolysis. The resulting hydrolysate was developed on 2-dimensional paper strip as well as l-dimensional. A paper strip which was not treated with ninhydrin solution was subsequently bioautographed on the surface litmus milk agar medium. When compared with $R_f$ values of authentic amino acids such as aspartic acid, alanine, glutamic acid, valine, lysine, proline and leucine were abundantly present in the hydrolysate (Fig. 4, 5 and 6). And it was thought that glutamic acid and phenylalanine were important for the stimulatory effect.

**Analysis of amino acids**

Based on the data in Table 3, the molecular weight of

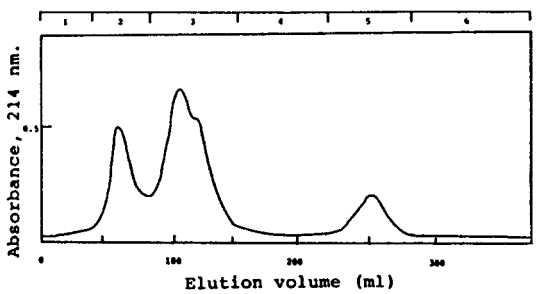


**Fig. 2. Chromatogram of filtrate on Sephadex G-25 column (2.0 × 60*cm*)**

Eluted solution; N/10-$NH_4OH$, Flow rate; 1 *ml*/min. Eluate was pooled into 6 fractions as shown.

**Table 2. Effect of heat treatment of the cell-free filtrate of *L. helveticus* YM-1 on the growth of *S. thermophilus* CH-1 in milk.**

| Fractions | Titratable acidity | | | Growth(%), 6 hr |
|---|---|---|---|---|
| | 0 hr | 3 hr | 6 hr | |
| Control | 1.60 | 3.0 | 5.0 | 100 |
| No-treated | 1.60 | 4.5 | 7.0 | 140 |
| Heat-treated | 1.60 | 4.2 | 6.8 | 136 |

Cell-free filtrate of *L. helveticus* YM-1 was held at 100°C for 15 min, followed by cooling and then added to 10% non-fat milk solid, 4 *ml*, for titratable acidity measurement.

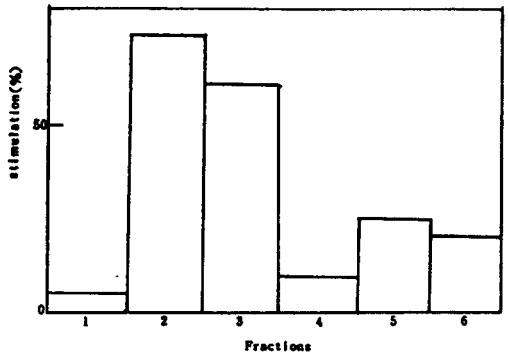


**Fig. 3. Effect of each fractions by Sephadex G-25 column chromatography on the growth of *S. thermophilus* CH-1 in milk**

Each fractions was added separately to 10% non-fat milk solid and incubated at 42°C for 6hr. Stimulation of growth was expressed as a percentage of the stimulation produced by equivalent concentration (10%, v/v) of the cell-free filtrate which was taken to represent 100% stimulation.

the stimulatory peptide was calculated to be approximately 5000. The approximation were in good agreement with the elution profiles of the gel filtration column which was packed by SephadexG-25. However it should be noted that acid hydrolysis may have destroyed certain amino acids such as tryptophan and cysteine present in the original peptide.

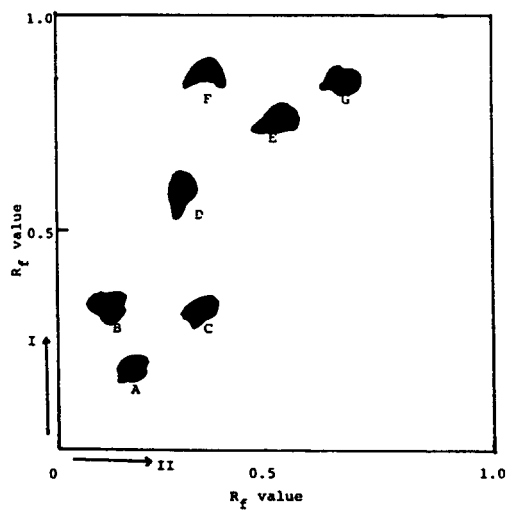


**Fig. 4. Paper chromatogram of acid-hydrolyzed stimulatory fraction (Fraction 2 and 3)**

: Moving direction of solvent system
I : n-Butanol-Acetic acid-Water (5:1:4, v/v)
II: Phenol-Water (8:2, v/v)
A: Aspartic acid, B: Glutarmic acid, C: Alanine, D: Valine, E: Lysine, F: Proline, G: Leucine

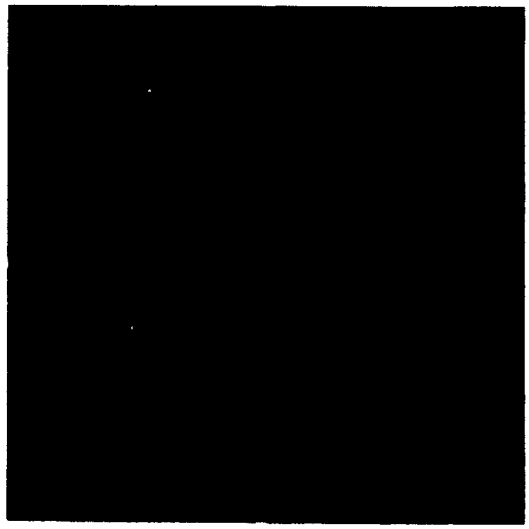

**Fig. 5. Effect of free amino acids on the growth of *S. thermophilus* CH-1 on litmus milk agar medium**

## Discussion

Stimulation of bacterial growth by peptides is well known phenomenon since the discovery of strepogenin by Woolley (1945).[10] Sandine et al (1956)[11] admitted that peptides stimulated the growth of bacteria, particularly the lactic acid bacteria. Merrifield and Woolley (1958)[12] argued that at least penta-peptide should be necessary for the strepogenin activity. Moss and Speck (1966)[13] identified the stimulatory peptide from *E. coli*

**Table 3. Amino acid composition of the stimulatory peptide (Fraction 2 and 3)**

| Amino | Content(μmole/*ml*) | No. of residues |
|---|---|---|
| Aspartic acid | 3.17 | 2.9 |
| Theronine | 1.48 | 1.4 |
| Serine | 2.10 | 1.9 |
| Glutamic acid | 7.76 | 7.0 |
| Glycine | 1.57 | 1.4 |
| Alanine | 3.43 | 3.1 |
| Valine | 3.21 | 2.9 |
| Isoleucine | 1.83 | 1.6 |
| Leucine | 4.44 | 4.0 |
| Phenylalanine | 1.09 | 1.0 |
| Lysine | 6.61 | 6.0 |
| Arginine | 2.89 | 2.7 |
| Total | | 35.9 |

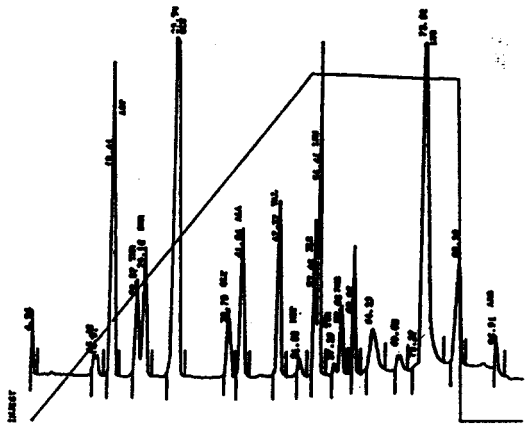

**Fig. 6. High performance liquid chromatogram of acid hydrolysed stimulatory peptide (Fraction 2 and 3)**

cells and Berg et al (1981)[14] also from yeast extract. But Selby Smith et al (1975)[15] reported tryptophan was a key amino acid to bacterial growth rather than peptides. Phenylalanine has been reported to be an important amion acid for the growth of *Lactobacillus casei* by Zuraw et al (1960).[16] They thought the stimulation by free phenylalanine was probably the result of inability of the organism to obtain this amino acid from the milk protein at a rate sufficient for its need. In the other hand, Dahiya and Speck (1962)[17] and Koburger et al (1963)[18] had identified bases such as adenine, xanthine as stimulatory factor from milk. In this study, it could be calculated that stimulatory compound in milk culture was mainly peptides containing a few amino acids abundantly such as aspartic acid, valine, glutamic acid, alanine, lysine, proline, leucine. This study emphasizes the need for a better understanding of factors involved in mixed culture of yoghurt starters for the used in the manufacture of yoghurt.

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## 요 약

환원탈지유배지에서 자란 *Lactobacillus helveticus* YM-1의 cell-free filtrate로부터 *Streptococcus thermophilus* CH-1의 생육촉진을 확인한 결과 이 물질은 분자량이 5,000정도의 peptide로 나타났다. 이 peptide 중에는 aspartic acid, lysine, proline, leucine, valine, alanine, glutamic acid등의 amino산이 비교적 풍부하였으며, 그중에서 glutamic acid 함량이 가장 높았다. 또 이 peptide가 생육 촉진효과를 나타내는데 가장 중요한 역할을 담당하는 것은 glutamic acid와 phenylalanine으로 판단되었다.

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