Identification of a Stimulant for *Lactobacillus casei* Produced by *Streptococcus lactis*

A. L. BRANEN AND T. W. KEENAN

Food Sciences Institute, Purdue University, Lafayette, Indiana 47907

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A compound stimulatory to the growth of *Lactobacillus casei* was isolated from cell extracts of *Streptococcus lactis*, purified, and characterized. The stimulant was identified as a small peptide with a molecular weight of approximately 4,500 daltons. The purified peptide gave negative tests for nucleic acids, phosphorus, glucosamine, and carbohydrates. Sixteen amino acids were detected in acid hydrolysates of this peptide. Serine, proline, glycine, alanine, leucine, and glutamic acid were present in hydrolysates in greatest abundance.

In an early report, Hansen (6) demonstrated that sonically prepared extracts of *Streptococcus lactis* and *S. cremoris* were stimulatory to the growth of *Lactobacillus casei* and concluded that this effect could play an important role in the ripening of cheese. We have previously confirmed and extended those observations (1). Cell extracts of several *Streptococcus* species were observed to stimulate growth of those lactobacilli prevalent in ripening Cheddar cheese. The stimulatory principle was dialyzable and partially inactivated by heat. In the present communication, we report the isolation and identification of a compound produced by *S. lactis* which is stimulatory to growth of *L. casei*.

**MATERIALS AND METHODS**

**Organisms.** *S. lactis* C2-F was used as the source of stimulant, and *L. casei* 393 was employed as the assay organism. The source of these organisms and methods for routine propagation and preparation of cell-free extracts have been described previously (1). For preparation of cell-free extracts, cells were harvested by centrifugation at 5°C and washed twice with amounts of sterile 0.85% saline equivalent to the volume of original culture (1).

**Growth assay.** A modification of the bioautographic method of Cogan et al. (3) was employed to follow the isolation of the stimulant. The method was modified by pouring the milk-agar medium into sterile petri plates and allowing it to harden. Sterile assay discs were dipped into sterile solutions to be tested and placed on the hardened medium; the plate was incubated at 40°C. Stimulatory zones were observed after 6 to 12 hr as areas of red color around and under the disc. To confirm the presence of the stimulant in isolated fractions, growth curves were determined for the test organism in the presence and absence of isolated fractions as described previously (1).

**Isolation of stimulant.** Cell-free extracts were prepared and dialyzed (1), and the dialysate was dried in vacuo, dissolved in minimal volumes of water, and applied to a Sephadex G-25 column (3 by 56 cm). Elution was accomplished with 0.05 m phosphate buffer (pH 7.4). Fractions were combined according to the absorbance spectrum at 260 nm, evaporated in vacuo, sterilized, and tested for stimulation. The active fraction obtained was applied to a column of Sephadex G-10 and eluted with distilled water. Fractions containing stimulant were combined, concentrated to a few milliliters, and applied to a column of diethylaminoethyl (DEAE) Sephadex A-50 (3 by 15 cm); elution was accomplished with a gradient of 0.02 to 0.1 m ammonium carbonate buffer. Active fractions were combined, concentrated, and applied to a column of carboxymethylcellulose. The stimulant was eluted with a gradient of 0.1 to 0.2 m NaCl in 0.01 phosphate buffer (pH 7.1). After concentration and desalting of the active fraction over Sephadex G-10, ethanol was added to achieve final concentrations of 30, 60, and 90%. The precipitate formed at each step was recovered by centrifugation, dried in vacuo to remove the last traces of ethanol, and dissolved in distilled water. The stimulant, recovered in the 90% ethanol fraction, was finally purified by chromatography on 500-μm layers of silica Gel G or cellulose MN 300 (Brinkman Instruments, Inc., Great Neck, N.Y.). Chromatoplates were developed with butanol-acetic acid-water (5:1:4, by volume), and a strip along one edge of the plate was exposed and sprayed with ninhydrin. Nihydrin-positive zones were marked on the unstained portion of the chromatoplate, scraped off, eluted with distilled water, concentrated, sterilized, and tested for stimulation of the test organism. At this point, the stimulant appeared homogeneous on paper electrophoresis in a Durrum-type cell (Spinco) at both pH 3.6 (acetate buffer) and 8.6 (barbiturate buffer).

**Amino acid analysis.** Samples were dissolved in an approximately 200-fold excess of 6 N HCl, refluxed for 15 hr, dried in vacuo, and redissolved in a small volume of 0.2 m sodium citrate buffer, pH 2.75. A portion of the hydrolysate was placed on the column of a
Technicon amino acid analyzer with 0.2 μmole of norleucine as an internal standard. Separation was accomplished according to standard procedures, and the relative molar concentrations of the amino acids were calculated.

**Chemical analyses.** Phosphorus content of both hydrolyzed and unhydrolyzed fractions was determined by the method of Rouser et al. (12); ninhydrin-positive material, by the method of Moore and Stein (9); ribonucleic acid (RNA), by the method of Von Euler and Hahn (15); and deoxyribonucleic acid (DNA) by the method of Ceriotti (2). Carbohydrate was determined by reaction with anthrone (14), protein by the biuret method (5), and glucosamine by the method of Elson and Morgan (4).

**RESULTS**

Analysis of the concentrated cell-free dialysate of *S. lactis* revealed the following composition: protein, 1.3 mg/ml; RNA, 1.4 mg/ml; and DNA, 0.36 mg/ml. Three major fractions were obtained by separation of the dialysate on Sephadex G-25, with peak 2 containing the majority of the stimulatory material (Fig. 1). The active fraction was excluded as one major peak from Sephadex G-10 and yielded only one major peak when chromatographed on both DEAE-Sephadex and carboxymethylcellulose (Fig. 2). Thin-layer chromatography of the carboxymethylcellulose eluate showed a number of ninhydrin-positive zones; however, the 90% ethanol precipitate, which contained the stimulant, yielded only one ninhydrin-positive zone when chromatographed on both silica gel ($R_F = 0.21$) and cellulose ($R_F = 0.41$).

**Fig. 1.** Elution profile of the stimulatory fraction through Sephadex G-10 (solid line) and Sephadex G-25 (broken line) columns. With G-25, elution was with 0.05 M phosphate buffer. The active fraction was collected and applied to a G-10 column which was eluted with distilled water. Arrows designate the active fractions.

**Fig. 2.** Elution profile of the stimulatory fraction on DEAE-Sephadex A-50 (solid line) and carboxymethylcellulose (broken line) columns. The active fraction obtained from Sephadex G-10 was applied to DEAE-Sephadex and eluted with a gradient of 0.02 to 0.1 M ammonium carbonate. The active fraction obtained was applied to the carboxymethylcellulose column and eluted with a gradient of 0.1 to 0.2 M NaCl in 0.01 M phosphate buffer. Arrows designate the active fractions.

The active fraction recovered from chromatograms gave negative tests for RNA, DNA, carbohydrate, organic phosphorus, and glucosamine and was homogeneous by electrophoresis. The final traces of RNA were removed from the stimulatory fraction after passage over carboxymethylcellulose, and the final traces of DNA were removed by thin-layer chromatography. The absence of nucleic acid in the final fractions was confirmed by the absence of absorbance maxima at 260 nm.

The stimulant appeared to be peptidyl in nature since it was precipitated by ethanol and yielded a ninhydrin-positive reaction, the magnitude of which was increased by hydrolysis. Sixteen amino acids were detected in this peptide, those present in greatest quantity included serine, glutamic acid, proline, glycine, alanine, and leucine. Sulfur amino acids were not detected. It should be noted that hydrolysis may have destroyed certain amino acids present in the original peptide.

Assuming that the number of residues in Table 1 is correct and rounding off these values to the nearest whole residue, the approximate molecular weight of the peptide is calculated to be 4,700. This is in agreement with its behavior on Sephadex columns, at which point the peptide was included by Sephadex G-25 (exclusion = 5,000) and excluded by Sephadex G-10 (exclusion = 700).

The same pattern of stimulation of growth of *L. casei* was observed when either the isolated
peptide or the original dialysate was added to broth cultures of this organism (Fig. 3). This suggests that the isolated peptide is responsible for the major portion of the stimulation of the test organism by cell-free extracts of *S. lactis*.

**DISCUSSION**

Peptides have long been known to stimulate the growth of bacteria, particularly the lactic acid bacteria (13). Kihara and Snell (7) outlined several conditions which would reduce the availability of an essential amino acid for bacterial growth, thereby enhancing the ability of appropriate peptides to promote growth. These authors were of the opinion that peptides play no special role in metabolism. Moss and Speck (10, 11) found that peptides present in Trypsin and in frozen *Escherichia coli* cell extracts enhanced the recovery of *E. coli* cells injured by freezing. They suggested that these peptides manifested their activity by stimulating cell repair and growth during the lag phase.

The stimulatory peptide characterized in the present study is similar to a series of peptides isolated by Moss and Speck (10, 11) from Trypsin and *E. coli* cells in Sephadex G-25 elution pattern, molecular weight, and amino acid composition. Stimulatory peptides isolated from pancreas also have an amino acid composition very similar to the peptide we isolated from *S. lactis* (8, 13). These similarities suggest that stimulatory peptides of rather constant amino acid composition are widespread in nature.

Since stimulation of the test organism by an amino acid mixture approximating the composition of the peptide was not evaluated, we cannot conclude that the integrity of the peptide is essential for activity. However, in view of the work discussed above, it seems logical that the peptide itself, and not its component amino acids, is necessary. Since exhaustively washed whole *S. lactis* cells, cell extracts (1), and the purified peptide were observed to stimulate growth of *L. casei* in milk culture, it can be concluded that this peptide is produced by the organism itself. Stimulation in milk culture implicates the peptide as a possible factor stimulating the development of lactobacilli in Cheddar cheese during ripening.

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