Characterization of Growth Stimulants in Corn Steep for Lactic Streptococci

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Received for publication 18 November 1970

The production of acid in milk cultures of lactic streptococci was stimulated by the addition of corn steep liquor. Separation by ion-exchange and paper chromatography indicated the presence of four major stimulatory components in the corn steep. Some variation was noted in the response of the lactic streptococci to the individual stimulatory components. The four components were further purified by paper and column chromatography. One of the four stimulatory components stained positively on chromatograms with ninhydrin. The remaining stimulatory components were detectable only by bioautography. All four components were unstable to acid hydrolysis and absorbed ultraviolet light between 230 and 275 nm in aqueous solution. The stimulatory components did not contain pentoses, suggesting that they were not nucleotides or nucleosides; however, they might be purine or pyrimidine bases.

Metabolic activity of lactic streptococcus starter cultures in milk can be stimulated by the addition of various extracts of plant and animal origin (5, 6, 7, 8, 9, 11, 12; W. Orme-Johnson III, Ph.D. Thesis, Univ. of Texas, Austin, 1964). The stimulatory activity of these complex substances has been generally attributed to their being sources of readily available nutrients (10). Many of the stimulants have not been identified; however, portions of the stimulatory components in pancreas and liver extract have been characterized as peptides (8, 9; W. Orme-Johnson III, Ph.D. Thesis). Nucleic acid derivatives in pancreas extract (7) and in tomato juice (2) have also been identified as stimulatory components. The stimulation of milk cultures of lactic acid bacteria by corn steep (a by-product from the manufacture of corn starch) was first reported by Kennedy and Speck (6). Heimbuch et al. (5) isolated and characterized a nucleotide in corn steep which was stimulatory for Lactobacillus casei, and Zuraw et al. (12) identified phenylalanine in corn steep as a stimulant for the same organism. The objective of the present study was to isolate and characterize components of corn steep which stimulate acid production by the lactic streptococci.

MATERIALS AND METHODS

Culture maintenance. All cultures were maintained by subculturing weekly in sterile litmus milk with a 1% inoculum and 18 hr of incubation at 22 C. The cultures were stored at 3 C and subcultured at least three times at daily intervals before being used experimentally.

Corn steep preparation. The corn steep (Corn Products Refining Co., Argo, Ill.) was diluted to approximately 20% solids with distilled water and centrifuged for 20 min at 30,000 X g. The supernatant was filtered through a sterile 0.45-μm membrane filter and stored at 3 C.

Acid production. Acid production by the cultures in 10% reconstituted nonfat milk solids (NFMS) was measured by continuously monitoring the pH by using the method described by Gilliland and Speck (4), except that the inoculated milk was dispensed in 19-ml quantities into sterile glass tubes (1.7 by 15 mm) containing 1 ml of the desired concentration of test material or 1 ml of sterile water. The inoculated milk was incubated in a water bath at 32 C.

Bioautographic analysis. Stimulatory zones were located on paper chromatograms by a bioautographic method similar to that reported by Speck et al. (11). The medium for each bioautographic assay consisted of three components: 160 ml of 4% agar, 160 ml of 20% reconstituted NFMS, and 5 ml of 20% aqueous litmus. All components were sterilized separately by autoclaving for 15 min at 121 C. The components were tempered to 45 C, mixed together on a magnetic stirrer, inoculated with 1% of the test organism, and poured into a Plexiglas plate (14 by 60 by 2.5 cm). (The Plexiglas plate was rinsed thoroughly with ethanol followed by sterile distilled water before pouring the bioautograph medium.) Strips of the chromatograms (2 cm in width) to be assayed were placed on the surface of the solidified medium beside a strip from a control chromatogram (no sample) and incubated at 22 C. Stimulatory zones were observed through the bottom of the plate as white areas.
(reduced litmus) on a blue background appearing within 4 to 8 hr.

Cation-exchange chromatography. A 10-ml sample of corn steep was passed through a column (2.2 by 41 cm) of the cation-exchange resin [AG50W-X8 (H^+), 100 to 200 mesh (BioRad)]. The sample was washed through with distilled water at a rate of 1 ml per min until a negative Molisch test for sugar was obtained. The column was then eluted with 6.5 bed volumes of 2 N NH4OH. Excess NH4OH was removed from the eluate by repeated dilution and evaporation as described by Cogan et al. (2). The NH4OH-free eluate was concentrated at 42 C under reduced pressure to 10 ml and passed through a sterile 0.45-μm membrane filter. A portion of the material was stored at 3 C and the remainder lyophilized.

Paper chromatography. Stimulatory components were isolated by using descending paper chromatography on sheets (46 by 37 cm) of Whatman 3MM chromatography paper. The chromatograms were developed for 15 hr with one of the following solvent systems: (I) n-butanol–acetic acid–water (4:1:1), (II) n-butanol–acetic acid–water (250:60:250), and (III) n-propanol–water (7:3). Stimulatory components were located by bioautographic assay of marker strips cut from the chromatograms. Marker strips were also stained with 0.25% ninhydrin in acetone or scanned for ultraviolet light-absorbing zones under a 254-nm emitting lamp. Zones on the remaining portion of the chromatograms which corresponded to the stimulatory zones on the bioautographed marker strips were excised and eluted in distilled water for 1 hr. The aqueous solution was then concentrated to 5 ml under reduced pressure at 42 C and stored at 3 C.

Gel chromatography. The components eluted from the chromatograms were applied to a column of G-25 Sephadex (1.3 by 33 cm; Pharmacia) equilibrated at room temperature with 0.1 N NH4OH. The column was eluted with 0.1 N NH4OH at a rate of 0.6 ml per min at room temperature. Fractions of 2 ml were collected and assayed for absorbance at 260 nm by using a Beckman DU spectrophotometer.

Acid hydrolysis. Evacuated and sealed glass vials containing the samples in 6 N HCl were heated at 110 C for 24 hr. The HCl was removed by evaporating the samples to dryness and rediluting to 4 ml.

Corn extraction. Twenty grams of whole corn of the same grade used for corn starch manufacture was ground to 20 mesh in a Wiley mill, added to 200 ml of distilled water at 50 C, and agitated in a shake water bath at 50 C for 1 hr. The extract was filtered through a Whatman no. 4 filter paper and centrifuged for 20 min at 5,800 × g; the supernatant was concentrated to 3 ml at 42 C under reduced pressure and stored at 3 C.

RESULTS

The production of acid by single-strain lactic streptococci in milk was stimulated by corn steep (Table 1). The amount of stimulation was recorded as the difference in the time required for the culture to reach pH 5.0 in the control milk and in the milk containing 0.05% corn steep. The amount of stimulation for four of the five cultures tested ranged from 0.9 to 2.0 hr, whereas S. lactis H1, which produces acid quite slowly in milk, was stimulated for 11.1 hr.

The active components in corn steep were absorbed on cation-exchange resin, and the active material was eluted from the column with NH4OH. A portion of the lyophilized eluate (0.6 g) was dissolved in 6.6 ml of distilled water and applied to 22 sheets of chromatography paper. After development with solvent system I, marker strips cut from each sheet were assayed by bioautographic analysis. The results (Table 2) revealed stimulatory zones for S. lactis AC2 at RF values of 0.18, 0.29, 0.40, and 0.54, which were designated A, B, C, and D, respectively. Bioautographic assays were also conducted with other single-strain lactic streptococci and mixed-strain commercial Cheddar cheese starter cultures. S. lactis C10 and S. cremoris C8 responded to the four stimulatory components observed with S. lactis AC2. S. cremoris AC11 responded to zones corresponding to B, C, and D and, in addition, responded to a different stimulatory zone having a slightly higher RF value (0.58) than D. Mixed strain commercial cheese starter cultures MD and MU responded to zones having RF values similar to those of components A, B, C, and D, whereas cultures VT3 and VT7 responded to zones having RF values comparable to components B and D. In addition, VT7 responded to a zone at RF 0.73.

Components A, B, C, and D were selected for further isolation and characterization. The zones containing the stimulatory components were eluted from the paper with distilled water and concentrated to 5 ml, and each was applied to 16 additional sheets of chromatography paper. The chromatograms for components A and B were developed with solvent system II; solvent system III was used for components C and D. Bioautographic analysis of marker strips from these chromatograms revealed a single stimulatory zone for each component. The RF values were as follows: 0.35 for A, 0.33 for B, 0.56 for C, and

| Culture              | Time (hr) to reach pH 5.0 | Stimulation (hr) |
|----------------------|---------------------------|------------------|
| Control              |                           |                  |
| 0.05% Corn steep     |                           |                  |
| S. lactis AC2        | 7.2                       | 6.0              |
| S. cremoris C8       | 6.6                       | 5.7              |
| S. cremoris ML1      | 6.5                       | 4.5              |
| S. cremoris AE8      | 7.5                       | 6.3              |
| S. lactis H1         | 20.0                      | 8.9              |
|                       |                           | 11.1             |
TABLE 2. Bioautographic response of single- and mixed-strain lactic streptococci to paper chromatograms of cationic eluate

| Culture                        | R_F of stimulatory zones |
|--------------------------------|--------------------------|
|                                | A | B   | C   | D   |
| Single-strain cultures        |   |     |     |     |
| S. lactis AC2                  | 0.18 | 0.29 | 0.40 | 0.54 |
| S. lactis C10                  | 0.17 | 0.27 | 0.39 | 0.54 |
| S. cremoris C11                | 0.19 | 0.28 | 0.39 | 0.54 |
|                                |   |     |     |     |
| Commercial cheddar cheese starters |   |     |     |     |
| MD                             | 0.16 | 0.26 | 0.35 | 0.50 |
| MU                             | 0.15 | 0.26 | 0.32 | 0.47 |
| VT7                            |   | 0.30 |     | 0.55 |
| VT2                            |   | 0.28 |     | 0.53 |

0.63 for D. None of the active zones absorbed or fluoresced under ultraviolet light. The active zone of component D was the only one which corresponded to a ninhydrin-positive zone on the chromatogram. Attempts to visualize the active zones with reducing agents, sulfuric acid ashing, or iodine were unsuccessful. Because of the lack of a chemical visualization method, the bioautographic assay was used to locate the active components to be eluted from the chromatograms.

Sephadex gel chromatography was employed to remove impurities eluted from the chromatography paper along with the stimulatory material. Preliminary investigations indicated that the stimulatory components eluted from chromatograms absorbed ultraviolet light at 260 nm; therefore, the eluate from the Sephadex column was monitored at 260 nm. One major peak was observed for each component (A, B, C, and D) along with several minor peaks. Analysis of the fractions by spot assay (similar to the bioautographic assay) indicated that the tubes containing the fraction under the major peak in each case contained the stimulatory activity. Paper controls were prepared for each component. For these controls, equal amounts of paper (based on weight) were excised from blank paper chromatograms which had been developed in the appropriate solvent systems used in the second paper chromatographic separation of the stimulatory components. The paper controls were eluted, concentrated, and passed over the Sephadex column in the same manner as used for the stimulatory material. For each paper control, the tubes corresponding to the stimulatory fraction from the respective component were pooled and treated in a manner similar to that of the stimulatory components. These samples were lyophilized and used for further characterization.

Aqueous solutions (0.75 mg/ml) of each stimulatory component were heated at 100°C for 10 min and then assayed for stimulatory activity. The results indicated that the components were resistant to boiling.

The acid hydrolysates of the stimulatory components and equivalent amounts of unhydrolyzed components were applied to chromatography paper strips and developed with solvent system I. The unhydrolyzed samples exhibited stimulatory zones having R_F values comparable to those observed in previous bioautographic assays. However, the stimulatory components were apparently not stable to acid hydrolysis in that no stimulatory zones were observed for the hydrolyzed samples. No ninhydrin-positive zones were evident on the chromatograms of the hydrolyzed samples.

The ultraviolet-absorption spectra of the stimulatory components revealed absorption in the 220- to 300-nm range. The maximal and minimal wavelengths in 0.1 N HCl and 0.1 N NaOH for each component are presented in Table 3. The spectra of the stimulatory components were suggestive of nucleic acid derivatives; however, they did not coincide closely with the spectra which have been reported for the common nucleic acid derivatives. Analyses of the components.
with orcinol ferric chloride reagent (1) revealed the absence of pentoses, thus eliminating the possibility that the components are nucleosides or nucleotides, or both.

In an attempt to determine the origin of the stimulatory material, an aqueous extract of ground corn was chromatographed on paper with solvent system I, and the resulting chromatograms were bioautographed with S. lactis ACs as the test culture. The results revealed stimulatory zones at Rf values of 0.15 and 0.37. These were similar to the Rf values for components A and C, indicating that at least two of the stimulatory components originated in the corn.

DISCUSSION

The measurements of stimulation in this study were based on the ability of the test culture to produce acid or to lower the oxidation-reduction potential of the growth medium. No attempt was made to correlate these measurements with growth of the organisms. Kennedy and Speck (6) reported that corn steep stimulated the growth as well as the acid production by L. casei. The results of the present study involving stimulation of single-strain lactic streptococci and multiple-strain Cheddar cheese starter cultures indicated some variation among cultures with respect to the amount of stimulation and to the number of stimulatory components in corn steep. The results, however, indicate that the components designated as A, B, C, and D were the major stimulatory components for the lactic streptococci.

It was not possible to locate the stimulatory components A, B, and C on paper with chemical detection methods. Only component D was located in this manner; it stained weakly with ninhydrin. This ninhydrin zone remained associated with the stimulatory zone during the second paper chromatographic separation. Since they were not stained with ninhydrin, stimulants A, B, and C apparently do not contain free amine groups or else were present in amounts below the detection limits of ninhydrin. None of the stimulatory components exhibited ultraviolet light-absorbing or fluorescing zones on the paper chromatograms, suggesting that they were not nucleic acid derivatives or that they were not present in sufficient quantities for visualization.

Heimbuch et al. (5), with L. casei as the test culture, isolated a stimulatory component from corn steep which possessed a pentose and exhibited an ultraviolet light-absorption spectral maximum at 265 nm and minimum at 245 nm. They postulated that the factor could be a nucleoside and further showed that several known nucleic acid derivatives were slightly stimulatory to L. casei. Zuraw et al. (12) studied the stimulatory effect of corn steep liquor on L. casei and S. lactis and isolated three stimulants for L. casei. The purified stimulants were not stimulatory for S. lactis and possessed ultraviolet light absorption maxima and minima between 230 and 290 nm. One of the stimulatory compounds was identified as phenylalanine. The other factors were postulated as being free aromatic amino acids or peptides containing aromatic amino acids.

In the present study, the isolated stimulatory substances retained their activity after boiling but lost activity when acid-hydrolyzed. If the stimulatory components were peptidic as has been shown for some materials that stimulate the streptococci (8, 9, 10), the hydrolyzed product may or may not contain stimulatory components. Stimulant D, which stained with ninhydrin before hydrolysis, was not stimulatory after hydrolysis and did not reveal additional ninhydrin-positive zones. Most amino acids are resistant to acid hydrolysis, although a few are partially destroyed. The ultraviolet absorption spectra and other results obtained for stimulant D are indicative of an aromatic amino acid. However, further characterization studies are needed before making a definite classification.

Components A, B, and C had ultraviolet absorption spectra similar to D but did not stain with ninhydrin. Since these spectra are characteristic of nucleic acid derivatives, a pentose determination was employed in an attempt to implicate nucleotides or nucleosides, but the results were negative. Purine and pyrimidine bases would not be eliminated by these results. Component D could also fit into this group. Certain nucleic acid derivatives are known to be stimulatory for lactic streptococci (3, 7). Vitamins could also be involved, since some exhibit ultraviolet light absorbance in the range found for the stimulants. Such compounds on paper chromatograms might be detected by bioautography and not by chemical tests if they were active in catalytic amounts.

Knowledge of the origin of the stimulatory components found in corn steep is desirable for evaluating its use as a food additive. Two of the components apparently originated in the whole corn. The remaining components may have been synthesized during the fermentation that occurs in the steeping process used in the manufacture of corn starch or they may be hydrolysis products of some nonstimulatory component of corn.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service training grant ES-61 from the Division of Environmental Health Sciences.
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