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Bitter Peptide Isolated from Milk Cultures of *Streptococcus cremoris*[1]

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ABSTRACT

GORDON, D. F., JR. (North Carolina State of the University of North Carolina, Raleigh), AND M. L. SPECK. Bitter peptide isolated from milk cultures of *Streptococcus cremoris*. Appl. Microbiol. **13**:537-542. 1965.—Certain cultures of *Streptococcus cremoris* produced a bitter taste that occurred in the whey portion of milk cultures. Whey from a culture which produced bitterness was fractionated on Sephadex. The fraction in which the bitter taste was concentrated was chromatographed successively on paper with butanol-acetic acid-water (5:1:4), and then butanol-2-butanone-water (2:2:1). In each instance, the bitter component was in the most rapidly moving band that gave a positive ninhydrin test. The bitterness was observed to be caused by a peptide containing the following numbers of each amino acid: arginine, 1; glutamic acid, 2; glycine, 2; isoleucine, 2; leucine, 2; phenylalanine, 1; proline, 5; and valine, 4. N-terminal amino acids could be detected by coupling with 2,4-dinitrofluorobenzene or phenylisothiocyanate, or by hydrolysis with leucine aminopeptidase. When treated with carboxypeptidase, only leucine and valine appeared at the C-terminal end, and these were detected simultaneously.

Of the various compounds capable of causing a bitter taste in milk products, it has been reported that proteolytic products resulting from starter culture growth are the causative agents in cheese (Kelly, 1932; Czulak, 1959; Czulak and Shimmin, 1961; Emmons et al., 1962*a*, *b*; Jago, 1962). Only limited attempts have been made to isolate and characterize these compounds. Raadsveld (1953) isolated bitter compounds from Gouda cheese and found that they exhibited characteristics of polypeptides. Murray and Baker (1952) and Carr, Loughheed, and Baker (1956) found that bitter peptides accumulated when casein was treated with various proteolytic enzymes. Carr et al. (1956) isolated and characterized a bitter peptide. Ichikawa, Yamamoto, and Fukumoto (1959) isolated a bitter peptide after incubation of a neutral proteinase from *Bacillus subtilis* with casein.

We have found (Gordon and Speck, 1965) that strains of *Streptococcus cremoris* capable of producing the bitter component in milk cultures possess greater proteolytic activity than "nonbitter" strains of the same species. The present investigation was undertaken to isolate, identify, and characterize the bitter component(s) produced by one of the bitter strains.

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MATERIALS AND METHODS

Cultures. Strains of *S. cremoris* designated HP, C13, E8, and ML1 were obtained from G. L. Hills of the Commonwealth Scientific and Industrial Research Organization, Dairy Research Section, Victoria, Australia. The organoleptic analysis of these cultures was reported previously (Gordon and Speck, 1965). All strains were included in this study; however, preparations from milk cultures of strain HP were used as the source for the isolation of the bitter component.

Isolation of peptide. The strains were cultured in reconstituted nonfat milk (10% solids) and incubated for 1 day at 22 C and then for 3 days at 32 C. Each culture was mixed in a Waring Blendor and then centrifuged at 6,000 × *g* in a refrigerated centrifuge. The supernatant fraction was filtered through Whatman no. 42 filter paper before it was concentrated (12:1) at 45 C on a flash evaporator. A portion of the concentrated whey (20 ml) was treated with 1.8 to 2.0 ml of concentrated ammonium hydroxide and then centrifuged at 30,000 × *g* for 15 min; the supernatant fraction was applied to the bed surface of a G-25 Sephadex column. Sephadex (100 g) was treated with 0.05 M NaCl (Gelotte, 1960), the fines were removed, and it was then packed into a column (4 by 60 cm) according to Flodin's (1961) procedure. The column was

equilibrated for 24 hr with the eluent (0.1 N NH_4OH) before the sample was applied. The column was mounted on a fraction collector at room temperature, and 7 to 10 ml of eluate were collected per tube at a flow rate of 1.5 to 2 ml per min. The optical density of every other tube was measured at 265 mμ with a Beckman DU spectrophotometer. The contents of certain groups of tubes were combined on the basis of optical density, and each fraction was concentrated under reduced pressure to 5 ml and then tasted.

A 15-ml amount of bitter concentrate was spotted as a band on 60 Whatman no. 3 MM chromatography strips (3.8 by 60 cm). The material was placed on a line 6 cm from one end, and the strips were equilibrated for 5 to 6 hr and then developed for 14 to 16 hr at room temperature with butanol–acetic acid–water (5:1:4). The lower phase of the solvent was used for equilibration, and the upper was used for developing the strips. After drying, a narrow strip was cut from the edge of each chromatogram and stained with ninhydrin (0.25% in acetone); the ninhydrin-positive areas were marked on the unstained portion of each chromatogram. Comparable zones were removed from the 60 chromatograms, combined in a column (2 by 40 cm), and eluted with 800 ml of distilled water. The eluates were concentrated to 5 ml under reduced pressure and were tasted, and the bitter sample was rechromatographed on 20 chromatography strips. These strips were equilibrated and developed with butanol–2-butanone–water (2:2:1) as described by Mizell and Simpson (1961). Comparable ninhydrin-positive areas were removed, combined, and eluted with 400 ml of distilled water. The eluates were concentrated; samples of the bitter material were examined for the presence of peptide linkages with the biuret test, and for the presence of carbohydrates (Morris, 1948) with anthrone reagent. The bitter concentrate was dried over Drierite and P_2O_5 in a vacuum desiccator.

High-voltage electrophoresis. A portion (0.1 to 0.2 mg) of the purified bitter concentrate was dissolved in 0.15 ml of buffer and applied as a band (4 cm) in the center of a sheet of Whatman no. 3 MM filter paper. The pyridine–acetic acid–water (1:10:289) buffer (*p*H 3.6) described by Katz, Dreyer, and Anfinsen (1959) was applied to the paper evenly up to the center line so that the sample was covered simultaneously from both sides. The paper was then placed in the tank of a high-voltage electrophoresis apparatus (High Voltage Electrophorator, model D; Gilson Medical Electronics, Middleton, Wis.) containing buffer (*p*H 3.6) covered with Varsol. Prior to and during operation, the tank was maintained at 21 to 24 C by circulating a coolant (3 C) through coils contained in the Varsol. A potential of 3,000 v and a current of 160 ma was maintained for 3 hr. The paper was removed and dried, and a 6-cm lengthwise strip was cut off and stained with ninhydrin. Ninhydrin-positive zones on the remainder of the sheet were then located, removed, and eluted. The eluate was made 6 N with HCl and hydrolyzed for 6 hr at 121 C. The resulting amino acids were tentatively identified by paper chromatography with butanol–acetic acid–water (5:1:4).

Amino acid identification. Positive identification of the amino acids in hydrolysates of the bitter component was made with the use of an amino acid analyzer (model 120B, Spinco Division, Beckman Instruments Inc., Palo Alto, Calif.). The height × width (HW) method was used for integration of the recorded peaks. HW constants were calculated for 1-μmole quantities of known amino acids. Dividing these constants into the values for the unknown amino acids gave the micromole quantities present. The number of residues in the original peptide was estimated by taking the lowest micromole value as one and dividing this into the other micromole values.

Preparation of 2,4-dinitrofluorobenzene derivatives. Approximately 1 mg of peptide or known amino acid was placed in a screw-capped tube (16 by 120 mm) fitted with a Teflon-lined cap. Derivatives were prepared by procedures described by Schroeder and LeGette (1953), except that, after the reaction, 5 ml of distilled water were added, and the solution was extracted five times with 10-ml portions of ethyl ether. The hydrolysis step was omitted when the known derivatives were prepared. Dinitrophenyl-arginine was not soluble in ether and remained in the acid fraction.

Amounts of 10 μliters of known derivatives and 20 to 30 μliters of the unknown ether- and acid-soluble fractions were spotted on sheets (18 by 48 cm) of Whatman no. 3 MM chromatography paper, which were then equilibrated for 1 hr and developed for 6 to 8 hr with water-saturated butanol (Mellon, Korn, and Hoover, 1953). The developed chromatograms were dried and treated with hydrochloric acid vapors as recommended by Sanger and Thompson (1953). This treatment bleached out yellow artifacts present on the chromatograms.

Preparation of phenylthiohydantoin derivatives. The procedure followed was essentially that of Margoliash (1962). Trimethylamine was replaced with 1 drop of 1 N NaOH, as proposed by Edman (1950). The derivatives were dissolved in 0.5 ml of ethyl acetate, and 50 μliters of the unknown and 10 μliters of the known were spotted on Whatman no. 1 chromatography paper sheets (18 by 48 cm), impregnated with starch. The chromatograms were developed with heptane-pyridine (7:3), and the derivatives were detected by the procedure of Sjöquist (1953).

Treatment of peptide with leucine amino peptidase. A 5-mg amount of partially purified preparations of leucine amino peptidase (Nutritional Biochemicals Corp., Cleveland, Ohio) was activated according to the procedure reported by Hill and Smith (1957). The bitter peptide (0.5 to 1.0 mg) was dissolved in 1 ml of buffer and equilibrated at 40 C before addition of the activated enzyme preparation. When incubation was longer than 6 hr, a few drops of toluene were added to

each preparation to prevent bacterial growth. Samples (0.2 ml) were removed at zero time and at selected time intervals, and were boiled to stop enzyme activity. Samples (100 μliters) were spotted on chromatography strips, which were then equilibrated and developed with butanol–acetic acid–water (5:1:4). The chromatograms were examined for any differences in amino acid patterns and were compared with chromatograms of known amino acids.

Treatment of peptide with carboxypeptidase. The C-terminal amino acids were determined by use of carboxypeptidase (Nutritional Biochemicals Corp.). A procedure described by Neurath (1955) was adopted for use in the present study. Carboxypeptidase (0.1 g) was dissolved in 50 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.5) containing 0.1 M sodium chloride; 2 ml were equilibrated at 25 C, and the remaining enzyme solution frozen and stored at −20 C. Between 0.5 and 1 mg of bitter peptide was dissolved in 1 ml of buffer and equilibrated for 15 min at 25 C. A 1-ml amount of the enzyme preparation was mixed with 1 ml of the substrate, and the mixture was incubated at 25 C up to 24 hr. Toluene was added as a preservative for incubation periods over 6 hr. Samples (0.2 ml) were removed at zero time and at intervals up to 24 hr. Enzyme activity was stopped by boiling, and 100 μliters of each sample were spotted on chromatography paper strips. The chromatograms were developed with butanol–acetic acid–water (5:1:4), and the amino acid patterns were compared before and after enzyme action. Chromatograms of known amino acids were included each time.

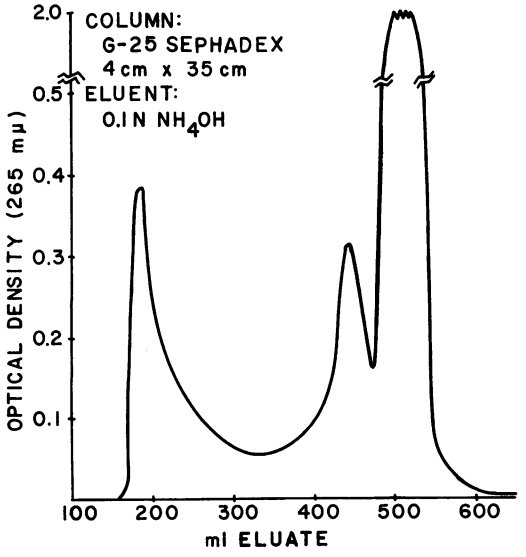

FIG. 1. *Elution pattern of concentrated whey after treatment with ammonium hydroxide.*

TABLE 1. *Organoleptic analysis of Sephadex fractions* of concentrated whey from culture HP*

Fraction	Eluate†	Organoleptic analysis
	ml	
1	170–230	Bland
2	230–340	Bitter
3	340–400	Sweet-salty
4	400–475	Very salty
5	475–600	Slight salty

* See Fig. 1 for optical density readings at 265 mμ and details of the elution pattern.

† Eluent was 0.1 N ammonium hydroxide.

RESULTS

The concentrated whey preparations from milk cultures of the different strains were fractionated on Sephadex. The absorption patterns (at 265 mμ) of the eluates from all cultures were comparable (Fig. 1). The five fractions selected were tasted by two to four individuals, and the composite evaluation of each fraction is listed in Table 1. Fractions comparable to those indicated in Table 1 were tasted for all cultures. The bitter taste was present in the one fraction (230 to 340 ml) from culture HP. Concentrated whey preparations from culture C13 also exhibited a bitter fraction, and the bitterness appeared in the same fraction as for culture HP. None of the fractions obtained from cultures E8 and ML1 were bitter. The fraction in which the bitter taste occurred contained little if any carbohydrate, but high amounts of proteins or peptides, or both.

Isolation of bitter component. The chromatograms of the bitter fraction from culture HP were developed with butanol–acetic acid–water, and all ninhydrin-positive zones were eluted from the paper strips. Only the fastest moving band (R_F 0.83) was bitter. This material also gave a positive biuret test. Preparations from strains C13, ML1, and E8 also were similarly chromatographed and developed with butanol–acetic acid–water; the most rapidly moving ninhydrin-positive zones were at R_F 0.74, 0.71, and 0.71, respectively. The material present at these R_F values was eluted. The preparation from culture C13 was slightly bitter and gave a weak biuret test, whereas those from strains E8 and ML1 were neither bitter nor biuret-positive.

Four ninhydrin-positive zones were detected when the bitter fraction (HP) was rechromatographed with the butanol–2-butanone–water solvent. The zone at R_F 0.36 was bitter and gave a positive biuret test. Separation of compounds with this solvent by descending chromatography was not distinct unless the solvent was allowed to flow off the strips. The bitter compound was lo-

cated as the foremost band which stained only lightly with ninhydrin. This band was distinctly separated from other ninhydrin-positive material.

The eluted, dried material was yellow-brown in color, very bitter, and also possessed a burned taste. Although the paper strips were dried in a hood, apparently the solvents or reactants of paper with solvent (Schwane and Kowkabany, 1963) were not removed completely. To ascertain whether material from the solvents or paper was causing the bitterness, eluates of developed paper strips without samples were tasted and found not to be bitter, although there was a distinct burned flavor. Most of the colored material could be removed by passing the concentrated bitter eluate through a second Sephadex column.

Amino acid composition. Components of the bitter peptide were identified by paper chromatography as arginine, glutamic acid, glycine, isoleucine, leucine, phenylalanine, proline, and valine. The R_F values of known amino acids and compounds in the acid hydrolysate of the bitter component are presented in Table 2. Chromatography of alkaline hydrolysates indicated that tryptophan was not present in the peptide. Throughout the study, a portion of each bitter preparation isolated was hydrolyzed and found to contain the same amino acids.

The micromole quantities of the amino acids and probable number of residues of each, as determined with the use of an amino acid analyzer, are presented in Table 3. The two samples analyzed were isolated from two different milk culture preparations of strain HP. Assuming the probable number (i.e., 19) of residues to be correct, the peptide would have a molecular weight of approximately 2,350. This value may be high, since estimation of whole-number residues for leucine and valine could possibly be lower by one.

TABLE 2. *R_f values of known amino acids and those obtained by acid hydrolysis of bitter peptide**

Amino acid	R_f values	
	Known sample	Bitter peptide hydrolysate
Arginine	0.14	0.12
Glycine	0.19	0.18
Glutamic acid	0.23	0.23
Proline	0.31	0.30
Valine	0.47	0.48
Phenylalanine	0.63	0.63
Isoleucine	0.63	0.63
Leucine	0.67	0.67

* Chromatograms developed with butanol–acetic acid–water (5:1:4).

TABLE 3. *Amino acid composition of acid hydrolysates of bitter peptide as determined by the amino acid analyzer*

Amino acid	First prepn		Second prepn		Probable residues
	Amt	Residues*	Amt	Residues*	
	μmoles		*μmoles*		
Arginine	0.255	1	0.248	1	1
Glutamic acid	0.496	1.9	0.551	2.2	2
Glycine	0.496	1.9	0.563	2.3	2
Isoleucine	0.443	1.7	0.432	1.7	2
Leucine	0.430	1.7	0.342	1.4	2
Phenylalanine	0.274	1.1	0.304	1.2	1
Proline	1.305	5.1	1.249	5.0	5
Valine	0.890	3.5	0.890	3.6	4

* Residues determined by dividing the micromole values for arginine into the other micromole values.

Attempts were made to determine both C-terminal and N-terminal amino acids. Under the conditions of this study, N-terminal amino acids were not detected. Examination of the peptide before and after treatment by the chemical methods indicated that no reaction had occurred, since examination of acid or alkaline hydrolysates of the material showed all amino acids still to be present in approximately the same quantities. With leucine aminopeptidase, control chromatography strips contained some ninhydrin-positive bands; however, there was no increase in intensity of color in these bands, nor were any new bands apparent when enzyme-peptide samples were chromatographed.

Both leucine and valine appeared almost immediately when the peptide was treated with carboxypeptidase, and no additional amino acids were detected during a 24-hr reaction time. As with leucine aminopeptidase, there were some ninhydrin-positive bands present in enzyme controls; however, their intensity did not increase during the incubation period. Substrate controls for both enzyme reactions revealed only one ninhydrin-positive zone with an R_F value comparable to that of the bitter peptide.

The results obtained by high voltage electrophoresis indicated that only one definite ninhydrin-positive compound was present in the bitter material obtained from the chromatography strips. Under the conditions employed, this material migrated 17.5 cm toward the cathode. When this material, and questionable ninhydrin-positive areas on either side, were hydrolyzed, iden-

tical amino acid patterns were obtained for all three areas (Table 2).

DISCUSSION

Previously, Gordon and Speck (1965) suggested that the bitter component in milk cultures of *S. cremoris* resulted from the breakdown of casein rather than from other milk proteins, and that bitter strains HP and C13 possessed greater proteolytic activity in milk cultures than "nonbitter" strains. The present study indicates that the bitter component is a peptide containing 19 amino acid residues representing 8 different amino acids. It is not known whether the bitter compound produced by lactic streptococci from casein is a primary breakdown product, is a result of the accumulation of secondary breakdown products, or is the product of a coupling reaction involving peptides and amino acids.

The bitter peptide isolated by Carr et al. (1956) from tryptic hydrolysates of casein contained aspartic acid, glutamic acid, glycine, alanine, valine, proline, tyrosine, and leucine or isoleucine, or both. They found that the C-terminal amino acid was leucine, followed by either valine or glutamic acid. N-terminal amino acids were not detected. The authors concluded that the bitter component was a single polypeptide, possibly possessing a cyclic portion with a side chain containing a C-terminal amino acid. Ichikawa et al. (1959) isolated a bitter peptide after treatment of casein with a proteinase from *B. subtilis*. This peptide contained alanine, arginine, glutamic acid, glycine, leucine, methionine, phenylalanine, proline, serine, threonine, and tyrosine, but the authors were unable to correlate the properties of the amino acids present with the bitter flavor.

The results of the present study are comparable to those of Carr et al. (1956). N-terminal amino acids were not detected, and leucine and valine appeared to be released almost simultaneously from the C-terminal end. Since the probable number of residues for these two amino acids was questionable, it is quite possible that one or both of these amino acids would not be present on the C-terminal end in all molecules of the bitter peptide. This seems reasonable since, on either side of the definite ninhydrin-positive zone (representing the bitter peptide) obtained by high-voltage electrophoresis, there were questionable ninhydrin-positive areas. When these particular areas were eluted and hydrolyzed, traces of all the amino acids present in the bitter peptide were detected. This could mean that differences in one or two amino acids in the bitter peptide would lead to small differences in mobility under high-voltage electrophoresis.

The simultaneous release of leucine and valine would tend to indicate two C-terminal amino acids and, therefore, two terminal ends. However, this is questionable since these are the only two amino acids released after prolonged incubation, and N-terminal amino acids were not detected. According to Gladner and Neurath (1953), the relative rates of liberation of amino acids do not necessarily indicate the position of the amino acids in a polypeptide chain. For this to be true, the amino acids would have to be arranged, from the C-terminal end, in decreasing order of susceptibility to carboxypeptidase. A situation could exist where the C-terminal bond is hydrolyzed more slowly than the adjacent one; therefore, both amino acids could be liberated at the same rate, since hydrolysis of the original C-terminal bond would be the rate-limiting step. After removal of leucine and valine, the next amino acid may not be susceptible to hydrolysis by carboxypeptidase. This could be due to the presence of an amino acid, such as proline (Hirs, 1960), or to secondary peptide bonds.

Bitter peptides that have been isolated and characterized by others were obtained from casein. Glutamic acid, glycine, proline, and leucine are found in the peptide described in the present study and in those isolated by Carr et al. (1956) and Ichikawa et al. (1959). Whether or not these amino acids could be the basis of a specific structure responsible for bitterness is not known. The origin of bitter peptides in a common protein suggests, however, that these amino acids may be involved in a specific structure that possesses bitterness.

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