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Hydrolysis of $\alpha_{s,1}$-Casein B by *Streptococcus lactis* Membrane Proteinase[1]

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The membrane-associated proteinase of *Streptococcus lactis* strain 3 hydrolyzed $\alpha_{s,1}$-casein B into 11 peptide fragments. Eight of the 11 peptides were purified and partially characterized. Each peptide contained several, but not all six, essential amino acids required for growth. The culture was able to utilize one peptide as the sole source for the essential amino acid leucine. Leucine, serine, valine, and glycine were found to be NH_2-terminal residues. Two of the peptides were phosphopeptides. The data support the functional role of the membrane-associated proteinase as being involved in the initial breakdown of proteins to peptides.

Streptococcus lactis strain 3 requires phenylalanine, glutamic acid, proline, valine, leucine, and lysine for growth (17). It has been suggested that the lactic group of streptococci grown in milk acquires necessary amino acids via degradation of milk proteins (12). The utilization of protein by these microorganisms would necessitate their having an active proteinase enzyme system. The proteinase system of *S. lactis* 3 has been described as containing two proteinase enzymes, one intracellular and one associated with the membrane (2). The importance of the membrane-associated proteinase to the nitrogen economy of the cell has been described (3). The present investigation was undertaken to elucidate the hydrolytic action of the membrane-associated proteinase on protein and to further substantiate its importance in the nitrogen economy of the cell.

MATERIALS AND METHODS

Enzyme. The membrane-associated proteinase of *S. lactis* 3 was purified as described by Cowman et al. (3). The homogeneity of the purified enzyme preparations was determined by polyacrylamide gel and cellulose-acetate strip electrophoresis in 0.05 M sodium phosphate buffer at *p*H 6.0, 7.0, and 8.0. Only those preparations showing one electrophoretic band at each *p*H, with corresponding activity (eluted from cellulose-acetate strips), were used experimentally.

Purification of $\alpha_{s,1}$-casein B. Milk from cows homozygous for $\alpha_{s,1}$-casein B was collected and pooled, and the $\alpha_{s,1}$-casein was purified by the method of Thompson and Kiddy (15). The purification was monitored by using polyacrylamide gel electrophoresis at *p*H 9.1 in the presence of 4.5 M urea (16).

Dipeptide substrates. Dipeptides which were tested as potential substrates were obtained commercially (Nutritional Biochemical Corp). Prior to use, the homogeneity of the peptides was determined by using descending paper chromatography with 1-butanol-acetic acid-water (5:1:4). Qualitative analysis for hydrolysis of various dipeptides by the membrane-associated proteinase was performed by using descending paper chromatography. The dipeptides were dissolved in 0.05 M sodium phosphate buffer, *p*H 6.0, to give a final concentration of 10 mg/ml. The enzyme assays were performed by incubating 1.0 ml of peptide substrate and 0.05 mg of enzyme at 37 C. Heat-denatured enzyme incubated with dipeptide served as a control. At intervals, 100-μliter samples of hydrolysis mixture were removed from incubation and spotted on Whatman no. 1 paper strips. The resulting strips were chromatographed by descending chromatography with 1-butanol-acetic acid-water (5:1:4) as mobile phase. After chromatography, the frontal boundary was marked, and the strips were air-dried and then developed with ninhydrin. Hydrolysis of the peptide was measured by comparing the R_F values of the resulting spots with those of nonhydrolyzed peptide and its constituent amino acids chromatographed simultaneously.

Proteolytic digestion of $\alpha_{s,1}$-casein B. The substrate (4-5 mg) was dissolved in 3.0 ml of 0.015 M sodium phosphate buffer, *p*H 6.0. Five milliliters of

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enzyme solution (0.5–1.0 mg/ml) was added to the substrate solution and incubated at 37 C for 12 hr. After incubation, the reaction mixture was adjusted to *p*H 4.6 to stop the reaction, concentrated in vacuo to 1.0 ml, and frozen.

Peptide mapping. The α_{s_1}-casein B hydrolysates were mapped by electrophoresis-chromatography as described by Bennett (1). High-voltage electrophoresis was performed at 2500 v (30–50 ma) for 75 min in a tank-type Gilson high-voltage electrophorator in 0.6 M formic-2 M acetic acid buffer (*p*H 1.9) with Whatman no. 3 MM chromatography paper. The solvent for descending paper chromatography in the second dimension was propanol-water (7:3). Peptides were observed after spraying the dried papers with ninhydrin (0.25% in acetone) and heating 15 to 20 min in an oven at 90 C.

Preparative isolation and purification of peptides. Initial separation of the peptides was achieved by applying pooled hydrolysate (12–14 mg/sheet) to the entire width of prewashed chromatography paper. High-voltage electrophoresis was performed as described above. The separated peptides were located and eluted as described by Moss and Speck (10). Each isolated peptide-containing zone was reapplied to chromatography paper and separated by descending chromatography using 1-propanol-water (7:3). The peptides were located and eluted.

TLC. Cellulose MN-300G layers were prepared by the method of Pataki (11). The peptides after separation by paper chromatography were applied to two thin-layer chromatography (TLC) plates. The plates were developed by ascending chromatography; one plate being developed with 1-butanol-acetic acid-water (4:4:1), and the other with 1-propanol-water (7:3). After air-drying, the plates were sprayed with ninhydrin (0.25% in acetone) and heated at 110 C for 15 min.

Silica Gel G layers, for chromatography of dansyl derivatives, were prepared by the method of Pataki (11). The plates were heated for 1 hr at 100 C prior to use.

Amino acid analysis. Identification of the amino acids in acid hydrolysates of the peptides was made with an amino acid analyzer by the method of Spackman (14). The sample to be analyzed was placed in a hydrolysis vial, and the neck of the vial was drawn out in a gas flame to a small opening. Hydrochloric acid (5.7 N, constant boiling) was then added, and, after degassing, the vial was sealed under vacuum. The sample was hydrolyzed for 24 hr at 110 C in a toluene bath. After hydrolysis, the vial was opened and the contents were dried *in vacuo* over NaOH and phosphorus pentoxide. The hydrolysate was dissolved in 1 ml of *p*H 2.2 sodium citrate buffer. Tryptophan and cysteine content was not determined.

NH_2-terminus analysis. The dansyl-chloride technique described by Gray (6) was used to prepare the NH_2-terminal derivative in each of the purified peptides. The dansyl-derivatives were chromatographed simultaneously with known dansyl-amino acids on Silica Gel G layers. The development of the plates was accomplished by ascending chromatography with chloroform-benzyl alcohol-acetic acid (100:30:5). Relative migration values (R_F) were calculated and compared to those of the known dansyl-amino acids.

Leucine aminopeptidase (LAP; Mann Research Laboratories) hydrolysis of the peptides was used to confirm, when necessary, the NH_2-terminal residues identified by dansylation. The enzyme was activated by the method of Hill and Smith (8). The enzymatic hydrolysis of the peptides was started by mixing 2.0 ml of activated enzyme (50 μg/ml) and peptide (500 μg/ml) solution with incubation at 40 C. At intervals, samples were removed from incubation and pipetted into a test tube in boiling water to stop the reaction.

The samples were prepared for amino acid analysis on the amino acid analyzer by the method of Pataki (11). After analysis, the micromoles of each amino acid were plotted as a function of time. The NH_2-terminal residue was defined as that amino acid in highest quantity in the shortest length of time. In cases where only one time of hydrolysis was used, the NH_2-terminal residue was taken as the amino acid in the highest concentration at that time.

Phosphoric ester determination. The organic phosphate content of each peptide was determined by using the reagent described by Haines and Isherwood (7). Amounts of each peptide (20 and 40 μliters) were spotted on a filter paper strip and allowed to air-dry. The papers were sprayed with reagent and heated for 7 min at 85 C. Final development was attained by suspending the papers for 5 to 10 min in a container of dilute hydrogen sulfide gas (Aitch-Tu-Ess cartridges). Phosphorus-containing peptides were identified as intense blue spots.

Growth and plating media. The chemically defined medium of Reiter and Oram (13) was used as a growth medium in testing the efficacy of α_{s_1}-casein B, or peptides derived from it, to serve as a source of nitrogen for growth. Lactic agar served as the plating medium.

RESULTS

Enzymatic hydrolysis of dipeptides. The hydrolytic activity of the membrane-associated proteinase toward several dipeptides was surveyed. No hydrolysis was observed within 48 hr at 37 C for DL-alanyl-phenylalanine, DL alanyl-tyrosine, glycylglycine, glycyltryptophan, glycyl-DL-methionine, glycyl-L-asparagine, glycylalanine, L-leucyl-L-leucine, glycylleucine, glycyl-L-tyrosine, and glycylserine. DL-alanyl-glycine, DL-alanyl-leucine, and DL-alanyl-valine were hydrolyzed. Most of the nonreactive peptides were glycyl peptides. Also, a leucyl peptide was not hydrolyzed. The data suggested that alanine was the preferred carboxyl-producing residue. However, bulky residues on the carboxyl side of alanine are not conducive to hydrolysis, as evidenced by nonhydrolysis of alanyl-tyrosine and alanyl-phenylalanine.

Hydrolysis of $\alpha_{s,1}$-casein B. Hydrolysis of $\alpha_{s,1}$-casein B at 37 C was followed by measuring the increase in absorbancy of 12.0% trichloroacetic acid filtrates (0.5 ml of digest: 3 ml of 12% trichloroacetic acid) at 280 nm. After 2 hr of incubation with enzyme, the absorbancy increased to about 0.18 (Fig. 1). A maximal absorbancy increase of 0.29 was obtained after 12 hr of incubation. The absorbancy of the control, consisting of $\alpha_{s,1}$-casein B and boiled enzyme, did not increase significantly during the 24 hr of incubation.

To ascertain whether hydrolysis of the substrate was maximum at 12 hr, portions of a 12- and 24-hr hydrolysate were subjected to high-voltage electrophoresis. After electrophoresis, both hydrolysates were observed to contain the same seven ninhydrin-positive zones which all migrated toward the anode. In another control, high-voltage electrophoresis of the enzyme alone and $\alpha_{s,1}$-casein B alone after 12- and 24-hr incubation periods revealed only one ninhydrin-staining zone at the origin.

The membrane-associated proteinase hydrolyzed $\alpha_{s,1}$-casein B into 11 different peptides, based on peptide mapping and positive biuret reaction (Fig. 2). After electrophoresis only, the hydrolysate was separated into seven ninhydrin-positive zones I through VII, all migrating toward the anode. After paper chromatography, zone IV had an R_F of 0.43; zone V, 0.33; zone VI, 0.22; and zone VII, 0.19 (Table 1). These four zones were not separated into additional zones. Zones I and II, however, were each separated into two zones. Zone III was separated into zones III_1, III_2, III_3.

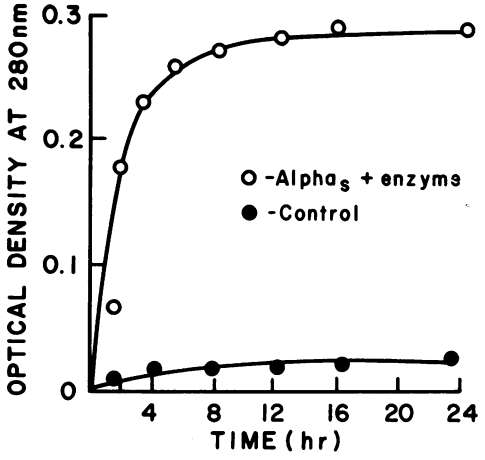

FIG. 1. *Hydrolysis of purified $\alpha_{s,1}$-casein B by the membrane-associated proteinase of Streptococcus lactis 3 (0.015 M sodium phosphate buffer, pH 6.0, 37 C).*

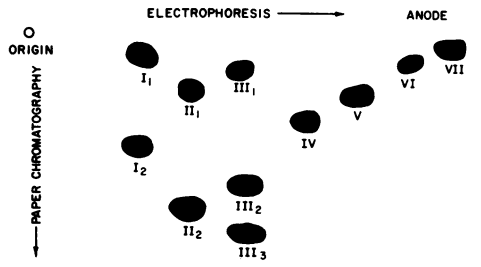

FIG. 2. *Representation of a two-dimensional chromatogram of enzymatic hydrolysate of purified $\alpha_{s,1}$-casein B after incubation for 12 hr, 37 C; electrophoresis performed at pH 1.9, 2,500 v, 75 min followed by chromatography in propanol-water (7:3).*

TABLE 1. *Migration distances[a] and R_F values[b] of zones separated by high-voltage electrophoresis and paper chromatography*

Zone	R_F	Migration distance (cm)
I_1	0.30	16.2
I_2	0.54	16.2
II_1	0.40	18.3
II_2	0.61	18.3
III_1	0.34	21.6
III_2	0.59	21.6
III_3	0.71	21.6
IV	0.43	24.4
V	0.33	29.3
VI	0.22	34.5
VII	0.19	37.5

[a] High-voltage electrophoresis, *p*H 1.9, 2,500 v, 75 min.

[b] Solvent, propanol-water (7:3).

Isolation and purification of peptides. Eight (I_2, II_2, III_1, III_3, IV, V, VI, VII) of the 11 tentatively identified peptides were obtained in larger quantities by preparative high-voltage electrophoresis and paper chromatography. The homogeneity of each isolated peptide was ascertained by TLC (MN-300G cellulose layers) with butanol-acetic acid-water and propanol-water as solvents. Peptide I_2, for example, gave one ninhydrin-positive zone in both solvents with an R_F of 0.35 in butanol-acetic acid-water and 0.72 in propanol-water (Table 2). Peptide VI had an R_F of 0.31 in butanol-acetic acid-water but 0.19 in propanol-water. Based on the occurrence of one ninhydrin-positive zone for each peptide in both solvents, each of the peptides was considered to be homogeneous.

Amino acid composition of peptides. The amino acid composition of each of the eight

TABLE 2. *Relative migration (R_F) values in two different solvents of isolated peptides of α_{s_1}-casein B hydrolysate after thin-layer chromatography on MN 300 G cellulose layers*

Peptide	R_F values in solvents:	
	Butanol-acetic acid-water (4:1:1)	Propanol-water (7:3)
I_2	0.35	0.72
II_2	0.33	0.86
III_1	0.32	0.24
III_3	0.33	0.78
IV	0.33	0.44
V	0.34	0.55
VI	0.31	0.19
VII	0.34	0.08

peptides is presented in Table 3. Each peptide contained some, but not all, of the essential amino acids required by *S. lactis* 3 for growth. The amino acids required are glutamic acid, valine, leucine, proline, lysine, and phenylalanine. Each of the peptides differed not only in the composition but also the ratio of acids in the peptides varied.

NH_2-terminus. The eight peptides were further characterized as to NH_2-terminus. The dansyl derivative of the NH_2-terminal residue of peptide I_2 was observed to have an R_F of 0.63 ± 0.01 (Table 4), close to 0.64 ± 0.02 for dansyl-valine. Valine also was tentatively identified as the NH_2-terminus of peptide II_2 by dansylation (Table 4) and confirmed by treatment with LAP.

The NH_2-terminal dansyl derivative of peptide III_1 was within experimental error for known dansyl-serine or dansyl-threonine (Table 4). Serine was substantiated as the NH_2-terminus after hydrolysis of the peptide with LAP. Leucine was confirmed as the NH_2-terminus of peptide III_3 by both dansylation and hydrolysis of the peptide by LAP. By both procedures, serine was identified as the NH_2-terminal residue of peptide IV.

The NH_2-terminal residue of both peptides V and VI after dansylation migrated as dansyl-glycine. When the two peptides were subjected to LAP hydrolysis, both glycine and serine were released (Fig. 3). For peptide V, glycine was liberated first by the enzyme, with serine being cleaved at a slightly lower rate. Glycine was confirmed as the NH_2-terminus, with serine most likely being the adjacent residue to glycine. Serine was released initially from peptide VI by LAP; however, after 2 hr of hydrolysis, glycine was present in a greater quantity. Since glycine was identified as the NH_2-terminus by dansylation, it probably was the NH_2-terminus. This peptide was observed to contain phosphate. Peptide VII was found to have NH_2-terminal leucine based on dansylation; this peptide also was observed to contain phosphate.

The ability of the eight peptides to support growth of the culture was tested in a chemically-defined medium. During the first 12 hr of incubation at 22 C, the culture made 3.3 generations. During the next 12 hr, one additional generation was produced. The viability of the culture in the control medium (no peptides) decreased during incubation. To test further the ability of the peptides to support growth, peptide III_3, highest in leucine, was selected to serve as the sole source of the essential amino acid leucine in the growth medium. The remaining required amino acids were added to the medium as free amino acids. In this medium, the cells underwent 5.7 generations in 12 hr of incubation at 22 C. Viability of the control culture was observed to decrease during incubation.

DISCUSSION

Hydrolysis of α_{s_1}-casein B by the membrane-associated proteinase was considered maximal after 12 hr of incubation as evidenced by no additional increase in absorbance of the trichloroacetic acid filtrates. This was supported by the appearance of seven ninhydrin-

TABLE 3. *Amino acid composition of peptides I_2, II_2, III_1, III_3, IV, V, VI, and VII from α_{s_1}-casein B*[a]

Amino acid	Peptides (nmoles applied to column)[b]							
	I_2	II_2	III_1	III_3	IV	V	VI	VII
Lysine	37	1	8	2	40	39	25	101
Histidine	[c]	[c]	[c]	1	6	[c]	12	10
Arginine	[c]	[c]	[c]	[c]	16	27	60	42
Aspartic acid	7	10	8	6	109	21	6	4
Threonine	7	[c]	5	4	25	5	[c]	8
Serine	13	1	27	12	18	16	18	16
Glutamic acid	32	3	22	12	[c]	58	21	20
Proline	10	[c]	[c]	5	25	[c]	[c]	11
Glycine	28	10	19	20	71	59	17	21
Alanine	14	2	11	12	[c]	20	7	9
Valine	7	10	[c]	[c]	[c]	8	[c]	[c]
Methionine	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[c]
Isoleucine	1	[c]	[c]	7	19	12	5	10
Leucine	12	4	[c]	67	18	20	20	15
Tyrosine	75	[c]	[c]	2	9	5	[c]	11
Phenylalanine	4	22	[c]	9	9	17	[c]	[c]

[a] Peptides were acid hydrolyzed 24 hr at 110 C; see text for details.

[b] Nanomoles of amino acid residues present in the amount of each peptide as applied to the column.

[c] Indicates a negligible quantity.

TABLE 4. *Relative migration values[a] (R_F) of known dansyl(DNS)-amino acids and dansyl-derivatives of NH_2-terminal residues of purified peptides on Silica Gel G thin-layer chromatography[b]*

Determinations	R_F
Known DNS amino acid	
Isoleucine	0.75 ± 0.02[c]
Leucine	0.73 ± 0.01
Valine	0.64 ± 0.02
Glycine	0.34 ± 0.02
Serine	0.16 ± 0.02
Aspartic Acid	0.08 ± 0.01
Phenylalanine	0.76 ± 0.01
Arginine	0.00 ±
di-Lysine	0.00 ±
Alanine	0.52 ± 0.02
Threonine	0.12 ± 0.02
Tyrosine	0.25 ± 0.02
DNS derivative of peptide	
I_2	0.63 ± 0.01[c]
II_2	0.63 ± 0.02
III_1	0.18 ± 0.02
III_3	0.71 ± 0.01
IV	0.15 ± 0.02
V	0.36 ± 0.01
VI	0.37 ± 0.02
VII	0.70 ± 0.02

[a] Average of triplicate independent trials.
[b] Solvent, chloroform-benzyl alcohol-acetic acid (100:30:5).
[c] Standard deviation of the mean.

positive zones from 12- and 24-hr hydrolysates subjected to high-voltage electrophoresis. $\alpha_{s,1}$-Casein B or the enzyme alone after 12 or 24 hr of incubation gave only one ninhydrin-positive zone; therefore, the isolated peptides did not result from autolysis of the enzyme or impurities bound to the casein.

Based on "peptide mapping," $\alpha_{s,1}$-casein B was hydrolyzed by the proteinase into 11 ninhydrin-positive fragments identified as peptides. Peptides IV, V, VI, and VII could be purified by electrophoresis. The results of electrophoresis and chromatography were consistent with the amino acid compositions given in Table 3. For example, peptide VII migrated the greatest distance on electrophoresis. Based on amino acid analysis, a calculated ratio of acidic to basic amino acids of 0.16 was obtained. Conversely, peptide IV, which exhibited the smallest migration rate of these four peptides, yielded a calculated ratio of 1.76. The separability of peptides I_2, II_2, III_1, III_3 from I_1, II_1, and III_2 was apparently related to the relative polarity of each peptide; for example, in agreement with amino acid analysis, the hydrophobicity of peptide III_3 was confirmed by a large R_F for this peptide in the relatively nonpolar solvent propanol-water.

Based on hydrolysis of small substrates (dipeptides), the enzyme hydrolyzed certain peptides containing leucine, valine, and glycine as potential NH_2-terminal residues. The action of this enzyme was not unlike that previously noted for the intracellular proteinase from *S. lactis* 3 in which dipeptides as well as proteins are hydrolyzed (5). Valine, leucine, and glycine were identified as NH_2-terminal residues of the peptides obtained from the hydrolysis of $\alpha_{s,1}$-casein B by the enzyme. Serine also was identified as a NH_2-terminal residue. Based on the primary sequence of $\alpha_{s,1}$-casein B as presented by Mercier et al. (9), there are 51 potential sites of hydrolysis which could lead to leucine, glycine, valine, and serine as NH_2-terminal residues. Since only 11 peptides were recovered, obviously not all potential sites were hydrolyzed. Studies are presently underway to identify the COOH-terminal residues of the peptides to further ascertain the specificity on the membrane-associated proteinase.

Cowman and Speck (2, 3) have suggested

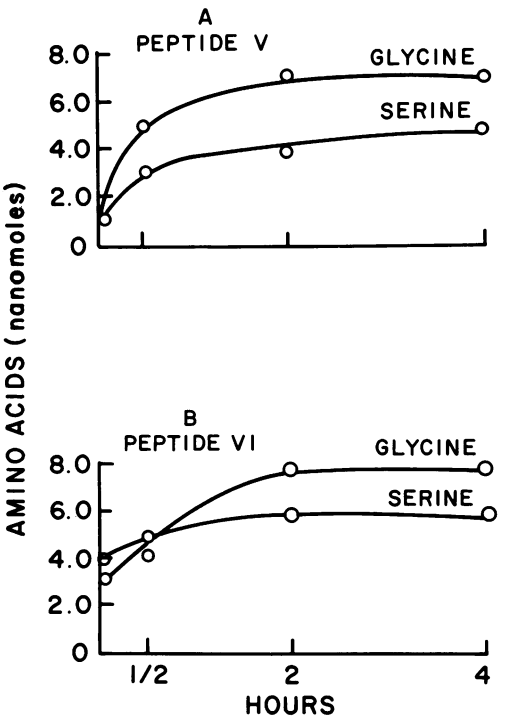

FIG. 3. *Leucine aminopeptidase treatment of peptides V and VI as a function of time in 0.005 M tris-(hydroxymethyl)aminomethane-hydrochloride buffer containing 0.0025 M $MgCl_2$, at 40 C.*

that the membrane-associated proteinase of *S. lactis* 3 may function to hydrolyze protein to peptides only. The intracellular proteinase (4), acting as a peptidase, hydrolyzes the incorporated peptides to amino acids. The data of the present study would support the functional role of the membrane proteinase as suggested by Cowman and Speck (2, 3) in the nitrogen economy of *S. lactis* 3. The utilization of the eight peptides, and especially peptide III_3, for growth further supports the importance of the enzyme in the utilization of nitrogen by the cell. The intracellular proteinase has been reported to hydrolyze the peptide bond at alanyl, aspartyl, and asparaginyl residues (5). Peptides derived from α_{s_1}-casein B will be used as substrates for the intracellular proteinase to further elucidate the importance of the proteinase enzyme system in the nitrogen economy of the lactic streptococci.

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