Fractionation of Dipeptidase Activities of *Streptococcus lactis* and Dipeptidase Specificity of Some Lactic Acid Bacteria

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Proteins in sonic extracts of *Streptococcus lactis* were separated by starch-gel electrophoresis at high voltage. Each slab was sliced longitudinally, and half was stained for peptidases in a mixture containing a peptide, L-amino acid oxidase (snake venom), peroxidase, and o-dianisidine; the other half was stained in amido black for protein. In addition to sonic treatment, trypsin also released enzyme from acetone-treated cells. Glycyl-L-phenylalanine, L-phenylalanyl-glycine, L-alamyl-L-phenylalanine, and L-phenylalanyl-L-alanine served as substrates in characterizing the enzymes. Five different fractions of various specificities appeared in the gels. Broad-range substrate specificities were found for sonic extracts of *S. lactis*, *S. cremoris*, *S. durans*, and *Lactobacillus acidophilus*.

Research in the past decade has clarified substantially the nature of proteinase activities in the cell envelope and in the cytoplasm of *Streptococcus lactis* (5–7). Characterization of the intracellular enzyme of this species by Cowman, Yoshimura, and Swaisgood (7) also revealed its activity towards several dipeptides and some of the bonds in oxidized insulin. The proteinase from a "slow acid" mutant of this strain expressed differences in specificity (20). Sasaki and Nakae (13) reported the presence of extracellular proteinase from *S. lactis*. Williamson, Tove, and Speck (23) investigated the extracellular principle but did not indicate the specific nature with regard to the nature of the peptide bonds hydrolyzed.

Prior to these findings, Van der Zant and Nelson (19) found peptidases in extracts from *S. lactis* to be active towards glycyl-L-leucine and DL-alanyl-glycine. These enzymes were not induced by a protein medium, they were rather heat-stable, and their rates of catalysis increased in the presence of some metallic ions, a behavior characteristic of some peptidases. Brandsaeter and Nelson (4) observed cleavage of dipeptides by *Lactobacillus casei* extracts. Again, cations influenced the rates, and in both of these investigations pH optima were in the range from 7.0 to 8.0.

Extensive use of lactic acid bacteria in starter cultures for cheeses and other fermented products makes a description of all of the details of protein and peptide turnover by these organisms of general interest. The above complete presentation of references on dipeptidase activities in lactic acid bacteria indicates a lag of efforts on this branch of the problem, but the main obstacles to such studies are now in great measure eliminated. We have broad information on proteinases of some of these species. Methods for the separation of protein mixtures on a microscale are well developed, and we also have a very sensitive assay for peptidases which allows the staining of these enzymes in gels. The last requirement was filled by Lewis and Harris (9) with their procedure using L-amino acid oxidase, peroxidase, and o-dianisidine.

This investigation was initiated to test the application of these experimental tools to a study of the peptidase activities of lactic acid bacteria and to analyze the results in view of the background information we now have. (A report of this work was presented at the 67th Annual Meeting of the American Dairy Science Association, Blacksburg, Va., 26–29 July 1972).

**MATERIALS AND METHODS**

**Cultures.** The strains used, *S. lactis* I, *S. lactis* II, *S. cremoris* I, *S. durans* I and *L. acidophilus* I, were
characterized in this laboratory after isolation from daily products (H. Djøve, and R. Bredholt, personal communication).

Media. Stock cultures were routinely kept at 3 to 4 C in ultrahigh-temperature pasteurized milk after propagation until slight coagulation. This medium was steamed for 30 min after distribution in sterile tubes. One percent inocula were used and three transfers preceded cell production on a larger scale. A medium to induce high proteolytic activity in S. lactis was developed for proteinase studies in this laboratory. A protein-derived base is required for this purpose (18, 22). Some amino acids and several di- and tripeptides were reported to repress synthesis of extracellular proteinase of a related microorganism (14). For these reasons, Proton (Difco), an enzyme digest of casein characterized by medium-length peptides, was chosen; furthermore, it can be treated to allow turbidimetric measurement of growth. A fairly low concentration of glucose was used because this compound can inhibit proteinase production (17). Sodium thioglycollate was added to give a reduced condition which is thought to enhance proteolytic activity (6, 10, 19). Small amounts of oxygen or carbon dioxide as well as glucose and galactose increase proteolysis by S. faecalis var. liquefaciens (15). Composition of the medium was 0.20% glucose, 0.30% yeast extract (Difco), 1.0% Proteose, 0.35% KH₂PO₄, 0.10% K₂HPO₄, 0.20% NaCl, 0.008% MgSO₄·7H₂O, 0.004% FeSO₄·7H₂O, 0.00012% MnCl₂, and 0.01% thioglycollate. Proton at 10% was steamed for 15 min and then filtered before sterilization in an autoclave. Thioglycollate was filter-sterilized as a 0.50% solution. Glucose (4.0%) was sterilized separately, as were yeast extract (3.0%), the mixture of KH₂PO₄, K₂HPO₄, and NaCl at 10 times medium concentration, and the mixture of the remaining salts at 200 times the concentration in the final medium. The buffered salts provided an initial pH of 6.2.

Cell production. Culture conditions were pH 6.3 and 30 C, and harvesting was in late logarithmic phase. Substantially higher cell yields of S. lactis have been obtained when the pH is maintained at 6.5 than without control of acidity (1–3). Harvey (8) reported a maximal growth rate at pH 6.3 for S. lactis. Agitation did not influence growth yield or growth rate to any extent (6). Glass components from Quickfit and Quarts, England, were used to assemble a fermentor of 10-liter capacity. The shaft sealing was filled (30 mm) with glycerol. The centrifugal stirrer (ST 1/3) was driven at 100 to 120 rpm by an electric motor. The complete unit, including distilled water to bring the medium to final volume, was sterilized in a vertical autoclave. The electrode (Radiometer, GK 20211 C, Copenhagen, Denmark) with a special long stem (300 mm) was treated separately in aqueous ethylene oxide, 1.0% (16), for 1 h at 4 C and then for 24 h at room temperature to drive off the gas. The same procedure was applied to the tubings to be used except that the liquid was circulated continuously by a peristaltic pump. Shortly before cultivation, the medium components were added. The vessel was placed in a vat filled with water and regulated at 30 C by a bridge thermostat (GP 15-12, Messergitte-Werk, Lauda, Lauda-Tauber, West Germany). A Beckman K Autotitrator (Beckman Instruments, Inc., Fullerton, Calif.) had been modified to suit the sterile condition. Sterile sodium hydroxide, 0.40 N, served in controlling the pH. A 1% inoculum from a culture in logarithmic phase in the same medium was used, and growth was checked by measurement of optical density at 650 nm.

Harvest. Late logarithmic phase cells were sedimented in a Sharples Super Centrifuge. (The Sharples Co., Philadelphia, Pa.) at 40,000 rpm. The cell paste from 5 liters was resuspended in 50 ml of water, centrifuged, and then was washed once with an ultrahigh-temperature pasteurized milk (–20 C) acetone. Final drying in a cold room was under a jet of nitrogen. The cell powder was then stored under nitrogen in a closed container at 3 to 4 C.

Cell disintegration. Cell envelopes were broken by ultrasonic treatment. The suspension of 0.40 g of acetone-dried powder in 4.0 ml of 0.5% NaCl in a glass tube was immersed in an ice bath for cooling. An MSE 7100, 100-W Ultrasonic Disintegrator (Measuring & Scientific Equipment, Ltd., London, England) with a titanium probe 0.75 inch (1.9 cm) in diameter was used at a setting of 5, giving an amplitude of 3 to 5 μm peak-to-peak. Periods of treatment for 2 min were followed by cooling for 2 min to avoid heating the suspension above 5 to 7 C. Total sonic treatment time regularly was 25 min, which resulted in 80 to 90% cell breakage. The final solution was centrifuged for 30 minutes at 25,000 × g. The supernatant fluid was then stored at 3 to 4 C. Protein in extracts was determined by the biuret method (12). Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) served as standard.

Trypsin treatment. Digestion of acetone-dried cells with trypsin was explored as one way to release protein or, more specifically, proteinases or peptidases. Enzymes being excreted at time of harvest would quite possibly be released from the cell envelope by such treatment, and the cells, previously influenced by acetone, might lose membrane and intracellular proteins. Great variation was observed between batches when supernatant fluids were analyzed for proteinase with casein as substrate. These were the best conditions found: 1.00 ml of cell suspension at 0.05 g/ml, 0.60 ml of trypsin solution (type III, twice crystallized from hog pancreas, Sigma Chemical Co.) at 0.0012 mg/ml, and 0.40 ml of 0.10 M sodium phosphate buffer, pH 7.6. The suspension was kept at 4 C for 12 hr with occasional stirring. Cells were sedimented by centrifugation, and the nearly clear supernatant fluid was kept at 3 to 4 C.

Electrophoresis. Starch-gel electrophoresis at high voltage was used as suggested by Wieme (21) and Pert and Pinteric (11). Hydrolyzed starch was prepared according to Zweig and Whitaker (24) starting from commercial potato starch. The yield varied from 270 to 290 g based on 300 g of initial material. Gels were prepared in 0.05 M sodium phosphate, pH 7.6, in a way slightly modified from the procedure of the same authors. Gel slabs were
prepared in troughs 15 mm wide and 250 mm long as follows. The trough was made from stacks of glass plates. The thickness of a single glass plate was one-half that desired in the final starch-gel. After the suspension was poured, the solidifying gel was covered with plastic and allowed to stand overnight. The two top layers of glass were then removed to expose a starch-gel layer about 3.5 mm thick. This layer was sliced off with a thin sharp knife. The next two layers of glass were removed, and the process was repeated until all of the gel layers had been cut.

The gel slabs were transferred immediately to the hydrophobic glass plate of a high-voltage electrophoresis apparatus (AB Analyseteknik, Vallentuna, Sweden). Efficient cooling was maintained by a streaming layer of cold water-ethanol mixture sprayed up below the glass plate from numerous orifices. A cryomate (Kryomate TK 30 D, Messgeräte-Werk Lauda, Lauda-Tauber, West Germany) provided cooling of liquid and the necessary pumping. Starch-gel strips were placed parallel to one another, the ends being covered by filter-paper bridges (J. H. Munktell 302) which were wrapped in cellophane membrane. One end of the filter-paper bridge extended into the electrode tray. Buffer in trays and bridges was 0.20 M sodium phosphate, pH 7.6. Samples were applied in filter-paper pieces (Whatman 3 MM) the size of a slab cross section. In some cases the aqueous sample volume exceeded the capacity of the filter piece. The sample solution was then added stepwise with a Pasteur pipette, the excessive water being evaporated each time in a jet of nitrogen. Finally, the pieces were inserted into the cross-cut starch-gel slabs. Samples were 3 mg of protein each in sonic extracts and an amount in supernatant fluids after trypsin treatment representing the same amount of cell material.

Potential was then increased gradually to 700 to 800 V to give 23 to 25 mA per strip of 200 mm effective length. Routinely, 3 h provided good separation. Shrinkage of gels occurred at the cathodic end, but all proteins migrated toward the anode. After electrophoresis, strips were marked at the end and along the sides for identification and comparisons of stained bands. Longitudinal slicing followed to give comparative slabs for protein and enzyme staining.

**Protein stain.** The method recommended by Zweig and Whitaker (24) with amido black was chosen. The dye was Amido Schwartz 10 B (Merck, für Electrophorese).

**Peptidase stain.** To detect peptidase activity in proteins separated by starch-gel electrophoresis, the procedure of Lewis and Harris (9) was followed. The method depends upon the reaction of L-amino acids freed by peptidase activity with L-amino acid oxidase from *Crotalus adamanteus* venom. L-Amino acid oxidase converts certain L-amino acids to the corresponding keto acid, ammonia, and peroxide. Peroxide reacts with o-dianisidine in the presence of peroxidase to produce a brown stain (oxidized dianisidine). According to the procedure of Lewis and Harris (9), the peptide substrate L-amino acid oxidase, peroxidase, and o-dianisidine hydrochloride are combined in agar and poured on the cut surface of the gel. The starch-gel with its agar overlay is then incubated at 37 C. The composition of the agar mixture was identical to that used by Lewis and Harris (9) except that the manganese chloride was omitted. The incubation time was 6 h for L-peptides or up to 20 h with DL-peptides. With L-peptides as substrates, the reaction usually was complete within 1 h. Peptides, *Crotalus adamanteus* venom, and o-dianisidine were purchased from Sigma Chemical Co., and peroxidase (POD III) was from Boehringer Corp. Ltd., Mannheim, West Germany.

**RESULTS**

Table 1 gives a survey of the dipeptidase activities in starch gels for sonic extracts of lactic acid bacteria against a series of dipeptides. Electrophoresis was conducted for 10 min in these cases to allow the proteins just to enter the gels. Each of the substrates was chosen to include at least one of the amino acids acted upon by the L-amino acid oxidase preparation (9). The use of pairs of peptides representing the two possible arrangements of the amino acids would in some cases prevent erroneous interpretation if only one of the peptides was degraded. The experiment provided some of the necessary results for the selection of substrates in the following studies, and it also represented a check on the effect of the fractionation procedure on activities. Complete separations followed by staining without dipeptide left the gels with a light-brown coloring throughout.

Three factors later influenced the selection of substrates: stains had to be clearly developed in the specificity experiment, one of the dipeptides or its reverse counterpart should preferably be among those used by Cowman et al. (7) with *S. lactis* proteinase, and they must be available as a reagent-grade L-amino acid derivative.

Figure 1 is a photograph of gels after electrophoresis for 3 h and dipeptidase staining for 2 h. Incisions along the sides of gels were made prior to slicing.

The pairs of starch gels in Fig. 2 to 5 were sliced from individual slabs. Determination of the position of a dipeptidase band among the protein bands was done within each pair. The matching incisions along the sides of gels in a pair were the best direct, positional markers to enable these decisions to be made because the gels shrink differently in the two staining procedures. Pairs are designated by roman numerals, protein fractions by arabic numerals, and the dipeptidase bands by capital letters.

DL-Alanyl-DL-phenylalanine was used in the
experiment for the comparison of four streptococci (Fig. 2). This substrate was hydrolyzed by the intracellular enzyme of Cowman et al. (7). All four extracts developed one band each after electrophoresis. The introduction of DL-amino acid peptides in this staining procedure where L-amino acid oxidase is included means that more substrate should be used than with peptides consisting of only L-amino acids or that a longer incubation time should be used. However, the presence of a DL-substrate did not interfere with the distinctness of staining.

For the following experiments (Fig. 3–5), L-alanyl-L-phenylalanine was chosen to be a substrate in common with those acted upon by the intracellular proteinase of Cowman et al. (7). Glycyl-L-phenylalanine was selected as a substrate which was not attacked by this enzyme. The two dipeptides of the reverse amino acid sequences were used to evaluate the importance of such changes of arrangements around the peptide bond. Clearly separated bands were developed from sonic extracts and from supernatant fluids after trypsin treatment, although protein fractions from the supernatant fluids were fewer and less distinctive because of the partial release of protein from cells after trypsin treatment.

A trypsin blank with three times the concentration of enzyme used in digesting cells provided the dipeptidase band in slab 8 (Fig. 5).

**DISCUSSION**

We note from Table 1 the broad-range specificities from all organisms; in only three cases was there no cleavage of either one dipeptide or its reverse counterpart. Tryptophan is a common part of these, and in particular the combination of tryptophan with glycine was difficult to degrade. Tryptophan is reported to react extensively with L-amino acid oxidase in *Crotalus adamanteus* venom, which should exclude a possible secondary effect of the amino acid in the staining process (9). *S. durans* extracts worked on all substrates and at comparatively high activity levels. There are

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**Table 1. Dipeptidase stains of supernatant fluids from sonic treatment of cells of lactic acid bacteria developed in starch gels according to the L-amino acid oxidase method of Lewis and Harris (9)**

| Bacterial strain          | Time (h) | Ala-Leu | Ala-Phe | Ala-Try | Ala-His | Leu-Ala | Phe-Ala | Try-Ala | Gly-Leu | Gly-Phe | Gly-Try | Gly-His | Leu-Gly | Phe-Gly | Try-Gly |
|---------------------------|----------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| *Streptococcus lactis I*  | 1        | +       | +       | 0       | 0       | 0       | +       | +       | 0       | +       | 0       | 0       | +       | +       | +       |
|                           | 6        | +++     | +++     | +       | +       | +       | +++     | +++     | +       | +++     | ++      | +       | +       | +       |
| *S. cremoris I*           | 1        | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       |
|                           | 6        | +++     | +++     | +       | +       | +       | +++     | +++     | +       | +++     | ++      | +       | +       | +       |
| *S. durans I*             | 1        | +       | +       | 0       | 0       | 0       | +       | 0       | +       | 0       | 0       | 0       | 0       | 0       |
|                           | 6        | +++     | +++     | +       | +       | +       | +++     | +++     | +       | +++     | ++      | +       | +       | +       |
| *Lactobacillus acidophilus*| 1        | +       | +       | 0       | 0       | 0       | +       | 0       | 0       | +       | 0       | +       | +       | +       |

* All peptides were composed of L-amino acids: Ala, alanine; Leu, leucine; Phe, phenylalanine; Try, tryptophan; His, histidine; Gly, glycine; +, very weak; ++, distinct band; ++++, strong color development.

**Fig. 1. Zymograms of supernatant fluid from sonic treatment of acetone-dried cells of *Streptococcus lactis I*. Details are as in Fig. 2, except that the substrates for dipeptidase activities were (I) L-alanyl-L-phenylalanine, (II) L-phenylalanyl-L-alanine, (III) glycyl-L-phenylalanine, and (IV) L-phenylalanyl-glycine. Photography was with Adox KB 17 in a Leica F III.**
more resistant to attack than the latter. This did not occur when the extracts of *S. durans* and *L. acidophilus* were used.

Figure 2 represents the protein and dipeptidase stains of four streptococci. All three group N strains have closely related protein patterns. For both *S. lactis* strains, the position of the enzyme band corresponds with the protein fraction indicated by number 4. This differs for *S. cremoris*, with which the protein band designated 3 and the catalyst stain come closest. The protein pattern of *S. durans* is markedly different, so that although one active fraction was expressed, comparison with the group N streptococcal separations may not be valid. There is consistency among the four strains of streptococci used in that the extract from each developed only one dipeptidase band with DL-alanyl-DL-phenylalanine.

Figure 3 shows the results for four substrates

**FIG. 2.** Proteinograms and zymograms of supernatant fluids from sonic treatment of acetone-dried cells of lactic acid bacteria. Electrophoretic separation in starch gels at 20 V/cm. Proteins were stained in slabs 1, 3, 5, and 7 with Amido Schwartz 10B. Dipeptidase bands were developed in gels 2, 4, 6, and 8 with L-amino acid oxidase according to Lewis and Harris (9) with DL-alanyl-DL-phenylalanine as substrate. The width of a band is represented by the line width in the graphs. Where cross-hatching is used, a less distinctive band appeared. The bacterial strains were (I) Streptococcus lactis I, (II) *S. lactis* II, (III) *S. cremoris* I, and (IV) *S. durans* I.

sufficient differences between this strain of *S. durans* and the two group N streptococci to consider some basic variations in the peptidase activities. The *S. lactis* strain and the *S. cremoris* strain coincide enough in their peptidases to reflect their close relationship. Only with L-tryptophanyl-glycine can we distinguish between *S. lactis* and *S. cremoris*. *L. acidophilus* extracts were generally less active and never gave visible responses towards the tryptophan-containing peptides. A certain directional specificity for the pair glycyl-L-leucine/L-leucyl-glycine can be seen for both *S. lactis* and *S. cremoris* in that the former peptide is much

**FIG. 3.** Proteinograms and zymograms of supernatant fluid from sonic treatment of acetone-dried cells of Streptococcus lactis I. Details were as indicated in Fig. 2, except that the substrates for dipeptidase activities varied: (I) L-alanyl-L-phenylalanine, (II) L-phenylalanyl-L-alanine, (III) glycyl-L-phenylalanine, and (IV) L-phenylalanyl-glycine.
L-Phenylalanyl-L-alanine was not used by Cowman et al. (7). The fraction developed in slab 4 which positionally deviates from the two previous ones may therefore be a peptidase. The use of L-phenylalanyl-glycine, a substrate not introduced by Cowman et al., resulted in two enzyme fractions appearing in the same gel, slab 8 (Fig. 3). One coincides with enzyme fraction C in slab 6, but the other deviates from all three of the above and is therefore named D, a possible peptidase. Modifications of a parent proteinase might give electrophoretically diverse variants, but it is questionable whether this would cause significant changes of substrate specificites. A conceivable approach will therefore be to consider the appearance of at least two peptidases in this part of the study.

Trypsin was shown to release proteinase from acetone-dried cells of S. lactis I. Figure 4

![Diagram](image)

**Fig. 4.** Proteinograms and zymograms of supernatant fluid from trypsin treatment of acetone-dried cells of Streptococcus lactis I. Details were as in Fig. 2, except that the substrates for peptidase activities varied: (I) L-alanyl-L-phenylalanine, (II) L-phenylalanyl-L-alanine, (III) glycyl-L-phenylalanine, and (IV) L-phenylalanyl-glycine.

applied after electrophoresis of sonic extracts from S. lactis I. Cowman et al. (7) found that the intracellular proteinase of S. lactis was active against L-alanyl-L-phenylalanine. It seems justified to think of the enzyme band in slab 2 as their proteinase. Slab 6 also gives one peptidase band. This substrate (glycyl-L-phenylalanine) was not attacked to any degree by the proteinase of Cowman et al. (7). However, Westhoff (20) reported that the enzyme from a "slow acid" mutant of the same strain cleaved this bond in oxidized insulin. This leaves the question whether we have both the intracellular proteinase of the Cowman group and the proteinase from the "slow acid" mutant in our S. lactis strain, in which case the two enzymes differ in electrophoretic mobility, or whether we have the former proteinase plus a specific peptidase in our strain for glycyl-L-phenylalanine.

![Diagram](image)

**Fig. 5.** Proteinograms and zymograms of supernatant fluid from sonic and trypsin treatments of acetone-dried cells of Streptococcus lactis I. Details were as in Fig. 2, except that L-alanyl-L-phenylalanine was used as substrate for peptidases. Gels were charged with: (I) supernatant fluid after trypsin treatment, (II) supernatant fluid after sonic treatment, (III) mixture of I and II, and (IV) trypsin blank.
represents the protein-dipeptidase combinations for supernatant fluids after trypsin treatment of whole cells. The figures for protein bands are chosen from a comparative and combined separation which will be discussed later. Slabs 1 and 2 closely corresponded to the results with L-alanyl-L-phenylalanine and sonic extracts in the preceding experiment. However, the next three pairs reveal activities toward the rest of the substrates at the same position. It will be interesting to learn about the substrate specificity of the membrane proteinase of Cowman et al. (6). The best interpretation at this time is that the enzyme responsible in this experiment is a different one from those in the sonic extracts, and presumably it can be either a cell wall-bound enzyme released by the trypsin or Cowman’s membrane proteinase. The active fraction in the supernatant fluid after trypsin treatment is named E.

Pairs I and II in Fig. 5 are good repeats of previously viewed separations, and slab 5 gives the evidence for correct comparative numbering of bands from sonic extracts and from supernatant fluids after trypsin treatment. Peptidase fractions A and E here coincide positionally in slab 6.

A blank for trypsin is shown in gel 8 at three times the concentration used in previous developments. This separation included only 0.12 μg of crystallized trypsin, demonstrating the sensitivity of this assay and its implications for proteinase studies. The band observed in gel 8 may be from trypsin or some minor impurity.

Table 2 is a summary of the fractionation studies. The strict specificity of the various fractions from sonic extracts is striking, although a limited number of substrates were used. The specialization of the different enzymes is also clearly expressed, since peptide pairs of the same amino acids require two catalysts for the breakdown. These results lead us to consider an extensive structural integrity in the cell or an elaborate regulatory system for protein breakdown, or both. In either case, it may be a reflection of highly controlled release of free amino acids inside the cell if we assume these enzymes to be cytoplasmic. There is the possibility that one enzyme cannot catalyze the cleavage of all peptide bonds. This need not necessarily be due to complexity alone but could reflect the fact that specialization may benefit the cell. Complex peptidase patterns were shown for human red cells (9), but no comparison will be made because specificities were differently distributed and variations of substrates also occurred between the investigations. Developed specialization may be empha-

| Enzyme fraction | Substrate* |
|-----------------|------------|
|                 | L-Ala | L-Phe | L-Ala | L-Phe | Gly | L-Phe | Gly |
| A               | +     |       | +     |       |     |       |     |
| B               |       |       | +     |       |     |       |     |
| C               |       |       |       |       | +   |       |     |
| D               |       |       |       | +     |     | +     |     |
| E               | +     |       | +     |       | +   |       | +   |

* Stain developed with the substrate is indicated by +. Ala, alanine; Phe, phenylalanine, Gly, glycine.

sized for peptidases in sonic extracts by comparison with the one fraction in supernatant fluids from trypsin treatment active on all four substrates. The latter enzyme possibly originates from the cell envelope and need not have the restrictions of a peptidase in some specific cytoplasmic location.

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