Physiological Roles of Pneumococcal Peptidases

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A methionyl-specific dipeptidase from *Streptococcus pneumoniae* has been described. This enzyme and the pneumococcal tripeptidase have been shown to be intracellular, soluble, and constitutive. In addition to their function in cleavage of peptide nutrients, these peptidases may play a role in protein synthesis and turnover.

Bacteria are often able to utilize peptides as a source of amino acids as well or better than utilizing the amino acids themselves. Hydrolysis often occurs via intracellular exopeptidases (12). It is not known to what extent such peptidases may play other roles in bacterial physiology, e.g., in protein turnover and synthesis. Some insight into the function of enzymes is obtained through a knowledge of the mechanisms that regulate them. Simmonds (11) has discussed the need for investigation of the problem of peptidase regulation with systems of several readily separable enzymes. This paper presents the results of such a study, using the dipeptidase and tripeptidase of *Streptococcus pneumoniae*, an organism with multiple amino acid requirements. In addition, the localization of the peptidases and the substrate specificity have been examined in an attempt to elucidate the function or functions of these enzymes.

MATERIALS AND METHODS

Preparation of cell-free extracts. Cultures of *Streptococcus pneumoniae* (type XXI) were grown, harvested, and used for preparation of cell extracts as described previously (5). In addition to Trypticase soy broth (TSB), cells were grown in a casein hydrolysate medium which contained 17 g of Casamino Acids (Difco), 2.5 g of K_2HPO_4, and 5 g of NaCl per liter. After adjusting the pH to 7.8 with 1 M NaOH, the medium was autoclaved at 121 C for 13 min and to it were added 2.5 ml of glutamine (10 mg/ml), 10 ml of 25% glucose, and 10 ml of 20% yeast extract (all sterilized by filtration). The semisynthetic medium contained 2.5 g of K_2HPO_4 and 5 g of NaCl in 450 ml of water. After autoclaving at 121 C for 15 min, 2.5 ml of glutamine (10 mg/ml), 10 ml of 25% glucose, 2 ml of 0.1% choline, and 2.5 ml of 20% yeast extract were added. As suggested by Tomasz (personal communication), the following L-amino acids were added (milligrams per liter): group I (sterilized by autoclaving together in solution)—glycine, 47.5; alanine, 87.5; valine, 180; isoleucine, 190; leucine, 250;

proline, 290; serine, 147.5; threonine, 112.5; methionine, 77.5; aspartic acid, 180; glutamic acid, 550; cysteine, 37.5; lysine, 217.5; and phenylalanine, 137.5; group II (sterilized by filtration after dissolving in water)—histidine, 80; tryptophane, 35; arginine, 100; tyrosine, 15; and asparagine, 50. Growth factors (sterilized by filtration) were added as follows (micrograms per liter): biotin, 0.6; nicotinic acid, 600; pyridoxine-hydrocloride, 700; calcium pantothenate, 2,400; thiamine-hydrochloride, 640; and riboflavin, 280. The total volume following these additions was 1,000 ml. The yeast extract used in these media was free of peptides hydrolyzable by pneumococcal peptidases, since it yielded no increase in ninhydrin-reactive material upon incubation with crude pneumococcal extract.

Separation of peptidases. Crude extract (30 ml) was fractionated with ammonium sulfate, and the material precipitating between 45 and 70% saturation was dissolved in 5 ml of 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (pH 7.5). After 2 h of dialysis against this buffer, the material was applied to a column (2.5 by 90 cm) of Sephadex G-200 in the same buffer. Fractions (3 ml) were eluted with 0.05 M Tris-hydrochloride buffer (pH 7.5) by upward flow and assayed for activity on leucyl-glycine (LG) and leucyl-glycyl-glycine (LGG). Protein content of the column eluate was estimated at 280 nm. Chemical measurement of protein concentrations was performed by the method of Lowry et al. (7). Specific activity was calculated as units per milligram of protein. A typical G-200 eluate had a specific activity of 194, reflecting a 30-fold purification.

Conditions for peptidase assay. The purification and properties of the tripeptidase have been described previously (5). Tripeptidase assay was performed in 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 0.01 M L-LGG at 37 C. Ninhydrin determinations were made on duplicate 25-μliter samples of duplicate reaction mixtures. Optimal conditions for assay of dipeptidase were studied at 37 C with the G-200 eluate, with a combination of those fractions having activity on LG but not on LGG. The effect of pH was examined in 0.05 M potassium phosphate buffer between pH 5.5 and 7.0 and with 0.05 M Tris-hydrochloride buffer between pH 7.0 and 9.0. The effects of

metal ions were determined in 0.05 M Tris-hydrochloride buffer at pH 7.8, with the metal ions added as chlorides and using enzyme preparations that had been dialyzed for 4 h against 4,000 volumes of deionized water. The dialysis procedure did not result in any loss of activity. The effect of ethylenediaminetetraacetic acid was determined under the same conditions. In all cases, the substrate (LG unless otherwise noted) concentration was 0.01 M, the final assay volume was 1.0 ml, and activity was measured by following increase in ninhydrin-reacting material on 25-μliter samples. A unit of dipeptidase activity hydrolyzes 1 μmol of LG per ml of reaction mixture in 60 min at 37 C. Since the tripeptidase had very little activity on LG (5), and the dipeptidase did not cleave LGG, both enzymes could be determined in crude extracts by use of the specific substrate. All peptides were obtained from Sigma Chemical Co. except methionyl-glycyl-glycine, which was obtained from Schwarz/Mann.

Localization of peptidase activity. Cells (5 g wet weight) were suspended in a total volume of 10 ml of either 0.05 M Tris-hydrochloride buffer (pH 7.5) or TSM buffer (0.01 M Tris containing 0.472 g of succinic acid and 0.01 M magnesium acetate buffer, pH 7.6). After disruption with a French press, the preparation was centrifuged twice at 3,000 × g for 5 min. The pellets were discarded, and the supernatant fluid (crude extract) was centrifuged at 20,000 × g for 30 min. This pellet was suspended in 10 ml of buffer, and the centrifugation was repeated. Resuspension of the resulting pellet in 10 ml of buffer yielded the fraction termed 20p30. Supernatant fluid (4 ml) from the first 20,000 × g spin was then centrifuged in a Spinco model L ultracentrifuge for 90 min with the no. 50 rotor at 144,000 × g. The supernatant fluid from this centrifugation was designated 144s90. The pellet was washed by suspension in buffer and centrifugation, and was then suspended in 4 ml of buffer (144p90). A portion of each fraction (0.1 ml) was assayed for activity on LG and LGG. The results shown are typical of three replicate experiments.

RESULTS

Separation of dipeptidase and tripeptidase activity. A previously reported study of pneumococcal tripeptidase (5) had revealed that the cell extracts also contained an enzyme active on the dipeptide L-leucyl-glycine. All of the dipeptidase activity, like that of the tripeptidase, was found to be precipitated by ammonium sulfate between 45 and 70% saturation. When this material was applied to a Sephadex G-200 column, the tripeptidase activity (i.e., activity on LGG) was separated from the dipeptidase activity (measured on LG), as shown in Fig. 1.

Properties of the dipeptidase. Measurements of dipeptidase activity in the pH range from 5.5 to 9.0 showed a broad optimum between pH 6.5 and 8.0. Activity was not stimulated by inclusion of $MgCl_2$ or $MnCl_2$ in the
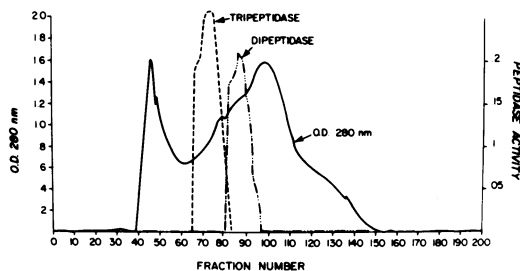

FIG. 1. *Separation of dipeptidase and tripeptidase on Sephadex G-200. Peptidase activity is expressed as increase in ninhydrin reacting material per 0.025-ml sample from 1 ml of reaction mixture containing 0.1 ml of column eluate and incubated for 15 min (for LGG) or 60 min (for LG).*

TABLE 1. *Activators and inhibitors of dipeptidase activity*

Addition	Activity[a]
5×10^{-3} M MgCl	104
5×10^{-3} M MnCl	106
5×10^{-3} M CaCl	82
5×10^{-3} M CoCl	23
5×10^{-4} M HgCl	18
5×10^{-6} M EDTA[b]	0

[a] Percentage of activity observed with no additions. Source of enzyme was G-200 eluate.

[b] EDTA, Ethylenediaminetetraacetic acid.

reaction mixture, and activity was inhibited by $CaCl_2$, $CoCl_2$, $HgCl_2$, and ethylenediaminetetraacetic acid (Table 1). These results were obtained with dipeptidase eluted from the Sephadex column; however, experiments performed with the crude extract as enzyme source gave similar results.

The results of experiments on substrate specificity of the dipeptidase are presented in Table 2. Methionyl dipeptides (especially those in which methionine appeared in the NH_2-terminal position) were hydrolyzed much more rapidly than were other dipeptides. The three leucyl dipeptides containing NH_2-terminal leucine were hydrolyzed at equal rates. However, the rate of hydrolysis of L-leucyl-glycine was considerably greater than that of glycyl-L-leucine. L-Alanyl-L-alanine and glycyl-glycine were also hydrolyzed at relatively low rates, and no activity was observed with tripeptides.

Effect of cultural conditions on peptidase levels. The results shown in Table 3 (typical, in each case, of at least two experiments) indicate that variation in the level of glucose initially present in the growth medium did not affect enzyme levels. Cells grown in a medium in

TABLE 2. *Substrate specificity*

Substrate[a]	Activity[b]
Methionyl-glycine	526
Methionyl-alanine	434
Methionyl-serine	336
Methionyl-methionine	327
Glycyl-methionine	145
Leucyl-leucine	101
Leucyl-alanine	98
Phenylalanyl-glycine	43
Glycyl-glycine	14
Glycyl-leucine	14
Alanyl-alanine	13
Methionyl-glycyl-glycine	0
Leucyl-glycyl-glycine	0

[a] All amino acids were L isomers.
[b] Relative to activity on leucyl-glycine set at 100. Enzyme source was G-200 eluate (0.1 ml hydrolyzed 1.2 to 2.0 μmol of LG per h per ml of reaction mix). Time of incubation was adjusted to assure measurement of initial rates.

TABLE 3. *Effect of variation in cultural conditions on peptidase levels in crude extracts*

Variable	Sp act (U/mg of protein)	
	Dipeptidase	Tripeptidase
Glucose level[a]		
High	4.3	3.9
Low	4.2	3.8
pH		
Initial 7.9; final 5.8	4.9	6.8
Maintained at 7.3 to 7.5	5.0	6.2
Medium composition[b]		
Peptide medium-TSB	4.9	6.7
Amino acid medium	6.2	7.4
Peptide medium-TSB	5.5	5.4
Amino acid medium (semi-synthetic)	10.0	8.9

[a] High, 10 ml of 25% glucose added with inoculum; low, 2 ml of 25% glucose added at five intervals.
[b] See Materials and Methods.

which the pH was maintained at 7.3 to 7.5 had the same level of enzyme activities as those grown in a medium of continually decreasing pH.

The effects of variation in the nitrogen source of the growth medium on peptidase content of the cells was investigated by growing cells in media containing amino acids and comparing the enzyme content with that observed in the peptide-rich TSB medium. Two such media were employed, one containing Casamino Acids

and the other a semisynthetic medium to which the individual amino acids were added. Peptidase levels were not reduced by growth in amino acid-containing medium; in fact, enzyme levels were significantly higher in cells grown in the semisynthetic medium (not due, necessarily, to the amino acid content, as there were many other differences between the semisynthetic and TSB media) (Table 3). In each case the "reference" cells (those grown in TSB medium) were grown and harvested at the same time and stored for the same interval as the cells grown in other media, since variation in the specific activity of different cultures has been observed at times, probably due to the length of storage time.

Enzyme localization. The data obtained in experiments on peptidase localization are presented in Table 4. No activity on LG or LGG was detected in the concentrated cell-free culture fluid. All the cellular peptidase was associated with the 144s90 fraction, with none being sedimentable at the forces employed. The data shown were obtained with cells fractionated in Tris-hydrochloride buffer, but the same pattern was observed when TSM buffer was used.

DISCUSSION

There are some similarities in the properties of the pneumococcal dipeptidase described herein and the tripeptidase studied previously (5), namely in pH optimum, sensitivity to Co^{2+} and Hg^{2+}, and lack of stimulation by Mg^{2+}. Differences were noted with respect to response to Mn^{2+} (which inhibited the tripeptidase but not the dipeptidase) and Ca^{2+} (which inhibited the dipeptidase but not the tripeptidase). The sensitivity to ethylenediaminetetraacetic acid (also noted with the tripeptidase) remains unexplained in the absence of stimulation by metal ions.

Similar substrate specificities were also ob-

TABLE 4. *Localization of peptidases*

Fraction[a]	Activity (U/ml)	
	Dipeptidase	Tripeptidase
Culture fluid	0	0
Crude extract	42.0	36.1
20s30	41.0	36.3
20p30	0	0
144s90	38.6	33.8
144p90	0	0

[a] Each fraction was made up to the same volume as the crude extract. For assay of culture fluid, 0.1 ml of ammonium sulfate-precipitated material (suspended in 0.005 of the original volume) was used.

served, since both enzymes were extremely active on methionyl peptides. (Of course, a major difference exists in that the tripeptidase acted much more readily on tripeptides and the dipeptidase acted only on dipeptides.) With the dipeptidase, the results established that activity on methionyl peptides with NH_2-terminal methionine was greater than that observed when the methionine occupied the COOH-terminal position. The hydrolysis of leucyl (NH_2-terminal) peptides was less rapid, and the COOH-terminal position in this case was not critical, as all the NH_2-terminal leucyl peptides were hydrolyzed at the same rate. Dipeptides with smaller NH_2-terminal residues (alanine and glycine) were hydrolyzed only slowly.

Since no extracellular peptidase was detected, it is clear that the intracellular enzymes serve a nutritional function, cleaving peptides of the medium after their transport into the cell. The fact that peptidase levels were not reduced upon cultivation of cells in peptide-free medium shows that these are constitutive enzymes and suggests that these enzymes may also serve other functions. In *Aeromonas proteolytica* (6), the level of aminopeptidase (which, because of its exocellular location, can be assumed to have only a nutritional function) was increased upon addition of peptides to the growth medium.

The specificity of the peptidases for methionyl linkages suggests a role for these enzymes in protein synthesis. Although protein synthesis in bacteria is initiated with *N*-formyl methionine (3), most proteins synthesized in vitro are not found to be formylated and, furthermore, many do not bear an NH_2-terminal methionine residue (1). Thus an exopeptidase capable of splitting off the terminal methionine (after deformylation) may function in protein maturation. The pneumococcal peptidases, like the enzyme for *E. coli* studied by Brown (2), have specificity for methionyl linkages. However, all of these enzymes act in vitro only on small peptides. A methionyl-specific peptidase studied by Matheson and Dick (8) and Matheson et al. (9) has been reported to hydrolyze tetrapeptides and to be associated with ribosomes. Vogt (13) has suggested the apparent ribosomal localization resulted from the low ionic strength buffer used by Matheson and Dick. Moreover, it is not clear whether cleavage of methionine takes place on the ribosome (as suggested by the work of

Housman et al., 4) or after release of the nascent protein (10). In any case, the pneumococcal peptidases described herein were clearly not ribosome associated, even when the TSM buffer of Matheson and Dick (8) was employed.

It is also possible that these peptidases are involved in protein turnover which occurs in bacteria at variable levels reported between 2 and 7% (12). The specificity for methionyl peptides may reflect a need for rapid turnover of methionine peptides produced by intracellular proteolysis.

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