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Peptidase Mutants of *Salmonella typhimurium*

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Six peptidase activities have been distinguished electrophoretically in cell extracts of *Salmonella typhimurium* with the aid of a histochemical stain. The activities can also be partially separated by chromatography on diethylaminoethyl-cellulose. These peptidases show overlapping substrate specificities. Mutants (*pepN*) of the parent strain *leu-485* lacking one of these enzymes (*peptidase N*) were obtained by screening for colonies that do not hydrolyze the chromogenic substrate L-alanyl-β-naphthylamide. The absence of this broad-specificity peptidase in *leu-485 pepN*⁻ mutants allowed the selection of mutants unable to use L-leucyl-L-alaninamide as a leucine source. These mutants (*leu-485 pepN*⁻ *pepA*⁻) lack a broad-specificity peptidase (peptidase A) similar to aminopeptidase I previously described in *Escherichia coli*. Mutants (*pepD*) lacking a dipeptidase (peptidase D) have been isolated from a *leu-485 pepN*⁻ *pepA*⁻ parent by penicillin selection for mutants unable to use L-leucyl-L-glycine as a leucine source. Mutants (*pepB*) lacking a fourth peptidase (peptidase B) have been isolated from a *leu-485 pepN*⁻ *pepA*⁻ *pepD*⁻ strain by penicillin selection for failure to utilize L-leucyl-L-leucine as a source of leucine. Single recombinants were obtained by transduction for each of the peptidases missing in a *leu-485 pepN*⁻ *pepA*⁻ *pepD*⁻ *pepB*⁻ strain. The growth response of these recombinants to leucine peptides shows that all of these peptidases can function in the catabolism of peptides and that they display overlapping substrate specificities in vivo.

Salmonella typhimurium and the closely related organism *Escherichia coli* are known to contain peptidases (17). Such enzymes must function in the utilization of peptides as sources of amino acids (18) and as sources of carbon and nitrogen (6). Peptidases must also be involved in the processes by which intracellular protein is degraded to amino acids (14). Although a dipeptidase (3) and one broad-specificity aminopeptidase (20) from *E. coli* have been purified and studied in some detail, our knowledge of the number of peptidases present in these organisms and of the specificities and physiological role of individual peptidases is far from complete. Since several peptidases with overlapping specificities are present, the analysis of the physiological role of individual peptidases and even the purification of these enzymes should be facilitated by the availability of mutant strains which lack one or more peptidases. Only two such strains have been reported, one lacking the ability to hydrolyze glycylglycine (7) and the other defective in the hydrolysis of lysine peptides (16).

The selection of peptidase mutants is made more difficult by the presence of more than one enzyme capable of hydrolyzing most peptide substrates. Before attempting to obtain mutants unable to utilize peptides, it is necessary to have some rationale for choosing peptides that are hydrolyzed relatively specifically by only one enzyme—i.e., it is necessary to know how many peptidases are present and what their specificities are.

This paper reports (i) the results of a study of the substrate specificities of several *Salmonella* enzymes capable of hydrolyzing small peptides and amino acid amides, and (ii) the isolation of mutants that lack several of these peptidases.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are derived from *S. typhimurium* LT2 and are listed in Table 1.

Media and growth conditions. E medium (19) supplemented with 1.0% (solid medium) or 0.2% (liquid medium) glucose and, when required, with 0.3 mM L-amino acids was used as a minimal medium. Nutrient broth (Difco) containing 0.5% NaCl was used as a rich medium. Solid medium contained 1.5% agar (Difco). Liquid cultures were aerated by shaking. All incubations were at 37 C, unless otherwise specified. Growth was followed when necessary by reading the optical density of the cell suspension at 600 nm (1.0-cm cuvette) on a Gilford model 2000 spectrophotometer. The ability of various strains to utilize peptides as amino acid sources was tested by placing a

TABLE 1. *Bacterial strains*

Strain designation	Genotype
TN2	*leu-485 pepN10*
TN102	*leu-485 pepN10 pepA1*
TN163	*leu-485 pepN10*
TN164	*leu-485 pepA1*
TN213	*leu-485 pepN10 pepA1 pepD1*
TN215	*leu-485 pepN10 pepA1 pepD1 pepB1*
TN271	*leu-485 pepA1 pepD1 pepB1*
TN272	*leu-485 pepN10 pepD1 pepB1*
TN273	*leu-485 pepN10 pepA1 pepD1*
TN274	*leu-485 pepN10 pepA1 pepB1*

few crystals of the peptide on the surface of a minimal glucose plate overlayed with a soft-agar layer containing 0.1 ml of an overnight culture of the strain to be tested. Peptides were obtained from commercial sources and were used without further purification.

Mutagenesis and transduction. Mutagenesis with diethyl sulfate (Eastman Organic Chemicals) was carried out as described by Roth (15). Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) was performed by adding 9 ml of an overnight culture to 1 ml of a *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine solution (500 μg/ml in 1 M citrate, pH 5.5) and incubating for 30 min at 37 C.

Transduction was performed using P22-*int4* phage as described by Roth (15).

Isolation of L-alanyl-β-naphthylamide nonhydrolyzing mutants. Mutants unable to hydrolyze L-alanyl-β-naphthylamide (ANA) were obtained by a plate staining procedure similar to that used by Messer and Vielmetter (12) for the isolation of other types of mutants. Approximately 10^4 cells from an overnight nutrient broth culture which had been mutagenized with diethyl sulfate were plated in 2.5 ml of nutrient soft agar (0.7% agar) on a nutrient agar plate. The plates were incubated until colonies were barely visible to the naked eye. The plates were then stained for enzymatic activity by pouring a staining mixture containing 5 ml of 0.2 M tris-(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (pH 7.5), 0.2 ml of ANA (Sigma Chemical Co.) solution (10 mg/ml in dimethylformamide), and 10 mg of Fast Garnet GBC (Sigma Chemical Co.). Colonies showed a dark red color, usually in 1 to 2 min. Incubation with substrate alone produced no color. If diazonium salt alone was present, prolonged incubation (20 to 30 min) caused only a slightly yellow colony color. Nonstainers were easily distinguishable and were picked for restreaking. Single colonies of the mutants from the restreak plates were transferred to a master plate with sterile toothpicks, incubated overnight, and, after pouring a soft agar top layer over the plate, retested for staining ability.

Isolation of pepA, pepD, and pepB mutants. Mutants deficient in peptidase A were obtained from strain TN2 by penicillin selection (15) in medium containing 0.1 mM Leu-Ala-NH_2 as leucine source. *pepD* mutants were obtained from strain TN102 by penicillin selection in the presence of 1×10^{-4} M Leu-Gly as leucine source. Derivatives of strain TN213 with mutations in *pepB* were isolated by penicillin selection in the presence of 1×10^{-4} M Leu-Leu as leucine source.

Preparation of extracts. Crude extracts were prepared from late-log or stationary-phase nutrient broth cultures. Cells were harvested by centrifugation, washed once in 0.05 M Tris-hydrochloride buffer (pH 7.5), and resuspended in 0.05 M Tris-hydrochloride (pH 7.5, 0.1 M KCl). The cells were broken in a French pressure cell, and the resulting suspension was centrifuged for 50 min at 30,000 × *g* in a Sorvall RC2B centrifuge. The supernatant solution was dialyzed overnight against 0.05 M Tris-hydrochloride buffer (pH 7.5). Better resolution was obtained in polyacrylamide electrophoresis experiments if the dialyzed extracts were subjected to high-speed centrifugation (100,000 × *g*, 1 h, Beckman model L centrifuge) before electrophoresis.

Gel electrophoresis. Polyacrylamide gels (1.5 by 7.0 mm or 1.5 by 10.0 mm, 7.5% acrylamide and 0.2% bisacrylamide) were prepared as described by Davis (5). No sample or stacking gel was used, and the 50-μliter sample (centrifuged, crude cell-free extract containing approximately 4 to 8 mg of protein per ml) was applied directly to the top of the gel. Electrophoresis was allowed to proceed at 1 mA per tube until the sample had entered the gel and then at 3 mA per tube for approximately 90 min. The electrophoresis was carried out at 4 C.

Gel stain for peptidase activity. Peptidase activity on the gels after electrophoresis was detected by the method of Lewis and Harris (9). The reaction mixture was prepared by dissolving 5 mg of peptide in 2 ml of 0.2 M phosphate buffer (pH 7.5), containing 0.5 mg of L-amino acid oxidase (Sigma Chemical Co., type I), 0.8 mg of horseradish peroxidase (Sigma Chemical Co.), 0.08 ml of an aqueous solution of *o*-dianisidine dihydrochloride (5 mg/ml; Sigma Chemical Co., crystalline), and 0.04 ml of 0.1 M $MnCl_2$. This solution was then mixed with an equal volume of 2% agar (at 50 C), and the resulting solution was poured into a test tube containing the gel. Peptidase activities could be detected as brown bands after 10 to 60 min of incubation at room temperature. (Note that only peptides containing amino acids that are substrates of L-amino acid oxidase can be used.) Hydrolytic activity toward ANA and other amino acid-β-naphthylamides could be detected by incubating the gel in a mixture containing 0.1 ml of a dimethylformamide solution of the naphthylamide (10 mg/ml) and 10 mg of Fast Garnet GBC in 5 ml of 0.2 M Tris-hydrochloride buffer (pH 7.5). Hydrolysis of L-leucine-*p*-nitroanilide was detected as a yellow band of *p*-nitroaniline when the gel was incubated in 5 ml of Tris-hydrochloride buffer (pH 7.5) containing 0.1 ml of substrate solution (20 mg/ml in dimethylformamide). R_f values were calculated as the ratio of the distance travelled by the band of activity to that of the tracking dye.

Assay of ANA hydrolysis. The ability of crude extracts to hydrolyze ANA was determined by a procedure similar to that of Lee et al. (8) for following the hydrolysis of L-leucyl-β-naphthylamide. ANA was

dissolved in 0.5 ml of 95% ethanol, and the solution was diluted to 25 ml with 0.05 M Tris-hydrochloride buffer (pH 7.5). The reaction was started by adding 10 to 50 μliters of enzyme to 3.0 ml of substrate solution contained in a spectrophotometer cuvette and was followed at 340 nm in a Gilford model 2000 recording spectrophotometer with a cuvette compartment maintained at 25 C. The rate was proportional to the enzyme concentration over the range of extract concentrations used (0.05 to 1.5 mg of protein/ml) and was constant to at least 20% hydrolysis. Specific activities were calculated from the observed absorbance changes using $\Delta\epsilon$ = 1,780 (8). Protein concentrations were determined by the method of Lowry et al. (10) with crystalline bovine serum albumin as standard.

RESULTS

Detection of peptidase activities after electrophoresis of cell-free extracts. After electrophoresis of a crude extract of *S. typhimurium* LT2 in a polyacrylamide gel and staining of the gel for hydrolytic activity toward Leu-Gly, four bands of activity were observed (Fig. 1a). (Peptide abbreviations are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [J. Biol. Chem. **247**:977-983, 1972].) The gels were also stained for activity with chromogenic peptidase substrates such as amino acid β-naphthylamides and *p*-nitroanilides. By using such substrates as well as peptides other than Leu-Gly in the gel-staining mixture, a qualitative specificity profile for the enzymes was determined. Data with a series of diagnostic substrates are presented in Table 2. Figure 2 is a schematic drawing of a gel showing the positions of all of the different bands of activity. The following conclusions can be drawn from these and other similar data. (i) The enzyme that appeared at the top of the gel (band 1) shows activity toward dipeptides (Leu-Gly), tripeptides (Leu-Gly-Gly), amino acid

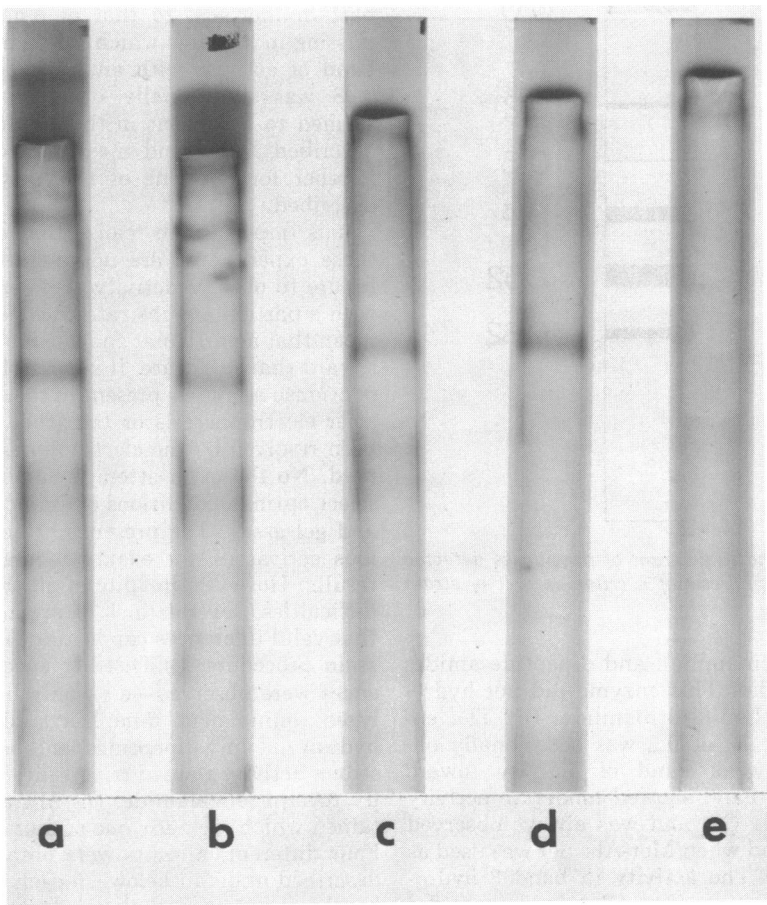

FIG. 1. *Hydrolysis of Leu-Gly after gel electrophoresis of crude extracts. (a) Strain Leu-485, (b) TN2 (leu-485 pepN10), (c) TN102 (leu-485 pepN10 pepA1), (d) TN213 (leu-485 pepN10 pepA1 pepD1), and (e) TN215 (leu-485 pepN10 pepA1 pepD1 pepB1).*

TABLE 2. *Specificity patterns of peptidase activities observed after electrophoresis*

Substrate	Peptidase activity					
	A	–	B	–	D	N
	1[a]	2	3	4	5	6
Leu-Gly	+	±	+	–	+	+
Leu-Gly-Gly	+	+	+	+	–	+
Leu-Leu-Leu	+	±	+	+	–	+
Met-Ala-Ser	+	+	+	+	–	+
Leu-Ala-NH_2	+	–	±	–	–	+
Leu-NH_2	+	–	±	–	–	+
Ala-β-naphthylamide	–	–	–	–	–	+
Leu-*p*-nitroanilide	–	–	–	–	–	+
Cbz-Gly-Phe	–	–	–	–	–	–
Hippuryl-Phe	–	–	–	–	–	–

[a] Gel band.

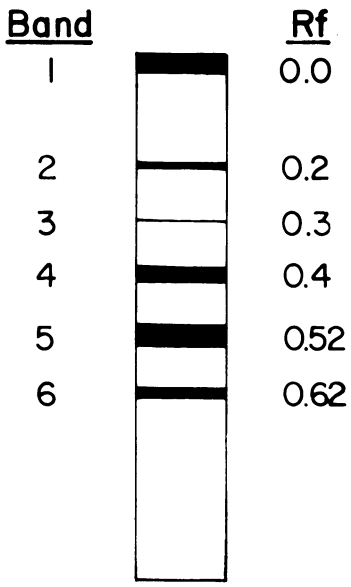

FIG. 2. *Schematic diagram of peptidases detected after gel electrophoresis of a crude extract of strain leu-485.*

amides (L-leucinamide), and dipeptide amides (Leu-Ala amide). This enzyme did not hydrolyze ANA or Leu-*p*-nitroanilide. (ii) The enzyme with an R_f of 0.2 was occasionally observed as a weak band of activity toward Leu-Gly. This band showed moderate activity toward Leu-Gly-Gly and was always observed as a strong band when Met-Ala-Ser was used as substrate. (iii) The activity in band 3 hydrolyzed both dipeptides (Leu-Gly) and tripeptides (Leu-Gly-Gly) and showed weak activity toward L-leucinamide. (iv) The enzyme in band 4 hydrolyzed only tripeptides (e.g., Leu-Gly-Gly) of the compounds tested. (v) The enzyme in band 5 appeared to hydrolyze only dipeptides. A variety of other dipeptides were hydrolyzed by this enzyme; Gly-Met, Gly-Leu, Ala-Phe, and Met-Gly are hydrolyzed, whereas Leu-Pro and Val-Pro are not. No activity was observed with such tripeptides as Leu-Gly-Gly, Leu-Leu-Leu, or with leucinamide. (vi) The enzyme responsible for the activity observed in band 6 was the only one detectable that could hydrolyze amino acid β-napthylamides or L-leucine *p*-nitroanilide. This enzyme also can hydrolyze dipeptides (Leu-Gly), dipeptide amides (Leu-Ala-NH_2), tripeptides (Leu-Gly-Gly), and amino acid amides (leucinamide). Apparently the enzymes detected on the gels required free *N*-terminal groups since neither Cbz-Gly-Phe nor Hippuryl-Phe are hydrolyzed.

A band of activity was frequently observed between bands 1 and 2 but had a specificity pattern identical to that of band 1 and was missing in mutants which lack band 1. Another band of activity with an R_f of approximately 0.48 was occasionally observed. This band seemed to be absent in the quadruple mutant described below and may therefore represent another form of one of the enzymes already described.

It is important to realize that the results of these experiments are not quantitative, since failure to observe activity at a certain position with a particular substrate does not necessarily mean that a particular enzyme has zero activity toward that substrate. It is possible that not all peptidase activities present in the cell are active after electrophoresis or that they have not all been resolved by the electrophoresis conditions used. No thorough attempt has been made to select optimal conditions of extract preparation and gel assay. The presence or absence of various activators, for example, might affect the results. However, in spite of all these potential difficulties, several lines of argument indicate that valid inferences can be drawn from the gel-stain procedure. (i) Distinct specificity differences were observed—e.g., only band 6 hydrolyzed amino acid β-naphthylamides, band 5 hydrolyzed only dipeptides, and band 4 showed strong activity toward tripeptides but no activity toward leucinamide. (ii) Mutants were obtained which lack any one of four of the bands. Four different mutants were obtained and are described in detail below. In each case only one band was absent and all substrates which show activity with that band in wild-type extracts showed none in the position corresponding to

the band after electrophoresis of the mutant extract. The ability of each mutant strain to utilize peptides for growth was consistent with the specificity patterns seen on the gels. It appears, therefore, that the pattern of activity bands on the gel does give some idea of the number of enzymes present in the cell capable of hydrolyzing small peptide substrates and that the specificities of these enzymes can be partially determined using the gel electrophoresis-activity stain procedure.

The peptidases seen after gel electrophoresis can also be partially separated by chromatography on diethylaminoethyl-cellulose. Material from fractions showing activity can be subjected to electrophoresis to determine which enzymes are present. The results of such an experiment are depicted schematically in Fig. 3. Under these conditions several distinct fractions containing activity toward Leu-Gly were identified. Each of these fractions contained one or two of the activities identifiable after electrophoresis of the crude extract. These observations further support the contention that the gel bands do represent distinct enzymatic activities.

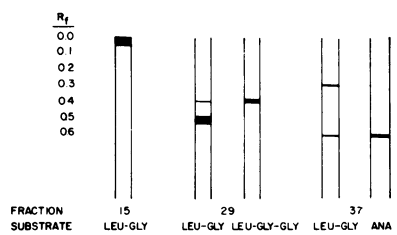

FIG. 3. *Electrophoresis of fractions from diethylaminoethyl-cellulose chromatography. A 2.0-ml portion of a crude extract (21 mg of protein per ml) was applied to a column (0.9 by 30 cm) of diethylaminoethyl-cellulose (Whatman DE-52) equilibrated with 0.01 M Tris-hydrochloride (pH 7.6). The column was eluted with a linear KCl gradient (0 to 0.4 M) in this buffer, and 3.0-ml fractions were collected. Active fractions were identified by incubating a 0.1 ml sample of each fraction with 0.1 ml of a mixture containing 0.8 ml of 0.05 M Tris-hydrochloride (pH 7.60), 0.1 ml of 0.1 M Leu-Gly, and 0.1 ml of* 2×10^{-3} *M* $MnCl_2$*, all contained in the wells of a plastic depression plate. After 15 min at room temperature, 0.01 ml of a mixture containing 12 mg of horseradish peroxidase, 6 mg of* L*-amino acid oxidase, and 0.2 ml of a 5 mg/ml solution of o-dianisidine dihydrochloride, all dissolved in 5.0 ml of 0.05 M Tris-hydrochloride, (pH 7.6) was added to each well. Activity was indicated by the formation of brown oxidized dianisidine. Material from the most active fractions (100 μliters) was subjected to electrophoresis, and the resulting gels were stained for peptidase activity.*

Isolation of mutants which lack peptidase N. Only one enzyme capable of hydrolyzing amino acid β-napthylamides was detected after electrophoresis. If this enzyme is not vital it should be possible to obtain mutants specifically unable to hydrolyze these compounds. Derivatives of β-naphthol and β-naphthylamine have been widely used as histochemical reagents for determing the localization of enzyme activities in various mammalian tissues (4). β-Naphthol derivatives have been used to screen for *E. coli* mutants deficient in β-galactosidase or alkaline phosphatase (12). Using a plate staining procedure we have shown that in the presence of ANA as substrate and Fast Garnet GBC as diazonium salt, colonies of *S. typhimurium* and *E. coli* quickly (1 to 2 min) acquired a dark red color. When plates containing colonies of a mutagenized culture were tested, nonstaining mutants were easily detected.

A number of independent nonstaining mutants were obtained and characterized further. No hydrolytic activity toward ANA was detected in crude extracts of most nonstainers. Leaky mutants can be distinguished; the colonies of one mutant strain stain light pink, and extracts of this strain had approximately 5% of the wild-type specific activity. After electrophoresis crude extracts of the nonstaining mutants also showed no detectable activity in the position corresponding to band 6 toward any of the substrates tested. This result indicates that only one enzyme is responsible for the hydrolysis of all of the substrates that are hydrolyzed in the region of the gel expected for peptidase N and that this enzyme is absent in the nonstaining mutants.

Isolation and growth properties of mutants that lack peptidase A. Only two of the gel bands (bands 1 and 6) showed strong activity toward Leu-Ala-NH_2. Since the *pepN* mutants had lost band 6, it seemed reasonable to attempt to select mutants deficient in the enzyme observed in band 1 by penicillin selection for mutants unable to utilize Leu-Ala-NH_2, starting with a *pepN*$^-$ strain. Mutant derivatives of strain TN2 (*leu-485 pepN10*) unable to use Leu-Ala-NH_2 were obtained in this way. The absence of gel band 1 in such a strain (TN102) is shown in Fig. 1c.

The ability of strain TN102 (*leu-485 pepN10 pepA1*) to utilize a series of leucine peptides was determined by crystal tests. Strain TN102 did not grow on Leu-Ala-NH_2, Leu-Trp-NH_2, or Leu-NH_2 as sources of leucine. The parent of our strains (LT2) grew on leucinamide

as sole nitrogen source. Strain TN102 did not utilize leucinamide as sole nitrogen source. Both strains TN2 and TN102 grew in nutrient broth and in minimal glucose leucine medium at rates indistinguishable from their parent, *leu-485*.

Strains containing only peptidase A or peptidase N can be constructed from strain TN102. In a transduction cross with strain *leu-485* as donor and strain TN102 (*leu-485 pepN10 pepA1*) as recipient, Leu-Ala-NH_2-utilizing recombinants were selected. Two size classes of recombinants were visible on the plate. Representatives of both classes were picked, purified, and tested for the presence of peptidase N (by the plate staining procedure) and for their ability to utilize leucinamide as sole nitrogen source. All of the larger transductants were *pepN*$^+$ (stained with ANA) and failed to grow on leucinamide as sole nitrogen source. All of the smaller transductants did not stain with ANA but did grow on leucinamide as sole nitrogen source. Crude extracts were prepared from a representative of each class of transductant. When an extract of strain TN163 (a nonstaining, leucinamide-utilizing "small transductant") was subjected to electrophoresis on a polyacrylamide gel and the gel was stained for activity toward Leu-Gly, a strong band at the position expected for peptidase A (at the top of gel) was seen. No activity was observed at the position expected for peptidase N (R_f 0.6). No bands of activity toward ANA were observed. When an extract of strain TN164 (a "large transductant") was subjected to electrophoresis and the gel stained for activity toward Leu-Gly, the peptidase N band (R_f 0.6) was present. No activity was observed at the top of the gel. When the gel was treated with the ANA reaction mixture, one band of activity with an R_f of approximately 0.6 was observed.

TABLE 3. *Utilization of leucine peptides*[a]

Leu source	Strain				
	leu-485	TN2[b]	TN102[b]	TN213[b]	TN215[b]
Leu	+	+	+	+	+
Leu-Gly	+	+	+	−	−
Leu-Arg	+	+	+	−	−
Leu-Trp	+	+	+	−	−
Leu-Leu	+	+	+	+	−
His-Leu	+	+	+	+	−
Gly-Leu	+	+	+	+	+
Pro-Leu	+	+	+	−	−
Leu-Pro[c]	+	+	+	+	+
Leu-Gly-Gly	+	+	+	+	−
Leu-Ala-NH_2	+	+	−	−	−
Leu-Trp-NH_2	+	+	−	−	−
Leu-NH_2	+	+	−	−	−

[a] Utilization was determined by crystal tests.

[b] Genotypes: TN2, *leu*$^-$ *pepN*$^-$; TN102, *leu*$^-$ *pepN*$^-$ *pepA*$^-$; TN213, *leu*$^-$ *pepN*$^-$ *pepA*$^-$ *pepD*$^-$; TN215, *leu*$^-$ *pepN*$^-$ *pepA*$^-$ *pepD*$^-$ *pepB*$^-$.

[c] Hydrolysis of Leu-Pro depends on the presence in these strains of either peptidase P or peptidase Q (11).

The enzyme identified here as peptidase A is probably similar or identical to the aminopeptidase I described by Vogt (19) in *E. coli*. Aminopeptidase I is a high-molecular-weight (320,000), broad-specificity enzyme which is characteristically heat stable (70 C, 5 min). According to Vogt, aminopeptidase I is the only peptidase activity in *E. coli* stable at high temperature. Since Vogt has reported that this enzyme aggregates at low ionic strength, we speculate that the band of activity that does not enter the gel may represent an aggregated form of this enzyme and that the band that is frequently observed at R_f 0.1 may be due to the unaggregated form of this enzyme. Both of these bands showed identical substrate specificity profiles, and both were absent in strain TN102. The heat stability of the enzyme in crude extracts was similar to that expected for aminopeptidase I. Figure 4 shows the stability of the Leu-Ala-NH_2 hydrolyzing activities present in strains TN164 (*pepN*$^+$ *pepA*$^-$) and TN2 (*pepN*$^-$ *pepA*$^+$). Extracts of strain TN102 (*leu-485 pepN10 pepA1*) contain no activity toward Leu-Ala-NH_2 detectable under these assay conditions. These results show that peptidase N is rapidly inactivated at 70 C and that peptidase A retains approximately 95% of its original activity after 5 min at 70 C. We believe that this temperature stability and the observed substrate specificity patterns suggest strongly that our peptidase A is probably very similar to aminopeptidase I and that the behavior observed on gel electrophoresis is consistent with this assignment.

Isolation of mutants lacking a dipeptidase. One band of activity observed after electrophoresis of a crude cell-free extract of the wild-type strain hydrolyzed only dipeptides. Fractions from diethylaminoethyl-cellulose column chromatography of the wild-type extract containing this enzyme (as identified by gel electrophoresis) showed greater activity under the assay conditions used (0.05 M Tris-hydrochloride, pH 7.6, 0.01 M substrate) toward Leu-Gly than other fractions. Since strain TN102 lacks two broad specificity peptidases, both capable of hydrolyzing Leu-Gly, we reasoned that even though the gel patterns indicated the presence

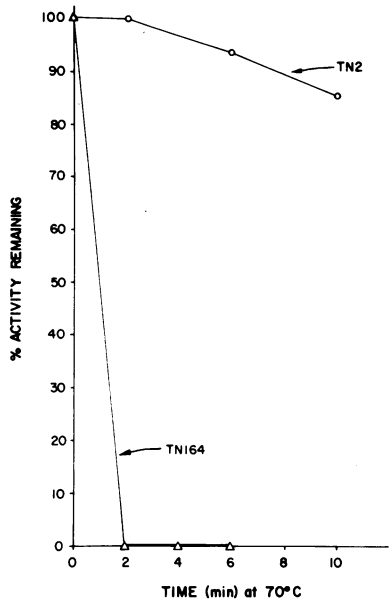

FIG. 4. *Heat stabilities of Leu-Ala-NH_2 hydrolyzing enzymes. A series of tubes containing 0.05 ml of crude extract (17 mg of protein per ml), 1.5 ml of buffer (0.02 M Tris-hydrochloride [pH 8.4], 1 × 10^{-5} M $MnCl_2$, and 0.1 M KCl), and 0.35 ml of distilled water were heated in a water bath at 70 C. Zero time was taken as the time the contents of the tube reached 70 C. Individual tubes were withdrawn at 2-min time intervals and cooled in ice. Leu-Ala-NH_2 hydrolysis was assayed by a modification of the procedure of Binkley et al. (2). To each tube equilibrated at 37 C, 0.1 ml of 0.1 M Leu-Ala-NH_2 was added at zero time. Samples (0.1 ml) of this reaction mixture were withdrawn at appropriate times into 0.9 ml of 0.05 M HCl to stop the reaction. After all samples had been collected, 2.0 ml of copper-borate reagent (5% sodium borate decahydrate, 0.6% copper sulfate pentahydrate) was added to each tube, 0.5 ml of a 0.4% solution of trinitrobenzenesulfonate (Pierce Chemical Co.) was then added, and the contents of the tubes were mixed thoroughly. After 20 min of incubation at 37 C in the dark, 0.5 ml of 5 M HCl was added to each tube, the contents were mixed, and the absorbance at 420 nm was read on a Gilford spectrophotometer. The rate of hydrolysis was determined from linear plots of absorbance versus time.*

of at least one other enzyme in addition to the dipeptidase with Leu-Gly-hydrolyzing activity, a mutant lacking the dipeptidase might grow poorly on this substrate as a source of leucine. If so, it should be possible to isolate mutations leading to loss of the dipeptidase in strain TN102 by penicillin selection for Leu-Gly nonutilizers. Therefore, strain TN102 was mutagenized with diethyl sulfate, and a penicillin selection for mutants unable to grow on 1 × 10^{-4} M Leu-Gly was carried out. Such mutants were obtained. The absence of the dipeptidase band in a crude extract of one of these mutants was confirmed by gel electrophoresis (Fig. 1d).

Strains lacking the dipeptidase (which we have called peptidase D) as well as peptidase N and peptidase A failed to grow on some, but not all, leucine-containing dipeptides (Table 3). The ability of these strains to utilize some dipeptides but not others is apparently not due to leakiness of the *pepD* mutations, since several independent *pepD* strains showed identical growth patterns and no band of activity was observed at the dipeptidase position (R_f 0.52) for any of the peptides listed in Table 2.

Isolation of strains lacking peptidase B. Mutants lacking the band of peptidase activity with an R_f in gel electrophoresis of 0.3 were obtained from strain TN213 (*leu-485 pepN10 pepA1*) by penicillin selection in the presence of Leu-Leu. The absence of this enzyme in one of these strains (TN215) is shown in Fig. 1e. This strain lacks four of the peptidase bands seen after electrophoresis: the band at the top of the gel (peptidase A), the 0.3 band (which we have called peptidase B), the 0.52 R_f dipeptidase (peptidase D), and the naphthylamidase, peptidase N (R_f 0.62). Strain TN215 (*leu-485 pepN10 pepA1 pepD1 pepB1*) failed to grow on a variety of peptides as sources of leucine (Table 3). After gel electrophoresis of crude extracts of this strain, no bands of activity toward Leu-Gly could be detected. Two remaining bands of peptidase activity (R_f 0.2 and 0.4) could be seen with Leu-Gly-Gly or Leu-Leu-Leu as substrate.

The sequence of steps through which strain TN215 was isolated is illustrated in Fig. 5.

Strain TN215 lacks four peptidases. Strains containing only one of these four have been constructed (Table 4). In transduction crosses using phage grown on strain *leu-485* as donor and strain TN215 as recipient, Leu-Gly-utilizing recombinants were selected in one experiment and Leu-Gly-Gly utilizing recombinants were selected in another. These recombinants could be classified as shown in Table 4. These classes are defined by the ability of a recombinant to hydrolyze ANA, utilize leucinamide as sole nitrogen source, and utilize peptides as sources of leucine. The presence of the indicated enzymes in representatives of each class was confirmed by gel electrophoresis. The growth properties of these strains clearly illustrate the overlapping specificities of these enzymes. The presence of any one of the enzymes coded for by the genes *pepN*, *pepA*, or *pepD* is sufficient to allow

growth on Leu-Gly. Leu-Gly-Gly will support the growth of strains containing either peptidase N, peptidase A, or peptidase B.

DISCUSSION

The results presented in this paper show that *S. typhimurium* contains several peptidases, each capable of hydrolyzing a variety of small peptides. A characteristic specificity pattern can be assigned to each of the enzymes separated by gel electrophoresis.

The isolation of mutants lacking several of these enzymes indicate that they are dispensable for growth under conditions where utilization of exogenously supplied peptides is not required. It is possible, of course, that some of the mutations affecting the abilities of the enzymes we have studied to hydrolyze small peptides may result in the production of altered proteins still capable of performing a vital function. Multiply peptidase-deficient strains (e.g., TN251) do grow more slowly than the wild type in minimal glucose medium (C. Yen, unpublished observations). It therefore seems likely that these enzymes are involved in processes other than hydrolysis of exogenously supplied peptides.

Each of these enzymes can clearly function in the catabolism of peptides. The growth properties of strains lacking all four enzymes (peptidases N, A, D, or B) compared to those of strains containing only one of the four show clearly that each one of these peptidases can function in the utilization of peptides as sources of amino acids. These results also clearly demonstrate the functional overlapping of these enzymes; a strain containing either peptidase N, peptidase A, or peptidase D can utilize Leu-Gly as a leucine source, but in the absence of all three enzymes this peptide will not support growth of a *leu*$^-$ strain.

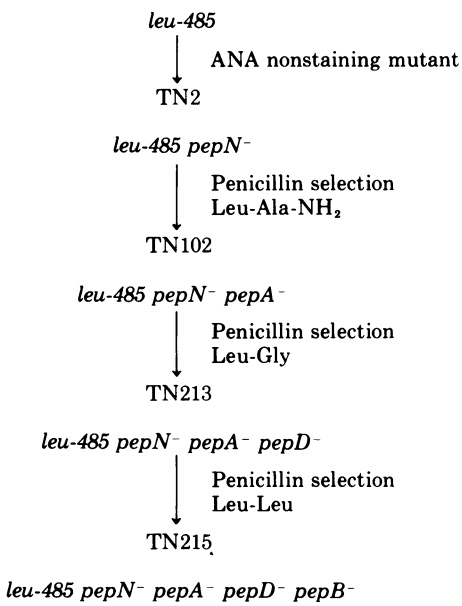

FIG. 5. *Isolation of peptidase mutants.*

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TABLE 4. *Recombinant classes from transduction cross leu485 × TN215*[a]

Selection	Class	ANA hydrolysis	Growth on leucinamide (sole N source)	Growth on Leu-Gly	Growth on Leu-Gly-Gly	Proposed genotype	Representative strain
Select Leu-Gly-Gly utilization	1	+	−	+		*pepN*$^+$	TN271
	2	−	+	+		*pepA*$^+$	TN272
	3	−	−	−		*pepB*$^+$	TN273
Select Leu-Gly utilization	1	+	−		+	*pepN*$^+$	
	2	−	+		+	*pepA*$^+$	
	3	−	−		−	*pepD*$^+$	TN274

[a] TN215 (*leu*$^-$ *pepN*$^-$ *pepA*$^-$ *pepD*$^-$ *pepB*$^-$).

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