Tripeptide-Specific Aminopeptidase from *Escherichia coli* AJ005†

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ABSTRACT: A tripeptidase, TP, from the ribosome-free fraction of *Escherichia coli* AJ005, a peptidase-deficient mutant of strain K-12, has been obtained using gel electrophoresis and chromatography on DEAE-Sephadex A-50, hydroxylapatite, and Sephadex G-200. Characterization studies on tripeptidase TP, freed of other detectable peptidases, indicate that this enzyme is capable of cleaving an amino-terminal leucine, lysine, methionine, or phenylalanine residue from certain tripeptides. Only one band of activity toward several tripeptides (and no activity toward dipeptides) was detected following gel electrophoresis of this preparation. Tripeptidase TP, the only strain AJ005 peptidase known to attack trilysine, was inactive toward all dipeptides, peptide amides, substituted peptides, esters, and tetrapeptides tested as substrates. Trilysine cleavage is optimal at about pH 8.5, as determined in Tris, borate, or phosphate buffers. Tripeptidase TP activity tested under a number of conditions was not inhibited by soybean trypsin inhibitor (3 mg/mL), phenylmethanesulfonyl fluoride (25 μM), or iodoacetate (9 mM). *p*-Mercuribenzoate (10 μM), divalent copper, cobalt, calcium (2.5 mM), zinc (25 μM), and mercury (10 μM) are inhibitory. Based on Sephadex G-200 chromatography tripeptidase TP has a particle weight of approximately 80 000 daltons. An apparent K_m of 5.3 mM was determined for methionylglycylglycine cleavage.

In microorganisms peptidase deficiency can result in atypical growth, exemplified in part by the abnormally long lag time characterizing the growth cycle of *Escherichia coli* strain AJ005 (Simmonds et al., 1976), a mutant isolated by Sussman & Gilvarg (1970) which lacks one of the two Lys_3-cleaving peptidases present in strain K-12. In their initial description of *E. coli* AJ005 Sussman & Gilvarg (1970) reported that this strain apparently contains only one enzyme capable of cleaving Lys_3. Subsequently, Simmonds et al. (1976) demonstrated that this Lys_3-cleaving activity was a function of strain K-12 tripeptidase (TP), an enzyme showing aminopeptidase activity toward several tripeptides. The present study was undertaken to free TP of other detectable peptidases in this strain and to further characterize the enzyme in vitro as an initial step in investigations to delineate its in vivo roles.

Materials and Methods

E. coli strain AJ005 (Lys$^-$Thr$^-$Leu$^-$thiamine$^-$oligopeptidase$^-$) was supplied by Dr. Charles Gilvarg, Princeton University (Simmonds et al., 1976; Sussman & Gilvarg, 1970).

Peptides, amino acid esters, and substituted peptides composed of L-amino acids (Cyclo Chemical, Bachem, Sigma, and Schwarz/Mann Companies) were checked for purity by TLC[1] followed by ninhydrin treatment prior to use. Soybean trypsin inhibitor affinity resin (STI resin) was the gift of Dr. Fred Yost, Jr., Duke University Medical Center. HA was provided by Dr. Franta Kalousek, Yale University. Tris, Trizma 8.0, Trizma 8.3, STI, $PhCH_2SO_2F$, snake venom (crude *Crotalus adamanteus*) and other reagents used in the amino acid oxidase coupled reactions were from Sigma.

TLC was performed on silica gel (Eastman Kodak) or ITLC-SAF sheets (Gelman Instrument Co.). Disc gel electrophoresis was performed in tube or slab gels in the Tris–glycine system of Davis (1964); stacking gels were omitted. Protein concentration was determined (Lowry et al., 1951; McKnight, 1977) using bovine plasma albumin as standard.

DEAE-Sephadex A-50 was acid and base washed prior to use. HA was washed extensively with water. Sephadex G-200 chromatography columns were calibrated for molecular weights using γ-globulin, apoferritin, bovine albumin, ovalbumin, cytochrome *c*, and hemoglobin (from Schwarz/Mann).

Enzyme Assays. Peptidase activity was assayed using a modification of the procedure of Simmonds et al. (1976): enzyme solution and buffer salts were incubated 15 min, and peptide solution (50 μL) was added to provide the standard *reaction mixture* (200 μL) containing enzyme preparation, 6.3 mM Trizma 8.0 or 8.3, 0.13 mM $MnSO_4$, 40 μM EDTA, 2 mM peptide, and where noted 7 mM mercaptoethanol.

Liberation of Lys from Lys_2 and Lys_3 were measured by the method of Shimura & Vogel (1966). With substrates other than Lys-containing peptides, the extent of hydrolysis was measured by the ninhydrin procedure of Matheson & Tattrie (1964). An amino acid oxidase coupled reaction (see Lewis & Harris, 1967; Auricchio et al., 1971), as modified by Shoaf et al. (1974), was used to detect liberation of Met, Leu, or Phe in the absence of mercaptoethanol (which prevents color development). Color production (530 nm) was proportional to the amount of susceptible amino acid in the range of 4 to 160 nmol. The oxidase method can also be adapted to a convenient microassay performed in wells of disposotrays (Hermsdorf, 1978).

TLC was used to ascertain the nature of hydrolysis products and to estimate enzymatic activity. Colors (commercial ninhydrin spray) and R_f values (Table I) facilitated identification of spots; appropriate peptide and amino acid standards chromatographed with samples permitted estimation of the extent of hydrolysis. Routinely, 2 or 5 μL of each reaction mixture (4 to 10 nmol of initial substrate) was spotted on the plate. Other

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[1] Abbreviations used: TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; Trizma, [tris(hydroxymethyl)aminomethane and hydrochloride]; STI, soybean trypsin inhibitor; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; ITLC-SAF, silicic acid gel-impregnated glass fiber; IAc, iodoacetate; pMCB, *p*-mercurichlorobenzoate; AP and L, aminopeptidases AP and L; DP, dipeptidase DP; OP, oligopeptidase OP; TP, tripeptidase TP; HA, hydroxylapatite; AS, ammonium sulfate; mol wt, molecular weight.

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TABLE I: R_f Values of Peptides and Potential Hydrolysis Products On TLC Systems Routinely Used.[a]

solvent 1[b]	solvent 2[c]	solvent 3[d]	solvent 4[e]	solvent 5[f]
Leu-Gly	Lys-Ala-Ala	Met-Gly-Gly	Gly_4	Leu_2
Gly (0.64)	Lys (0.08)	Gly-Gly (0.47)	Gly_4 (0.27)	Leu (0.49)
Leu (0.75)	Lys-Ala (0.28)	Gly (0.54)	Gly_3 (0.31)	Leu (0.61)
Leu-Gly (0.82)	Lys-Ala-Ala (0.29)	Met-Gly-Gly (0.61)	Gly_2 (0.39)	
	Ala (0.54)	Met (0.73)	Gly (0.42)	
	Ala-Ala (0.69)			
Lys-Gly-Gly	Lys-Gly-Gly	Met-Leu-Gly	Met-Gly-Gly	Phe-Gly
Lys (0.35)	Lys (0.06)	Gly (0.57)	Gly_2 (0.39)	Gly (0.25)
Lys-Gly-Gly (0.44)	Lys-Gly-Gly (0.11)	Leu-Gly (0.73)	Gly (0.42)	Phe-Gly (0.45)
Gly (0.64)	Lys-Gly (0.16)	Met (0.75)	Met-Gly-Gly (0.49)	Phe (0.48)
Gly-Gly (0.71)	Gly (0.35)	Met-Leu-Gly (0.76)	Met-Gly (0.58)	
	Gly-Gly (0.52)	Leu (0.76)	Gly-Met (0.60)	
			Met (0.62)	
Lys_2 or Lys_3	Lys-Tyr		Leu_2	
Lys_3 (0.04)	Lys (0.04)		Leu (0.71)	
Lys_2 (0.20)	Lys-Tyr (0.37)		Leu_2 (0.85)	
Lys^2 (0.38)	Tyr (0.52)			

[a] R_f values, shown in parentheses for each peptide substrate and its potential hydrolysis products, vary slightly from experiment to experiment depending on the conditions used, i.e., size of TLC sheet, size of chromatography jar, and temperature. TLC matrix, composition of solvent system, and time required for development of chromatogram are summarized in footnotes *b–f*. [b] ITLC-SA; chloroform:methanol:ammonium hydroxide (2:2:1; v:v); rapid development (18 min). [c] ITLC-SA; chloroform:methanol:ammonium hydroxide (4:4:1; v:v); rapid development (15 min). [d] ITLC-SA; butanol:pyridine:water (2:1:1; v:v); very slow development (130 min). [e] ITLC-SA; butanol:acetic acid:water (4:1:2; v:v); slow development (35 min). [f] Silica gel; butanol:acetic acid:water (70:25:100; v:v; top layer); very slow development (115 min).

detection methods used were iodine vapor, iodine/starch (Rydon & Smith, 1952), sulfuric acid/dichromate (Ehrhardt & Cramer, 1962), and $KMnO_4$/HCl (Ertel & Horner, 1962).

Enzymatic activity following gel electrophoresis was detected by the Enzyme Assays procedure after the gels had been sliced and suspended in buffered 0.02 M KCl. When the Matheson & Tattrie (1964) ninhydrin procedure was used each gel-slice eluent was dialyzed to remove glycine (derived from electrophoresis buffer) prior to assay. Peptidases in electrophoresis gels also were localized by overlaying the gels with 1% agar containing 0.13 mM $MnSO_4$, 40 μM EDTA, 50 mM Trizma 8.0, 2 mM peptide, and, per mL, 0.1 mg of dianisidine, 0.2 mg of peroxidase, and 0.1 mg of snake venom. After detection of the activity and removal of the overlay, peptidases could be eluted and further characterized using TLC.

Enzyme Preparation. Ribosome-free extracts in which aminopeptidase I remained ribosome bound (Vogt, 1970; Simmonds, 1972) were prepared from late log-phase cells using 25 mM KCl buffer as described by Simmonds et al. (1976). The extract was filter-sterilized and stored at 4 °C.

Although AS (55% saturation) precipitation of the ribosome-free extract removed most of the DP (supernate) from TP (precipitate) variable loss of TP activity resulted. Hence, this procedure was omitted from the purification scheme described under Results.

After adjustment to 0.17 M KCl and filter-sterilization, the ribosome-free extract of *E. coli* AJ005 (395 mL, 910 mg) was fractionated on DEAE Sephadex A-50 (Figure 1). The effluent was assayed for activity toward Lys_3, Leu_2, and Lys_2 using TLC.

[For comparative purposes, a preparation from strain K-12 comparable to the region of fractions 175–350 of Figure 1 was also subjected to chromatographic analysis; the pooled material was diluted to 0.2 M KCl, applied to Sephadex A-50, and eluted with 0.25 to 0.40 M KCl as above (Figure 2).]

After fractions from the strain AJ005 column exhibiting Lys_3-cleaving activity had been pooled and filter sterilized, 84 mL (19 mg of protein) of this pool was fractionated on a HA column (Figure 3). Fractions containing Lys_3-cleaving activity were used in tests for TP susceptibility to several substances known to inhibit peptide-cleaving enzymes.

Material from this column containing Lys_3-cleaving activity was further purified by two passages through Sephadex G-200. Material from the descending limb of the TP activity peak (first passage, Figure 4) was rechromatographed on Sephadex G-200 to remove traces of AP. Fractions containing TP (from the second Sephadex G-200 column) were used for characterization studies. Because TP increased in lability as its purity increased, the characterization tests were carried out immediately following Sephadex G-200 chromatography.

Inhibitor Studies. Tests were performed on strain AJ005 TP preparations obtained by AS precipitation followed by chromatography on Sephadex A-50 and HA as well as by the procedure in which the AS fractionation step had been omitted. In these preparations TP was the only detectable peptidase capable of cleaving Lys_3 and no enzymes capable of cleaving Lys_2 were present. The TP preparation (undiluted and diluted 4-, 16-, or 64-fold) and potential inhibitor were combined and incubated for 15 min or overnight. Salts and Lys_3 were added (final concentrations as in the standard reaction mixture) and the extent of hydrolysis was determined as a function of time (TLC procedure). Final potential inhibitor concentrations were 0.3 and 3.0 mg/mL STI, 25 μM $PhCH_2SO_2F$, 40 μM to 9.0 mM IAc, 0.48 μM to 1.5 mM $HgCl_2$ and pMCB, 0 to 25 mM EDTA, and 0.2 μM to 2.5 mM divalent Cu, Co, Ca, and Zn.

pH Studies. The pH optimum for Lys_3-cleaving activity was determined in assay mixtures containing 12.5 mM borate, 12.5 mM Tris, or 62.5 mM potassium phosphate, respectively, plus peptidase preparation, 2 mM Lys_3, 0.13 mM $MnSO_4$, 3.5 mM mercaptoethanol, 2.1 mM Trizma 8.0, and 0.04 mM EDTA.

Substrate Specificity Studies. Enzyme solutions were tested initially at several concentrations (i.e., undiluted and diluted 2-, 4-, or 8-fold) in the standard mixture containing Trizma 8.3 buffer but no mercaptoethanol. Routinely, incubation of one set of standard assay mixtures containing the test sub-

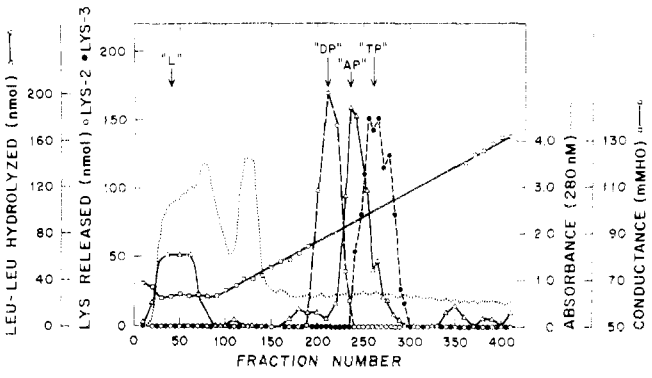

FIGURE 1: DEAE-Sephadex A-50 chromatography of the ribosome-free fraction from *E. coli* AJ005. After the extract was applied, the column (2 × 20 cm) was washed with 100 mL of buffer containing 0.2 M KCl, 0.1 mM $MnSO_4$, 7.1 mM mercaptoethanol, 6 mM Trizma 8.0, then developed with a linear KCl gradient (above buffer containing 0.2 to 0.4 M KCl) at a flow rate of 75 mL/h. Fractions (5 mL) were collected. Highest activities per μL of undiluted column effluent were: Leu_2 (△), 72 nmol hydrolyzed/h at 25 °C; Lys_2 (○), 20 nmol hydrolyzed/h at 25 °C; and Lys_3 (●), 24 nmol of Lys released/h at 37 °C. Activity toward Lys_2 was measured in the presence of added mercaptoethanol.

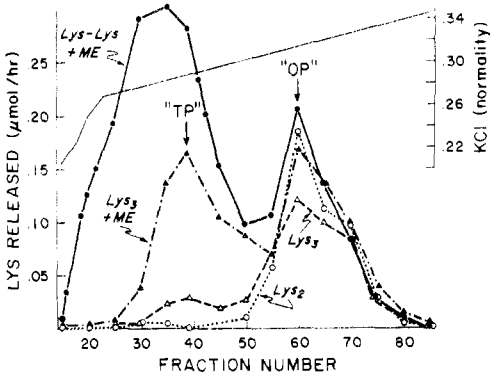

FIGURE 2: DEAE-Sephadex A-50 chromatography of *E. coli* K-12 extract. Material from strain K-12 corresponding to fractions 175–350 of Figure 1 (adjusted to 0.2 M KCl) was applied and then eluted with a 0.25–0.40 M KCl gradient in buffer as above. Activities of fraction 60 per μL of undiluted fraction at 37 °C were: in the absence of added mercaptoethanol, 0.9 nmol of Lys_2 hydrolyzed/h (○), and 1.2 nmol of Lys released from Lys_3/h (△); in the presence of added mercaptoethanol, 1.0 nmol of Lys_2 hydrolyzed/h (●), and 1.7 nmol of Lys released from Lys_3/h (▲).

strates was stopped at the time when hydrolysis of the reference substrate, Met-Gly-Gly, was approximately 50%; longer incubation periods were also used for the slowly hydrolyzed substrates. Results for each substrate were normalized to the activity determined simultaneously on Met-Gly-Gly.

Determination of K_m. The K_m for Met-Gly-Gly cleavage in the absence of mercaptoethanol was determined using the amino acid oxidase assay to measure Met liberation.

Results

Peptidases L, DP, AP, and TP were demonstrable in both strains AJ005 (Figure 1) and K-12 (Figure 2) when ribosome-free extracts were fractionated on DEAE-Sephadex A-50. As noted by Simmonds et al. (1976), TP appeared to be the only enzyme of the strain AJ005 extract capable of cleaving Lys_3 since this strain lacks the K-12 enzyme OP (Sussman & Gilvarg, 1970, 1971).

Electrophoretic analysis (assay of gel slices) of fractions from the strain K-12 column (Figure 2) indicated that fraction 25 (DP) contained mercaptan-stimulated activity toward

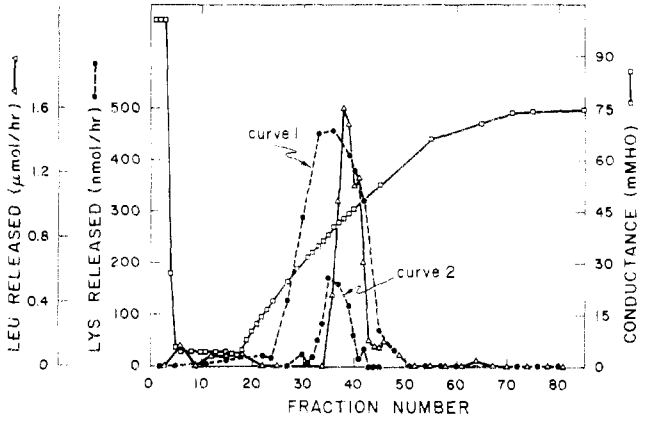

FIGURE 3: HA chromatography of partially purified TP preparation. After material from pooled fractions 237–297 of Figure 1 was applied, the column (10 mL) was washed with buffer (50 mL) containing 6 mM Trizma 8.3 and 5 mM phosphate and then developed (in the absence of Trizma) with a linear gradient of 5–250 mM potassium phosphate (pH 8.0; 100 mL of each phosphate solution). Fractions 1 to 11 contained 5 mL each; fractions 12 to 85 each had 2.5 mL. Highest activities per μL of undiluted column effluent were: Leu_2 (△), 80 nmol hydrolyzed/h (25 °C), and Lys_3 (●), 17.4 nmol/h (37 °C). For Lys_3 the standard 200 μL of assay mixtures contained 100 μL of column effluent fractions which were incubated for 1 h for curve 1, releasing a maximum of 450 ± 50 nmol of Lys; for curve 2, 10 μL of column fractions was similarly incubated for 30 min, releasing a maximum of 87 nmol of Lys (fraction 35). Since TLC indicated total disappearance of Lys_3 and appearance of Lys and Lys_2 in the presence of fractions 35 and 36 of curve 1, and, since 100% hydrolysis of 400 nmol of Lys_3 produces 1200 nmol of Lys, the results of these assays indicate that TP does not completely degrade Lys_3.

Phe-Gly and Lys_2 banding at R_f 0.6, fraction 40 (TP) had activity toward Lys_3 at R_f 0.5, and fraction 65 (OP) had activity toward both Lys_2 and Lys_3 at R_f 0.9.

The effect of mercaptoethanol on Lys_2 cleavage by DP was at least a 60-fold stimulation (Figure 2, fraction 35), while mercaptan stimulation of OP activity appeared to be 1.1-fold with Lys_2 and 1.4-fold with Lys_3 (fraction 60) and 1.8-fold for TP (fraction 39).

Fractionation of strain AJ005 extracts on DEAE-Sephadex A-50 (Figure 1) resulted in an estimated 76% yield of Lys_3-cleaving activity, with the most active fractions showing a 22-fold increase in specific activity (Table III); this activity was stable.

When strain AJ005 material containing AP and TP (pooled fractions 237–297, Figure 1) was further fractionated on HA, the Lys_3- and Leu_2-cleaving activities were only partially resolved (Figure 3). Material not retained on this column (approaching 100% applied protein) was found to lack Lys_3-cleaving activity. [Neither AP nor TP was retained on CM-50 resin columns.]

As shown in Figure 3 even at high enzyme concentration and long incubation time (curve 1), the amount of Lys released was equivalent only to 100% hydrolysis of Lys_3 to Lys plus Lys_2; TLC verified that Lys_3 had been completely hydrolyzed and that only Lys and Lys_2 had been produced. Electrophoresis of column effluent fractions 33 to 40 indicated the presence of two bands possessing activity ($R_f \cong 0.28, 0.47$) toward Met-Gly-Gly, Met-Ala-Met, and Met-Gly-Met, only one of which ($R_f \cong 0.47$) showed the characteristic TP activity toward Lys_3. No protein could be detected (using Coomassie Brilliant Blue R-250) at R_f 0.47 following electrophoresis of a 200-μL sample. Electrophoresis often resulted in a great loss of TP enzymatic activity; AP appeared less labile than TP upon electrophoresis.

The Lys_3-cleaving activity observed in HA column effluents was not inhibited by STI resin, STI (3 mg/mL), $PhCH_2SO_2F$

(25 μM), or IAc (9 mM). Both $HgCl_2$ and pMCB at final concentrations of $\geq$10 μM caused significant inhibition. At a final concentration of 2.5 mM, Mg^{2+} appeared slightly inhibitory and Ca^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} were highly inhibitory; Zn^{2+} inhibited even at 25 μM.

Although HA chromatography increased the specific activity toward Lys_3 35-fold, it also resulted in considerable loss of activity (58%), due in part to increased lability following dilution of TP by this chromatographic procedure. However, recovery of AP (Leu_2-cleaving activity) following the fractionation was essentially 100%.

When material from HA fractions containing Lys_3-cleaving activity was chromatographed on Sephadex G-200 peptidases AP and TP were readily separable. Following Sephadex G-200 chromatography in the absence of molecular weight markers, protein content in fraction 76 was less than 1 μg/mL (Figure 4); only 40% of the Lys_3-cleaving activity (TP) was recovered in the eluent but recovery of AP activity was almost 100%. (By increasing the amount of material applied and/or including molecular weight marker proteins the recovery of TP activity could be increased to 80%.)

When column effluent fractions contain well-resolved AP and TP activities (e.g., Figure 4), comparisons of TP and AP action can readily be made. Thus, using Leu-Leu, Met-Leu-Gly, and Met-Gly-Gly with the amino acid oxidase coupled reaction, assignment of peak AP activity to fraction 61 was made on the basis of activity toward Leu_2; the relative rates of hydrolysis in the presence of this fraction were in accord with the known preference of AP for Leu_2 and Met-Leu-Gly over Met-Gly-Gly (Simmonds, 1972). For fraction 76, the relative rates of tripeptide hydrolysis and the absence of activity toward Leu_2 provided evidence that it contained a tripeptidase (TP) for which Met-Gly-Gly is the better substrate (Table II). (In relation to work with enzymes like AP that are capable of completely degrading tripeptides it is important to recognize that the amino acid oxidase coupled assay system only indicates that amino acids suseptible to the oxidase have been produced. TLC is required to ascertain the nature of the hydrolysis products.) Scans of this column for the presence of peptidases

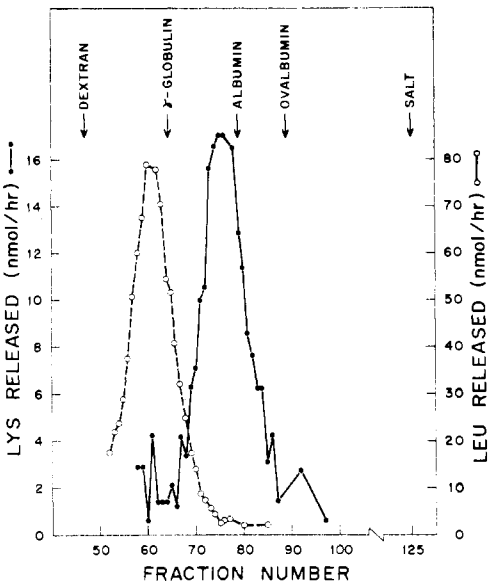

FIGURE 4: Sephadex G-200 chromatography of partially purified TP. Material from fraction 35 of Figure 3 was fractionated on Sephadex G-200 (1.5 × 90 cm column; buffer containing 0.1 M $MnSO_4$, 0.02 M KCl, and 6 mM Trizma 8.0). Highest activities per μL of column effluent were 0.79 nmol of Leu_2 hydrolyzed/h at 37 °C (fraction 60) and 0.17 nmol of Lys released from Lys_3/h at 37 °C (fraction 76). Rechromatography on Sephadex G-200 of material from pooled fractions 76–85 yielded TP free of detectable activity toward Leu_2. Scans of this column effluent for activity toward other peptides are discussed in text.

capable of liberating Lys from Lys-containing peptides (using TLC) indicated activity only in fractions corresponding to AP or TP and as follows: Lys-Ala-Ala is a relatively good substrate for TP, but a poor one for AP; Lys-Gly-Gly, an even better substrate for TP, is essentially resistant to attack by AP; Lys-Trp-Lys is cleaved very slowly by TP and even more slowly by AP; Lys-Tyr-Glu and Lys-Tyr-Thr are hydrolyzed only slowly by AP but appear to be better substrates for that enzyme than for TP. Under conditions permitting 100% hydrolysis of

TABLE II: Peptide Specificity Data for Strain AJ005 Tripeptidase.[a]

act. toward tripeptides and peptide amides[b]				
compound tested	rel act	compound tested	rel act.	other compounds tested and found to be inactive as substrates[c]
Glutathione	0	Lys-Tyr-Thr	4	Ala-NH_2; Ala-A, Ala-B, Ala-C
Gly-Gly-Gly	0	Met-Ala-Met	104	Gly-NH_2; Gly-B, Gly-C, Gly-Val
Gly-Phe-NH_2	0	Met-Ala-Ser	93	Leu-NH_2; Leu-A, Leu-B, Leu-C, Leu-Pro, Leu-Tyr
Leu-Ala-NH_2	0	Met-Gly-Gly	100	Lys-NH_2; Lys-A, Lys-B, Lys-Glu, Lys-Lys, Lys-D
Leu-Gly-Gly	70	Met-Gly-Met	126	Met-NH_2; Met-Ala, Met-B, Met-C, Met-Ser
Leu-Gly-Leu	70	Met-Leu-Gly	44	Met-Gly-Met-Met
Leu-Leu-Gly	13	Met-Phe-Gly	8	Phe-NH_2; Phe-Ala, Phe-B, Phe ethyl ester
Leu-Phe-NH_2	0	Met-Phe-Met	8	Pro-Leu; Trp-Lys; Val-Leu
Lys-Ala-Ala	26	Phe-Ala-NH_2	0	acetyl-Leu, acetyl-Phe, and their amides
Lys-Gly-Gly	60	Phe-Gly-NH_2	0	acetyl-Tyr ethyl ester, acetyl-Trp ethyl ester
Lys-Lys-Lys	8	Phe-Gly-Gly	37	CBZ derivatives of Gly, Gly_2, Gly_3, and their amides
Lys-Phe-Ile-Gly-Leu-Met-NH_2	0	Pro-Leu-Gly-NH_2	0	CBZ derivatives of Gly-Gly-Ala, Gly-Gly-Met, Gly-Gly-Phe
Lys-Tyr-Glu	4	Trp-Met-Asp-Phe-NH_2	0	

[a] Tests were made using TP preparations obtained from the ribosome-free cell extract by sequential column chromatography on DEAE-Sephadex-A50 and hydroxylapatite followed by two passages through Sephadex G-200. After gel electrophoresis, only one band of activity toward Met-Gly-Gly (R_f 0.47) was detected in the gel; no activity toward Leu-Leu was observed. Compounds initially found to be resistant to hydrolysis were likewise inactive in tests using larger amounts of the TP preparation and prolonged incubation periods (see text). [b] For tripeptides shown as active substrates, the extent of hydrolysis was also found to be proportional to the amount of enzyme present and the time of incubation. TLC of reaction mixtures showed hydrolysis to be limited to removal of the amino-terminal residue. [c] In dipeptides, A is Ala and β-naphthylamide, B is Gly and Leu, C is Met and Phe, and D is Trp and Tyr.

TABLE III: Summary of Purification Steps Described in Figures 1, 3, and 4.

figure no. and column resin	material applied to column: sample applied to column	total act.[b] (units)	protein (mg/mL)	spec act.[b] (units/mg of protein)	material in column effluent[a]: total recovered act.[b] (units)	yield (%)	peak fraction protein (mg/mL)	peak fraction spec act.[b] (units/mg of protein)
1; Sephadex A-50	ribosome-free cell extract	74[c]	2.3	0.081	56	76	0.23	1.74[d]
3; hydroxyl-apatite	pooled fract. 237–297 in Fig 1	14.5	0.23	0.75	6.1	42	0.011	26.4[d]
4; Sephadex G-200	fract. 35 in Fig 3	0.15	0.011	26.4	0.06	40	nd[e]	nd

[a] Total recovery values were estimated from activity in all fractions showing activity towards trilysine. Peak fractions were: no. 265 in Figure 1, no. 35 in Figure 3, and no. 76 in Figure 4. [b] One unit is defined as activity effecting release of 1 μmol of lysine from trilysine in 1 min at 37 °C. [c] May include lysine liberation by action of DP on dilysine formed by TP action on trilysine. [d] Purification over sample applied was 22-fold for Sephadex A-50 column and 35-fold for hydroxylapatite column. [e] Specific activity could not be determined because the protein content was too low.

Leu_2 by AP, neither AP nor TP liberated detectable Lys from Lys-Glu or Lys-Val.

Rechromatography on Sephadex G-200 of material from the descending limb of the TP activity peak (as in fractions 77 to 84 of Figure 4) resulted in a preparation devoid of contamination by AP. Gel electrophoresis of TP-containing fractions from the second gel filtration column indicated the presence of only one peptidase, banding at R_f ca. 0.47 and having activity toward a number of tripeptides but none toward Leu_2 or other dipeptides. Material obtained in this manner was used for the TP substrate specificity studies (Table II and text).

Enzyme Characterization. Substrate specificity of TP toward a variety of peptides and related compounds in the absence of mercaptoethanol is presented in Table II. The extent of tripeptide hydrolysis (if less than 65%) was approximately proportional to the amount of enzyme used and to the time of incubation with peptide. For susceptible peptides, except those containing Lys, both the Matheson & Tattrie (1964) and the oxidase method assays indicated similar extents of hydrolysis. However, the data presented were obtained by the oxidase method which is more sensitive, especially when the extent of hydrolysis is low (i.e., peptides as well as free amino acids give color with ninhydrin).

All the substrate specificity work on TP was done using material freed of other known *E. coli* peptidases such as AP because even slight contamination could result in cleavage of dipeptides produced by TP action. The complete absence of Leu_2-cleaving activity in the TP preparation indicates the absence of AP contamination.

Data from TLC indicated that TP cleaved tripeptides only to a free amino acid and a dipeptide. In the series X-Y-Z and X-Y-Y, where X = Met, Leu, Phe, or Lys, the only hydrolysis products observed (TLC) were the amino acid X and the dipeptides Y-Z or Y-Y; in no case was free Z or free Y observed. Addition of AP (e.g., material present in fractions 57 to 60 in Figure 4) to an assay mixture containing TP and Met-Gly-Met-Met did not result in Met liberation. Since Met-Met and Met-Gly are substrates for AP, it is clear that none of the peptide bonds in the tetrapeptide is hydrolyzed by TP.

In addition to assays of the TP preparation per se, the Sephadex G-200 column effluent fractions from which the TP used in these studies was derived were tested (by TLC and/or the amino oxidase system) for activity toward each peptide which appeared to serve as a TP substrate. In each instance the activity was symmetrically distributed around fraction 76 (as in Figure 4). For compounds listed in Table II as inactive, assay mixtures containing 160 nmol of substrate and TP sufficient to completely degrade Lys_3 to Lys_2 and Lys in 2 h were incubated for 24 h or longer. No hydrolysis products were observed by any of the detection methods mentioned in the previous section when the entire assay mixture was chromatographed on TLC plates. Nor were any ninhydrin-positive products or oxidase-assay color produced when TP was incubated with albumin, γ-globulin, or ovalbumin. After long-term incubation of TP in the presence of albumin followed by gel electrophoresis, no additional protein bands arising from albumin degradation were observed on the gels.

As shown by the Lineweaver–Burk plot of TP activity toward Met-Gly-Gly (Figure 5) an apparent K_m value of 5.3 mM was determined for this substrate. In tests of TP activity in the standard reaction mixture supplemented with EDTA, no hydrolysis of Lys_3 was detected when the EDTA concentration exceeded the Mn^{2+} concentration; however, inclusion of some EDTA (40 μM) in the standard reaction mixture was necessary for hydrolysis to occur, indicating that EDTA may be necessary to chelate an inhibitory trace metal contaminant. Also, the TP-catalyzed hydrolysis of Met-Gly-Gly was immediately terminated by addition to the standard mixture of the amino acid oxidase reagent solutions containing *o*-phenanthroline, a potent metal-ion chelator. In the absence of *o*-phenanthroline and phosphate (pH 7.0), hydrolysis proceeded normally following addition of other components of the oxidase reagent solution. Figure 6 shows the profile of Lys_3-cleavage by TP as a function of pH using borate, Tris, and phosphate buffers. The activity in phosphate appears less than in the other buffers, and activity appears to be optimal between pH 8 and 9.

Discussion

In Figure 1 (AJ005 extract), where DP and TP were better resolved than they were for the K-12 extract (Figure 2), the absence of Lys_2 cleavage by TP-containing fractions in the presence of mercaptoethanol supported the view that Lys_2 is not hydrolyzed by TP. Also, Figure 3 indicates that TP lacks appreciable ability completely to degrade Lys_3 to Lys.

The earlier observation that AP lacked demonstrable ac-

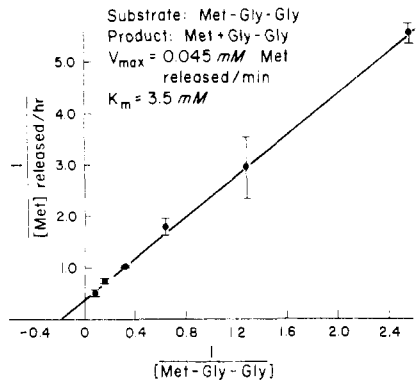

FIGURE 5: Lineweaver–Burk plot for Met-Gly-Gly cleavage at 37 °C by TP. The peptide concentration was varied from 0.195 to 12.15 mM. The assay was performed in triplicate. Error bars indicate the standard deviation. Material used in this assay had been fractionated by chromatography on A-50, HA, and 2 G-200 columns. No AP was detectable by assay toward Leu-Leu in solution or on electrophoretic gel assays. Electrophoresis of material used here indicated one band of activity toward Met-Gly-Gly (R_f 0.47).

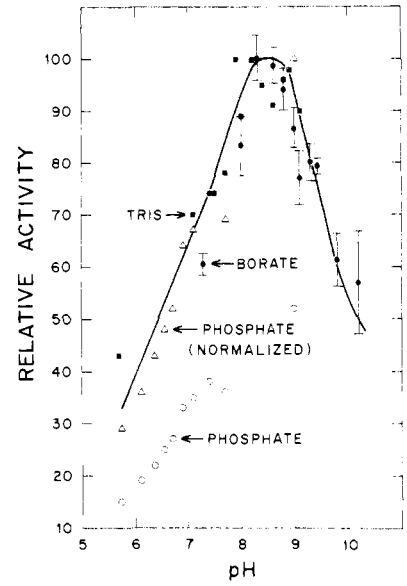

FIGURE 6: Effect of pH on TP activity toward Lys_3. Activity of TP was measured in the standard reaction mixture modified to contain phosphate (observed (○); normalized to other buffers (△)), Tris (■), or borate (●), as described in Materials and Methods. The error bars shown for borate indicate the standard deviations calculated from four assays.

tivity toward Lys_3 (Simmonds et al., 1976) raised the question of its ability to attack other substrates containing an amino-terminal Lys residue. TLC scans of Sephadex G-200 column eluents containing well-resolved AP and TP indicated AP is capable of cleaving some Lys-containing TP substrates (e.g., Lys-Ala-Ala, Lys-Trp-Lys), but does so only with great difficulty.

Similar behavior of AP and TP on several types of chromatography columns indicates similarities in properties. However, based on Sephadex G-200 elution profiles they differ in size (Figure 4): AP, mol wt ≈230 000; both OP (not shown) and TP, mol wt ≈80 000. These results are not completely in accord with those of Sussmann & Gilvarg (1970) on *E. coli* AS013 which indicated a molecular weight similar to that reported here for OP but a larger molecular weight for TP. No explanation for the difference can be offered at this time. Although TP and OP appear to have similar molecular weights, they differ in electrophoretic mobilities (R_f ≈0.47 for TP and R_f ≈0.9 for OP).

Specificity data (Table II) support the initial suggestions of Sussman & Gilvarg (1970) and Simmonds et al. (1976) that strain AJ005 contains a tripeptide-specific peptidase. Data (Table II) indicate that TP acts on tripeptides as an aminopeptidase capable of removing Leu, Lys, Met, and Phe residues. Under the assay conditions used, no dipeptides, amides, esters, N-substituted derivatives, or compounds containing more than three amino acid residues were found that could be cleaved by TP. The strict tripeptide specificity is emphasized not only by the failure of TP to release Lys from dilysine or tetralysine (Sussman & Gilvarg, 1970) and from the eledoisin related hexapeptide amide, Lys-Phe-Ile-Gly-Leu-Met-NH_2 (Table II), but also by its inability to hydrolyze Met-Gly or Met-Gly-Met-Met, although Met-Gly-Met is rapidly degraded to Met plus Gly-Met. Also, although Phe-Gly-Gly is cleaved, neither Phe-Gly nor Phe-Gly-amide is a substrate. The available specificity data suggest that in susceptible tripeptides, the presence of Gly or Ala as the second residue was preferred over a larger or more hydrophobic amino acid. In contrast, the structure of the carboxy-terminal residue appeared to have little effect on the relative rate of hydrolysis.

This apparent strict specificity of TP for tripeptides distinguishes it from other *E. coli* peptidases, and, although several tripeptides cleaved by TP can also be cleaved by *E. coli* aminopeptidases I (Vogt, 1970) and L and AP (Simmonds et al., 1976), these enzymes display both di- and tripeptidase activity.

Aminopeptidase I differs from TP in several respects including its dipeptidase activity, large particle size, and ability to bind to ribosomes, but its inhibition by Zn^{2+} and activity toward Met-containing tripeptides (Vogt, 1970) resembles that of TP.

While the strain AJ005 ribosome-free fraction contains DP and TP, capable of cleaving di- and trilysine, respectively (Figure 1), the parental strain K-12 contains these enzymes plus OP (Figure 2), active toward Lys_2, Lys_3, and Lys_4 (Sussman & Gilvarg, 1970). Both strains contain an endopeptidase (Sussman & Gilvarg, 1970) which may be identical with *E. coli* B dipeptidocarboxypeptidase DCP, an enzyme inactive toward tripeptides but which releases Lys_2 from the carboxy-terminal end of Lys homopolymers (Yaron et al., 1972).

Although TP is not inhibited by the protease inhibitor STI, $PhCH_2SO_2F$, or IAc under the conditions tested, the inhibition observed in the presence of mercury salts and some divalent cations, the inactivation by *o*-phenanthroline, and the inhibitory effect of high concentrations of EDTA on TP activity toward Lys_3 and Met-Gly-Gly suggest that TP is a metalloenzyme.

The bell-shaped curve (Figure 6) observed for Lys_3-cleaving activity of TP as a function of pH (optimum pH 8.5) and the apparent K_m value of 5.3 mM for Met-Gly-Gly cleavage (Figure 5) are similar to values found for the action of other peptidases (Lorand, 1976; Perlmann & Lorand, 1970; Schiller et al., 1977).

It is of interest that Miller & MacKinnon (1974) were able to produce mutants of *Salmonella typhimurium* deficient in a number of peptidases which appear to be analogous to several of the *E. coli* peptidases studied here [See also, Simmonds et al. (1976)]. Yet Miller & MacKinnon did not obtain mutants lacking activity ascribable to *E. coli* TP, which in *Salmonella* appears to be associated with a band of activity variably appearing at about R_f 0.4 in electrophoresis gels. The data for *Salmonella* and the obvious similarities between *Salmonella*

and *E. coli* raise the possibility that TP may be a peptidase essential for survival and poses interesting questions as to roles TP may play in vivo in addition to being used for nutritional utilization of tripeptides. However, the existence of multiply-deficient peptidase mutants in *Salmonella* (Miller & MacKinnon, 1974) raises the question whether any given peptidase, including TP, is indeed essential.

In a search for peptidase- deficient *E. coli* mutants, inhibition of growth by Lys_3 was used as the screening procedure that led to the isolation of strain AJ005 (Sussman & Gilvarg, 1970). Although Lys_3 can be slowly cleaved by TP, and, under certain conditions, can serve as an exogenous source of Lys for AJ005 (Simmonds et al., 1976), it does appear to be inhibitory to growth of strain AJ005 in liquid cultures even when adequate Lys is provided (Sussman & Gilvarg, 1970). If this Lys_3 toxicity is caused by inhibition of protein synthesis at the translational level due to accumulation of tripeptides with multicharged side chains, as suggested by Gilvarg & Levin (1972), then tripeptides such as Lys-Gly-Gly and Lys-Ala-Ala may not be toxic. Both peptides are cleaved by TP faster than is Lys_3 (Table II) and may serve as readily utilized exogenous sources of Lys for strain AJ005. From the fact that TP appears to be the AJ005 peptidase capable of rapidly cleaving Lys-Gly-Gly, failure to utilize this peptide as a Lys source for growth might provide a useful test to screen for mutants which lack TP.

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