

Proline-Specific Endopeptidases from Microbial Sources: Isolation of an Enzyme from a *Xanthomonas* sp.

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An extensive screening among microorganisms for the presence of post-proline-specific endopeptidase activity was performed. This activity was found among ordinary bacteria from soil samples but not among fungi and actinomycetes. This result is in contrast to the previous notion that this activity is confined to the genus *Flavobacterium*. A proline endopeptidase was isolated from a *Xanthomonas* sp. and characterized with respect to physicochemical and enzymatic properties. The enzyme is composed of a single peptide chain with a molecular weight of 75,000. The isoelectric point is 6.2. It is inhibited by diisopropylfluorophosphate and may therefore be classified as a serine endopeptidase. The activity profile is bell shaped with an optimum at pH 7.5. By using synthetic peptide substrates and intramolecular fluorescence quenching it was possible to study the influence of substrate structure on the rate of hydrolysis. The enzyme specifically hydrolyzed Pro-X peptide bonds. With Glu at position X, low rates of hydrolysis were observed; otherwise the enzyme exhibited little preference for particular amino acid residues at position X. A similar substrate preference was observed with respect to the amino acid residue preceding the prolyl residue in the substrate. The enzyme required a minimum of two amino acid residues toward the N terminus from the scissile bond, but further elongation of the peptide chain by up to six amino acid residues caused only a threefold increase in the rate of hydrolysis. Attempts to cleave at the prolyl residues in oxidized RNase failed, indicating that the enzyme does not hydrolyze long peptides, a peculiar property it shares with other proline-specific endopeptidases.

Over the past 20 years a number of serine and thiol endopeptidases that cleave specifically on the carboxyl side of particular amino acid residues in peptides and proteins have been identified. These enzymes should be distinguished from the special-purpose endopeptidases (involved in, e.g., hormone processing and blood clotting) that recognize several amino acid residues in the substrate. Enzymes with specificity for cleavage at the carboxyl side of Asp and Glu (6, 11, 18), Glu (25, 31), Lys (13), Arg (7), Val (1), Gly (5), and Pro (2, 10, 20, 21, 23, 34, 36-38, 40) have been described. These enzymes are of interest from the point of view of understanding the nature of their high specificity, but they have also been used for specific cleavage of fusion proteins and for synthesis of peptide bonds.

Due to the unusual structure of proline, most endopeptidases hydrolyze Pro-X bonds at extremely low rates; this has increased the interest in proline-specific enzymes. Such enzymes are commonly found in small quantities in plants (21, 37, 38) and in mammalian organs (2, 10, 20, 23, 29, 34), where they appear to function in the degradation of numerous proline-containing peptide hormones (12, 15, 26-28). In contrast, they have been reported to be rare in microorganisms; extensive screening only identified a single genus, *Flavobacterium*, with such activity (39). We here report the result of a new screening that revealed members of other bacterial genera that produce proline-specific endopeptidases, the isolation of a proline-specific enzyme from a *Xanthomonas* sp., and a detailed description of its enzymatic properties.

The binding site notation used herein is that of Schechter and Berger (22). Accordingly, the binding sites are denoted S₁, S₂, . . . S_i in the amino-terminal direction from the

scissile bond and S₁', S₂', . . . S_i' in the C-terminal direction. The substrate positions are denoted with a corresponding P.

MATERIALS AND METHODS

Materials. Morpholineethanesulfonic acid (MES), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), Bicine [*N,N*-bis(2-hydroxyethyl)glycine], Ches [2-(*N*-cyclohexylamino)ethane sulfonic acid], Caps [3-(cyclohexylamino)-1-propane sulfonic acid], Tris, carbohydrates, and FAST GARNET were obtained from Sigma Chemical Co. The growth media were from Difco Laboratories. Chromatographic materials and polyacrylamide gels were obtained from Pharmacia, Sweden. Z-Gly-Pro-β-naphthylamide (BNA), (Z-Gly-Pro-*p*-nitroanilide (pNA), and amino acid derivatives for peptide synthesis were purchased from Bachem, Switzerland. All other reagents and solvents were from Merck, Germany. *Streptomyces* and *Nocardia* strains were gifts from J. Wiczorek, Wroclaw, Poland, and *Aspergillus*, *Penicillium*, and *Fusarium* strains were gifts from O. Filtenborg, Copenhagen, Denmark. Yeast strains were obtained from the Slovak Academy of Science, Yeast Strain Collection, Czechoslovakia.

Isolation of microorganisms and fermentation. All pure lines of microorganisms from culture collections were cultivated in an organic medium (1% Bacto-Peptone [Difco], 0.5% yeast extract, 0.2% Tryptone, 0.5% NaCl [pH 6 to 7]) at 28°C for 24 to 48 h with reciprocal shaking. After cultivation, the filtrates and cell suspensions were assayed for activity toward Z-Gly-Pro-βNA as described below.

To isolate bacteria that produce post-proline-specific endopeptidase (PPSE), soil samples were spread over agar plates (1% Bacto-Peptone, 0.2% yeast extract, 0.2% tryptone, 0.6% NaCl [pH 7.2]). After incubation for 2 to 3 days at 28°C, cultures were replica plated and the growth was

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allowed to continue until the colonies had reached a sufficient size. Then 4 ml of 0.6% melted agar–0.25 M Tris-HCl (pH 7.5) and 2 ml of 0.25 mM Z-Gly-Pro-βNA in 40% dimethyl sulfoxide were mixed and poured onto the agar plate. After incubation for 30 min at 25°C, 6 ml of FAST GARNET (3 g/liter) was spread over the petri dish. The evolution of a red color indicated the presence of activity toward the selected substrate, and the corresponding colonies were consequently picked from the master plate and replated on new agar plates. The isolated microorganisms were kept at –20°C in 20% glycerol. Selected strains were identified at the Czechoslovak Collection of Microorganisms, Brno, and at the National Collection of Industrial Bacteria, Aberdeen, Scotland.

A 100-μl sample of a *Xanthomonas* sp. (identified at the Czechoslovak Collection of Microorganisms) cell suspension (in glycerol) was transferred directly from the glycerol into 100 ml of culture medium consisting of 1% tryptone, 0.5% soybean flour, 0.5% NaCl, 0.2% CaCl₂, MnSO₄ (2 μg/ml), and ZnSO₄ (1 μg/ml) (pH 7.2). The first preculture was grown to the late-logarithmic phase (24 h) on a rotary shaker and shifted to 1 liter of the same medium. After an additional 24 h the second preculture was transferred to a 20-liter Chemap fermentor type CF with a 15-liter working volume. The cultures were grown at 28°C with an aeration rate of 60 liters per h at 400 rpm. The initial pH was 7.2 and was not adjusted further. After 28 h, the cells were harvested by centrifugation and stored frozen at –20°C.

Enzyme assays and protein concentration. Cultivation filtrates were assayed against 2.45 ml of 0.02 M Tris-HCl–1 mM EDTA (pH 7.5), 20 μl of Z-Gly-Pro-βNA (5 mM in methanol), and 30 μl of enzyme solution at 25°C. The increase in fluorescence at 410 nm after excitation at 340 nm was monitored with time.

During purification the following spectrophotometric assay mixture was used: 970 μl of 0.02 M Tris-HCl–1 mM EDTA (pH 7.5), 10 μl of Z-Gly-Pro-pNA (8 mM in methanol), and 20 μl of enzyme solution at 25°C. The increase in A_{410} was monitored with time. A difference in the extinction coefficient at 410 nm ($\Delta\epsilon_{410}$) of $10,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used. One unit is the amount of enzyme necessary to cleave 1 μmol of substrate in 1 min at 25°C. The protein concentration was determined spectrophotometrically at 280 nm. An A_{280} of 2.1 was considered to indicate a protein concentration of 1 mg/ml; this value was derived from the amino acid composition of the purified enzyme.

Purification of the enzyme. The harvested *Xanthomonas* cells were suspended in 500 ml of 0.02 M Tris-HCl–1 mM EDTA–20% sucrose–1 mg of lysozyme per ml (pH 8.5). The suspension was stirred for 30 min and then centrifuged. The cell contents were extracted by the addition of 150-ml portions of buffer (initially 0.02 M Tris-HCl, 50 mM EDTA, 0.1% Triton X-100 [pH 7.5]) and then 10 consecutive extractions with 0.02 M Tris-HCl–1 mM EDTA (pH 7.5). Polyethyleneimine (6.67 ml) was added to the extract to precipitate nucleic acids, and after 10 min the precipitate was removed by centrifugation. Ammonium sulfate was added to 40% saturation, and the mixture was stirred for 16 h. The concentration of ammonium sulfate in the supernatant was increased to 80% saturation, and after centrifugation the precipitate was dissolved in 250 ml of 0.02 M Tris-HCl (pH 7.5) and then applied to a Phenyl-Sepharose CL-4B column (5 by 30 cm) equilibrated with 0.02 M Tris-HCl–0.6 M ammonium sulfate (pH 7.5). The column was washed with the equilibration buffer until the A_{280} was <0.01, and elution was accomplished with a gradient from 0.6 to 0 M ammo-

nium sulfate in the same buffer (two 3-liter volumes). The enzyme eluted around 0.25 M ammonium sulfate, and the fractions containing the activity were pooled, concentrated with a 300-ml ultrafiltration cell and a YM 10 membrane from Amicon, and then dialyzed against 0.02 M Tris-HCl (pH 8.5). The sample was applied to a DEAE-Sepharose Fast Flow column (5 by 25 cm) equilibrated with 0.02 M Tris-HCl (pH 8.5). The column was washed with the same buffer until the A_{280} was <0.01. Elution was accomplished with a gradient from 0 to 0.05 M NaCl in the same buffer (two 3-liter volumes). The enzyme eluted at around 0.01 M; the fractions containing activity were pooled, adjusted to 1 M ammonium sulfate (pH 7.5), and applied to a Butyl-Fractogel column (1.6 by 23 cm) equilibrated with 0.02 M Tris-HCl–1 M ammonium sulfate (pH 7.5). The column was washed until the A_{280} was <0.04 and then eluted with a gradient from 1 to 0 M ammonium sulfate (two 500-ml volumes). The enzyme eluted at around 0.6 M ammonium sulfate, and the fractions containing activity were dialyzed against 0.02 M Tris-HCl (pH 8.5). The sample was applied to a DEAE-Sepharose Fast Flow column (2.6 by 15 cm) equilibrated and eluted with a salt gradient as described above (two 500-ml volumes). The enzyme was concentrated with equipment from Amicon and kept at 4°C.

Enzyme characterization. The molecular weight of the native enzyme was determined by gel filtration chromatography on Sephadex G-150. The column (2.6 by 90 cm) was equilibrated with 0.02 M Tris-HCl–0.1 M NaCl (pH 7.5) and calibrated with standard proteins. The purity and molecular weight of the isolated enzyme with or without reduction with dithiothreitol were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with a 10 to 15% polyacrylamide gradient gel. The isoelectric point of the pure enzyme was determined in a polyacrylamide gel (1 mm thick, 10% glycerol slabs, Pharmlyte, pH 3 to 9 and 5 to 8). The amino acid composition was determined after hydrolysis with 6 N HCl at 110°C for 24, 48, and 72 h.

The stability and pH profile of the activity toward Z-Gly-Pro-pNA were studied with 0.025 M buffer–0.1 M NaCl and the following buffers: pH 4 to 5, sodium acetate; pH 5.5 to 6.5, MES; pH 6.5 to 7.7, HEPES; pH 7.8 to 8.5, Bicine; pH 8.8 to 9.7, Ches; pH 9.9 to 10.7, Caps. The stability of the enzyme was estimated by incubating the enzyme at 25°C in these buffers and monitoring the activity over time. The stability at elevated temperatures was studied in 0.02 M Tris-HCl–0.1 M NaCl (pH 7.5).

The K_m for the hydrolysis of Z-Gly-Pro-pNA was estimated from initial rates at different substrate concentrations. The substrate preference of the enzyme was determined by hydrolysis of a series of peptides containing an anthraniloyl (ABz) and a Tyr(NO₂) group, a recently described donor-acceptor pair for intramolecular fluorescence quenching (14), allowing peptide cleavage to be monitored by the release of ABz fluorescence. The substrates were synthesized as previously described (14) and exhibited a purity exceeding 95%. The assay mixture consisted of 2.45 ml of 0.025 M HEPES, 0.1 M NaCl, 1 mM EDTA (pH 7.5), 50 μl of substrate (approximately 20 μM in dimethylformamide), and 5 to 50 μl of enzyme solution. The fluorescence at 420 nm after excitation at 320 nm was monitored with time. k_{cat}/K_m values were determined from initial rates using the formula $k_{cat}/K_m = v_0/(S_0 E_0)$, which is valid when S_0 is much less than K_m . The determination was performed at a minimum of two different concentrations to secure that the employed substrate concentration (0.1 to 0.4 μM) is far below the K_m . The cleavage site was determined with

TABLE 1. Purification of PPSE from a *Xanthomonas* sp.

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	% yield	Purification (fold)
Crude extract	25,300	2,300	0.090	100	1
Dissolved ammonium sulfate precipitate	2,500	1,700	0.69	74	7.5
Phenyl-Sepharose	1,290	1,500	1.1	65	13
DEAE-Sepharose	38	1,100	30	48	320
Butyl-Fractogel	4.4	600	140	26	1,500
DEAE-Sepharose	2.7	440	160	19	1,800

ABz-Gly-Ala-Phe-Pro-Ala-Tyr(NO₂)-Asp-OH by separation of the products by high-performance liquid chromatography (HPLC) with equipment from Waters Vydac and a C₁₈ reverse-phase column and trifluoroacetic acid-CH₃CN as the eluent. The reaction was performed as follows. A 50- μ l sample of the substrate (5 mM in dimethyl formamide) was added to 500 μ l of 0.025 M HEPES-0.1 M NaCl-1 mM EDTA (pH 7.5), and 10 μ g of PPSE was added; when about 75% of the substrate had been consumed, the reaction products were separated and identified by amino acid analysis.

Oxidized RNase prepared as previously described (4) was digested as follows: 1.6 mg of oxidized RNase was dissolved in 250 μ l of 0.05 M Bicine (pH 8.0), 15 μ l of PPSE (0.3 mg/ml) was added, and aliquots were taken after 1 and 24 h for separation by HPLC as described above.

The effect of inhibitors on enzymatic activity was investigated by incubating the enzyme in 0.02 M Tris-HCl (pH 7.5) containing 0.1 mM diisopropylfluorophosphate, 3 μ M HgCl₂, 1 mM *o*-phenanthroline, or 1 mM EDTA. After 30 min, Z-Gly-Pro-pNA was added, and the enzyme activity was determined.

RESULTS AND DISCUSSION

The search among yeasts, filamentous fungi, and *Streptomyces* and *Nocardia* spp. did not result in identification of any organism that produced enzymes with activity toward Z-Gly-Pro- β NA in spite of the high sensitivity of the test. However, six bacterial strains with PPSE activity were isolated from soil samples and identified as *Bacillus sphaericus*, *Flavobacterium multivorum*, *Xanthomonas* sp., *Serratia liquefaciens*, *Pseudomonas* sp., and *Staphylococcus epidermidis*. This suggests that the distribution of such activities is not as narrow as previously reported; in a previous study the activity was only found in species of the genus *Flavobacterium* (39). On the basis of the media used for the screening of soil samples, the growth conditions were optimized with the *Xanthomonas* sp. Mono- and disaccharides, amino acids, Bacto-Peptone, and yeast extract suppressed the production of enzyme, whereas tryptone and soybean flour had a beneficial influence (data not shown). Under the optimal conditions, the level of enzyme was increased eightfold.

The intracellular *Xanthomonas* enzyme with activity toward Z-Gly-Pro- β NA was extracted from the lysed cells by treatment with Triton X-100 and EDTA. The enzyme was subsequently purified to homogeneity by a combination of ammonium sulfate fractionation, hydrophobic interaction chromatography, and ion-exchange chromatography. The purification was 1,800-fold; 2.7 mg of enzyme was isolated, corresponding to a yield of 19%, from the cells obtained from 15 liters of culture broth (Table 1).

The isolated enzyme showed a single band on SDS-polyacrylamide gel electrophoresis with a mobility corresponding to a molecular weight of 75,000 in the presence or absence of dithiothreitol (Fig. 1). The molecular weight of the native enzyme was 74,000 as determined by gel filtration on Sephadex G-150, indicating that the enzyme is a monomer with a molecular weight of around 75,000. This value corresponds to the 68,000 to 77,000 previously determined for proline-specific enzymes from plants (21, 37, 38), mammalian tissues (10, 20, 23, 36), and *Flavobacterium meningosepticum* (32, 40). The isoelectric point was 6.2, as compared with 9.6 for the *Flavobacterium* enzyme (32). The amino acid composition (Table 2) was similar to that of the *Flavobacterium* enzyme (32), suggesting that the two enzymes are related. The sequence determination on PPSE failed to produce any phenylthiohydantoin amino acids, suggesting that the enzyme is N-terminally blocked.

PPSE was stable at 25°C for at least 4 h at pH values between 6 and 8.5. At pH 5 and 9.5 the enzyme lost its activity with half-lives of 6 and 12 h, respectively. The rate of hydrolysis of Z-Gly-Pro-pNA increased with pH, reached a maximum at pH 7.5, and then declined (Fig. 2). Lack of substrate solubility prevented accurate determination of the K_m , but at pH 7.5 it was estimated to be larger than 0.3 mM; the profile therefore reflects the pH dependence of k_{cat}/K_m . The pH profile is consistent with the hydrolysis being dependent on the deprotonation of a group on the enzyme with a pK_a around 6.2 and on the protonation of a group with a pK_a around 9.2. A similar pH profile was observed with the enzymes from rat brain (2) and *F. meningosepticum* (40).

PPSE was inhibited by diisopropylfluorophosphate, like other proline-specific endopeptidases (10, 20, 21, 23, 30, 34-37, 40), and it may therefore be classified as a serine protease. The activity of PPSE appears not to be dependent on thiol groups, since it was not sensitive to mercuric ions. This corresponds to the *Flavobacterium* enzyme (40) but differs from the mammalian enzymes, in which there is apparently a thiol group at the active site (2, 12, 17, 19, 23, 24, 30), although it appears not to be essential (19). These mammalian proline specific endopeptidases may belong to



FIG. 1. SDS-polyacrylamide gel electrophoresis of the purified PPSE. Silver staining was used.

TABLE 2. Amino acid composition of PPSE from a *Xanthomonas* sp.

Amino acid	Amino acid residues per 75,000
Asp	85
Thr ^a	48
Ser ^a	48
Glu	67
Gly	68
Ala	39
Val ^b	42
Met	6
Ile ^b	36
Leu	47
Tyr	36
Phe	32
His	10
Lys	57
Arg	17
Pro	24
Trp ^c	13
Cys ^d	4

^a Extrapolated to zero time.^b Value after 72 h of hydrolysis.^c Determined by the method of Goodwin and Morton (8).^d Determined as cysteic acid after oxidation with performic acid (9).

the group of thiol-dependent serine endopeptidases, e.g., proteinase K from *Tritirachium album* (3).

The substrate specificity of PPSE was investigated by the release of the intramolecularly quenched fluorescence after hydrolysis of synthetic peptides containing the ABz and Tyr(NO₂) groups (14) (Table 3). Substrates without Pro but with Ser, Ala, Phe, Arg, or Glu at potential cleavage positions were not hydrolyzed by PPSE. Thus, PPSE appears to be specific for Pro as opposed to other proline-specific enzymes, which have been reported to slowly hydrolyze at Ala as well (15, 17, 24, 33, 40). Substrates containing Pro were readily hydrolyzed by the enzyme; amino acid analysis of the isolated products from the hydrolysis of ABz-Gly-Ala-Phe-Pro-Ala-Tyr(NO₂)-Asp-OH (Fig. 3) showed that the site of cleavage was only on the carboxyl side of Pro. PPSE accepted hydrophobic and basic amino acid residues at the P₁' and P₂ positions, whereas substrates with Glu in these positions were hydrolyzed with much

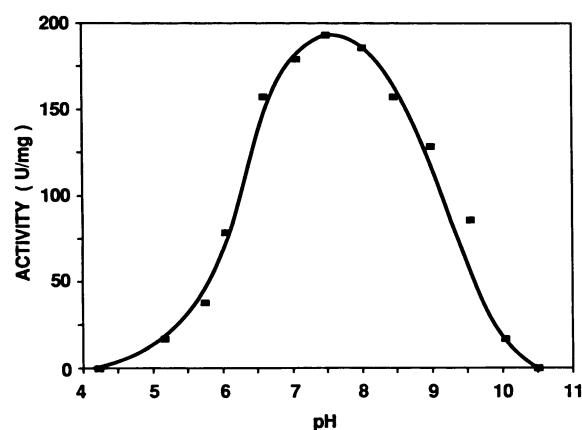


FIG. 2. Influence of pH on the PPSE-catalyzed hydrolysis of Z-Gly-Pro-pNA.

TABLE 3. Substrate preference of PPSE

Substrate	k_{cat}/K_m (min ⁻¹ mM ⁻¹)
Substrates without proline	
ABz-Gly-Ala-Ser-Ala-Phe-Gly-Tyr(NO ₂)-Asp-OH	<10
ABz-Gly-Ala-Ala-Arg-Phe-Gly-Tyr(NO ₂)-Asp-OH	<10
ABz-Gly-Ala-Ala-Glu-Phe-Gly-Tyr(NO ₂)-Asp-OH	<10
ABz-Gly-Ala-Ala-Phe-Phe-Gly-Tyr(NO ₂)-Asp-OH	<10
Substrates with substitutions at the P ₁ ' position	
ABz-Gly-Ala-Ala-Pro-Gly-Tyr(NO ₂)-Asp-OH	29,300 ± 2,100
ABz-Gly-Ala-Ala-Pro-Ala-Tyr(NO ₂)-Asp-OH	29,000 ± 1,100
ABz-Gly-Ala-Ala-Pro-Val-Tyr(NO ₂)-Asp-OH	43,000 ± 1,900
ABz-Gly-Ala-Ala-Pro-Phe-Tyr(NO ₂)-Asp-OH	71,400 ± 2,700
ABz-Gly-Ala-Ala-Pro-Glu-Tyr(NO ₂)-Asp-OH	4,420 ± 270
ABz-Gly-Ala-Ala-Pro-Gln-Tyr(NO ₂)-Asp-OH	31,800 ± 1,800
ABz-Gly-Ala-Ala-Pro-Arg-Tyr(NO ₂)-Asp-OH	41,900 ± 2,600
Substrates with substitutions in the P ₂ position	
ABz-Gly-Ala-Ala-Pro-Ala-Tyr(NO ₂)-Asp-OH	29,000 ± 1,100
ABz-Gly-Ala-Phe-Pro-Ala-Tyr(NO ₂)-Asp-OH	31,800 ± 1,400
ABz-Gly-Ala-Glu-Pro-Ala-Tyr(NO ₂)-Asp-OH	2,750 ± 190
ABz-Gly-Ala-Arg-Pro-Ala-Tyr(NO ₂)-Asp-OH	42,500 ± 2,100
ABz-Gly-Ala-Pro-Pro-Ala-Tyr(NO ₂)-Asp-OH	47,400 ± 1,000
Substrates with different chain lengths	
ABz-Pro-Ala-Tyr(NO ₂)-Asp-OH	<10
ABz-Ala-Pro-Ala-Tyr(NO ₂)-Asp-OH	16,400 ± 1,300
ABz-Ala-Ala-Pro-Ala-Tyr(NO ₂)-Asp-OH	14,400 ± 700
ABz-(Gly-Ala) ₁ -Ala-Pro-Ala-Tyr(NO ₂)-Asp-OH	29,000 ± 1,100
ABz-(Gly-Ala) ₃ -Ala-Pro-Ala-Tyr(NO ₂)-Asp-OH	45,900 ± 1,100

lower rates. Similar studies have not previously been performed with other proline-specific endopeptidases.

The peptide ABz-Pro-Ala-Tyr(NO₂)-Asp-OH was not hydrolyzed. Elongation of the peptide chain by a single amino acid residue, i.e., ABz-Ala-Pro-Ala-Tyr(NO₂)-Asp-OH,

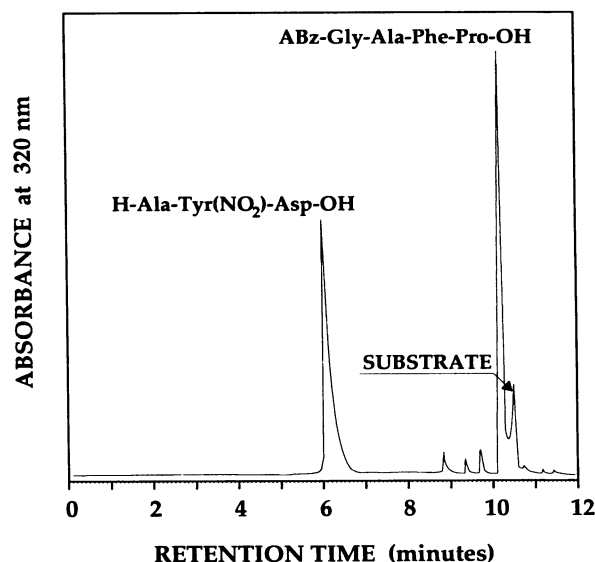


FIG. 3. HPLC separation of the products from the PPSE-catalyzed hydrolysis of ABz-Gly-Ala-Phe-Pro-Ala-Tyr(NO₂)-Asp-OH. A linear gradient from 0.1% trifluoroacetic acid–10% CH₃CN to 0.1% trifluoroacetic acid–90% CH₃CN over 10 min and a flow rate of 1 ml/min were used. The separation was monitored at 320 nm.

caused a dramatic increase in the rate of hydrolysis, but further elongation of the peptide chain had only a small beneficial effect on the rate of hydrolysis. Thus, it appears that interactions between the enzyme and the P₂-P₃ region of the substrate is particularly important for catalysis, whereas interactions more remote from the scissile bond appear less important. Similar results were previously obtained with the proline-specific enzyme from lamb kidney (29) and *F. meningosepticum* (40).

Oxidized RNase containing four Pro residues among the 121 amino acid residues was not hydrolyzed by PPSE, suggesting that the enzyme does not act on long peptides. Similar results were obtained with enzymes from mammalian sources (15, 16, 26, 34). With the enzyme from porcine muscle, substrate molecular weights below 3,000 were required to obtain hydrolysis (16). It has been speculated (15) that this property of the proline-specific endopeptidases from mammalian sources limits their action in vivo to relatively short peptide hormones containing proline and prevents digestion of longer peptides and proteins. A similar function of a bacterial enzyme is not probable. It is more likely that the lack of activity toward long peptide substrates is due to conformational requirements that only can be met by short peptides.

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