A Novel Assay Method for Aminopeptidase P and Partial Purification of Two Types of the Enzyme in *Escherichia coli*[†]

Tadashi YOSHIMOTO, Nobuhiro MURAYAMA and Daisuke TSURU

*School of Pharmaceutical Sciences, Nagasaki University,
Nagasaki 852, Japan*

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A new assay for aminopeptidase P was established by coupling with proline iminopeptidase using Gly-Pro-chromogen (e.g. Gly-Pro- β -naphthylamide, Gly-Pro-*p*-nitroanilide, or Gly-Pro-4-methyl coumarin amide) as the substrate. With each substrate, a linear relationship was established between the enzyme amounts and color development or fluorescence due to the chromogen released. This assay method did not suffer from interference by materials in culture broth. By using this assay method, aminopeptidase P was partially purified from *Escherichia coli* HB101 by chromatographies on DEAE-Sephadex and high performance liquid chromatography (HPLC). On the chromatogram with a DEAE-Sephadex column, two peaks of aminopeptidase P were observed and were named APP-I and APP-II. APP-I was further purified by HPLC using DEAE-5PW and Phenyl-5PW columns. Optimum pHs of APP-I and APP-II were 8.0 and 9.0, respectively. In contrast to APP-I which was stable around pH 10, APP-II was stable at pH 8 to 9. After incubation for 30 min at pH 8.0, fifty percent of the remaining activity of APP-I and APP-II were observed at 60°C and 50°C. APP-I and APP-II were activated 3-fold by the addition of 5 and 30 μ M Mn²⁺. They were inhibited by EDTA, and reactivated by adding Mn²⁺. The molecular weights of APP-I and APP-II were 350,000 and 210,000, respectively. Each enzymes released the amino terminal amino acid when proline is at the penultimate position. The velocity of hydrolysis by the enzymes was not significantly different for most X-Pro bonds (X=amino acid) of peptides except for Pro-Pro bond. APP-II hydrolyzed penta-(Pro-Pro-Gly) at a much higher rate than APP-I, suggesting the aminopeptidase P reported by Yaron and Mlynar (*BBRC*, 32, 658 (1968)) to be APP-II.

Aminopeptidase P (EC 3.4.11.9) is a peptidase which hydrolyzes the amino acid-proline bond when proline is in the penultimate position from the amino terminal. The enzyme has been found in *Escherichia coli*,¹⁾ *Neisseria gonorrhoeae*²⁾ and pig kidney.^{3,4)} Yaron *et al.*¹⁾ and Dehm and Nordwig³⁾ assayed the enzyme activity by the ninhydrin method using poly-proline and Gly-Pro-Hyp as substrates, respectively. Chen and Buchanan²⁾ detected enzyme activity by high voltage electrophoresis. Color development by

ninhydrin reaction is sometimes very troublesome, especially when enzyme preparations are crude, and high voltage electrophoresis is not fit for a routine assay method of the enzyme activity. Due to these reasons, the enzymatic characteristics and protein structure have remained unclear.

We have previously reported that aminopeptidase N in *E. coli*⁵⁾ has not only arylamidase activity but also peptidase activity which is supposedly concerned with peptide catabolism in *E. coli*. However, this enzyme

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Abbreviations: MCA, 4-methyl-coumaryl-7-amide; 2-NNap, γ -naphthylamides; pNA, *p*-nitroanilide; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethane sulfonylfluoride; DFP, diisopropylphosphoro fluoridate; EDTA, ethylenediaminetetra acetate; Hyp, hydroxyproline.

All optically active amino acids are L-configuration.

cannot hydrolyze amino acid-proline bonds and, therefore, cooperation of aminopeptidase P and proline iminopeptidase seems necessary for complete hydrolysis of proline-containing peptides. From this point of view, aminopeptidase P seemed to be important for processing amino terminal amino acids.

This paper describes the enzyme assay method and partial purification of aminopeptidase P from *E. coli*, adopting this method to assay the activity.

MATERIALS AND METHODS

Materials. *p*-Chloromercuribenzoate (PCMB) and proline iminopeptidase from *Bacillus coagulans*⁶⁾ were purchased from Nakarai Chemicals. Bovine pancreas trypsin and Fast Garnet GBC salt were from the Sigma Chemical Co. Gly-Pro- β -naphthylamide (Gly-Pro-2-NNap) was obtained from Novabiochem. Gly-Pro-4-methyl-coumaryl-7-amide (Gly-Pro-MCA), Gly-Pro-*p*-nitroanilide (Gly-Pro-pNA), bestatin, amastatin, and peptides were from the Protein Research Foundation, Minoh. Horse heart muscle catalase, bovine pancreas chymotrypsinogen, and rabbit heart muscle aldolase were obtained from Boehringer-Mannheim. Ala-Pro-pNA, Glu-Pro-pNA, His-Pro-pNA, Lys-Pro-pNA, and Ser-Pro-pNA were kindly supplied by Dr. A. Barth, Martin-Luther University. Dipeptidyl aminopeptidase IV from lamb kidney⁷⁾ and proline-specific endopeptidase from *Flavobacterium meningosepticum*⁸⁾ were the same samples used previously. Hydroxyapatite was prepared by the method of Bernardi.⁹⁾

Assays of aminopeptidase P Activity.

(A) *Gly-Pro-2-NNap substrate.* To 700 μ l of 20 mM Tris-HCl buffer, pH 8.0, were added 100 μ l of 2 mM Gly-Pro-2-NNap dissolved in the same buffer and 100 μ l of enzyme sample. After 15 min of incubation at 37°C, enzyme reaction was stopped by heating in boiling water for 2 min. Proline iminopeptidase (0.3 unit) in 100 μ l of 20 mM Tris-HCl buffer, pH 6.8, was added to the solution. After 30 min, 500 μ l of Fast Garnet GBC solution (1 mg/ml) containing 10% Triton X-100 in 1 M acetate buffer (pH 4.0) was added, and the reaction mixture was left for 25 min at 37°C. The absorbance was measured at 550 nm. One unit of the enzyme activity was defined as the amount of the enzyme that released 1 μ mol of β -naphthylamine per min under the standard conditions.

(B) *Gly-Pro-pNA substrate.* The reaction mixture was composed of 0.2 mM Gly-Pro-pNA and test enzyme in 1 ml of 20 mM Tris-HCl buffer, pH 8.0. After 15 min of incubation at 37°C, the enzyme reaction was stopped by adding 1 ml of 20 mM EDTA in 200 mM Tris-HCl buffer, pH 6.8, then 1 ml of proline iminopeptidase (0.4 unit) was

added. After incubation at 37°C for 30 min, absorbance was measured at 410 nm.

(C) *Gly-Pro-MCA substrate.* To 700 μ l of 20 mM Tris-HCl buffer, pH 8.0, were added 100 μ l of 2 mM Gly-Pro-MCA dissolved in the same buffer and 100 μ l of enzyme sample. The enzyme reaction was stopped by adding 1 ml of 20 mM EDTA in 200 mM Tris-HCl, pH 6.8. After adding 100 μ l of proline iminopeptidase (0.5 units), the solution was incubated at 37°C for 30 min, the fluorescence of 7-amino-4-methylcoumarin released being monitored using a spectrofluorometer with excitation at 370 nm and emission at 440 nm.

Cultivation of *E. coli* HB101. *E. coli* was cultivated in a bouillon medium containing 1% Polypepton, 1% meat extract, and 0.5% NaCl, pH 7.0, at 37°C for 20 hr using a Micro Fermentor, New Brunswick Co. Cells were harvested by centrifugation at 8,000 rpm for 15 min, washed with cold 50 mM borate-Na buffer, pH 9.0, and were used for the enzyme purification.

Purification of Aminopeptidase P. All the purification procedures were done at 4°C. The washed cells (40 g wet weight) were suspended in 200 ml of 20 mM borate-Na buffer, pH 9.0, and disrupted for 10 min with glass beads in a Dyno-Mill. The glass beads were removed by decantation and the disrupted cell suspension was centrifuged to remove intact cells. The intact cells were again treated as above, and the supernatants were combined. After centrifugation at 8,000 rpm for 15 min, the supernatant was fractionated by ammonium sulfate at 40 to 80% saturation. The precipitate was dissolved in a small volume of 20 mM borate-Na buffer, pH 9.0, and desalted by gel filtration on a Sephadex G-25 column, and the enzyme fraction was put on a column (2.5 $\times$ 30 cm) of DEAE-Sephadex equilibrated with the same buffer. The adsorbed enzyme was eluted with an increasing linear gradient of NaCl concentration to 0.7 M. Two peaks (APP-I and APP-II) of active fractions were separately combined and the enzymes were precipitated by 80% saturation of ammonium sulfate. Each enzyme, APP-I and APP-II, was

FIG. 1. Activity Assay Method for Aminopeptidase P Using Gly-Pro-Chromogens as Substrate.

repeatedly chromatographed on DEAE-Sephadex. APP-I was further purified by HPLC after being desalted by Sephadex G-25 (1×40 cm) and concentrated by Amicon ultrafiltration: the enzyme fraction was chromatographed by HPLC using a DEAE-5PW column (7.5×75 mm) with a linear gradient system of NaCl in 20 mM Tris-HCl buffer, pH 7.8, and hydrophobic chromatography on a Phenyl-5PW column (7.5×75 mm) equilibrated with the same buffer. The active fractions were combined and kept at 4°C until use.

Substrate specificity. Hydrolyses of amino acid-Pro-2-NNap and amino acid-Pro-pNA were assayed by the standard assay method. Hydrolyzing sites of peptides and the above substrates were analyzed by TLC using *n*-BuOH-AcOH-H₂O (4:1:2) as a solvent and comparing with authentic markers. The products were visualized by spraying with ninhydrin solution.

Estimation of molecular weight. Molecular weight was estimated by gel filtration using HPLC with a TSK G-3000SW column. Thyroglobulin (669,000), apoferritin (443,000), β -amylase (200,000) and alcohol dehydrogenase (150,000) were used as protein markers.

RESULTS

Assay of aminopeptidase P

The principle of the enzyme activity assay is shown in Fig. 1. The products released by the action of aminopeptidase P were further hydrolyzed by proline iminopeptidase and the chromogenic substances liberated (β -naphthylamine, *p*-nitroaniline, and 4-methyl-7-coumarin) were followed spectrophotometrically. Figure 2 shows the concentration of proline iminopeptidase necessary for complete liberation of β -naphthylamine from Pro-2-NNap, the enzymatic product by the action of aminopeptidase P. One-third of a unit of proline iminopeptidase was found to be enough for assays of aminopeptidase P with three different substrates, Gly-Pro-2-NNap, Gly-Pro-pNA, and Gly-Pro-MCA.

Figure 3 shows the relationship between the amounts of aminopeptidase and β -naphthylamine liberated, indicating the linear relationship between them. The same good linearity was observed between enzyme concentrations and products released for the proline iminopeptidase-coupled methods with the other two chromogenic substrates. These methods

FIG. 2. Effects of the Concentration of Proline Iminopeptidase on Assay of Aminopeptidase P.

Enzyme activity was assayed by the standard assay method except for proline iminopeptidase activity.

made it possible to measure enzyme activity even when the enzyme preparations were crude. The enzyme activity of kidney extract that shows high dipeptidyl aminopeptidase IV activity was also measurable in the presence of DFP by these methods (data not shown).

Purification of aminopeptidase P

The purification procedure for aminopeptidase P is summarized in Table I. The enzyme was eluted at 0.25 and 0.4 M NaCl from a DEAE-Sephadex column (Fig. 4), the enzyme peaks were named APP-I and APP-II, respectively, and APP-I was further purified by HPLC. However, both enzyme preparations were still heterogenous: they were contaminated with three minor impurities, as seen by disc gel electrophoresis.

Enzymatic and physicochemical properties of two aminopeptidase P

The optimum pHs of APP-I and APP-II were 8.0 and 9.0, respectively. APP-I was stable around pH 10 and APP-II was stable at pH 8 to 9. After 30 min at pH 8.0, fifty percent of the remaining activities of APP-I and APP-II were observed at 60°C and 50°C (Table II). The molecular weights of APP-I and APP-II estimated by gel filtration were 350,000 and 210,000, respectively (Fig. 5).

FIG. 3. Relationship between Enzyme Amount and Product Liberation by the Standard Assay Methods Using Gly-Pro-2-NNap (A), Gly-Pro-pNA (B), and Gly-Pro-MCA (C) as Substrates.

See MATERIALS AND METHODS for the experimental procedures.

TABLE I. PURIFICATION OF AMINOPEPTIDASE P FROM *E. coli* HB101

Purification		Total activity (unit)	Specific activity (unit/mg)	Recovery (%)	Purification (fold)
Cell-free extract		106	0.005	100	1
Ammonium sulfate fractionation		95	0.0056	90	1.1
DEAE-Sephadex	APP-I	37.1	0.036	35	7.1
	APP-II	23.5	0.027	22	5.3
2nd DEAE-Sephadex	APP-I	26.7	0.14	25	20.8
	APP-II	14.8	0.08	14	16.1
DEAE-5PW (HPLC)	APP-I	2.61	0.14	2.4	27
Phenyl-5PW (HPLC)	APP-I	1.02	0.21	1	41

Effects of chemicals

Both enzymes were activated 3~4 fold by the addition of 5 μM Mn²⁺ (Fig. 6), and were inhibited by EDTA. The enzymes inactivated with EDTA were reactivated by adding Mn²⁺. Both enzymes were partially inhibited by PCMB but were not affected by DFP, iodoacetic acid, bacitracin, bestatin, leupeptin, pepstatin, puromycin, or E-64.

Substrate specificity

Both enzymes released the amino terminal amino acid from peptides when proline was in

the penultimate position. The velocity of peptide hydrolysis by the enzymes was not significantly different for most X-Pro bonds (X = amino acid) or peptides, except for Pro-Pro bonds. APP-II hydrolyzed (Pro-Pro-Gly)₅ much faster than APP-I.

DISCUSSION

The classical assay method by ninhydrin reaction with poly-proline had some problems: the precise assay of the enzyme activity is impossible, since crude preparations contain

FIG. 4. Chromatogram of Crude Enzyme Preparation of Aminopeptidase P from *E. coli* HB101 on DEAE-Sephadex.

The column (2.5 × 30 cm) equilibrated with 20 mM borate-Na buffer, pH 9.0, was used and the adsorbed enzyme was eluted with an increasing linear gradient of NaCl concentration to 0.4 M. ○---○, absorbance at 280 nm; ●—●, enzyme activity.

TABLE II. SOME ENZYMATIC AND PHYSICOCHEMICAL PROPERTIES OF TWO TYPES OF AMINO-PEPTIDASE P FROM *E. coli* HB101

	APP-I	APP-II
Optimum pH	8.0	9.0
pH stability ^a	10	8~9
Optimum temperature ^b	55°C	41°C
Thermal stability ^c	60°C	50°C
Molecular weight	350,000	210,000

^a 70% remaining activity after incubation at 37°C for 30 min.

^b At pH 8.0.

^c For 50% remaining activity after incubation for 30 min at pH 8.

large amounts of ninhydrin-positive substances and since the substrate is hydrolyzed by an intracellular proline iminopeptidase found in *E. coli*. On the contrary, our assay method using Gly-Pro-chromogen coupled

with proline iminopeptidase can be used not only for a routine assay but also screening for the enzyme activity in crude enzyme preparations. While Gly-Pro-chromogen is also a substrate for dipeptidyl aminopeptidase IV, this activity has not been detected in *E. coli*. These three procedures with three different substrates have some merits. β -Naphthylamine liberated from β -naphthylamide substrate shows a bright red color by coupling with Fast Garnet GBC. Thus, it is very easy to detect the enzyme activity in the crude preparation. The *p*-nitroanilide substrate can be monitored after enzymatic hydrolysis in their reaction tubes. The digestion product, 4-methyl-7-aminocoumarin, from the coumarin substrate is a highly sensitive fluorogenic compound. When this assay method is applied to the other sources which are contaminated with dipeptidyl aminopeptidase IV, it is possible to avoid the

FIG. 5. Estimation of the Molecular Weight of the Enzyme by Gel Filtration on HPLC Using a TSK 3000SW Column.

1, thyroglobulin (MW 669,000); 2, apoferritin (MW 443,000); 3, β -amylase (MW 200,000); 4, alcohol dehydrogenase (MW 150,000). Flow rate, 0.7 ml/min; pressure, 30 kg/cm; temperature, 25°C; solvent, 20 mM Tris-HCl buffer, pH 7.5 containing 0.1 M NaCl; detector, UV monitor at 280 nm.

TABLE III. EFFECTS OF SEVERAL INHIBITORS ON ENZYME FROM *E. coli* HB101

The reaction mixture consisting of 0.1 unit of enzyme, chemicals, or metal salts, in 100 μ l of 50 mM borate-Na buffer, pH 9.0, was incubated at 37°C. After 20 min, the remaining activities were assayed by the standard method using Gly-Pro-2-NNap.

Inhibitors	Conc. (mM)	Remaining activity	
		APP-I	APP-II
Control	—	100	100
EDTA	1	2	1
<i>o</i> -Phenanthroline	1	118	49
DFP	1	97	107
PCMB	0.06	35*	30*
Iodoacetic acid	1	92	86
Bacitracin	1	112	84
Bestatin	1	106	95
Leupeptin	1	87	74
Pepstatin	1	103	60
Puromycin	1	140	91
E-64	1	109	102

* Remaining activity was assayed by the ninhydrin method using (Pro-Pro-Gly)₅ as substrate.

FIG. 6. Effects of Mn on Enzyme Activity of Aminopeptidase P.

After the enzyme was incubated with various concentration of Mn^{2+} at pH 7.5 for 5 min, activities were assayed by standard assay method using Gly-Pro-2-NNap as substrate.

TABLE IV. SUBSTRATE SPECIFICITY OF AMINOPEPTIDASE P FROM *E. coli* HB101

Substrates	Relative rate of hydrolysis	
	APP-I	APP-II
Gly↓Pro-2-NNap	100	100
Gly↓Pro-pNA	170	241
Ala↓Pro-pNA	443	1131
Glu↓Pro-pNA	113	541
His↓Pro-pNA	149	453
Lys↓Pro-pNA	415	986
Ser↓Pro-pNA	150	466
Pro↓Pro-Gly-(Pro-Pro-Gly) ₄	18	354
Gly↓Pro-Ala	+	+
Gly↓Pro-Phe	+	+
Gly↓Pro-Leu-Gly	+	+

The following compounds were not hydrolyzed: Z-Gly-Pro-2-NNap, Z-Pro-2-NNap, Pro-2-NNap, Gly-Ala, Leu-Phe.

enzymatic hydrolysis by adding DEP.

Contrary to Yaron and Mlynar¹⁾ who reported only one enzyme in *E. coli*, we could find two types of the enzymes in *E. coli* HB101. It remains unclear whether this discrepancy is due to the differences in the strains used, purification methods, or enzyme assay methods, but it seems likely that Yaron and Mlynar failed to detect APP-I, since they used poly-proline, which will be a poor substrate for APP-I as seen with oligo Pro-Pro-Gly (Table IV).

Except for Pro-Pro bond hydrolysis by APP-I, the rates of peptide hydrolysis by both enzymes were not significantly different by the kind of amino acids at the P1 site of substrate. Moreover, the enzymes are thermally stable, and higher optimum pH and stability at higher pHs will help for removal of an amino terminal amino acid under denaturing conditions in alkaline conditions, indicating that the enzymes are good tools for processing amino terminals of peptides.

To study the details of enzymatic characteristics and protein structure, we attempted the cloning of the enzymes and succeeded in the expression of APP-II,¹⁰⁾ and are now studying the nucleotide sequencing.

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