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POST-PROLINE DIPEPTIDYL AMINOPEPTIDASE (DIPEPTIDYL AMINOPEPTIDASE IV) FROM LAMB KIDNEY

PURIFICATION AND SOME ENZYMATIC PROPERTIES

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Summary

Post-proline dipeptidyl aminopeptidase (dipeptidylpeptide hydrolase, EC 3.4.14.1), also known as glycylprolyl β-naphthylamidase or dipeptidyl aminopeptidase IV, was isolated and purified in an overall yield of 20% from autolyzed extracts of lamb kidney by CM-cellulose and column chromatography on DEAE-Sephadex and Sephadex G-200. Purified enzyme was homogeneous by disc gel electrophoresis and ultracentrifugal analysis and was most active at pH 7.8 using Gly-Pro β-napthylamide as substrate. The K_m values for Gly-Pro β-naphthylamide and Ala-Ala β-naphthylamide were 0.63 and 0.77 mM, respectively. The proline-containing peptides were hydrolysed more than 10-fold faster. By isoelectric focusing a pI of 4.9 was determined. The enzyme has a sedimentation coefficient of 10 S. The molecular weight of the enzyme was estimated to be 230 000 ± 15 000 by the sedimentation equilibrium method and sodium dodecyl sulfate polyacrylamide gel electrophoresis indicating that the enzyme is composed of two identical subunits with molecular weights of 115 000. It was inhibited by the active-site directed, irreversible inhibitor diisopropylphosphorofluorofluoridate. Post-proline dipeptidyl aminopeptidase, in contrast to the endopeptidase post-proline cleaving enzyme [9,10] (Walter R. (1976) Biochim. Biophys. Acta 422, 138—158, and Koida, M. and Walter, R. (1976) J. Biol. Chem. 251, 7593—7599) exhibits no endopeptidase activity. Instead it is an exopeptidase with a high specificity for NH_2-terminal-free peptides containing a proline residue in the penultimate position and releases the

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Abbreviations used follow the tentative rules and recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature for amino acids and peptides, J. Biol. Chem. (1972) 247, 977 and for enzyme inhibitors as circulated by the Office of Biochemical Nomenclature on July 17, 1975. All optically active amino acids are of L-configuration unless otherwise stated. 2-NNap: β-naphthylamide.

dipeptide with proline being the COOH-terminal moiety. The name "post-proline dipeptidyl aminopeptidase" is suggested.

Introduction

Since the discovery of glycylprolyl β-naphthylamidase by Hopsu-Havu and Glenner from rat liver [1], the enzyme has been purified from this tissue [2], from pig kidney [3], and human submaxillary gland [4] using as the first step the method of autolysis. McDonald et al. [5] confirmed and extended the substrate specificity reported for glycylprolyl β-naphthylamidase and referred to the enzyme as dipeptidyl aminopeptidase IV.

Recently, Kenney et al. [6] concluded that purified dipeptidyl aminopeptidase IV (dipeptidylpeptide hydrolase, EC 3.4.14.1) from pig kidney is a serine peptidase, that it exists as a dimer, and that, in addition to its dipeptidyl aminopeptidase activity, it also shows endopeptidase activity attacking the peptide chain at the carboxyl site of proline and alanine residues. These latter contentions raised the possibility that dipeptidyl aminopeptidase is similar or even identical to post-proline cleaving enzyme discovered in this laboratory in human uterus [7] as well as subsequently in other tissues [8] and purified from lamb kidney [9,10]. Post-proline cleaving enzyme has recently also been identified as a serine protease [11], exists as a dimer [10] and has a specificity for catalyzing the hydrolysis of the Pro-X peptide bond [9,10].

In order to be able to compare the two enzymes in detail dipeptidyl aminopeptidase IV has been purified from lamb kidney and some of its enzymatic properties have been determined. In this study we refer to dipeptidyl aminopeptidase IV as "Post-proline dipeptidyl aminopeptidase", which more specifically reflects its enzymatic properties and sets it apart from post-proline cleaving enzyme.

Materials and Methods

Lamb kidneys, frozen on dry ice, were purchased from Max Cohen, Newark, N.J. The following chemicals were purchased from Sigma Chemical Company: phenylglyoxal, 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs), *N*-acetylimidazole, *N*-ethylmaleimide, 1,10-phenanthroline, *p*-mercuribenzoate, 2-iodoacetamide, *o*-nitrophenyl sulfenylchloride, phenylmethylsulfonylfluoride ($PhCh_2SO_2F$), EDTA, Ala-Ala-Ala, Gly-Ala-Ala, Gly-Pro-Ala, Gly-Pro-Pro, Fast Garnet GBC, β-naphthylamine, Coomassie Brilliant Blue R, apoferritin, β-galactosidase, Sephadex G-200, DEAE-Sephadex A-50 and CM-cellulose. Sodium tetrathionate was obtained from K & K Labs, Inc. Aldolase, ovalbumin, chymotrypsinogen A and ribonuclease were obtained from Pharmacia Fine Chemicals. Ala-Pro-Gly, Z-Pro-Ala, Z-Pro-Asp, Z-Pro-Leu, Z-Pro-Glu, Z-Pro-Phe, Z-Pro-Leu-Gly, Ala-β-naphthylamide (Ala-2-NNap), Gly-Pro-2-NNap, Leu-2-NNap and Ala-Ala-2-NNap were brought from Vega-Fox Biochemicals Company. Gly-Pro-Leu-Gly was prepared from Z-Gly-Pro-Leu-Gly by hydrogenation in methanol according to standard procedures [12]. Z-Gly-Pro-Leu-Gly and Z-Gly-Pro-Leu-Gly-Pro were purchased from the Protein Research Foundation, Japan. Carrier

ampholite was purchased from LKB, diisopropylphosphofluoridate (iPr_2P-F) was obtained from Aldrich.

Purification of Post-proline dipeptidyl aminopeptidase. The cortical tissue of thawed lamb kidney (1.16 kg) was homogenized in 2 liters of water for 1 min by a Virtis 45 Homogenizer. After removing the precipitate by centrifugation (7500 × *g* for 15 min) the supernatant was adjusted to pH 3.8 with 3.2 M H_2SO_4 and then incubated at 37° C for 30 h. The autolysed mixture was then centrifuged (11 000 × *g* for 15 min) and the supernatant added to 150 g (wet weight) of CM-cellulose which was equilibrated with 10 mM sodium acetate buffer, pH 4.7. The mixture was stirred at 4° C overnight. Since the solution had a high viscosity the CM-cellulose was collected after centrifugation (7000 × *g* for 15 min) and washed with the same buffer. The enzyme was eluted by suspending the CM-cellulose-enzyme complex in one liter of 0.05 M phosphate buffer, pH 6.8. The CM-cellulose was removed by centrifugation (7000 × *g* for 15 min) and the enzyme precipitated from the supernatant with solid $(NH_4)_2SO_4$ at 80% saturation at 4° C overnight. The precipitate was collected by centrifugation (7000 × *g* for 15 min) and then desalted using a Sephadex G-25 column (2.5 × 77 cm) equilibrated with 20 mM Tris · HCl, pH 7.8. The desalted preparation was applied to a DEAE-Sephadex A-50 column (3.5 × 40 cm, flow rate 40 ml/h) and eluted with a linear NaCl-gradient (0 to 0.25 M). The fractions (10 ml each) containing the enzymatic activity were combined, the enzyme precipitated with solid $(NH_4)_2SO_4$, redissolved and rechromatographed. Eluted enzyme was precipitated with $(NH_4)_2SO_4$ at 80% saturation, collected by centrifugation, redissolved in 5 ml of 20 mM Tris · HCl, pH 7.8, containing 0.1 M NaCl and then applied onto a Sephadex G-200 column (2 × 100 cm, flow rate 16 ml/h) equilibrated with the buffer. The enzyme preparation was dialyzed for two days against 1 liter water (3 times changed) at 4° C, then lyophilized and stored at −60° C for use in subsequent experiments.

Enzyme activity assay. Enzyme activity was assayed by a slightly modified method of Hopsu-Havu et al. [13]. A 0.25 ml aliquot of enzyme solution was mixed with 0.25 ml of substrate (1 mM Gly-Pro-2-NNap in H_2O) and 1 ml of Tris · HCl buffer (0.2 M, pH 7.8). After incubation at 37° C for 5 min, 0.5 ml of diazonium salt Fast Garnet GBC (0.5 mg/ml in 1 M acetate buffer pH 4.2 containing 10% Triton X-100) was added to the reaction mixture. The color of the diazo dye was measured at 550 nm after incubation at 25° C for 30 min. One unit of enzyme activity is defined as the amount of activity which released 1 μmol of β-naphthylamine per min at 37° C. Protein concentration was assayed by the Lowry method [14] using bovine serum albumin as standard.

Disc electrophoresis. The purity of the enzyme preparation was examined by disc electrophoresis based on the method of Ornstein [15] and Davis [16]. A 7% separation gel in 0.37 M Tris · HCl (pH 8.9) was prepared using ammonium persulfate as the catalyst. For pre-electrophoresis a current of 2 mA/tube was applied to the gels for 1 h in 0.37 M Tris · HCl (pH 8.9). The enzyme preparation (125 μg in 50 μl) was applied onto the gel and a current of 2.5 mA per tube was applied for 2 h at approximately 6° C. Matched pairs of gels were prepared. One of the gels was stained for 30 min for protein in 0.05% Coomassie Brilliant Blue R and destained in 10% trichloroacetic acid overnight. The other was used to evaluate the location of the enzymatic activity. Post-proline dipep-

tidyl aminopeptidase specific staining was carried out by submerging the gel in the presence of 0.5 mM Gly-Pro-2-NNap and 25 mg Fast Garnet GBC in 20 ml of 0.2 M Tris · HCl, pH 7.8, for 5 min at 37°C.

Dependence of amidase activity of post-proline dipeptidyl aminopeptidase on pH. The substrates, Gly-Pro-2-NNap or Ala-Ala-2-NNap (1 mM in 0.25 ml), were incubated in a final volume of 1.5 ml 100 mM buffer of appropriate pH in the presence of the enzyme 94 μg/ml in the case of Gly-Pro-2-NNap (and 40 μg/ml with Gly-Pro-2-NNap) at 37°C for 5 min. The following buffers were used: acetate, pH 5—6; phosphate, pH 6—7; Tris · HCl, pH 7—9; borate, pH 9—10.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. The method of Weber and Osborn [17] was adopted. The enzyme (250 μg in 0.1 ml) was mixed with 0.1 ml of 0.02 M sodium phosphate buffer, pH 7.0, containing 2% sodium dodecyl sulfate, 2% β-mercaptoethanol. Incubation was carried out for 2 h at 37°C. Gels with half of the normal amount of crosslinker (0.3 g of methylenebisacrylamide per 100 ml solution) were used and electrophoresis was carried out at a constant current of 8 mA per gel for 4 h using bromphenol blue as running marker. Gels were stained with 0.28% of Coomassie Brilliant Blue R destained by washing with a mixture of acetic acid/methanol/water (7.5 : 50 : 875, v/v/v).

Isoelectric focusing. The method described by Vesterberg and Svensson [18] was adopted using a Uniphor Column Electrophoresis System 7900, LKB. 10 ml of enzyme solution (11 mg protein) was applied onto a column (250 ml) using carrier ampholite pH 3—10 and a sucrose density gradient from 0—50%. The electrofocusing run was carried out at 300 V for 46 h at 4°C.

Ultracentrifugal analysis. Enzyme was dialyzed against 20 mM Tris · HCl buffer, pH 7.8 containing 0.1 M NaCl at 4°C for 48 h. The dialysed enzyme solution at concentrations 1.2, 1.8, and 3.6 mg/ml (as determined by the biuret method of Gornall et al. [19] and the outer solution were used for the ultracentrifugal analyses using a Spinco Model E Ultracentrifuge equipped with Schlieren and Rayleigh interference optics. Sedimentation velocity experiments were carried out at 20°C at 42 040 rev./min using Schlieren optics and cells with 12 and 30 mm width. Sedimentation-equilibrium experiments were performed at 8°C at a speed of 24 630 rev./min using the 6-channels cells of Yphantis and an initial protein concentration of 1.2 mg/ml in 20 mM Tris · HCl buffer, pH 7.8, containing 0.1 M NaCl. The interference photographs were taken after 14, 16 and 18 h.

Effect of chemical reagents on enzymatic activity. Enzyme solution (10 μg, 0.125 ml) was mixed with 0.125 ml of Tris · HCl buffer, pH 7.8, in the absence or presence of chemicals (0.1 to 1 mM) and preincubated at 25°C for 30 min. Residual enzymatic activity was then determined by the standard assay method for post-proline dipeptidyl aminopeptidase using Gly-Pro-2-NNap as substrate.

Enzyme-catalyzed hydrolysis of peptides. (i) For the qualitative identification of products formed upon treatment with post-proline dipeptidyl aminopeptidase the particular peptide (10 mM in 45 μl of 20 mM Tris · HCl buffer, pH 7.8) was incubated for 4 h at 37°C with 15 μg of the enzyme in 5 μl of the same buffer. A 10 μl aliquot was then applied to high voltage paper electrophoresis (carried out for 2 h at 46 V/cm in pH 3.5 buffer comprised of 5% acetic acid and 0.5% pyridine). Appropriate markers (peptide(s) and/or amino acid)

were also subjected to electrophoretic separation. A cadmium-ninhydrin spray [20] was used to develop the chromatogram. (ii) In order to determine the rates of hydrolysis the reaction mixture containing peptide (1.6 mM) and enzyme (4 or 40 μg/ml) in 20 mM Tris · HCl, pH 7.0, was incubated at 37°C. Aliquots (25 μl) of the mixture were assayed by the ninhydrin method after 2, 5, 10, 20, 40, 60, 90, and 120 min incubation. The initial rate of hydrolysis was obtained for each peptide by plotting the amount of products formed as a function of time.

Results

Isolation and purification of Post-proline dipeptidyl aminopeptidase

The enzyme was isolated on a large scale according to Hopsu-Havu et al. [13] as improved by Kenny et al. [6] with some additional modifications. Following the solubilization of the enzyme from cortical tissue of lamb kidney by autolysis [2] the enzyme was partially purified by precipitation with CM-cellulose and subsequent elution with 0.5 M phosphate buffer, pH 6.8 (Table I). After desalting, the enzyme was subjected to ion exchange chromatography on two DEAE-Sephadex columns using a 0 to 0.25 M NaCl gradient for elution and eluted at a mean value of 0.14 M NaCl. The elution pattern obtained after the first chromatographic step is shown in Fig. 1. The enzymatic activity was present in fractions 115—145.

Low-molecular weight components were removed by gel filtration on Sephadex G-200. The enzyme was isolated from fractions 53—60 and, as can be seen from Fig. 2, the 280 nm absorbance curve is practically parallel to the enzymatic activity curve. Post-proline dipeptidyl aminopeptidase was purified about 100-fold with a recovery of 20% starting with the supernatant obtained by autolysis (Table I). The specific activity of purified enzyme under standard conditions was 30.0 units/mg using Gly-Pro-2-NNap as substrate (determined by the Lowry method). The specific activity of the lyophilized preparation was 23.0 units/mg (by dry weight).

Criteria for the purity of post-proline dipeptidyl aminopeptidase

After gel filtration on Sephadex G-200, the enzyme revealed upon disc elec-

TABLE I

PURIFICATION OF POST-PROLINE DIPEPTIDYL AMINOPEPTIDASE

		Volume (ml)	Protein (mg)	Enzyme activity		
				Total units · 10^3	Recovery (%)	Specific activity *
1)	Supernatant of autolysed extract	2100	28 240	7.6	100	0.27
2)	CM-cellulose	950	1 640	5.9	78	3.6
3)	1st DEAE-Sephadex	380	162	3.8	50	23.5
4)	2nd DEAE-Sephadex	290	106	2.7	36	25.5
5)	Sephadex G-200	28	50	1.5	20	30.0

* In units/mg protein.

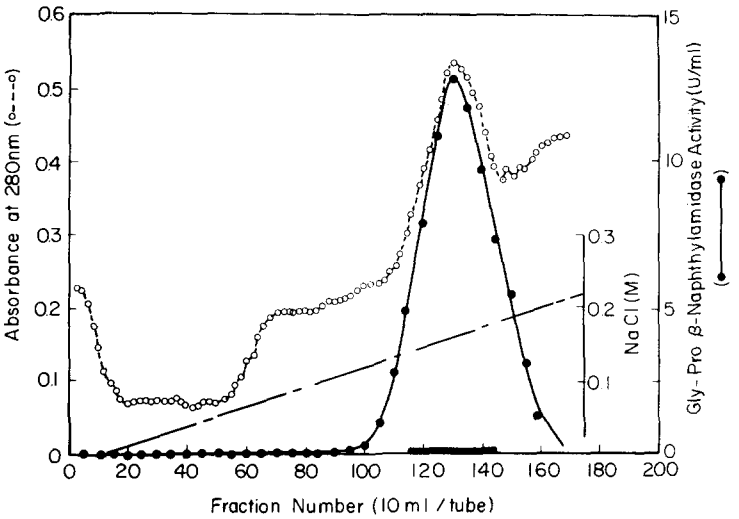

Fig. 1. Chromatpgraphy of post-proline dipeptidyl aminopeptidase on DEAE-Sephadex A-50. The column (3.5 X 40 cm) was equilibrated with 20 mM Tris · HCl buffer, pH 7.8. Elution was performed using a linear gradient of 0.0—0.25 M NaCl in 20 mM Tris · HCl buffer (— · — · —). Flow rate was 40 ml/h. Absorbance was measured at 280 nm (○———○). Enzyme activity was determined under standard conditions as described in the text using Gly-Pro-2-NNap as substrate (●———●).

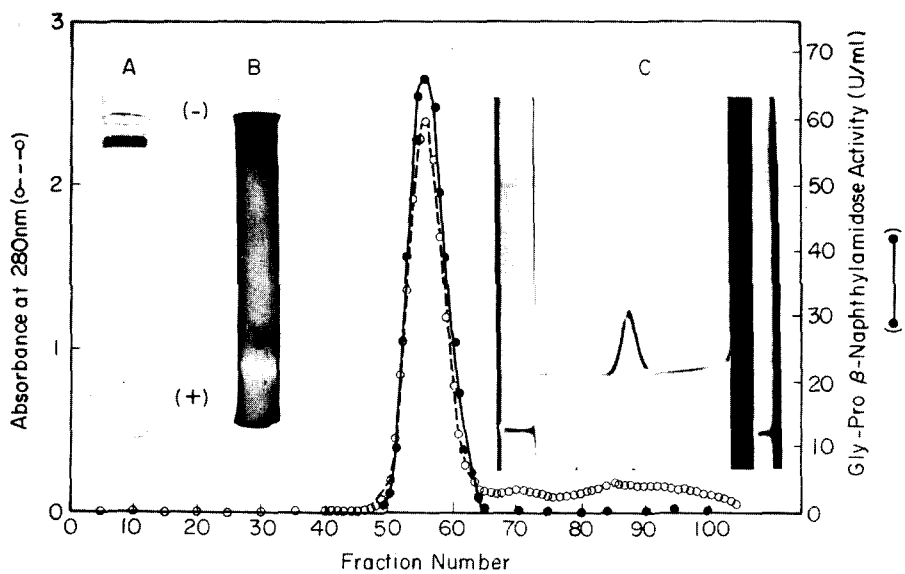

Fig. 2. Gel filtration on Sephadex G-200 and analysis of post-proline dipeptidyl aminopeptidase. The column size was 2 X 100 cm. The flow rate of the 20 mM Tris · HCl buffer, pH 7.8 was 16 ml/h and 3.1 ml-fractions were collected. Absorbance was measured at 280 nm (○- - - - - -○) and the amidase activity was determined using Gly-Pro-2-NNap as substrate (●———●). A, Analytical disc gel electrophoresis of the enzyme; protein concentration applied was 125 μg. Electrophoresis was carried out 2.5 mA for 2 h at 6°C. Protein was stained with 0.05% Coomassie Brilliant Blue R and destained with 10% trichlotoacetic acid; B, Determination of enzyme activity following disc gel electrophoresis as described in (A); enzyme, specific staining was carried out by treatment of the gel with 0.5 mM Gly-Pro-2-NNap solution and 25 mg Fast Garnet GBC in 20 ml of 0.2 M Tris-HCl, pH 7.8, for 5 min at 37°C; C, Ultracentrifugal sedimentation pattern of the enzyme taken after 45 min at a speed of 42 040 rev./min at 20°C. Protein concentration was 3.6 mg/ml.

trophoresis only a single and sharp band (Fig. 2, insert A). Using Gly-Pro-2-NNap as substrate, enzymatic activity is only detected in the very region of the single band (Fig. 2, insert B). As shown in Fig. 2 (insert C) a symmetrical Schlieren pattern was obtained by ultracentrifugal sedimentation.

Effect of pH on the activity of post-proline dipeptidyl aminopeptidase

The enzyme hydrolyzed Gly-Pro-2-NNap faster than Ala-Ala-2-NNap. For Gly-Pro-2-NNap the pH optimum was at 7.8 and for Ala-Ala-2-NNap at 7.5.

Substrate specificity of post-proline dipeptidyl aminopeptidase

The enzyme catalyzes the hydrolysis of the peptide bond at the carboxyl site of a Pro or Ala residue in the penultimate position of an unprotected peptide with at least three residues, of NH_2-terminal-free dipeptide amides or even the amino acid amide itself. K_m values obtained for Gly-Pro-2-NNap and Ala-Ala-2-NNap were 0.62 and 0.77, respectively. However, the rate of hydrolysis of Pro-containing peptides was more than one order of magnitude higher than that of the Ala-containing peptides (Table II). Z-Gly-Pro-Leu-Gly-Pro was not attacked by the enzyme, as were a number of other benzyloxycarbonyl N-protected peptides and Gly-Pro-Pro (Table II).

Physicochemical properties and subunit structure of post-proline dipeptidyl aminopeptidase

Measurements of absorbance and dry weight of the lyophilized enzyme revealed an extinction coefficient $A_{280}^{1\%} = 12.0$. Isoelectric focusing gave a p*I* value of 4.9 (Fig. 3). Using the velocity method a sedimentation constant $s = 10$ S was determined. The molecular weight of the enzyme was estimated by the sedimentation equilibrium method of Yphantis [21]. Plots of logarithmic fringe displacement, log ΔY, versus the square of radial distance, r^2, were linear. The apparent molecular weight of the enzyme was calculated to be 230 000 ± 15 000 adopting $\bar{v}$ as 0.73 based on protein determination by amino acid analysis. The diffusion constant D was calculated to be $4 \cdot 10^{-7}$ cm^2/s and the form factor (frictional coefficient) f/f_0 was found to be 1.3. The molecular weight of the enzyme was estimated to be 220 000 by gel fil-

TABLE II

HYDROLYSIS OF PEPTIDES BY POST-PROLINE DIPEPTIDYL AMINOPEPTIDASE

No detectable hydrolysis of the following peptides (defined as not more than 0.005 μmol/min/mg enzyme): Z-Gly-Pro-Leu-Gly-Pro, Gly-Pro-Pro, Z-Pro-Ala, Z-Pro-Asp, Z-Pro-Leu, Z-Pro-Leu-Gly, Z-Pro-Glu, Z-Pro-Phe, Leu-2-NNap.

Peptides	pH	Rate of hydrolysis (μmol/min/mg enzyme)
Gly-Pro-2-NNap	7.8	23.0
Gly-Pro-Leu-Gly	7.8	61.5
Gly-Pro-Ala	7.8	45.1
Ala-Pro-Gly	7.8	37.3
Ala-Ala-2-NNap	7.5	1.7
Ala-Ala-Ala	7.5	1.0
Gly-Ala-Ala	7.5	0.9
Ala-2-NNap	7.5	0.1

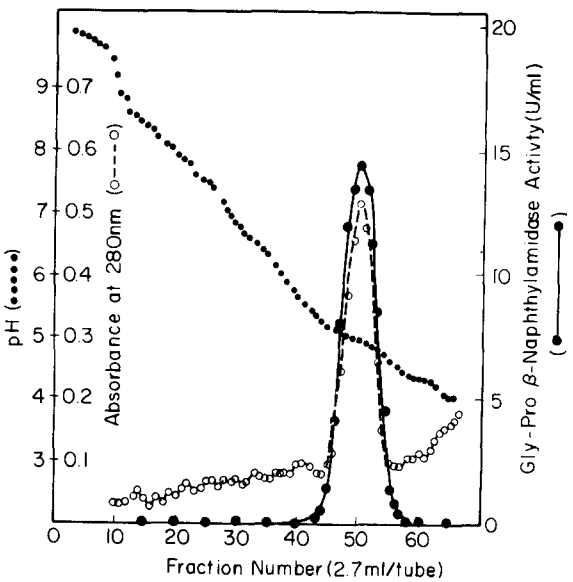

Fig. 3. Isoelectric focusing pattern of post-proline dipeptidyl aminopeptidase. The enzyme (11 mg) was applied onto an electrophoretic column (250 ml) using ampholite (pH 3—10) and a sucrose density gradient from 0.0—50%. Electrophoresis was performed at 4°C for 46 h at 300 V. Absorbance was determined at 280 nm (○- - - - -○) and the amidase activity was measured with Gly-Pro-2-NNap as substrate (●———●); pH gradient (●●●).

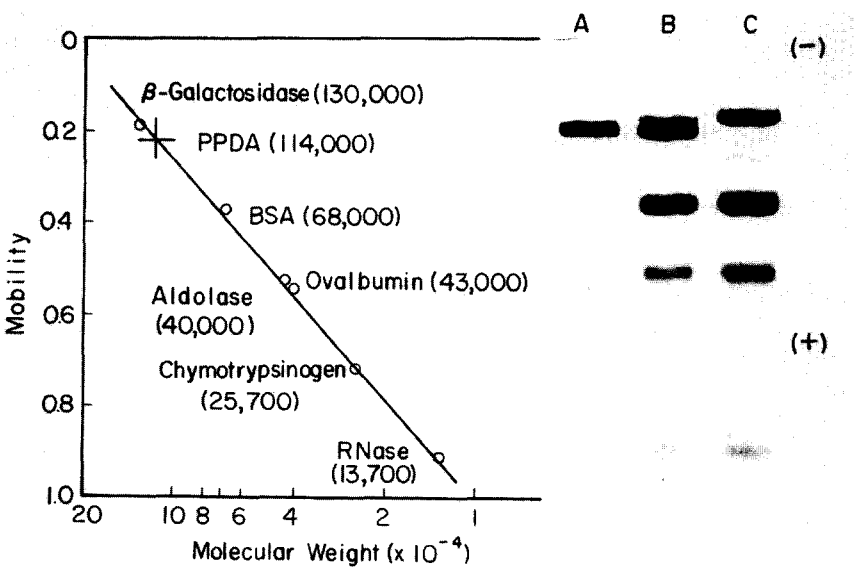

Fig. 4. Estimation of molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis of post-proline dipeptidyl aminopeptidase. For experimental detail see text. A, Electrophoretic pattern of enzyme alone; B, Electrophoretic profile in the presence of marker proteins; C, Electrophoretic pattern of marker proteins alone.

tration on Sepharose 4B using apoferritin, aldolase and chymotrypsinogen A as calibration markers. When the purified enzyme was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis an average molecular weight of 115 000 was obtained (Fig. 4).

Effect of chemical reagents on activity of post-proline dipeptidyl aminopeptidase

The enzyme was strongly inhibited by iPr_2P-F (0.1 mM = 69% inhibition; 1 mM = 91%) and only partially inhibited by CH_3CO_2Hg (0.1 mM = 48; 1 mM = 62%) and *p*-mercuribenzoate (1 mM = 53%). No significant inhibition was seen with 1 mM concentrations of *o*-nitrophenyl sulfonylchloride, $PhCH_2SO_2F$, *N*-acetylimidazole, 2-iodoacetamide, *N*-ethylmaleimide, phenylglyoxal, sodium tetrathionate, Nbs, EDTA and 1,10-phenanthroline.

Discussion

The most important finding in the present study is that post-proline dipeptidyl aminopeptidase isolated and purified from cortex of lamb kidney rapidly hydrolyzes Gly-Pro β-naphthylamide, which is in agreement with reports from other laboratories using enzyme obtained from different sources [2,4]. However, the enzyme obtained from lamb kidney fails to attack Z-Gly-Pro-Leu-Gly-Pro, which is in contrast to recent reports by Kenny et al. [6] who concluded that this enzyme exhibits in addition to its exopeptidase activity also endopeptidase activity. It is possible that the activity observed by Kenny et al. was due to a contamination of their preparation with the serine protease post-proline cleaving enzyme [9—11]. Post-proline cleaving enzyme has been shown to be very effective in catalyzing the hydrolysis of the peptide bond between Pro and Leu of Z-Gly-Pro-Leu-Gly-Pro [10].

Furthermore, the ability of post-proline dipeptidyl aminopeptidase to catalyze the hydrolysis of amide bonds of peptides with a free NH_2-terminal and a proline or alanine residue in the penultimate position has been confirmed [2—4,22]. It is apparent from the comparative data of Barth et al. [3] and those reported in this study (Table II) that the enzyme catalyzes the hydrolysis of proline-containing peptides much more rapidly than alanine-containing peptides. While none of the reported [6] endopeptidase activity was detected, the enzyme slowly released naphthylamine from Ala-β-naphthylamide (Table II). Thus, this exopeptidase exhibits a high specificity for proline-containing peptides and releases in a step-wise manner, starting from the NH_2-terminus, dipeptide units with a free proline carboxyl group. Therefore, in the present study this enzyme is referred to as post-proline dipeptidyl aminopeptidase.

In addition, the following observations were made in the course of this study. The highest yield of the enzyme was obtained by autolysis of cortex of lamb kidney at pH 3.8 as used by most other workers [2,4,6]. Barth et al. [3] who performed the autolysis of hog kidney preparation at pH 8 in the presence of Triton X-100 encountered difficulties with aminopeptidase contamination. In this study almost all of the aminopeptidase activity was removed with the CM-cellulose step. After the Sephadex G-200 step no aminopeptidase activity was detected. The CM-cellulose step was found to be most useful in that the

supernatant obtained after the autolysis was used directly without further treatment or adjustment of pH and in that the specific activity of the enzyme increased more than 10-fold (Table I). However, instead of performing CM-cellulose chromatography [6], which in our hands resulted in difficulties because of the high viscosity of the enzyme-containing supernatant, the enzyme absorbed on the CM-cellulose was isolated by centrifugal sedimentation and was recovered in high yield (Table I). Following Hopsu-Havu et al. [13] post-proline dipeptidyl aminopeptidase of lamb kidney was further purified by ion-exchange chromatography on DEAE-Sephadex (Fig. 1) and gel filtration on Sephadex G-200 (Fig. 2) to yield an enzyme with a specific activity of 23 units/mg using Gly-Pro β-naphthylamide as substrate. The enzyme sedimented as a single peak in the analytical ultracentrifuge (Fig. 2), which is a necessary albeit not a sufficient condition for monodispersity. By disc gel electrophoresis, enzyme-specific staining after disc gel electrophoresis and electrophoresis in the presence of sodium dodecyl sulfate, the enzyme behaved as a homogeneous protein. Gel filtration suggests an approximate molecular weight of 220 000 for the enzyme of lamb kidney, which appears to be in the same range found for this enzyme isolated from rat liver [2], hog kidney [3,6, 13] and human submaxillary gland [4]. The reciprocal of the sedimentation coefficients of the purified enzyme gave a linear extrapolation to zero protein concentration, giving a value of 10 S for the sedimentation constant. From sedimentation equilibrium studies an apparent molecular weight of 230 000 ± 15 000 was calculated. These results are in close agreement with those of Kenny et al. [6], who reported values of $s^0_{20,w}$ 10.12 ± 0.12 S and $M_{r(app)}$ = 239 700 ± 6700. Confirming results of Kenny et al. [6] using procine kidney, the enzyme of lamb kidney consists of a dimer with molecular weights of 115 000 as determined by sodium dodecyl sulphate gel electrophoresis (Fig. 4). However, in contrast to the enzyme isolated from pig kidneys, which has a subunit molecular weight of 130 000 and exhibits the same electrophoretic mobility as the marker β-galactosidase from *Escherichia coli*, the subunit molecular weight of the enzyme isolated from lamb kidney is lower and has an electrophoretic mobility slightly different from β-galactosidase (Fig. 4).

Using Gly-Pro β-naphthylamide as substrate, the enzyme of lamb kidney exhibits optimal activity at pH 7.8. The findings are identical to those of Hopsu-Havu et al. [13] and similar to those of Barth et al. [3], although in the latter case a different substrate was used. On the other hand, the enzyme isolated from human submaxillary gland shows a considerably higher pH optimum of 8.7 [4]. The p*I* of post-proline dipeptidyl aminopeptidase of lamb kidney is 4.9 (Fig. 3), while for that of hog kidney a p*I* of 6.4 was reported [3].

Kenny et al. [6] concluded that the enzyme of hog kidney is a serine enzyme and we also found the purified enzyme of lamb kidney to be irreversibly inhibited by diisopropylphosphorofluoridate, while a number of chemical reagents known to inhibit sulfhydryl and metal-activated enzymes failed to significantly effect this protease. The 50% inhibition by phenylmethylsulfonylfluoride observed by Barth et al. [3] could not be repeated in agreement with Kenny et al. [6]. A detailed comparison of the enzymatic properties and characteristics of post-proline dipeptidyl aminopeptidase and post-proline cleaving enzyme [9,10] is being carried out currently.

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