Biochimica et Biophysica Acta, 422 (1976) 170—178

BBA 67684

PURIFICATION AND SPECIFICITY OF PROLYL DIPEPTIDASE FROM BOVINE KIDNEY

AWNI F. AKRAWI and GRAHAM S. BAILEY

Department of Chemistry, University of Essex, Wivenhoe Park, Colchester (U.K.)

(Received March 14th, 1975)
(Revised manuscript received September 29th, 1975)

Summary

Prolyl dipeptidase (iminodipeptidase, L-prolyl-amino acid hydrolase, EC 3.4.13.8) was purified 180-fold from bovine kidney. The enzyme which was obtained in a 10% yield was completely separated from a number of known kidney peptidases including an enzyme of very similar substrate specificity, proline aminopeptidase (L-prolyl-peptide hydrolase, EC 3.4.11.5). The specific activity of the enzyme with L-prolylglycine as substrate is 1600 units of activity per mg protein. Optimum activity of the enzyme is at pH 8.75 and the molecular weight on gel filtration was estimated to be 100 000. The isoelectric point of the enzyme is pH 4.25. Studies of substrate specificity showed that the enzyme preferentially hydrolyzes dipeptides and dipeptidyl amides with L-proline or hydroxy-L-proline at the N-terminus. Longer chain substrates with N-terminal proline were not hydrolyzed.

Introduction

A number of peptidases are known to exist in kidney. Aminopeptidase (cytosol) (EC 3.4.11.1) is probably the best known example [1]. That enzyme is unable to attack imino acid bonds and therefore cannot hydrolyze substrates with L-proline or hydroxy-L-proline at the N-terminus. However, Sarid and co-workers [2] have described a kidney enzyme, proline aminopeptidase, which cleaves L-proline but not hydroxy-L-proline from the N-terminus of peptide chains of any length. Earlier, an enzyme preparation containing prolyl dipeptidase, capable of hydrolyzing dipeptides containing L-proline or hydroxy-L-proline at the N-terminus, had been partially purified from porcine kidney [3]. The degree of purification of the prolyl dipeptidase was very low and Mayer and Nordwig [4], using the same source, devised a new purification procedure. The purification procedure consisted of seven separate stages and

although the final enzyme preparation was very active it was only obtained in a 1% yield.

This paper reports the purification of prolyldipeptidase in a much higher yield from bovine kidney by a four-step procedure. Results of preliminary investigations of the substrate specificity of the bovine enzyme are also recorded.

Materials and Methods

Chemicals. All chemicals used were of analytical grade and were purchased from B.D.H. Chemicals Ltd, Poole, England or Sigma London Chemical Co. Ltd, Kingston-upon-Thames, England. All solutions were made in glass-distilled water.

Substrates. L-Prolylglycine, purchased from Sigma London Chemical Co. Ltd, was used as the main substrate for monitoring prolyl dipeptidase activity throughout the purification procedure. A second and reportedly more specific substrate for prolyl dipeptidase hydroxy-L-prolylglycine obtained from Bachem Inc., Calif., U.S.A., was also employed. The tripeptide L-prolylglycylglycine, obtained from Sigma, was used in the assay for proline aminopeptidase activity. The other substrates which were used in the specificity studies were purchased from various manufacturers as noted in Table III.

Chromatography resins. The gel filtration resin Sephadex G-100 was obtained from Pharmacia (G.B.) Ltd, London, England. The microgranular anion-exchange resin Whatman DE-52 was purchased from H. Reeve Angel and Co. Ltd, London, England.

Analytical electrophoresis. Disc electrophoresis at pH 9.4 was carried out on 10% polyacrylamide gels using the method of Davis [5]. Electrophoresis gels were stained with Amido Black 10B to reveal protein bands and were stained according to the procedure of Clarke [6] to reveal the presence of carbohydrate. Electrofocusing in the pH range 3.5—9.5 was carried out on thin-layer gels according to the manufacturer's instructions (LKB Instruments Ltd, South Croydon, England). The pH gradient was measured with the aid of an antimony electrode (Activion Glass Ltd, Fife, Scotland). The gels were stained with Coomassie brilliant blue R250 according to the method of Okavsky and Drysdale [7].

Equipment. In the purification of the enzyme by the column chromatographic procedures a refrigerated, automatic collector (LKB Instruments Ltd) was employed. The absorbance of the eluates from the columns was continuously measured at 280 nm using a Uvicord II photometer (LKB Instruments Ltd). Concentration of protein solutions was achieved by ultrafiltration under nitrogen using Diaflo membranes of the type PM10 in ultrafiltration cells of various volumes (Amicon Ltd, High Wycombe, England). De-salting and dialysis of fractions were carried out using hollow fibre devices of the type Bio-Fibre 50 according to the manufacturer's instructions (Bio-Rad Laboratories Ltd, Bromley, England). A Unicam SP 8000 or SP 800 spectrophotometer (Pye Unicam, Cambridge, England) or a Perkin-Elmer 402 spectrophotometer (Perkin-Elmer Ltd, Beaconsfield, England) were used in all spectrophotometric analyses. pH measurements were made with a pH-meter 51 (Radiometer, Copenhagen, Denmark).

Estimation of protein concentration. The concentration of protein in fractions was estimated by the method of Warburg and Christian [8] involving measurement of absorbance at 280 and 260 nm in 1-cm cells.

Enzyme assays. All enzyme assays were carried out at 37°C. Unless otherwise stated the buffer was 0.05 M Tris · HCl, pH 7.4, at 37°C, containing 1 mM $MnCl_2$. For enzyme dilutions in the buffer bovine serum albumin (2 mg/ml) was included to enhance stability. The incubation of enzyme with all substrates was carried out in the following manner: 10 µl of enzyme solution together with 40 µl of Tris buffer was incubated for 5 min with 50 µl of 100 mM substrate. For the measurements of proline-liberating activity the incubation was stopped by addition of 0.5 ml of aqueous trichloroacetic acid (6.25%, w/v). The amount of proline released by enzymic action was then determined using the Mayer and Nordwig [4] modification of the method of Messer [9]. For the measurement of enzymic activity against all other substrates the incubation was stopped by the addition of 1.9 ml of ethanol. The amounts of products formed by the enzymic action were then determined by the procedure of Josefsson and Lindberg [10]. In all cases one unit of enzymic activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 µmol of substrate per min.

Purification procedures. All operations were carried out at 2°C. However, the buffers were made at 20°C and therefore records of pH refer to that temperature. Whenever fractions had to be frozen sucrose was added to a final concentration of 0.25 M to increase stability.

Step 1: Homogenization. Bovine kidneys weighing 300—500 g were obtained from freshly slaughtered animals at a local abattoir. Adipose and connective tissue were carefully removed from each kidney. The cortex was cut into small pieces which were thoroughly washed with isotonic saline to remove blood and then dried. 100 g of segments of cortex were homogenized in 400 ml of 0.05 M Tris · HCl buffer, pH 7.75, containing 1 mM $MnCl_2$ and 0.25 M sucrose, for 2 min in a Waring blendor. The homogenate was centrifuged at 48 000 × *g* for 2 h in a Beckman centrifuge model J-21. The resulting 360 ml of supernatant was used in the further purification procedures.

Step 2: $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to the supernatant to give 40% saturation. After the precipitate had been removed by centrifugation further $(NH_4)_2SO_4$ was added to the solution to give 75% saturation. The resulting precipitate was collected by centrifugation and was dissolved in 80 ml of 0.05 M Tris · HCl buffer, pH 7.75, containing 1 mM $MnCl_2$. The salt was removed from the solution by dialysis against the same buffer using the hollow fibre system. Complete removal of the $(NH_4)_2SO_4$ took 6 h and the volume of the solution increased to 240 ml.

Step 3: Gel filtration on Sephadex G-100 resin. The material from Step 2 was processed by gel filtration on Sephadex G-100 resin in 12 batches. Each experiment was carried out in an identical manner. 10 ml of the fraction was added to the column (2.6 × 100 cm) of resin and was eluted by the Tris buffer containing 1 mM $MnCl_2$ using a flow rate of 24 ml/h and collecting 8-ml fractions. The fractions which were active against L-prolylglycine were pooled as indicated on the representative elution profile recorded in Fig. 1. The active material from all gel filtration experiments was 300 ml in volume and was

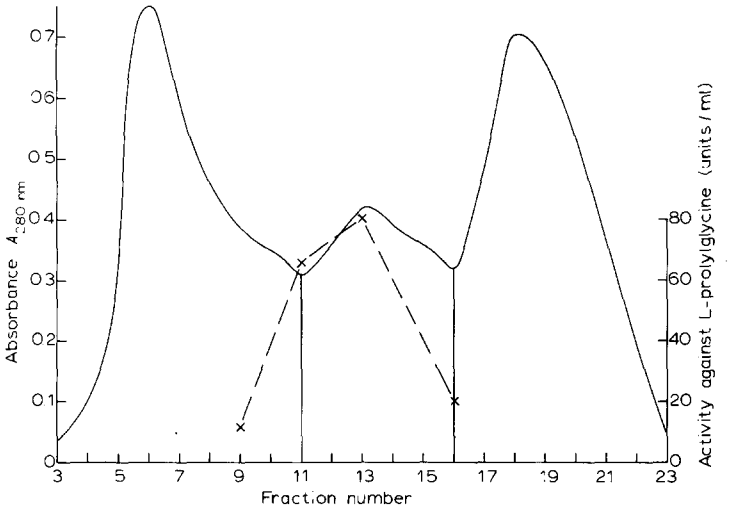


Fig. 1. Step 3 of the purification procedure: gel filtration on Sephadex G-100. ———, absorbance at 280 nm; - - - - - -, activity against L-prolylglycine.

concentrated to 54 ml by ultrafiltration. Gel filtration was also carried out for a series of standard proteins [11] under identical conditions to those used in the separation process in order to estimate the molecular weight of prolyl dipeptidase (Fig. 2).

Step 4: Anion-exchange chromatography on DEAE-cellulose. 6 ml of the concentrated sample obtained in the gel filtration step was dialyzed for 18 h

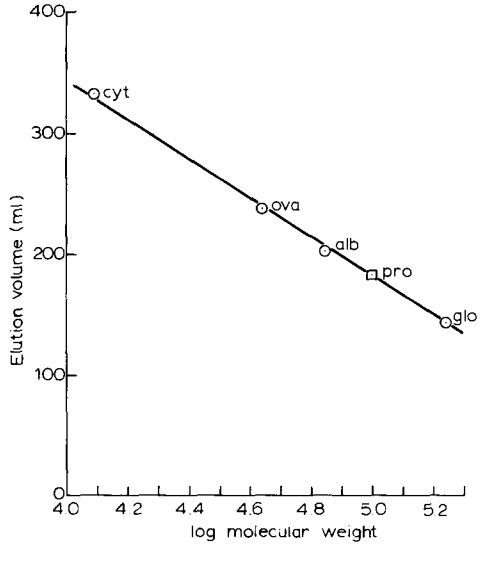


Fig. 2. A plot of elution volume versus log molecular weight for a series of standard proteins in order to estimate the molecular weight of prolyl dipeptidase. cyt, cytochrome *c*; ova, ovalbumin; alb, serum albumin; pro, prolyl dipeptidase; glo, γ-globulin.

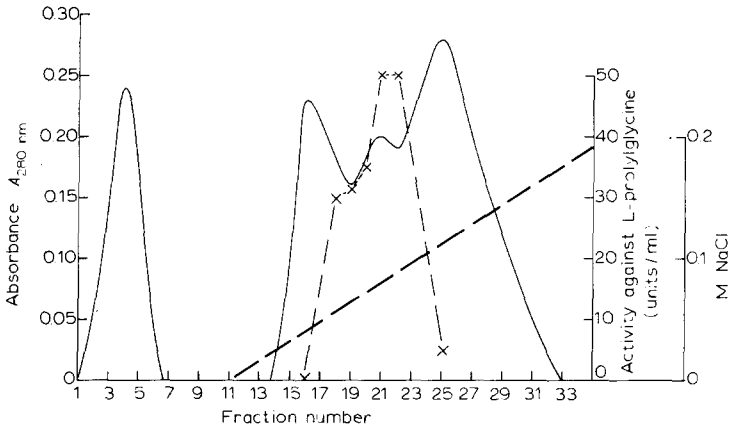


Fig. 3. Step 4 of the purification procedure: Anion-exchange chromatography on DEAE-cellulose. ———, absorbance at 280 nm; — — —, activity against L-prolylglycine; = = =, NaCl concentration.

against 500 ml of 0.005 M Tris · HCl buffer, pH 7.75, containing 1 mM $MnCl_2$, 2 mM succinic acid and 5 mM $MgCl_2$. The dialyzed sample which had increased in volume to 15 ml was applied to a column (2.6 × 10 cm) of Whatman DE-52 resin which had been equilibrated against the same buffer. Unadsorbed material was removed by the buffer using a flow rate of 28 ml/h and collecting 9.3-ml fractions. The adsorbed material was removed by application of a linear salt gradient (0—0.2 M NaCl in Tris buffer, total volume 240 ml).

All of the material from Step 3 was processed in an identical manner and a representative elution profile is recorded in Fig. 3.

Results and Discussion

(1) Purification procedure

The results of the purification procedures are summarized in Table I. The major purification step was anion-exchange chromatography on DEAE-cellulose. In fact a number of different resins and conditions were tested but the

TABLE I

PURIFICATION OF PROLYL DIPEPTIDASE FROM BOVINE KIDNEY CORTEX

The weight of cortex used in the purification process was 100 g.

Fraction	Volume (ml)	Total protein (mg)	Specific activity * (units/mg)	Total activity * (units)	Yield of activity * (%)	Purification (*n*-fold)
Supernatant	360	6840	8.5	58150	(100)	(1)
$(NH_4)_2SO_4$	90	1800	19.5	35100	60.4	2.3
Sephadex G-100	54	648	36.0	23330	40.1	4.2
DEAE-cellulose						
Fractions 21 and 22	126	3.8	1600	6050	10.4	188.0
Fractions 18—20	127	3.8	1200	4570	7.9	141.0

* Enzymic activity was measured with 50 mM prolylglycine as substrate.

procedure involving DEAE-cellulose gave the best results. The method used was a modification of one of the purification procedures employed by Mayer and Nordwig [4]. It was found necessary to include $MgCl_2$ in the buffer to increase the stability of the enzyme whilst it was attached to the resin. The purification procedure used by Mayer and Nordwig [4] was quite complex. It included three separate steps of ion-exchange chromatography and both ethanol and $(NH_4)_2SO_4$ precipitations. The most active material had a specific activity of 1800 units per mg protein but was only obtained in a 1% yield. In contrast, the procedure followed in this study was relatively simple and resulted in highly active material in a much higher yield.

However, it has been reported that L-prolylglycine is hydrolysed by both prolyl dipeptidase and proline aminopeptidase, and both enzymes are present in kidney [2]. Therefore, it was thought necessary in the present study to check the purity of the fractions at each stage of the procedure by the use of more specific substrates. Hydroxy-L-prolylglycine, reportedly only hydrolyzed by prolyl dipeptidase and L-prolylglycylglycine, only specifically hydrolyzed by proline aminopeptidase, were used as substrates. The results are recorded in Table II. It can be seen that the ratio of activities towards L-prolylglycine and hydroxy-L-prolylglycine decreased as the purification proceeded indicating the purification of prolyl dipeptidase. On the other hand the ratio of activities towards L-prolylglycine and L-prolylglycylglycine increased during the purification procedure indicating the removal of proline aminopeptidase. A molecular weight of 100 000 was found for prolinase by gel filtration on Sephadex G-100 resin (Fig. 2). Mayer and Nordwig [4] recorded a molecular weight of 300 000 for the corresponding enzyme from porcine kidney. The difference in molecular weights could be due to differences in the state of aggregation of the enzyme molecules, bearing in mind that the calibrated gel filtration was carried out on a relatively crude preparation of the bovine enzyme in contrast to the pure preparation used for the enzyme from porcine kidney.

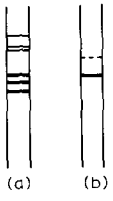
(2) Purity of the enzyme

The fraction of maximum enzymic activity was heterogeneous on disc electrophoresis at pH 9.4 (Fig. 4). Heterogeneity on analytical electrophoresis has been seen for several other purified peptidases including prolyl dipeptidase from porcine kidney [4], proline dipeptidase (EC 3.4.13.9) from porcine intestine [12], a soluble dipeptidase from porcine intestinal mucosa [13] and a

TABLE II

ENZYMIC ACTIVITIES OF FRACTIONS OBTAINED IN THE PURIFICATION PROCEDURE

Fraction	Specific activity against Hyp-Gly (units/mg)	Specific activity against Pro-Gly-Gly (units/mg)	Ratio of activity Pro-Gly / Hyp-Gly	Ratio of activity Pro-Gly / Pro-Gly-Gly
Supernatant	0.125	3.5	65.4	2.4
$(NH_4)_2SO_4$	1.38	7.1	14.2	2.7
Sephadex G-100	4.00	3.3	9.0	11
DEAE-cellulose fractions 21 and 22	370	0	4.3	∞



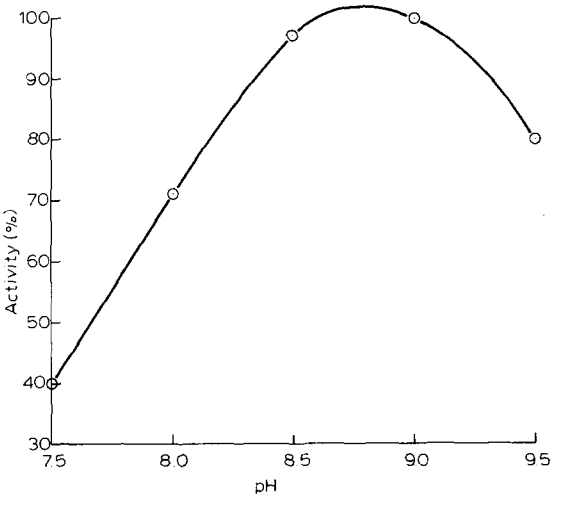


Fig. 4. Disc electrophoresis at pH 9.4 of prolyl dipeptidase. 3 mA/gel were applied for 0.5 h. (a) 25 μg prolyl dipeptidase. (b) 25 μg prolyl dipeptidase after incubation with 2-mercaptoethanol.

Fig. 5. Prolyl dipeptidase activity against L-prolylglycine as a function of pH. Activity was measured in the absence of Mn^{2+} to prevent precipitation of $Mn(OH)_2$ under alkaline conditions.

mouse ascites tumour dipeptidase [14]. However, in the present study, the heterogeneity was almost completely removed after incubation with 2-mercaptoethanol according to a modification of the procedure of Sjöström and co-workers [12]. Prolyl dipeptidase was incubated for 6 h at 20°C in 0.1 M 2-mercaptoethanol and was then dialyzed overnight at 2°C against 0.05 M Tris · HCl buffer, pH 7.75, containing 1 mM $MnCl_2$. It is possible that the heterogeneity was due to aggregated forms of the enzyme which were dissociated by treatment with 2-mercaptoethanol. The staining procedure of Clarke [6] was negative for the prolyl dipeptidase sample indicating that if carbohydrate was present it represented less than 10% by weight of the sample.

The enzyme, after it had been extensively dialyzed against 0.05 M Tris · HCl buffer of pH 7.75 to remove excess metal ions and succinic acid, showed only one component on gel electrofocusing. After elution from the gel the component was active towards L-prolylglycine. The isoelectric point was found to be pH 4.25.

(3) pH optimum and specificity of the enzyme

The optimum activity of the enzyme towards L-prolylglycine was at pH 8.75 (Fig. 5), a value which was also recorded for the enzyme from porcine kidney [4]. From the results of experiments on substrate specificity (Table III) it can be seen that the enzyme has a marked preference for dipeptides containing N-terminal L-proline. The ability of the enzyme to hydrolyze other dipeptides could be due to impurities of other dipeptidases. However, such impurities were not detected on gel electrofocusing. Also, Sjöström and co-workers [12] found that proline dipeptidase from porcine intestine could slowly hydrolyze dipeptides which did not contain L-proline at the C-terminus. So it would appear that prolyl dipeptidase of bovine kidney does possess some de-

TABLE III

SUBSTRATE SPECIFICITY OF BOVINE KIDNEY PROLYL DIPEPTIDASE

Substrate (L-form)	Manufacturer	Method of assay	Activity (%)
Pro-Gly	1	A	100
Pro-Ala	1	A	100
Pro-Met	1	A	100
Hyp-Gly	2	B	23
Gly-Pro	1	A	0.5
Val-Pro	1	A	3.0
Leu-Ala	3	B	2.0
Leu-Gly	1	B	7.5
Gly-Ala	3	B	14.5
Gly-Gly	3	B	6.3
Gly-Leu	3	B	8.0
Val-Gly	1	B	6.3
Pro-Gly-Gly	1	A	0
Leucinamide	1	C	0
Poly(Pro)	1	A	0
Pro-Leu-NH_2	4	A	38
Pro-Val-Gly	2	A	0
Pro-β-Naphthylamide	1	A	0

1, Sigma London Chemical Co. Ltd.
2. Bachem Inc. U.S.A.
3, B.D.H. Chemicals Ltd.
4, Fox Chemical Co. Ltd, U.S.A.

A, Mayer and Nordwig [4].
B, Josefsson and Lindberg [10].
C, Binkley and Torres [15].

gree of intrinsic activity towards dipeptides which do not contain proline.

The enzyme of the present study shows a very similar substrate specificity to that of the enzyme isolated by Mayer and Nordwig [4]. The ability of the porcine enzyme to hydrolyze L-prolyl-leucinamide was attributed to the presence of an impurity of a new type of peptidase capable of removing N-terminal proline. That enzyme was subsequently purified and characterized by Mayer and Nordwig [16]. It was found to hydrolyze dipeptidyl amides, tripeptides and longer chain peptides with N-terminal proline. The activity against L-prolyl-leucinamide was completely blocked by *p*-chloromercuribenzoate. In contrast, it was found that incubation of prolyl dipeptidase of bovine kidney with 2.5 mM *p*-chloromercuribenzoate at room temperature for 15 min produced only a 50% reduction in specific activity against both L-prolylglycine and L-prolyl-leucinamide. Also, the bovine enzyme did not hydrolyze the tripeptides tested, and it appeared to be pure on gel electrofocusing. Thus, it is believed that prolyl dipeptidase of bovine kidney possesses an intrinsic ability to hydrolyze dipeptidyl amides.

References

1 Himmelhoch, S.R. and Peterson, E.A. (1968) Biochemistry 7, 2085—2092
2 Sarid, S., Berger, A. and Katchalski, E. (1962) J. Biol. Chem. 237, 2207—2212
3 Davis, N.C. and Smith, E.L. (1953) J. Biol. Chem. 200, 373—384
4 Mayer, H. and Nordwig, A. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 371—379
5 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
6 Clarke, J.T. (1964) Ann. N.Y. Acad. Sci. 121, 428—436

7 Otavsky, W.I. and Drysdale, J.W. (1975) Anal. Biochem. 65, 533—536
8 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384—421
9 Messer, M. (1961) Anal. Biochem. 2, 353—359
10 Josefsson, L. and Lindberg, T. (1965) Biochim. Biophys. Acta 105, 149—161
11 Andrews, P. (1970) Methods of Biochemical Analysis (Glick, D., ed.), Vol. 18, pp. 1—53, Interscience Publishers, New York
12 Sjöström, H., Noren, O. and Josefsson, L. (1973) Biochim. Biophys. Acta 327, 457—470
13 Noren, O., Sjöström, H. and Josefsson, L. (1973) Biochim. Biophys. Acta 327, 446—456
14 Hayman, S. and Patterson, E.K. (1971) J. Biol. Chem. 246, 660—669
15 Binkley, F. and Torres, C. (1960) Arch. Biochem. Biophys. 86, 201—203
16 Mayer, H. and Nordwig, A. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 380—383