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PURIFICATION AND PROPERTIES OF A PEPTIDASE ACTING ON A SYNTHETIC SUBSTRATE FOR COLLAGENASE FROM MONKEY KIDNEY

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Summary

A peptidase cleaving a synthetic substrate for collagenase, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (designated as Pz-peptide) has been purified extensively (about 5200-fold) from a soluble extract of monkey kidney with a view to carrying out studies on its possible physiological role. The purified Pz-peptidase appeared essentially free of collagenase, nonspecific protease and di- and tri-peptidase activities. The properties of the purified Pz-peptidase resemble very much the granuloma enzyme. It is optimally active around pH 7.0. Its apparent K_m value for Pz-peptide is 0.72 mM and V is 10.1 μmol/mg protein/min. It is reversibly inhibited by *p*-hydroxymercuribenzoate and $HgCl_2$, whereas iodoacetamide does not affect the enzyme activity. *N*-Ethylmaleimide inhibited the enzyme partially (50%). Heavy metals like Cu^{2+}, Cd^{2+}, Ag^{+}, Pb^{2+}, Ni^{2+} and Zn^{2+} completely inhibited the enzyme activity, while the inhibition by Co^{2+} was only partial. Fe^{2+} did not exert any effect on the activity. The enzyme activity was completely inhibited by EDTA and was restored almost to the original value by metal ions like Mn^{2+}, Mg^{2+}, Ca^{2+} and Ba^{2+}. The approximate molecular weight of the purified enzyme was estimated to be 56 000.

Introduction

In spite of the widespread distribution of a peptidase acting on a synthetic substrate for collagenase, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (designated as 'Pz-peptide' in this paper), it has not so far been studied in detail [1—4] (for earlier papers see ref. 1). A partially purified enzyme from experimental granuloma tissue permitted limited studies on the

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nature of the enzyme [5]. In the present paper, the properties of an extensively purified monkey kidney enzyme have been described.

Materials and Methods

The following chemicals were obtained commercially as indicated: Pz-peptide (Fluka, Switzerland); dithiothreitol, *p*-hydroxymercuribenzoate, Tris, reduced glutathione and Azocasein (Sigma Chemical Co., U.S.A.); cysteine (Calbiochem, U.S.A.); protein markers, bovine serum albumin, ovalbumin, sperm whale myoglobin, horse heart cytochrome *c*, $(NH_4)_2SO_4$ (enzyme grade), Tris (ultra pure) (Schwarz Mann, U.S.A.); sodium diethyl dithiocarbamate (Riedel De Haenag Seelze-Hannover, Germany); α,α'-dipyridyl (E. Merck AG, Germany); *o*-phenanthroline (National Chemical Laboratory, Poona, India); Sephadex G-200, blue dextran 2000, CM-Sephadex C-50 and DEAE-Sephadex A-50 (Pharmacia, Sweden). Most of the dipeptides (total 75) and tripeptides (total 8) used in these studies were commercially obtained and a few others were gifts [6,7]. Other chemicals were commercially available reagent grade products. Calcium phosphate gel and hydroxylapatite were prepared as described by Keilin and Hartree [8] and by Levin [9], respectively.

Animals

Adult male and/or female monkeys (*Macaca radiata*) were anaesthetized with Nembutal and the kidneys were dissected out. The kidneys containing mainly the cortex tissue were stored in the frozen (−20° C) state until a sufficient quantity (usually 1 kg) was accumulated. Freezing did not have any deleterious effect on the enzyme upto 3 months. The frozen tissue was thawed, minced with scissors and homogenized as given below. The average weight of the two kidneys was about 15 g. Pz-peptidase assays were carried out as described earlier [5].

Non-specific protease activity

Nonspecific protease activity was assayed essentially according to the method of Charney and Tomarelli [10] using Azocasein as the substrate. A reaction mixture (1.5 ml) containing Azocasein (10 mg), Tris · HCl, pH 7.4 (100 μmol), $CaCl_2$ (7.5 μmol) and enzyme (30 μg) was incubated at 37° C for 2 h. After stopping the reaction with the addition of 2 ml of 12% perchloric acid and on further incubation for 30 min the absorbance of the supernatant fraction obtained on centrifugation (2200 × *g*, 30 min) of the reaction mixture was measured at 350 nm. This method was sensitive enough to detect even 50 ng of crystalline trypsin which on incubation under the same conditions with Azocasein gave an absorbance of 0.1 at 350 nm.

Di- and tri-peptidase activities

The di- and tri-peptidase activities were assayed by paper chromatography essentially as described elsewhere [7]. The enzyme (approx. 1.5 μg of protein containing 8 munits of Pz-peptidase) was incubated for 7 h at 37° C in a final reaction mixture of 0.05 ml containing substrate (0.6 μmol), Tris · HCl, pH 7.4 (5 μmol) and $CaCl_2$ (0.25 μmol). At the end of incubation, the reaction mix-

ture was chilled in ice and 0.02 ml aliquot used for identification of the split products of peptides by paper chromatography using *n*-butanol:acetic acid: water (4 : 1 : 1) solvent system.

Assay of collagenase

Collagenase activity was determined by measuring the radioactivity in the soluble fraction obtained from ^{14}C-labeled reconstituted collagen fibrils (400 μg) after incubation at 37°C for 8 h with the enzyme (12 μg of purified Pz-peptidase), essentially according to the method of Nagai et al. [11].

Purification of Pz-peptidase from monkey kidney

All operations were carried out at 0—4°C and all buffers contained dithiothreitol (0.01 mM), unless otherwise stated. The kidney tissue was homogenized with 1.15% KCl containing 1% glycerol and dithiothreitol (0.1 mM), in a Waring blendor for 2 min to obtain a 25% homogenate. The bulk of the kidney Pz-peptidase was found to be present in the 100 000 × *g* supernatant fraction. For purposes of purification given below, a 25% homogenate was prepared from 970 gm of tissue and was centrifuged at 12 000 × *g* for 1 h in a refrigerated centrifuge (Sorvall, Model RC2) and the supernatant fraction was used as the starting material.

Step I. Acid precipitation. To the supernatant fraction (2950 ml; total protein 70 g) 1 M acetic acid was added slowly with mechanical stirring to bring the pH to 5.1 and left for 15 min. After centrifugation at 12 000 × *g* for 20 min the pellet was discarded and the supernatant fraction was quickly adjusted to pH 7.4 by addition of 1 M Tris · HCl, pH 7.4 to a final concentration of 0.05 M.

Step II. $(NH_4)_2SO_4$ fractionation. To the supernatant fraction (2960 ml; total protein 39 g) from Step I, solid $(NH_4)_2SO_4$ (861 g) was added slowly with mechanical stirring to bring the saturation to 50% and stirring continued for an additional hour and then centrifuged at 10 000 × *g* for 20 min. The pellet was discarded and the $(NH_4)_2SO_4$ concentration in the supernatant fraction (3240 ml) was raised to 75% by further addition of $(NH_4)_2SO_4$ (515 g). The pellet collected by centrifugation at 10 000 × *g* for 20 min was dissolved in 0.01 M sodium-potassium phosphate buffer, pH 6.0 containing 1% glycerol ('phosphate-glycerol buffer') and then dialyzed against the same buffer (10 l each time) with 4 changes. The dialyzed enzyme was centrifuged at 10 000 × *g* for 20 min and the supernatant fraction was used for further purification.

Step III. CM-Sephadex chromatography. The fraction from the previous step (390 ml, total protein 13.3 g) was applied on a CM-Sephadex C-50 column (54 cm × 3.4 cm; bed vol. 490 ml) previously equilibrated with phosphate-glycerol buffer, pH 6.0. The first 150 ml of the breakthrough fraction which did not contain Pz-peptidase were discarded. The subsequent breakthrough fraction and one bed volume of washings (in 100 ml fractions) with the same buffer were collected and checked for activity. The pooled fraction (breakthrough fraction and the first 300 ml of washings) was used for further purification.

Step IV. DEAE-Sephadex chromatography. The enzyme fraction from the

previous step (540 ml, total protein 6.6 g) was dialyzed against 0.01 M Tris · HCl, pH 7.4 containing 1% glycerol ('Tris-glycerol buffer') and 0.07 M NaCl for 24 h with 3 changes of the buffer (10 l each time). The dialyzed fraction was applied on a DEAE-Sephadex A-50 column (2.3 cm × 35 cm; bed vol. 150 ml) previously equilibrated with the same buffer. After washing with three bed volumes of the equilibrating buffer, the column was eluted with 0.01 M Tris-glycerol buffer, pH 7.4 with a linear NaCl gradient (0.08—0.16 M) using a Varigrad (Technicon) device at a flow rate of 25 ml/h, and 10 ml fractions were collected. Pz-peptidase activity was eluted between 0.1 to 0.14 M NaCl and the fraction representing the major peak of the activity (fractions 108—136) was used for further purification.

Step V. Calcium phosphate gel adsorption. The enzyme fraction (290 ml; total protein 250 mg) from the previous step was concentrated by $(NH_4)_2SO_4$ precipitation and subsequent dialysis against 0.01 M sodium-potassium phosphate glycerol buffer, pH 6.0 (10 l, 8 h) and protein concentration adjusted to 10 mg/ml. This fraction was treated with calcium phosphate gel (20 mg solids/ml) using a protein to gel ratio of 1 : 1. A suspension of the calcium phosphate gel was added slowly with stirring to the protein solution. The suspension was stirred for 20 min and then centrifuged at 10 000 × *g* for 20 min. The pellet was eluted first with 25 mM sodium-potassium phosphate buffer, pH 6.6 and then with 0.2 M sodium-potassium phosphate buffer, pH 7.0. The volume of the eluant buffer was the same as the volume of the gel suspension originally used for obtaining the pellet. The 0.2 M phosphate buffer eluate (15.8 ml) containing the Pz-peptidase activity was used for further purification.

Step VI. $(NH_4)_2SO_4$ extraction. The enzyme fraction (15.8 ml, total protein 121 mg) from the previous step was precipitated with $(NH_4)_2SO_4$ (90% saturation). It was centrifuged at 12 000 × *g* for 1 h. The pellet was eluted stepwise according to the method of Jakoby [12] with 5.5 ml each of 75, 70, 65, 60, 55, 50 and 45% saturated $(NH_4)_2SO_4$ in 0.05 M Tris · HCl buffer, pH 7.4 containing dithiothreitol (0.1 mM). The Pz-peptidase of highest specific activity came in the eluates of 60% and 55% saturation of $(NH_4)_2SO_4$. These fractions were pooled and used for the purification in the next step.

Step VII. Hydroxyapatite chromatography The enzyme fraction from the previous step was concentrated by $(NH_4)_2SO_4$ precipitation and dialyzed against 10 mM sodium-potassium phosphate buffer, pH 7.0 to a small volume (5.2 ml, total protein 29.1 mg). This sample was applied to a hydroxyapatite column (1.2 cm × 5.5 cm, bed vol. 10 ml) previously equilibrated with 0.01 M sodium-potassium phosphate buffer, pH 7.0. After washing with the starting buffer (2 bed volumes) the column was eluted with the same buffer containing dithiothreitol (0.1 mM) with a linear phosphate gradient (0.01—0.1 M) using the Varigrad (Technicon) device. The flow rate was 4 ml/h and 3 ml fractions were collected. The enzyme was eluted between 0.05—0.07 M sodium-potassium phosphate buffer (Fig. 1).

Step VIII. Gel filtration on Sephadex G-200. The pooled enzyme fraction (61 ml, total protein, 1.76 mg) from the previous step was concentrated by $(NH_4)_2SO_4$ and applied in a sample volume of 2 ml on a Sephadex G-200 column (1.5 cm × 70 cm, bed vol. 125 ml) previously equilibrated with 0.05 M Tris · HCl, pH 7.4 (Tris, ultra pure) containing dithiothreitol (0.1 mM). The

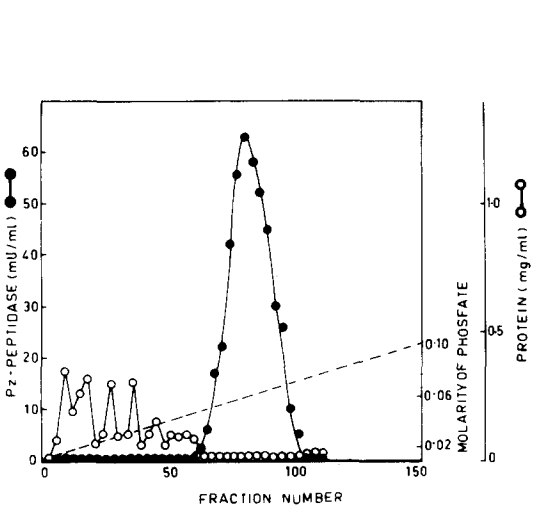

Fig. 1. The elution profile of Pz-peptidase from hydroxyapatite column. The experimental details of the column operation are given in the text.

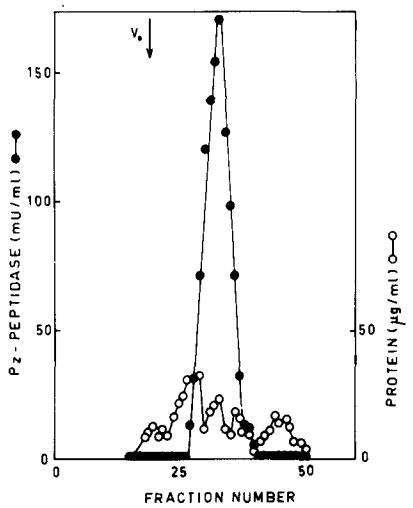

Fig. 2. The elution profile of Pz-peptidase by Sephadex G-200 gel filtration. The experimental details are given in the text.

same buffer was used for elution and 2 ml fractions were collected at a flow rate of 10 ml/h. The elution profile of Pz-peptidase on the Sephadex G-200 column is given in Fig. 2. Active fractions were pooled (15 ml, total protein, 0.45 mg) and was used for further studies.

A summary of the purification procedure is given in Table I. The enzyme was purified about 5200-fold with a rather low overall recovery of 3.2%. In view of the limited quantities of the final enzyme preparation only kinetic studies were made and a check on its homogeneity or otherwise of the enzyme could not be made conclusively. However, with 3 µg of protein, the purified

TABLE I

PURIFICATION OF Pz-PEPTIDASE FROM MONKEY KIDNEY

The specific activity of the enzyme is expressed as nmol of Pz-Pro-Leu liberated/min (munits) per mg protein.

Fraction	Total protein (mg)	Enzyme activity		Recovery (%)
		Total (munits)	munits/mg protein	
1. Tissue extract (12000 × *g* supernatant)	70000	93000	1.3	100
2. Acid-precipitated supernatant	39000	84000	2.2	90
3. $(NH_4)_2SO_4$ precipitation (50—75% satn)	13300	52000	3.9	56
4. CM-Sephadex breakthrough fraction	6600	39700	6.0	43
5. DEAE-Sephadex	250	14500	58.0	15.6
6. Calcium phosphate gel	121	10600	88.0	11.4
7. $(NH_4)_2SO_4$ extraction (60% and 55% satn eluates)	29.1	7000	240.0	7.5
8. Hydroxyapatite	1.76	3880	2200.0	4.3
9. Sephadex G-200	0.45	3000	6700.0	3.2

Pz-peptidase on acrylamide gel electrophoresis carried out according to Davis [16] gave a diffuse band. When the electrophoresis was done according to Weber and Osborn [17] in the presence and absence of β-mercaptoethanol with 30 μg protein and sodium dodecyl sulfate revealed three protein bands, two closely moving bands of equal intensity approximately corresponding to a molecular weight of 30 000—35 000 and a third faint band corresponding to 60 000.

Inclusion of glycerol (1%) in all the buffers improved the recovery of the enzyme especially in the DEAE step in which the recovery was about 35% more than that obtained in its absence. For the sake of convenience glycerol was included in the buffers in the earlier steps also.

Results

Properties of purified kidney Pz-peptidase

pH Optimum, K_m and V values. Within the limits of the assay procedure, the enzyme activity increased linearly with the time of incubation upto 1 h and with enzyme concentration upto 80 ng protein for the final preparation and upto 0.7 munits of enzyme at the earlier steps of purification. The pH optimum for the purified Pz-peptidase is around pH 7.0 (Tris-acetate, 0.2 M final). The enzyme is completely inactive below pH 5.0 and above 8.0. The K_m for the Pz-peptide was found to be 0.72 mM and V was 10.0 μmol of Pz-peptide hydrolyzed/mg protein per min estimated from the linear Lineweaver-Burk plot obtained.

Specificity

Other than its action on Pz-peptide the purified Pz-peptidase appeared to be essentially free of collagenase and nonspecific protease activities. It did not have any hydrolytic action towards a large number of dipeptides and tripeptides tabulated elsewhere [6,7] and also on the following: Gly-L-β-Ala, Gly-L-Ser, Gly-L-Tyr, Gly-L-Hyp, Gly-L-Asp, Gly-L-Asn, L-Ala-L-Pro, L-Val-L-Pro, L-His-Gly, L-His-Ala, L-Hyp-Gly, L-Pro-L-Ala, L-Pro-L-Phe, L-Pro-L-Met, Gly-L-Pro-L-Hyp, Gly-L-Pro-L-Ala, Gly-L-Leu-L-Tyr, Gly-L-Phe-L-Ala, L-Glu-Gly-L-Phe and Gly-L-Phe-L-Phe.

Effect of various metal ions and chelating agents on Pz-peptidase activity

The effect of various metal ions and chelating agents on the Pz-peptidase activity was studied at a final concentration of 0.1 mM under standard assay conditions. The results are given in Table II. It can be seen that heavy metals like Hg^{2+}, Ag^{+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} (all at 0.1 mM) completely inhibit the enzyme activity while the inhibition by Co^{2+} was only partial. Fe^{2+}, Ba^{2+}, Mn^{2+} and Ca^{2+} were not inhibitory.

At a concentration of 1 mM sodium diethyl dithiocarbamate and α,α′-dipyridyl do not affect the enzyme activity (Table III). However EDTA and o-phenanthroline (0.1 mM) were found to be inhibitory.

Effect of metal ions on the EDTA treated enzyme

The purified enzyme preparation was exhaustively dialyzed against 0.05 M Tris · HCl, pH 7.4 (Tris-Ultrapure) containing dithiothreitol (0.1 mM), and

TABLE II

EFFECT OF VARIOUS METAL IONS ON Pz-PEPTIDASE ACTIVITY

For assay conditions see text.

Metal added (0.1 mM)	Enzyme activity (munits)
None	1.13
Lead acetate	0.08
$CoSO_4$	0.54
$NiCl_2$	0.01
$HgCl_2$	0
$AgNO_3$	0.12
$CuSO_4$	0
$CdSO_4$	0.01
$ZnSO_4$	0.12

1 mM EDTA for 15 h. EDTA from this enzyme preparation was not removed further by dialysis against buffers which may contain tracers of heavy metal ions, and it was present at 0.02 mM in the assay mixture. This was done in order to prevent possible inhibition by heavy metals which severely inhibit the enzyme. Under these conditions the enzyme showed 16% of its original activity (Table IV). The effect of metal ions was studied in this system. Ca^{2+}, Mg^{2+}, Mn^{2+} and rather unusually Ba^{2+} (all at a final concentration of 1 mM) restored the enzyme activity almost to the original level before EDTA treatment.

Effect of sulfhydryl reagents

p-Hydroxymercuribenzoate (0.01 mM) inhibited the enzyme activity completely. The inhibition by *N*-ethylmaleimide was partial (50%) whereas iodoacetamide did not have any effect on the enzyme activity (Table III). The inhibition by *p*-hydroxymercuribenzoate or Hg^{2+} is reversed by sulfhydryl compounds like dithiothreitol, reduced glutathione or cysteine. These sulfhydryl compounds themselves did not affect the enzyme activity but appear to stabilize the enzyme during purification.

TABLE III

EFFECT OF CHELATING AGENTS AND SULFHYDRYL REAGENTS

For assay conditions see text.

Compound (1 mM)	Enzyme activity (munits)
None	1.3
EDTA	0.2
Diethyl dithiocarbamate	0.86
α,α'-Dipyridyl	1.2
p-Hydroxymercuribenzoate (0.01 mM)	0
o-Phenanthroline (0.1 mM)	0.05
N-Ethylmaleimide	0.66
Iodoacetamide	1.3

TABLE IV

EFFECT OF METAL IONS ON THE EDTA-TREATED ENZYME

The purified Pz-peptidase was exhaustively dialyzed against 0.05 M Tris·HCl, pH 7.4 containing dithiothreitol (0.1 mM) and 1 mM EDTA for 15 h. The activity regained on addition of metal ions (1 mM) to the incubation mixture is expressed as percentage of the activity of the undialyzed enzyme. The metal salts in all the cases were chlorides.

Description	Enzyme activity (% of control)
Enzyme before EDTA treatment	100
Enzyme after EDTA treatment	16
+ Ca^{2+} (1 mM)	98
+ Mg^{2+} (1 mM)	91
+ Mn^{2+} (1 mM)	114
+ Ba^{2+} (1 mM)	87

Molecular weight estimation

An estimate of the molecular weight of the purified enzyme was made by the gel filtration method using Sephadex G-100 column (57 cm × 2.2 cm, bed vol. 226 ml) according to Andrews [13]. The void volume of the column was determined with blue dextran 2000. The apparent molecular weight was estimated to be approximately 55 000.

Discussion

Partial purification and some properties of a Pz-peptidase from rat granuloma tissue were described earlier [5]. Limitations of this enzyme source prompted us to seek a tissue available in larger quantity and monkey kidney was found to be most suitable. The enzyme was extensively purified from monkey kidney to facilitate study of any possible physiological role of this enzyme in collagen degradation. The enzyme resembles the granuloma enzyme [5] in most of its properties.

Like the granuloma enzyme, monkey kidney enzyme also appears to be a sulfhydryl protein [5]. The effect of heavy metals like Hg^{2+} on the enzyme activity seems to closely resemble that of the granuloma enzyme. However, unlike the granuloma Pz-peptidase, the enzyme from monkey kidney was stimulated by metal ions such as Mn^{2+}, Ca^{2+}, Mg^{2+} or Ba^{2+}, and Mn^{2+} being the most effective under the conditions of the experiment. The synovial and chick embryo skin enzymes are reported to require Ca^{2+} for their enzyme activities [14,15].

The molecular weight of the purified Pz-peptidase from monkey kidney is around 55 000 on the basis of its elution from a calibrated Sephadex G-100 column, though confirmation by other methods is necessary. This molecular weight is very similar to that of the granuloma enzyme [5]. The properties of Pz-peptidase reported to be present in other systems have not been studied in detail.

There have been several reports in the literature suggesting a relationship between Pz-peptidase and the degradation of collagen in vivo [15,18,19]. Har-

ris and Krane [14] recently isolated a synovial protease which also exhibited Pz-peptidase activity. They showed that this enzyme acted on denatured collagen and on collagen fragments, TC_A and TC_B, derived from collagen by collagenase action, suggesting an involvement of Pz-peptidase in collagen breakdown. Preliminary work was, therefore, carried out with monkey kidney Pz-peptidase which was free of collagenase, non-specific protease and di- and tripeptidase activities. Collagen fragments, TC_A and TC_B were prepared from rat skin collagen by the action of rabbit skin collagenase by the procedure of Sakai and Gross [20] and the action of Pz-peptidase was followed by the disappearance of the characteristic Amido-black stained collagen protein bands [20] on acrylamide disc electrophoresis according to Nagai et al. [21]. These studies showed that the Pz-peptidase which has no action on collagen itself, acts on a preparation enriched with respect to its degradation products, TC_A and TC_B. These results suggest that Pz-peptidase may be involved in the breakdown of collagen after its initial cleavage by collagenase into TC_A and TC_B fragments. Further experimental evidence is needed to support these results.

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