BBA 66644

# PURIFICATION AND PROPERTIES OF A PEPTIDASE ACTING ON A SYNTHETIC COLLAGENASE SUBSTRATE FROM EXPERIMENTAL GRANULOMA TISSUE IN THE RAT

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(Received February 18th, 1972)

SUMMARY

An enzyme which acts on a synthetic collagenase substrate, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide) has been purified 250-fold from soluble extracts of experimental granuloma tissue of the rat and its properties studied. It is optimally active around pH 7.2. Its apparent $K_m$ value for Pz-peptide is 0.01 mM and $V$ is 100 nmoles/mg protein per min. It is reversibly inhibited by *p*-hydroxymercuribenzoate (PHMB) and $HgCl_2$, whereas iodoacetamide does not affect the enzyme activity. Heavy metals like $Cu^{2+}$, $Cd^{2+}$, $Ag^{+}$, $Ni^{2+}$ and $Zn^{2+}$ completely inhibit the enzyme activity while the inhibition by $Co^{2+}$ was only partial. $Fe^{2+}$, $Ba^{2+}$, $Mn^{2+}$, $Pb^{2+}$ and $Ca^{2+}$ did not exert any affect on the activity. Chelating agents like EDTA, sodium diethyl dithiocarbamate and $\alpha,\alpha'$-dipyridyl do not affect the enzyme activity. Approximate molecular weight of the purified enzyme was estimated to be 56 000.

## INTRODUCTION

The enzymatic cleavage of a synthetic collagenase substrate, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide) has been observed in a number of tissues and body fluids[1-13]. The exact role of this enzyme (designated Pz-peptidase in this paper) in collagen metabolism in animals is not known, except for the observation that collagen degradation is closely correlated with the Pz-peptidase in post partum uterus[1], tumour tissue[2] and in developing chick embryo skin[3]. Since the above substrate is rather resistant to a number of known proteases and peptidases of animal origin, the above findings would suggest that the Pz-peptidase may have some function in collagen metabolism.

Although some of the properties of a crude enzyme preparation have been reported[9], detailed information on a purified preparation is not available. In the present

Abbreviations: Pz-peptide, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg; PHMB, *p*-hydroxymercuribenzoate.

paper, the purification and properties of a Pz-peptidase from experimental granulation tissue in the rat are described.

## MATERIALS AND METHODS

The following chemicals were obtained commercially as indicated. Pz-peptide from Fluka, Switzerland; dithiothreitol and cysteine from Calbiochem., U.S.A.; *p*-hydroxymercuribenzoate (PHMB), Tris, reduced glutathione and protein markers, bovine serum albumin, bovine liver catalase, pepsin, bovine pancreatic trypsin, sperm whale myoglobin, soyabean trypsin inhibitor, horse heart cytochrome *c* from Sigma, U.S.A.; $(NH_4)_2SO_4$, enzyme grade (Mann., U.S.A.); $\beta$-mercaptoethanol, Eastman, U.S.A.; sodium diethyl dithiocarbamate, Riedel De Haenag Seelze-Hannover, Germany; $\alpha,\alpha'$-dipyridyl, E. Merck AG, Germany; *o*-phenanthroline, National Chemical Laboratory, Poona, India; Sephadex G-200, blue dextran 2000 and DEAE-Sephadex A-50 (Pharmacia, Sweden). Other chemicals were commercially available reagent grade products. Calcium phosphate gel and hydroxylapatite were prepared as described by Keilin and Hartree[14] and by Levin[15] respectively.

### *Induction of granuloma tissue*

Granuloma was induced by subcutaneous implantation of sterilized cotton wicks (7 cm × 0.5 cm) on either side of the abdominal region of 4-month-old albino rats according to a modified procedure[16] of the technique of Meier[17]. The rats were sacrificed on the 4th day of the implantation and the granuloma tissue around the cotton wicks was peeled off and used as the enzyme source.

### *Preparation of tissue homogenate*

Tissue which was frozen and thawed repeatedly was cut into small pieces with scissors and then homogenized with 3 vol. of 0.01 M Tris–HCl buffer, pH 7.4, containing 0.05 M NaCl and 5 mM $CaCl_2$ in a Sorvall Omni-Mixer for 15 min., the cup being immersed in ice during homogenization.

### *Enzyme assay*

The enzyme was assayed by the method of Wuensch and Heidrich[18] in a reaction mixture (0.5 ml) containing the substrate (400 $\mu$g Pz-peptide), $CaCl_2$ (2.5 $\mu$moles), Tris–HCl buffer, pH 7.4 (50 $\mu$moles). After incubation for 1 h, 3% citric acid (1 ml) was added to adjust the pH to around 3.0, and the split product, Pz-Pro–Leu was extracted into ethyl acetate (2 ml) and the absorption measured at 320 nm and the values calculated from a standard curve for Pz-Pro–Leu. In separate experiments, the product was initially identified by thin layer chromatography on silica gel using *n*-butanol–acetic acid–water (4:1:1, by vol.) system using a standard Pz-Pro–Leu.

### *Enzyme unit*

The unit of enzyme activity is defined as the quantity required to liberate 1 $\mu$mole of the product (Pz-Pro–Leu) per min. The specific activity is expressed in milliunits per mg protein.

Protein was determined by the method of Lowry *et al.*[19] using crystalline bovine serum albumin as the standard.

*Purification*

All operations were carried out at 0–4 °C, unless otherwise stated. The bulk of the Pz-peptidase was found to be present in the 100 000 × *g* supernatant fraction. For purposes of purification, the 25% homogenate 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. $(NH_4)_2SO_4$ fractionation*

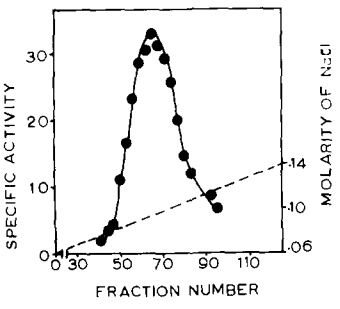
To the supernatant fraction (2000 ml) solid $(NH_4)_2SO_4$ (582 g) was added slowly with mechanical stirring to bring the saturation to 50%. The solution was stirred for an additional hour and then centrifuged at 10 000 × *g* for 20 min. The pellet was discarded and the $(NH_4)_2SO_4$ concentration in the supernatant fraction (2220 ml) was raised to 80% by further addition of $(NH_4)_2SO_4$ (431 g). The pellet was collected by centrifugation and was dissolved in 0.01 M Tris–HCl buffer, pH 7.4, containing NaCl (0.02 M), $CaCl_2$ (5 mM) and dithiothreitol (0.01 mM) (Tris–dithiothreitol buffer) and then dialyzed against the same buffer (36 h) with 4 changes of buffer (5 l each time). The dialyzed enzyme was centrifuged at 10 000 × *g* for 20 min and the supernatant was used for the further purification of Pz-peptidase activity.

*Step II. Calcium phosphate gel adsorption*

The protein concentration of the above fraction (269 ml) was adjusted to 15 mg/ml by the addition of Tris–dithiothreitol buffer and the pH was adjusted to 6.6 with 1 M acetic acid. The overall protein to gel ratio was 1:0.5. A suspension of calcium phosphate gel (20 mg solids/ml) was added slowly with stirring to the protein solution. After 54 ml of gel had been added, the suspension was stirred for 20 min, and then centrifuged at 10 000 × *g* for 20 min (Pellet 1). To the supernatant fraction was added 162 ml of the gel suspension and the process repeated (Pellet 2). To this supernatant fraction another 54 ml of gel was added and Pellet 3 was obtained. Each of the 3 pellets was eluted in two steps with sodium–potassium phosphate buffer, by thoroughly dispersing the pellets and stirring them for 1 h in the eluting buffers and then centrifuging the suspensions at 12 000 × *g* for 20 min. The first elution was with 0.05 M sodium–potassium phosphate buffer, pH 6.6, containing dithiothreitol (0.01 mM) and the second elution with 0.2 M sodium–potassium phosphate buffer, pH 8.0, containing dithiothreitol (0.01 mM). In each case, 1 ml of the buffer was used per ml of gel suspension used for obtaining the pellets. The eluates were dialyzed for 8 h against 10 l of Tris–dithiothreitol buffer to remove $PO_4^{2-}$ and then for an additional 8 h against Tris–dithiothreitol buffer (10 l) containing $CaCl_2$ (5 mM). Enzyme fractions with the highest specific activity were obtained in the 0.2 M buffer eluates of Pellets 2 and 3.

*Step III. DEAE-Sephadex chromatography*

The pooled gel fraction from the previous step (198 ml, total protein 2060 mg) was applied on a DEAE-Sephadex A-50 column (1.8 cm × 39 cm; bed volume 100 ml) previously equilibrated with 0.01 M Tris–dithiothreitol buffer, pH 7.4. After washing with 3 bed volumes of the same buffer, the column was eluted with Tris–dithiothreitol buffer, pH 7.4 with a linear NaCl gradient (0.05–0.14 M) using the Varigrad (Technicon) device and 2-ml fractions were collected. The enzyme was eluted between 0.09–0.12 M NaCl (Fig. 1). The enzymatically active fractions were pooled and used for further purification.



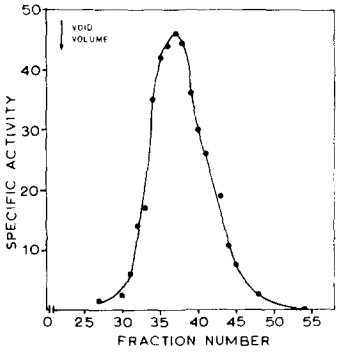




Fig. 1. The elution profile of Pz-peptidase by DEAE-Sephadex chromatography. The details of the column operation are given in the text. The specific activity of the enzyme is given as munits/mg protein.

Fig. 2. The elution profile of Pz-peptidase by Sephadex G-200 gel filtration. The experimental details are given in the text. The specific activity of the enzyme is given as munits/mg protein.

*Step IV. Hydroxylapatite column chromatography*

The pooled fraction from the previous step was dialyzed against water containing 0.01 mM dithiothreitol, concentrated by lyophilization to a small volume (10 ml), and dialyzed against 0.01 M sodium–potassium phosphate buffer (pH 7.0) containing dithiothreitol (0.01 mM). The dialyzed material was applied to a hydroxylapatite column (1.8 cm × 20 cm; bed volume 50 ml) previously equilibrated with 0.01 M sodium–potassium phosphate buffer (pH 7.0) containing dithiothreitol (0.01 mM). After washing with the starting buffer (2 bed volumes) the column was eluted stepwise with 2 bed volumes each of 0.1 M, 0.15 M, 0.2 M and 0.3 M, sodium–potassium phosphate buffer (pH 7.0) containing dithiothreitol (0.01 mM). The eluates were dialyzed exhaustively against 0.01 M Tris–HCl containing dithiothreitol (0.01 mM) and 1 mM $CaCl_2$. The enzyme was eluted with 0.1 M phosphate buffer.

*Step V. Gel filtration on Sephadex G-200*

The enzyme fraction from the previous step was concentrated by precipitation with $(NH_4)_2SO_4$ and the precipitate dissolved in 3 ml Tris–dithiothreitol buffer and applied on a Sephadex G-200 column (1.5 cm × 70 cm; bed volume 125 ml, void volume 44 ml) previously equilibrated with 0.01 M Tris–HCl containing 0.2 M NaCl, 1 mM $CaCl_2$ and 0.01 mM dithiothreitol (pH 7.4) and collected in 2-ml fractions at a flow rate of 15 ml/h. The pattern of Pz-peptidase on the Sephadex G-200 column is given in Fig. 2.

*Step VI. $(NH_4)_2SO_4$ extraction*

The pooled fraction from the above step was precipitated with $(NH_4)_2SO_4$ (90% satn). The precipitate was collected and extracted successively with 5 ml each of 75%, 65%, 55%, 45%, 35% and 25% saturated $(NH_4)_2SO_4$ in 0.05 M Tris–dithiothreitol buffer (pH 7.4). The Pz-peptidase of highest specific activity came in the eluates of 45%, 55%, and 65% saturation of $(NH_4)_2SO_4$. These fractions were pooled and concentrated by $(NH_4)_2SO_4$ and dialyzed against Tris–dithiothreitol buffer and stored at −20 °C at a protein concentration of 10 mg/ml.

The enzyme was thus purified about 250-fold with 22% overall recovery (Table I). This enzyme preparation was used to study the properties of the Pz-peptidase.

TABLE I

PURIFICATION OF Pz-PEPTIDASE FROM RAT GRANULOMA TISSUE

| *Fraction* | *Total protein (mg)* | *Enzyme activity* | | *Recovery (%)* |
|---|---|---|---|---|
| | | *Total (munits)* | *munits/mg protein* | |
| 1 Tissue extract (12 000 × *g* supernatant) | 36 000 | 16 000 | 0.45 | 100 |
| 2 $(NH_4)_2SO_4$ precipitation (50–80% satn) | 10 800 | 13 400 | 1.24 | 84 |
| 3 Calcium phosphate gel | 2 060 | 7 000 | 3.4 | 44 |
| 4 DEAE-Sephadex | 470 | 5 800 | 12.3 | 36 |
| 5 Hydroxylapatite | 295 | 5 900 | 20.0 | 37 |
| 6 Sephadex G-200 | 120 | 5 000 | 42.0 | 31 |
| 7 $(NH_4)_2SO_4$ extraction | 30 | 3 600 | 120.0 | 22 |

## RESULTS

### *Properties of purified Pz-peptidase*

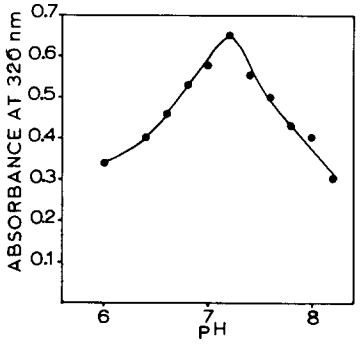
Within the limits of the assay procedure the enzyme activity increased linearly with the time of incubation (upto 1 h) and with enzyme concentration (upto about 10 μg protein).

### *pH optimum*

The pH optimum for the purified Pz-peptidase is in the neutral range (Fig. 3). An identical activity profile is obtained when it was studied at the crude state. The enzyme is completely inactive below pH 5.6 and above 8.4.

### *$K_m$ and V values*

The $K_m$ for the Pz-peptide was found to be 0.01 mM and $V$ was 100 nmoles/mg protein per min. (Fig. 4).



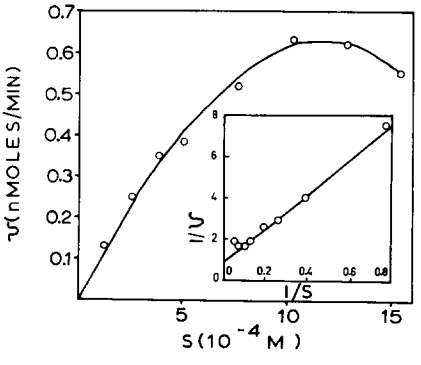




Fig. 3. Dependence of the enzyme activity on pH. Standard assay conditions were employed except that the final buffer concentration was 0.2 M.

Fig. 4. Effect of substrate concentration of Pz-peptidase activity. Standard assay conditions were employed with substrate concentration varied.

TABLE II

EFFECT OF VARIOUS METAL IONS ON Pz-PEPTIDASE ACTIVITY

| *Metal added (0.1 mM)* | *Enzyme activity (munits)* |
|---|---|
| None | 1.00 |
| Lead acetate | 0.87 |
| $CoSO_4$ | 0.45 |
| $NiCl_2$ | 0.15 |
| $HgCl_2$ | 0.10 |
| $AgNO_3$ | 0.03 |
| $CuSO_4$ | 0.02 |
| $CdSO_4$ | 0.02 |
| $ZnSO_4$ | 0 |

*Effect of metal ions, chelating agents and sulfhydryl reagents*

The effect of various metal ions 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 $Hg^{2+}$, $Ag^{+}$, $Cd^{2+}$, $Cu^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ completely inhibit the enzyme activity while the inhibition by $Co^{2+}$ was only partial. $Fe^{2+}$, $Ba^{2+}$, $Mn^{2+}$, $Pb^{2+}$ and $Ca^{2+}$ did not exert any effect on the activity.

At a concentration of 1 mM, EDTA, sodium diethyl dithiocarbamate and $\alpha,\alpha'$-dipyridyl do not affect the enzyme activity. However, *o*-phenonthroline (0.1 mM) was found to be inhibitory to the enzyme (Table III).

TABLE III

EFFECT OF CHELATING AGENTS AND SULFHYDRYL REAGENTS

| *Compound (1 mM)* | *Enzyme activity (munits)* |
|---|---|
| None | 1.0 |
| Diethyl dithiocarbamate | 0.9 |
| EDTA | 1.0 |
| $\alpha,\alpha'$-Dipyridyl | 1.2 |
| PHMB (0.01 mM) | 0.02 |
| Iodoacetamide | 1.0 |
| *o*-Phenanthroline (0.1 mM) | 0.1 |

PHMB inhibited the enzyme activity completely whereas iodoacetamide did not. The inhibition by PHMB or $Hg^{2+}$ is reversed by sulfhydryl compounds like dithiothreitol, GSH or cysteine (Table IV). These sulfhydryl compounds themselves did not affect the enzyme activity. However, they appear to stabilize the enzyme during purification and hence dithiothreitol at 0.01 M was used throughout the purification procedure.

*Gel filtration on Sephadex and approximate molecular weight*

The separation of Pz-peptidase and collagenase from tadpole tissue on Sephadex G-200 column was reported by Harper and Gross[12]. Fractionation of the Pz-peptidase from the rat granuloma tissue on a Sephadex G-200 column (1.5 cm × 70 cm, bed

TABLE IV

REVERSAL OF PHMB INHIBITION BY SULFHYDRYL COMPOUNDS

| *Addition* | | *Enzyme activity (munits)* |
|---|---|---|
| Enzyme | | 0.67 |
| Enzyme + PHMB (0.01 mM) | | 0.03 |
| + PHMB | + cysteine (1 mM) | 0.62 |
| + PHMB | + glutathione (redcued) (1 mM) | 0.67 |
| + PHMB | + dithiothreitol (1 mM) | 0.58 |
| + $HgCl_2$ (0.01 mM) | | 0.03 |
| + $HgCl_2$ | + cysteine (1 mM) | 0.58 |
| + $HgCl_2$ | + glutathione (reduced) (1 mM) | 0.78 |
| + $HgCl_2$ | + dithiothreitol (1 mM) | 0.68 |
| + cysteine (1 mM) | | 0.57 |
| + glutathione (reduced) (1 mM) | | 0.77 |
| + dithiothreitol (1 mM) | | 0.67 |

volume 125 ml, void volume 44 ml) under conditions identical to those employed by Harper and Gross[12] showed that the enzyme emerged earlier than the Pz-peptidase of tadpole tissue and closer to the emergence of tadpole collagenase activity (Fig. 2).

An estimation 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 volume 226 ml) according to Andrews[20]. The void volume of the column was determined with blue dextran 2000. The apparent molecular weight was estimated to be approx. 56 000 (Fig. 5). Preliminary studies indicate that the purified granuloma Pz-peptidase fraction has a weak collagenase activity towards native [$^{14}$C]glycine labeled collagen. This would require further substantiation.

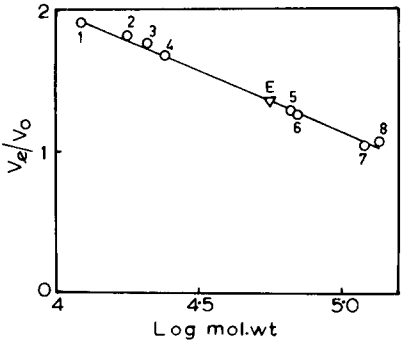



Fig. 5. Estimation of the molecular weight of Pz-peptidase on Sephadex G-100. The protein markers used were (1) cytochrome *c* (12 384); (2) myoglobin (17 800); (3) soyabean trypsin inhibitor (21 000); (4) trypsin (23 800); (5) bovine serum albumin (67 000); (6) pepsin dimer (70 000); (7) catalase dimer (120 000); (8) bovine serum albumin dimer (134 000); (E) Pz-peptidase .The experimental details are given in the text.

## DISCUSSION

Although the Pz-peptidase has been partially purified[3] the properties of the

enzyme have not been studied in detail. In the present paper, the properties and kinetics of a 250-fold purified enzyme are given.

The enzyme is optimally active at pH 7.2. The reported pH optimum for the crude enzyme from rabbit liver and spleen is 8.4[6]. The pH optimum was 7.6 for a partially purified chicken embryo skin enzyme[3] and in the case of rat liver there was a broad pH optimum in the range 8.0–9.0[9]. The human serum Pz-peptidase showed two pH optima[10], one in the range 7.0 and 7.3 and the other between 8.0 and 8.2. At present, it is not clear whether these enzymes constitute a group of closely related enzymes.

The enzyme is severely inhibited by PHMB (0.01 mM) and heavy metals like $Hg^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Cd^{2+}$ and $Ag^{+}$ (all at 0.1 mM) but iodoacetamide (1 mM) did not inhibit the activity. The inhibition by PHMB or $Hg^{2+}$ can be effectively reversed by sulfhydryl compounds like cysteine, reduced glutathione and dithiothreitol. These results would indicate that sulfhydryl groups are required for activity.

There was no activation of the enzyme by any of the metal ions tested. Inclusion of chelating agents like EDTA, sodium diethyl dithiocarbamate and $\alpha,\alpha'$-dipyridyl (all at 1 mM) in the reaction mixture did not affect enzyme activity. However, rather interestingly *o*-phenanthroline (0.1 mM) showed complete inhibition and this would require further investigation. The enzyme from chick embryo skin is reported to require $Ca^{2+}$ for activity[3]. The nature of the metal ion involved in the Pz-peptidase activity from granuloma tissue is still not known.

The apparent molecular weight of the purified Pz-peptidase was estimated to be approx. 56 000 on the basis of its elution from a calibrated Sephadex G-100 column, though confirmation by other methods is needed. Molecular weight of Pz-peptidase from any other source is not known.

Though the Pz-peptidase has been reported to be present in tadpole tissue[12], tumour tissue[2], post partum uterus[1], developing chick embryo skin[3], human serum[10], various tissues of rat[9] and of rabbit[6] and in a few other systems[4,5,7,8,11,13], its physiological significance is not clear. While studying the peptidase and collagenase activities in the invasion zones of tumours of the breast, Keiditsch and Strauch[2] found that the pattern of distribution of Pz-peptidase and collagenolytic activity was similar. With a growth culture of primary mouse fibroblasts and in the rat uterus during post partum involution, a close correlation between Pz-peptidase and collagen metabolism was observed[1]. In a similar study in developing chick embryo Woessner[3] reported that the peak of this peptidase activity, coincided with that of free hydroxyproline. Since the synthetic substrate is not acted upon by a number of known proteases and peptidases[1] but only by bacterial collagenase and by the specific Pz-peptidase from animal tissues described here and elsewhere[1–13], the above results would indicate a close relationship between the Pz-peptidase and collagen metabolism. In a recent report, Harper and Gross[12] have separated the Pz-peptidase from collagenase in tadpole tissue. In our preliminary studies, the crude granuloma extracts and the purified Pz-peptidase show weak collagenolytic activity towards native [$^{14}$C]glycine labeled collagen and was also eluted differently from the tadpole enzyme from Sephadex columns. This collagenolytic activity was not inhibited by soyabean trypsin inhibitor. However, these observations need further experimental substantiation.

ACKNOWLEDGEMENTS

The authors are grateful to Professor S. J. Baker for his keen interest. The project was supported by PL-480 funds under contract No. 01 329 01 and by Council of Scientific and Industrial Research, India.

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