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PURIFICATION AND PROPERTIES OF A COLLAGEN PEPTIDASE (PZ-PEPTIDASE) FROM RABBIT SERUM

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Summary

A peptidase cleaving a synthetic substrate for collagen peptidases, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (designated as PZ-peptide) has been purified 1200-fold from rabbit serum and characterized. The enzyme preparation is free of collagenase and unspecific proteinase activity. The natural substrates are denatured collagen and collagen peptides. The peptidase has a molecular weight of 124 000 and an isoelectric point at pH 5.1. The pH dependence curve exhibits two maxima, one at pH 7.1 and the other at pH 7.9. The enzymic reaction is completely inhibited by Zn^{2+} and to a slower degree by Hg^{2+}, Mn^{2+} and *p*-hydroxymercuribenzoate. It is not affected by EDTA and KCN but totally blocked by *o*-phenanthroline. Phenylmethylsulfonylfluoride is completely inhibitory and points to a serine residue in the active site.

Introduction

With the aid of the PZ-peptide of Wünsch and Heidrich [1] (4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg) enzyme activities have been determined in various sera and tissues. According to the present opinion such enzymes (PZ-peptidases, collagen peptidases) are involved in collagen degradation [2—8], possibly by cleaving the reaction products of collagenases [7,8]. Until now only three PZ-peptidases have been purified to homogeneity [8—10] and there is not yet enough information about the physiological role or more specific physico-chemical properties of these enzymes. In the present paper the

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purification and properties of a PZ-peptidase from rabbit serum have been described.

Methods

1. Purification

A typical example of the purification procedure is described in the following protocol. From 52 ml serum obtained by cardiac puncture of two female chinchilla rabbits (body weight approx. 2000 g) the enzyme is precipitated by 50% saturation with ammonium sulphate. The precipitate is dissolved in 0.05 M Tris-HCl buffer at pH 7.2 (Tris buffer) to a final volume of 14.5 ml and applied to two consecutive columns filled with Ultrogel AcA 44 (3 × 100 and 2.5 × 100 cm). 10-ml fractions are eluted with Tris buffer and all samples with a specific activity not less than 10 nmol · min^{-1} · mg^{-1} are combined. The conductivity of of the solution is adjusted to $6.0 \cdot 10^3$ μsiemens and the enzyme is adsorbed to a 2.5 × 30 cm column with DEAE-Sephadex A-50 in Tris buffer. Elution is performed by a linear NaCl gradient in Tris buffer; desorption begins at 0.12 M NaCl. Nearly all active fractions are combined to give a specific activity of 700 nmol · min^{-1} · mg^{-1}. The enzyme is precipitated with ammonium sulphate at 80% saturation and dissolved in 7.5 ml Tris buffer. Then it is run again on the two Ultrogel columns. 7-ml fractions are collected and all samples with a specific activity $\geqslant$350 nmol · min^{-1} · mg^{-1} are combined. The solution is dialyzed against 1% glycine adjusted to pH 7.2 with NH_4OH for 18 h. Then isoelectric focusing is performed in a flat bed (for details see Fig. 2). From the carrier gel the enzyme is eluted with 3 ml H_2O in special elution columns (LKB, Sweden). pH value and enzymic activity are determined and all active fractions are combined (25 ml). The enzyme solution is filled into a hollow fibre filter beaker (Dow, U.S.A., type b/HFD-1/20 Mini Beaker) and concentrated 10-fold by pumping a solution of 100 ml polyethyleneglycol 6000 through the fibres. Finally the PZ-peptidase is separated from salts and residual ampholytes on a Sephadex G-50 column (2.5 × 60 cm) equilibrated with Tris buffer. The purified enzyme is unstable and has to be stored in small portions at −20° C.

2. Assays

All enzymic reactions with the exception of those which served for the pH curve were performed in 0.05 M Tris-HCl buffer at pH 7.2. Special assay methods are indicated in Table IV. The PZ-peptidase assay was carried out with phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg as substrate (Fluka, Switzerland) on the basis of the method developed by Wünsch and Heidrich [1] with modifications [11]. Additionally with all peptides possible digestion was examined analyzing the incubation mixtures (weight ratio of enzyme/substrate 1 : 500, 16 h at 25° C) by thin-layer chromatography [12]. Collagenolytic activity was determined by the release of soluble radioactivity from ^{14}C-labeled reconstituted fibrils of guinea-pig skin collagen according to Nagai et al. [13]. The samples were incubated for 18 h at 37° C. Digestion of heat-denatured collagen (heated to 60° C for 10 min) was examined by gel electrophoresis [14] (Fig. 4). For discovering unspecific protease activity the enzyme was incubated together with the single proteins (weight ratio of

enzyme/substrate 1 : 100) for 2 h at 25°C. After adjusting to 1 M trichloroacetic acid and centrifugation the supernatants were analyzed for cleaving products by the Lowry method [15]. Parallel incubations were not stopped by trichloroacetic acid but immediately used for the determination of free ninhydrine-positive aminogroups [16]. For all assays controls were run with enzyme heated to 100°C, trypsin, clostridiopeptidase A, and buffer.

Results

1. Purification

The purification procedure is described in Table I. The 1200-fold purified enzyme fraction exhibits a single band in gel electrophoresis (Fig. 1), but as activity could not be demonstrated in the gel slices homogeneity requires further verification. The purified enzyme is unstable. Activity is completely lost after lyophilization and strongly diminished during exposure to room temperature.

2. Characterization of the purified enzyme

The isoelectric point of 5.1 is demonstrated in Fig. 2. The apparent molecular weight was estimated by gel filtration to be 124 000. Fig. 3 gives the pH curve of the peptide cleaving reaction. It exhibits two maxima, one at pH 7.1 and the other at pH 7.9. The presence of a second peptidase able to split the PZ-peptide is not probable because identical pH curves with a constant quotient of the activities at pH 7.1 and 7.9 were measured as well with a 200-fold as with a 1200-fold purified enzyme preparation. Table II shows the effect of various ions on the activity of an enzyme preparation treated with EDTA. Table III represents the effects of many other substances which are normally used to characterize peptidases and proteinases.

Concerning PZ-peptidases one of the most important problems is still the question of their natural substrate. We incubated the purified PZ-peptidase

TABLE I

PURIFICATION OF PZ-PEPTIDASE FROM RABBIT SERUM

The specific activity of the enzyme is expressed in nmol PZ-Pro-Leu liberated per minute (munits) per mg protein.

Fraction	Total protein (mg)	Enzyme activity		Recovery %
		Total (munits)	munits/mg protein	
1. Serum	4144	20 500	5	100
2. $(NH_4)_2SO_4$-precipitation	1262	17 600	14	85.9
3. Ultrogel AcA 44	169	18 100	107	88.3
4. DEAE-Sephadex A-50	16.5	11 500	703	56.1
5. $(NH_4)_2SO_4$-precipitation	12.4	6 900	556	34.5
6. Ultrogel AcA 44	5.84	6 800	1164	33.1
7. Isoelectric focusing *	—	2 600	—	12.7
8. Sephadex G-50	0.367	1 900	5180	9.3
9. Highest purification	0.138	1 300	6380	6.3

* Protein was not determined because of the presence of interfering ampholytes.

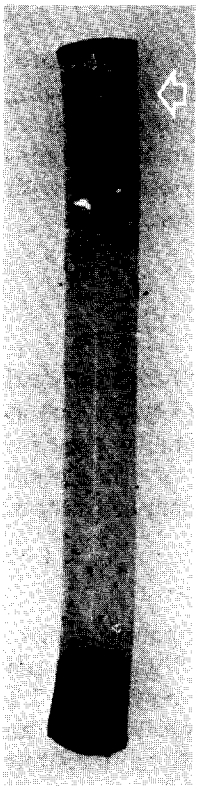

Fig. 1. Disc-electrophoresis of PZ-peptidase. Gel system No. 1 of Maurer [17] was used. 20 μg protein taken from a 1200-fold purified enzyme preparation was applied and run at 5 mA for 3 h. Staining was performed with Coomassie brilliant blue.

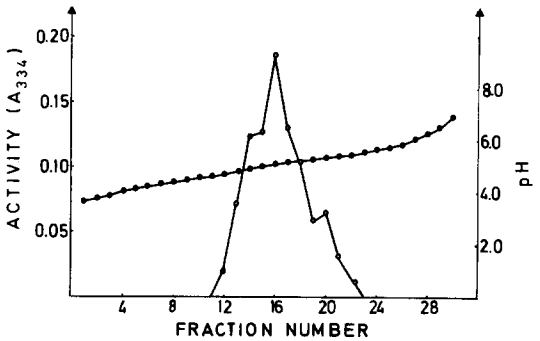

Fig. 2. Isoelectric focusing of PZ-peptidase. Focusing was carried out in a 22 × 11 × 0.5 cm flat bed of Ultrogel (LKB, Sweden) with a 6% ampholyte gradient (Serva, F.R.G.) at pH 4—6 and 20 W for 6 h. ●——●, pH gradient; ○——○, enzymic activity.

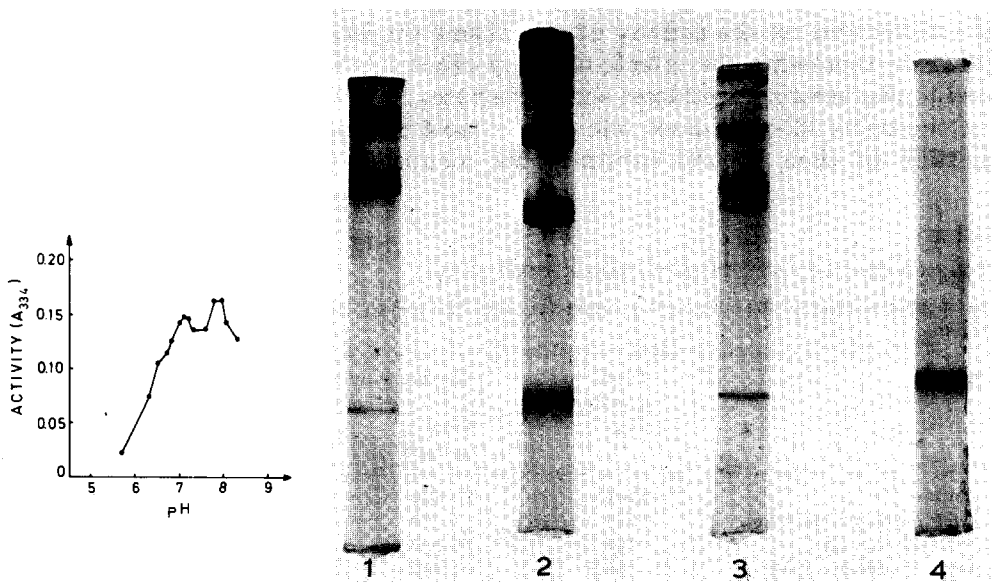

Fig. 3. pH dependence of the purified PZ-peptidase. All assays were performed in 0.05 M Tris-acetate buffer at different pH values. The exact pH-values of the incubation mixtures were determined with the aid of a microelectrode at the end of the incubation period.

Fig. 4. Effect of purified PZ-peptidase on native and heat-denatured collagen. 20 μg enzyme or 0.2 ml buffer was incubated together with 500 μg collagen in 0.05 M Tris-HCl buffer (pH 7.2) containing 0.2 M NaCl and 3 mM $CaCl_2$ for 18 h at 25°C. Then the mixtures were adjusted to 6 M urea and 30 μl each was analyzed by gel electrophoresis according to Nagai et al. [14]. 1, native collagen + buffer; 2, native collagen + PZ-peptidase; 3, denatured collagen + buffer; 4, denatured collagen + PZ-peptidase.

TABLE II

EFFECT OF VARIOUS IONS ON THE ACTIVITY OF THE PURIFIED PZ-PEPTIDASE

The enzyme solution was adjusted to 10 mM EDTA and then dialyzed against Tris buffer for 15 h to eliminate the chelator. The preparation was adjusted to 1 mM of each salt and incubated for 30 min at 25°C. Then the standard assay procedure was performed decreasing the concentration of the salts to half the initial amount.

Substance added	Enzyme activity (% of control)
none	100
EDTA	100
$MgCl_2$	108
$CaCl_2$	108
$MnCl_2$	50
$FeCl_2$	87
$CuCl_2$	103
$ZnCl_2$	0
$BaCl_2$	89
$HgCl_2$	19

from rabbit serum together with many proteins and peptides and came to the result that this enzyme is highly specific for damaged collagen cleaving the sequence -Pro-X-Gly-Pro-Y- at the X-Gly bond (Table IV, Fig. 4). Additionally to the compounds listed in Table IV we offered the following substances which turned out to be not cleaved by the enzyme: Leu-Gly-Gly, Gly-Hyp-Ala,

TABLE III

INFLUENCE OF VARIOUS COMPOUNDS UPON THE ACTIVITY OF PZ-PEPTIDASE

The enzyme was preincubated together with each compound (dissolved in the Tris buffer) for 30 min at 25°C. Reaction was then started by addition of substrate solution. By that the concentration of the compounds added was decreased to half the initial amount. Serum was directly added to the assay mixture.

Substance added	Concentration in standard assay	Activity (% of control)
none	—	100
Mercaptoethanol	1 mM	100
Dithiothreitol	10 mM	91
Cysteine	1 mM	100
Iodoacetamide	5 mM	96
p-Hydroxymercuribenzoate	0.05 mM	51
p-Hydroxymercuribenzoate + 5 mM dithiothreitol	0.05 mM	85
$HgCl_2$	0.5 mM	30
$HgCl_2$ + 5 mM dithiothreitol	0.5 mM	49
EDTA	5 mM	100
KCN	10 mM	100
o-Phenanthroline	5 mM	0
Diazoacetylnorleucine methyl ester	0.5 mM	100
Pepstatin	1 μg	100
Soybean trypsin inhibitor	10 μg	100
Phenylmethylsulfonylfluoride	1.5 mM	0
Penicillamine	1 mM	100
Human serum	100 μl	75
Rat serum	100 μl	77
Rabbit serum	100 μl	100

TABLE IV

SUBSTRATE SPECIFICITY OF THE PURIFIED PZ-PEPTIDASE

For details see Methods.

Substrate	Digestion	Reference
(A) Peptides		
PZ-Pro-Leu-Gly-Pro-D-Arg	+	1,11
N-CBZ-Gly-Pro-Gly-Gly-Pro-Ala	+	12,18
N-CBZ-Gly-Pro-Leu-Gly-Pro	+	12,19
Z-Pro-Ala-Gly-Pro-β-NA	—	12,20
N-α-tosyl-Gly-Pro-Arg-*p*-aniline	—	12,20
CBZ-Gly-Pro-Arg-*p*-aniline	—	12,20
(B) Proteins		
Azocasein	—	21
Casein	—	15,16
Albumin	—	15,16
Fibrinogen	—	15,16
Denatured hemoglobin	—	15,16
Native collagen	—	13,14
Denatured collagen	+	14

Gly-Pro, Ala-*p*-nitroaniline, Ala-Ala-*p*-nitroaniline, Ac-Ala-*p*-nitroaniline, CBZ-Ala-Ala-*p*-nitroaniline, benzyl-Arg-*p*-nitroaniline, Lys-*p*-nitroaniline, Phe-*p*-nitroaniline.

Discussion

The molecular weight of the PZ-peptidase is one of the highest reported and lies in the order of bacterial collagenases. Preliminary studies with SDS-electrophoresis make it probable that the enzyme consists of two subunits with a molecular weight of 60 000. The pH curve of the rabbit peptidase exhibiting two peaks was already demonstrated by Gries and Grasedyck [2], and was also observed in the case of human PZ-peptidases [2,5]. But these enzymes had not been purified. In spite of the 1200-fold purification of the rabbit enzyme, it was not possible to separate two PZ-peptidases of different pH optima like Hino and Nagatsu [22] did with a preparation from bovine dental follicle. In order to classify the PZ-peptidase we studied the influence of various effectors upon the purified enzyme. The lack of inhibition by pepstatin and diazoacetyl-norleucine methyl ester as well as the alkaline pH optima rule out carboxyl protease activity. In contrast to collagenases and most PZ-peptidases so far described, the rabbit enzyme does not seem to require metal ions. It is not affected in its activity by EDTA and KCN, but, rather surprisingly, completely inhibited by *o*-phenanthroline. Concerning this point it resembles a PZ-peptidase isolated from rat granuloma [23], but not the peptidases purified from monkey kidney and chick embryos [8,10]. Like other PZ-peptidases the rabbit enzyme contains SH groups. As the activity is only partly blocked by *p*-hydroxymercuribenzoate, we assume that they are not located in the active site. The complete inhibition by phenylmethylsulfonylfluoride suggests that a serine residue is involved in the cleaving reaction. Inhibition is not achieved by

soybean trypsin inhibitor and this again is a point of coincidence of the rabbit and the rat peptidase. The so far existing reports on PZ-peptidases make it obvious that in mammalians enzymes of high specificity towards collagen peptides exist which differ distinctly from collagenases. The enzyme purified from chick embryos [10] shows similar properties; it is limited in its action to collagen peptides containing 5—20 amino acid residues. The PZ-peptidase of rabbit serum, too, exhibits high specificity for the sequence -Pro-X-Gly-Pro-Y-. But in contrast to the chicken peptidase, it is able to attack the intact polypeptide chains of heat denatured collagen. Though not checked it surely takes also TC^A and TC^B, the reaction products of collagenase, as substrates. This was already proved in the case of the PZ-peptidase from human synovial fluid [7].

Our results show that there may exist great differences in the physico-chemical properties of PZ-peptidases, but nevertheless they confirm the conception that these enzymes find their main task in remodeling processes of the connective tissue specifically cleaving damaged collagen at a sequence which is poorly affected by other enzymes.

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