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SEPARATION OF TWO PZ-PEPTIDASES FROM BOVINE DENTAL FOLLICLE

MASAMI HINO and TOSHIHARU NAGATSU

Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, Nagoya 464 (Japan)

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Summary

Two PZ-peptidases (EC 3.4.-) (A and B) cleaving a synthetic substrate for collagenase, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-peptide) have been separated from the particulate fraction of bovine dental follicle. PZ-peptidase A had a molecular weight of 220 000, an optimum pH at 8.0—8.5, and a K_m value of 67 μM toward PZ-peptide at pH 7.1, whereas PZ-peptidase B had a molecular weight of 20 000, an optimum pH at 6.5—6.7, and a K_m value of 400 μM toward PZ-peptide at pH 7.1. Two similar enzymes were also isolated from the soluble fraction. Since the pH-activity curve of the crude tissue preparations such as homogenate, microsomes and soluble supernatant had two peaks at 6.5—6.7 and 8.0—8.5, both PZ-peptidase A and B may exist in situ as two independent active enzymes.

Introduction

Due to the synthesis of an artificial substrate for collagenase, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-peptide) which permits the assay of certain types of peptidase [1], many tissues were found to contain a peptidase (PZ-peptidase) (EC 3.4.-) which cleaves PZ-peptide [2]. PZ-peptidase is an enzyme distinct from animal collagenase which attacks native collagen [3], but there have been several reports suggesting a relationship between PZ-peptidase and the degradation of collagen in vivo [4—6]. We found a high activity of PZ-peptidase in the dental follicle beneath the deciduous tooth undergoing resorption [7], where collagen degradation is expected to be high and collagenase activity was found [8—10]. A PZ-peptidase has been highly purified from monkey kidney [11] but the properties of a purified enzyme in other sources have not been studied in detail. PZ-peptidase activity in human or avian serum or rabbit tissues was found to have two distinct pH optima, one at pH 7.2 and the other at pH 8.0, suggesting the presence of two

different forms of the enzyme [12—15]. However, there has been no report on the isolation of multiple forms of PZ-peptidase. During the purification of PZ-peptidase from bovine dental follicles, we have observed two pH-activity optima with crude tissue preparations such as homogenate, microsomes and supernatant fraction, and have isolated two PZ-peptidases with different molecular weights, pH optima and kinetic properties.

Materials and Methods

4-Phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-peptide) was purchased from Fluka (Switzerland); Fast Garnet GBC, from Sigma; fluorescamine from Nippon Roche, Tokyo; and acid soluble calf skin collagen from Worthington Biochem. Corp.

Bovine mandibles were obtained fresh, packed in ice, from a slaughter-house, and dental follicles of the unerupted premolars beneath the deciduous teeth were collected. The tissues were rinsed in cold saline, blotted and homogenized in 10 vols. of 0.25 M sucrose with an Ultra-Turrax homogenizer; the homogenate was filtered twice through gauze to remove tissue debris. The homogenate was centrifuged at 700 × *g* for 10 min, then at 5000 × *g* for 10 min, and finally at 100 000 × *g* for 60 min, to separate nuclear, mitochondrial, and microsomal fractions, respectively.

The activity of PZ-peptidase was measured as described by Wünsch and Heidrich [1]. The incubation mixture contained (in final concentrations) 40 mM Tris · HCl buffer, pH 7.1, 0.2 mM PZ-peptide, 5 mM $CaCl_2$, and enzyme plus water to a total volume of 1.5 ml. Incubation was carried out at 37°C for 30 min. Reaction was terminated by adding 0.5 ml of 1.5 M citrate buffer, pH 4.5; the product, PZ-Pro-Leu, was extracted with 2.5 ml of ethylacetate, and the absorbance at 320 nm was measured. For the blank incubation, the substrate was omitted during and added after the incubation. Incubated substrate controls without enzyme or with boiled (90°C, 10 min) enzyme was also run.

With purified enzyme preparations, PZ-peptidase activity was also measured fluorimetrically using fluorescamine [16] by measuring the appearance of an amino group in the product, Gly-Pro-D-Arg. Phosphate buffer, pH 8.0, was used instead of Tris · HCl buffer for the incubation, and $CaCl_2$ was omitted. The enzyme reaction was terminated by rapid addition of 0.5 ml of a fluorescamine dioxane solution (15 mg/50 ml). The fluorescence intensity of Gly-Pro-D-Arg released by enzymatic hydrolysis of the substrate was measured at 480 nm with the excitation light at 380 nm. Gly-L-Pro-L-Leu was used as a standard. The sensitivity of the fluorimetric assay system was at least 10-fold higher than the colorimetric assay system. As a blank, enzyme was omitted during and added after the incubation. This fluorescence assay gave identical values as those obtained by colorimetry.

The unit of enzyme activity is expressed as that amount which catalyses the hydrolysis of 1 nmol of the substrate per min.

Collagenase activity in the PZ-peptidase preparations was examined by a modification of the digestion of native collagen gel prepared from calf skin [17]. Collagen solution was prepared by dissolving 0.2% of collagen in cold 50 mM Tris · HCl buffer, pH 7.6, containing 5 mM $CaCl_2$ and 1.0 M NaCl, then

dialysed for 24 h against the same buffer. The dialysed collagen solution was incubated in ordinary moist air at 37°C for 3—4 h to make the collagen gel. After incubation, enzyme solutions were soaked into the filter paper (diameter, 5 mm), and each filter paper was placed on the collagen gel in a micro-slide chamber. The micro-slide chamber was closed with a glass plate, incubated in moist air at 37°C for 3—4 days, and observed daily for gel lysis on a black background using oblique illumination.

The approximate molecular weight was determined by gel filtration on Sephadex G-200 according to the method of Whitaker [18]. The molecular weights of apoferritin, γ-globulin, bovine albumin, chymotrypsinogen A, myoglobin, and cytochrome *c* were taken as 480 000, 160 000, 67 000, 45 000, 25 000 and 12 000, respectively.

Protein was measured by the method of Lowry et al., using bovine serum albumin as a standard [19,20].

Results

pH-Activity curve of PZ-peptidase in crude preparations of bovine dental follicle

About 30% of the activity of PZ-peptidase was in the 100 000 × *g* precipitate (microsomal fraction), and about 40% in the 100 000 × *g* supernatant (soluble fraction). The specific activity was the highest in the 100 000 × *g* precipitate. pH-activity curves of PZ-peptidase in the homogenate (Fig. 1, A), microsomal fraction (Fig. 1, B), and soluble fraction (Fig. 1, C), all had two pH-optimum peaks, at pH 7—8 and pH 8—9.

Separation of two PZ-peptidases from the microsomal fraction of bovine dental follicle

Purification of PZ-peptidase was carried out using the microsomal fraction as the enzyme source. All procedures were carried out at 0—4°C.

Step 1. Isolation of microsomes. 60 g of bovine dental follicles were homog-

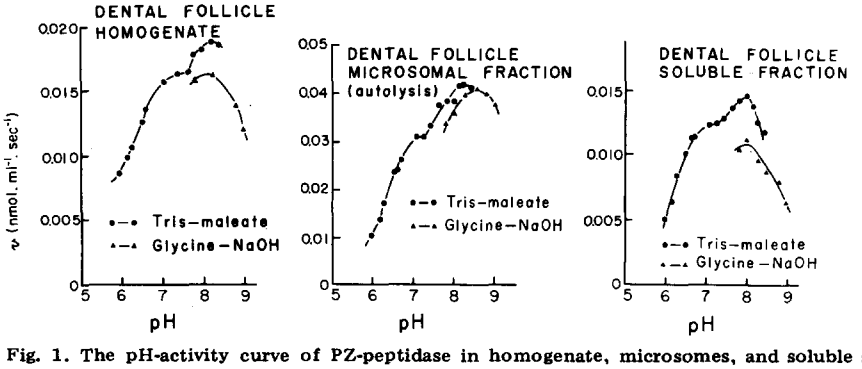

Fig. 1. The pH-activity curve of PZ-peptidase in homogenate, microsomes, and soluble supernatant of bovine dental follicle. The incubation mixture contained (in final concentrations) 40 mM buffers as indicated, 0.2 mM PZ-peptide, 5 mM $CaCl_2$, and enzyme plus water to a final volume of 1.50 ml. PZ-peptidase activity was assayed photometrically and expressed as nmol of PZ-Pro-Leu formed/ml enzyme per s after incubation at 37°C for 30 min.

enized in 10 vols. of 0.25 M sucrose using an Ultra-Turrax homogenizer. Microsomes were isolated by differential centrifugation as described in Materials and Methods. The microsomes were suspended in water to get a protein concentration of 10—15 mg/ml and stored frozen at −20°C.

Step 2. Solubilization. The microsomal suspension stored frozen at −20°C for more than 24 h was incubated at 37°C for 24 h, then centrifuged at 100 000 × *g* for 60 min. The supernatant was carefully removed. The residue was resuspended in water and stored at −20°C for 24 h. This autodigestion procedure was repeated (usually 3 times) to get nearly 100% recovery of activity and the supernatant fractions were combined. An example of this solubilization procedure is shown in Table I. In the experiments in Table I and in Table II, about 93% and 106% of the enzyme activity were solubilized by repeated autodigestion, respectively.

Step 3. $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to 62 ml of the extract from the microsomal fraction to 50% saturation. After mixing for 60 min, the solution was centrifuged at 10 000 × *g* for 20 min. The supernatant was removed and the precipitate discarded. To the supernatant, solid $(NH_4)_2SO_4$ was added to 70% saturation. After mixing for 60 min, the solution was centrifuged at 10 000 × *g* for 20 min. The supernatant was discarded, and the precipitate dissolved in a minimum volume of 20 mM phosphate buffer, pH 8.0, containing 0.01 mM dithiothreitol. The solution was dialyzed against a large volume of the same buffer. The dialyzed solution could be stored frozen at −20°C.

Step 4. Sephadex G-200 chromatography. The enzyme at Step 3 was passed through a column of Sephadex G-200 equilibrated previously with 20 mM phosphate buffer, pH 8.0, containing 0.01 mM dithiothreitol. As shown in Fig. 2, two peaks of the enzyme activity appeared; the first peak (PZ-peptidase A) between the 22nd and 31st fractions and the second peak (PZ-peptidase B) be-

TABLE I

SOLUBILIZATION OF PZ-PEPTIDASE FROM THE MICROSOMAL FRACTION OF BOVINE DENTAL FOLLICLE

The microsomal fractions were isolated from 40 g of bovine dental follicle and PZ-peptidase was solubilized by autodigestion as described in Results. Protein was determined by the method of Lowry et al., using bovine serum albumin as standard [19,20].

Preparation	Total protein (mg)	PZ-peptidase		
		Total activity		Specific activity (mU/mg)
		(mU)	(%)	
Microsomal fraction	242	208	100	0.9
Supernatant				
after 1st autolysis	25.0	40	19	1.6
after 2nd autolysis	10.2	139	67	13.6
after 3rd autolysis	4.3	14	7	3.3
Combined supernatant	39.5	193	93	4.9

TABLE II

PURIFICATION OF PZ-PEPTIDASE FROM MICROSOMES OF BOVINE DENTAL FOLLICLE

Protein was determined by the method of Lowry et al., using bovine serum albumin as standard [19,20].

Step	Protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification (-fold)
Homogenate *	2504	953	0.38	100	1
Microsomes	297	386	1.30	41	3
Extract	31.3	409	13.1	43	34
$(NH_4)_2SO_4$ (50—70%)	5.5	123	22.3	13	59
Sephadex G-200					
Peak A	1.6	11	6.9	1.2	18
Peak B	2.4	66	27.5	6.9	72

* Bovine dentol follicle, 60g.

tween the 36th and 47th fraction. These two fractions were combined, concentrated by ultrafiltration, and stored at −20°C. The purified enzymes were stable at least for 6 months at −20°C. The entire purification is shown in Table II.

Separation of two PZ-peptidases from the soluble fraction of bovine dental follicle

The enzyme in the soluble fraction was subjected to the purification procedure described above; $(NH_4)_2SO_4$ fractionation between 40—70% saturation and subsequent Sephadex G-200 chromatography. Two peaks of the enzyme activity were also separated.

Properties of purified PZ-peptidase from the microsomal fraction of bovine dental follicle

The properties of PZ-peptidases A and B purified from the microsomal fraction were examined.

Incubation was carried out with shaking in air, usually for 30 min, but the enzyme reaction proceeded linearly with time at least for 15 h at 37°C with PZ-

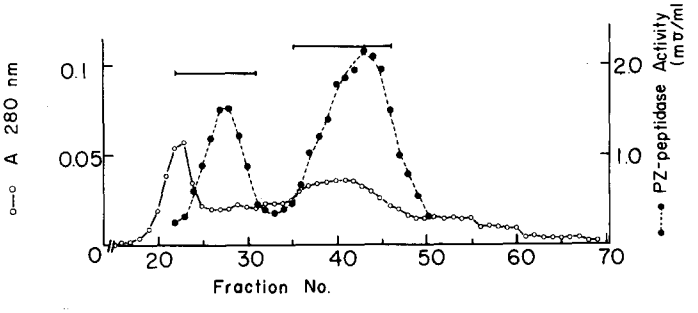

Fig. 2. Chromatography of PZ-peptidases from microsomes of bovine dental follicles on Sephadex G-200. For 5.5 mg of protein in 3.4 ml, a 1.5 × 90 cm column was used. Elution was carried out using the same buffer at a flow rate of 6—8 ml/h, and fractions of 2.2 ml each were collected. PZ-peptidase activity was determined photometrically, as described in Materials and Methods.

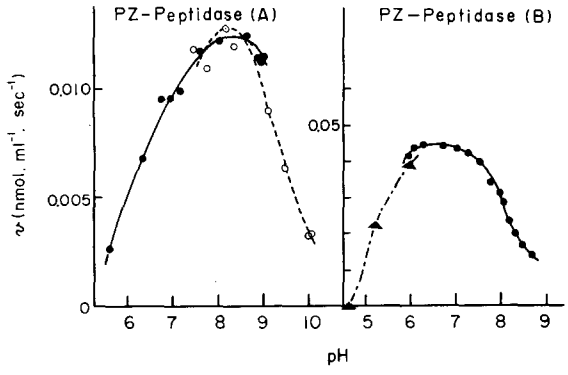

Fig. 3. pH-Activity curve of purified PZ-peptidase A and PZ-peptidase B from microsomes of bovine dental follicles. Conditions of assay of PZ-peptidase activity were the same as described in Fig. 1. ▲— · —▲, acetate buffer, ●——● Tris/maleate buffer, and ○– – –○, glycine/NaOH buffer. Final buffer concentrations were 40 mM.

peptidases A and B. For 15 h incubation, toluene (0.05 ml) was added to inhibit the growth of bacteria, and the amount of enzyme was reduced to get a net absorbance of about 0.6 which was equivalent to 130 nmol of the product and to 0.04% hydrolysis of the substrate. The approximate molecular weight was estimated by gel filtration on Sephadex G-200 in 20 mM phosphate buffer (pH 8.0) containing 0.01 mM dithiothreitol. Plots according to the method of Whitaker [18] indicated an approximate molecular weight of 220 000 for PZ-peptidase A and 20 000 for PZ-peptidase B. The pH-activity relationship was examined in acetate buffer, Tris/maleate buffer, and glycine buffer. As shown in Fig. 3, the optimum pH of PZ-peptidase A was at 8.0—8.5, and that of PZ-peptidase B at 6.5—7.0. The K_m value towards PZ-peptide substrate obtained from Lineweaver-Burk plots were 67 μM for PZ-peptidase A and 400 μM for

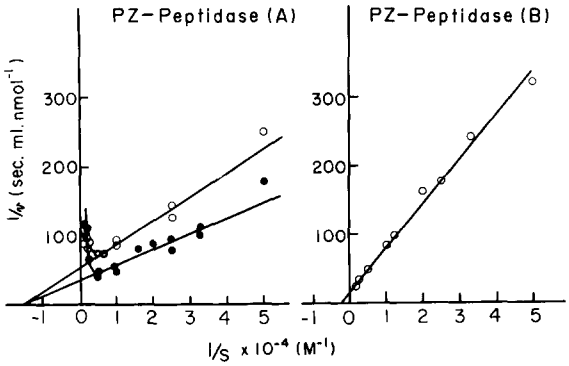

Fig. 4. Lineweaver-Burk plot of PZ-peptidase A and PZ-peptidase B from microsomes of bovine dental follicles. The activity was measured photometrically for PZ-peptidase A and B (○——○). PZ-peptidase A activity was also measured fluorimetrically (●——●). Incubation mixture contained (in final concentrations) 40 mM Tris · HCl buffer, pH 7.1, 0.2 mM PZ-peptide, 5 mM $CaCl_2$, and enzyme plus water to a final volume of 1.50 ml. For fluorimetry, $CaCl_2$ was omitted. Incubation was carried out at 37°C for 30 min. In photometry, the product, PZ-Pro-Leu was isolated by extraction and assayed at 320 nm [1]. In fluorimetry, the product, Gly-Pro-D-Arg, was assayed using fluorescamine [16].

TABLE III

AFFECTORS OF PZ-PEPTIDASE FROM MICROSOMES OF BOVINE DENTAL FOLLICLE

The affectors were added into the standard assay mixture, without $CaCl_2$, (total volume 1.50 ml) simultaneously with enzyme. In the case of serum, the volume to get a maximum activation was shown in parenthesis. The specific activities of enzyme A and B were 6.9 mU/mg and 27.5 mU/mg, respectively.

Affectors added (1 mM)	Enzyme activity (% of control)	
	Enzyme A	Enzyme B
– (control)	100	100
$CaCl_2$	98	92
$AgNO_3$	49	10
$CuSO_4$	44	0
$HgCl_2$	17	5
EDTA	70	4
EDTA + $CaCl_2$	100	5
α,α'-Dipyridyl	89	103
Cysteine	100	93
Glutathione (reduced)	99	86
Serum		
Human serum	143 (50 μl)	226 (15 μl)
Dialyzed human serum	139 (50 μl)	220 (15 μl)
Boiled human serum	100 (50 μl)	84 (15 μl)
Bovine serum	112 (30 μl)	236 (15 μl)

PZ-peptidase B, respectively (Fig. 4). In the case of PZ-peptidase A, the K_m value obtained by fluorimetry was identical with that obtained by photometry. Substrate inhibition was observed for PZ-peptidase A, but not for PZ-peptidase B. The effects of metals on PZ-peptidases A and B were examined (Table III). Ag^+, Cu^{2+} and Hg^{2+} inhibited both enzymes A and B; enzyme B was more susceptible than enzyme A; Ca^{2+} had been reported to activate PZ-peptidase from chick embryo skin [4], but had no effect on enzymes A and B. EDTA (1.0 mM) was inhibitory, especially for PZ-peptidase B. This inhibition reversed by Ca^{2+} in the case of enzyme A, but not in the case of enzyme B. Reduced glutathione or cysteine did not affect enzyme activity but appear to stabilize the enzyme during purification.

Both bovine and human sera (1 : 100—1 : 20 dilution) activated PZ-peptidases; PZ-peptidase B was activated more pronouncedly (about 2-fold). Dialyzed serum had similar activatory effects, but boiling of serum at 100°C for 10 min completely abolished the activatory effect (Table III).

Comparison of the PZ-peptidases in the microsomal and soluble fractions

As described above, two forms of PZ-peptidases were also present in the soluble fraction. Properties of PZ-peptidases in the first peak and the second peak in Sephadex G-200 chromatography from the soluble fraction were similar to those of PZ-peptidases A and B from the microsomal fraction, respectively.

The soluble fraction from bovine dental follicle had collagenase activity towards gelatin, but neither of the two PZ-peptidases purified from the soluble

fraction had collagenase activity towards gelatin. The results indicate that it was completely separated from the PZ-peptidases during purification.

Discussion

The presence of two pH optima of PZ-peptidase activity in crude preparations such as homogenate, microsomes and soluble supernatant of bovine dental follicles suggest the presence of at least two enzymes.

Two pH optima of PZ-peptidase had been reported in the literature in human and rabbit sera and tissues [12—15], but two enzymes with different pH optima have never been isolated.

In the present study, we have isolated two PZ-peptidases with different pH optima from microsomal and soluble fractions of bovine dental follicle: a higher molecular weight form and a lower molecular weight form. The enzymes in microsomes were designated as A and B, whereas those of the soluble fraction as I and II.

The properties of A and I and those of B and II were similar, thus A and I and B and II may be the same enzymes. The properties of enzymes A and B were quite different. Enzyme A had a higher molecular weight, a more alkaline optimum pH, and a lower K_m value towards PZ-peptide, whereas enzyme B had a lower molecular weight, a more acidic optimum pH, and a higher K_m value.

Animal collagenase is known to hydrolyze gelatin, but neither PZ-peptidase A nor B acted on gelatin. The soluble fraction of bovine dental follicle had collagenase activity on gelatin, indicating the presence of both animal collagenases and PZ-peptidases, but subsequent purification of PZ-peptidases separated collagenase activity from PZ-peptidases.

The recovery of enzymes at purification steps of $(NH_4)_2SO_4$ fractionation and Sephadex chromatography was low. Ca^{2+} was added in an attempt to increase the stability, but was not effective.

The significance of serum activation is not clear. The results indicate that the activation factor may be protein. The activation of PZ-peptidases by serum protein is in contrast to the inhibition of animal collagenase by serum α_2-macroglobulin [21].

Since enzymes A and B were solubilized from the microsomes by autodigestion the possibility that enzyme B is a fragment of enzyme A during autodigestion was also considered. However, since two similar forms are also found in the soluble fraction and since the pH optima of enzymes A and B correspond to the two peaks of optimum pH in the homogenate, microsomes, or soluble supernatant, it is most likely that A and B forms exist in situ.

As shown in Table III, Ca^{2+} had no effect on enzymes A and B, but EDTA inhibition on enzyme A was reversed by Ca^{2+}. Reversal of EDTA inhibition by Ca^{2+} was also reported with monky kidney enzyme [11].

PZ-peptidase has been found in many tissues, but has not been purified extensively. Aswanikumar and Radhakrishnan [11] have recently extensively purified a PZ-peptidase from monkey kidney. The properties of PZ-peptidase B in our work, such as K_m value, optimum pH, inhibition by heavy metals and EDTA are similar [11] to those purified from the monkey kidney. One significant different is that PZ-peptidase B from bovine dental follicle has a

lower molecular weight (20 000) than the monkey kidney PZ-peptidase (56 000). PZ-peptidase is supposed to act on the degradation of collagen in vivo. The physiological significance of two PZ-peptidases in vivo remains a topic for further investigation.

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