The metalloenzymic nature of collagenase-like peptidase of the rat testis

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Summary. Collagenase-like peptidase, an enzyme degrading synthetic collagenase substrate (PZ-pentapeptide), was purified from rat testes and its properties were examined. Its activity was strongly inhibited by chelating agents, such as EDTA and 1,10-phenanthroline. By chelation and exhaustive dialysis it was possible to obtain this enzyme in its inactive, metal-free form. The activity of the metal-free enzyme was partly recovered by treatment with zinc or manganese ions, while a combined zinc and manganese treatment resulted in complete recovery of enzyme activity.

Introduction

We have previously described a collagenase-like peptidase (CLP), an enzyme capable of degrading synthetic collagenase substrate, whose activity was detected in rat and bull testes and spermatozoa and in human spermatozoa (Koren & Milković, 1973). While investigating the role of CLP in the male reproductive system we found that purified CLP from rat testes degraded proteins from the rat seminal vesicle secretion, and could thus promote the release of spermatozoa from the copulatory plug (Koren, Lukač & Milković, 1974; Koren, Schön & Lukač, 1975). CLP from human semen and testes participates in the liquefaction of the human ejaculate (Lukač, 1976).

Enzymes that can degrade synthetic collagenase substrate have been detected in a number of different animal tissues (Sakai *et al.* 1967; Strauch & Vencelj, 1967; Cutroneo & Fuller, 1970; Rabadjija, Koren & Pende, 1971; Levdikova & Orekovich, 1972; Aswanikumar & Radhakrishnan, 1972, 1973) and are believed to play some role in the degradation of extracellular proteins, although their properties have not been studied in detail. Woessner (1970) reported that such an enzyme, obtained from chick embryos, required Ca^{++} for its activity, but no metal requirement was found for the enzyme from rat granuloma tissue (Aswanikumar & Radhakrishnan, 1972). We therefore investigated whether the CLP isolated from rat testes requires metal cations for its activity.

Materials and Methods

Chemicals

Synthetic PZ-pentapeptide (4-phenylazo-benzyloxy-carbonyl-L-prolyl-L-leucyl-L-glycyl-L-prolyl-D-arginine) and PZ-dipeptide (4-phenylazo-benzyloxy-carbonyl-L-prolyl-L-leucin) were purchased from Fluka, Switzerland, Na_2EDTA from BDH, U.K., dithiothreitol and 2-mercaptoethanol from Sigma Chemicals Co., U.S.A., 1,10-phenanthroline and cysteine from Merck, Germany. All the other chemicals were commercially available reagent-grade products.

Purification of CLP from rat testes

CLP was purified from rat testes by the acetone precipitation method described previously (Koren & Milković, 1973).

Preparation of the metal-free enzyme

CLP was dialysed in a small dialysing tube (A. H. Thomas Co., Philadelphia, U.S.A., 6 mm diam.) for 6 h against 500 ml 20 mM-EDTA. To remove EDTA the dialysis bag was then transferred to 500 ml deionized water, pH 7.5 (adjusted by tris). Dialysis was continued at 4°C for 48 h, with 6 changes of the outer solution, at constant magnetic stirring. To obtain the same ionic strength the control sample was prepared by dialysing CLP against deionized water.

Enzyme assay

The enzyme was assayed by the method of Wunsch & Heidrich (1963) in a reaction mixture containing 100 µg of enzyme preparation and 500 µg PZ-pentapeptide in 2 ml 0.05 M-tris-acetic acid buffer, pH 7.5, containing 0.05 M-NaCl. After 60 min incubation at 37°C the reaction mixture was stopped with 0.5 ml 10% citric acid, and the PZ-Pro-Leu released was extracted with 3 ml ethyl acetate. Absorption was measured at 320 nm.

The effects of various metal ions, chelators and sulphhydryl compounds on CLP activity were tested after mixing 0.9 ml enzyme solution with 0.1 ml agent solution (dissolved in 0.05 M-tris-acetic acid buffer, pH 7.5).

In the assay of the metal-free CLP, all metal cation solutions and substrate solutions were prepared with deionized water, pH 7.5 (adjusted by tris).

In all experiments the degradation product was identified by thin-layer chromatography on Silica Gel G using the *n*-butanol:acetic acid:pyridine:water (5:1:2.2:4, by vol.) solvent system. PZ-pentapeptide and PZ-dipeptide were employed as standards.

The unit of enzyme activity was defined as the amount of enzyme releasing 1 µg PZ-Pro-Leu under the above described conditions.

Results

Table 1 shows the effects of different bivalent cations on CLP activity. Heavy metals, such as Zn⁺⁺, Cu⁺⁺, Ni⁺⁺, Cd⁺⁺ and Hg⁺⁺, caused an almost complete inhibition of CLP activity in the concentration range of 0.1–1 mM. Magnesium ions showed no effect, while Ca⁺⁺ and Mn⁺⁺ stimulated CLP activity.

Chelating agents also caused inhibition of CLP activity (Table 1), the most effective inhibitor being 1,10-phenanthroline (1 mM).

At the concentrations tested (Table 1) the sulphhydryl compounds had no effect on CLP activity, but after inhibition with Hg⁺⁺ cysteine and dithiothreitol elicited recovery of enzyme activity (Table 2). Following inhibition with EDTA attempts were made to restore the enzyme activity with various metallic cations (Table 3), but even a five-fold excess of ions was ineffective.

After exhaustive dialysis against EDTA the enzyme remained almost completely inhibited, while the activity of the control CLP preparation (dialysed against deionized water only) underwent practically no change (Table 4).

As shown in Table 5 the activity of the CLP inactivated by dialysis against EDTA could be partly restored by Zn⁺⁺ ions. A zinc ion concentration of 1 µM restored about 40% of the enzyme activity, while higher concentrations caused an inhibitory effect. Almost complete restoration of CLP activity was achieved only by the combined action of zinc and manganese ions (Table 5).

Discussion

These results show that heavy metals have a strong inhibitory effect on the activity of CLP from rat testis. The fact that inhibition by Hg⁺⁺ can be effectively reversed by sulphhydryl compounds such as cysteine and dithiothreitol (Table 2) seems to indicate that sulphhydryl groups are required for the

Table 1. The effect of some metallic cations, chelating agents and SH agents on the activity of collagenase-like peptidase from rat testis

	mm	% of activity	No. of estimations
Cations*			
None	—	100	12
Zn⁺⁺	0.1	7	7
	1	5	7
Cu⁺⁺	0.1	5	4
	1	0	4
Cd⁺⁺	0.1	0	3
	1	0	3
Ni⁺⁺	0.1	0	3
	1	0	3
Hg⁺⁺	0.1	10	3
Mg⁺⁺	0.1	100	3
	1	100	3
Ca⁺⁺	0.1	100	7
	1	125	9
	5	142	9
	10	131	9
	50	122	6
Mn⁺⁺	0.1	127	3
	1	132	4
	5	149	4
	10	167	6
	50	159	4
Chelating agents†			
None	—	100	11
Na₂EDTA	1	74	4
	5	49	8
	10	36	8
	20	11	4
1,10-Phenanthroline	0.1	39	5
	1	28	9
SH agents†			
Dithiothreitol	10	100	4
Dimercaptoethanol	10	100	4
Cysteine	10	100	4

* In the chloride form.

† The incubation mixture contained 10 mM-CaCl₂.

activity of this enzyme. The inhibitory effect of the chelating agents is most probably due to the binding of the heavy metal. This interpretation can be supported by several pieces of indirect evidence: (a) 1,10-phenanthroline, which has a poor binding affinity to calcium and a markedly strong affinity to heavy metals (Sillén & Martel, 1964), is effective in low concentrations; (b) phenanthroline also has an inhibitory effect when there is a great excess of Ca ions; (c) after inhibition by EDTA, which

Table 2. Restoration of collagenase-like peptidase activity by SH agents after inhibition by Hg⁺⁺

Addition	% of activity	No. of estimations
None	100	4
0.1 mM-Hg ⁺⁺	10	4
0.1 mM-Hg ⁺⁺ + 1 mM-cysteine	92	4
0.1 mM-Hg ⁺⁺ + 1 mM-dithiothreitol	50	4

Table 3. Reversal of Na₂EDTA inhibition of collagenase-like peptidase activity by some metallic cations

Na ₂ EDTA added (mM)	Cation added (mM)	% of activity	No. of estimations
—	—	100	5
5	—	29	5
5	Ca ⁺⁺ 5	32	5
5	10	36	5
5	25	37	5
5	Mn ⁺⁺ 5	30	5
5	10	30	5
5	25	27	5
5	Zn ⁺⁺ 5	20	3
5	10	0	3

Table 4. The effect of Na₂EDTA inhibition (20 mM) and dialysis against deionized water on collagenase-like peptidase (CLP) activity

Treatment	Activity* (μg PZ-dipeptide/ ml incubation mixture)	% of activity
Uninhibited, undialysed CLP	32.0 ± 2.61*	100
Uninhibited, dialysed CLP	26.6 ± 3.13	83
Inhibited, dialysed CLP	3.8 ± 0.42	12

* Mean ± S.E.M. of 5 estimations.

Table 5. The effect of zinc and manganese ions on the activity of collagenase-like peptidase inactivated by chelation and exhaustive dialysis

Zn ⁺⁺ (M)	Mn ⁺⁺ (M)	% of activity*
—	—	12
10 ⁻⁷	—	15
10 ⁻⁶	—	41
10 ⁻⁵	—	24
10 ⁻⁶	10 ⁻⁵	41
10 ⁻⁶	10 ⁻⁴	60
10 ⁻⁶	10 ⁻³	81
10 ⁻⁶	10 ⁻²	80

Each number represents the mean values of 5 estimations.

*100% activity is that of uninhibited, undialysed CLP (Table 4).

also prefers heavy metal ions, the enzyme activity cannot be reversed even with a great excess of Ca⁺⁺ or Mn⁺⁺ (Table 3); (d) following dialysis against EDTA, the enzyme activity can be partly restored only by Zn⁺⁺, while Ca⁺⁺ or Mn⁺⁺ alone remain ineffective.

It appears that a heavy metal is firmly bound to the enzyme molecule, since CLP activity cannot be diminished by dialysis against deionized water alone, and because the inactive enzyme could be obtained only by dialysis against EDTA (Table 4). The results (Table 5) indicate that complete CLP activity requires two metals: one, most probably Zn⁺⁺, for its catalytic function, and another (Ca⁺⁺ or Mn⁺⁺) for the stabilization of the enzyme molecule.

The effect of Ca⁺⁺ on collagenases has been described in connection with bacterial and animal collagenases. In the case of human and rat skin and uterus collagenase (Seltzer, Welgus, Jeffrey &

Eisen, 1973), Ca^{++} is necessary for thermostabilization, while in clostridial collagenase A it appears to participate in the binding of the enzyme to the substrate (Seifter & Harper, 1970). In addition to their requirement for extrinsic Ca^{++} , some collagenases appear to contain an intrinsic metal ion. Clostridial collagenase A is known to contain Zn^{++} (Seifter & Harper, 1970), and the same metal is most probably present also in some animal collagenases (Berman & Manabe, 1973). Although CLP can not be considered a 'true collagenase' (it does not degrade native collagen), it is obviously similar to collagenases, not only because it is capable of degrading a synthetic collagenase substrate and collagen peptides (Koren & Milković, 1973), but also in respect to its metal requirement. The fact that zinc is one of the metals inhibiting CLP activity (Table 1) does not exclude the possibility that this metal is present at the active site of the enzyme, since it is known that a metal present at the active centre of an enzyme molecule may exert an inhibitory effect when it is also bound to some other part of the same molecule (Vallee, 1960).

The results obtained indicate that CLP from rat testes is a true metalloenzyme with a tightly bound intrinsic metal necessary for its activity.

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