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SEPARATION OF COLLAGENASE AND PEPTIDASE ACTIVITIES OF TADPOLE TISSUES IN CULTURE*

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SUMMARY

Collagenase and peptidase activities in the crude powder obtained from the culture media of tadpole tissues have been separated from each other by three different procedures. The peptidase cleaves synthetic peptides which have been used in the assay of *Clostridium histolyticum* collagenase, but has no effect on native collagen, whereas the collagenase degrades native collagen but does not attack the synthetic peptides.

It is concluded that synthetic peptides are not suitable substrates for identifying or assaying for collagenolytic activity in animal tissues, that only native collagen should be used for this purpose.

INTRODUCTION

In recent years collagenolytic enzymes have been detected in a number of living amphibian and mammalian tissues by their lytic action on native collagen substrates in tissue culture at neutral pH (refs. 1–16). These enzymes have been isolated from liquid culture media, partially purified and their mode of attack on native collagen molecules at physiologic pH described[9,12,14,18]. In only one instance, that of human polymorphonuclear leucocytes, has a similar enzyme been isolated directly from the cells by extraction[17,18]. All these enzymes have a neutral or slightly alkaline pH optimum and attack native collagen at temperatures below the substrate denaturation temperature, by severing one-quarter of the molecule without degradation or denaturation of the two fragments. The collagenases of rat uterus[11] and salamander regenerating limb[19] remove two additional small fragments at 20–28°. At 37° fibrils are rapidly degraded to dialyzable peptides.

The action of the best characterized animal collagenase, that from tadpole, liberates only COOH-terminal glycine and NH_2-terminal leucine and isoleucine from

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Abbreviations: Z, benzyloxycarbonyl; Pz, phenylazobenzyloxycarbonyl.

native collagen at 20–28° (ref. 20). The attack is more extensive on denatured collagen, releasing COOH-terminal glycine only and NH_2-terminal leucine, isoleucine, valine, alanine and phenylalanine[20]. This mode of action is entirely different from that of *Clostridium histolyticum* collagenase (Clostridio peptidase A, EC 3.4.4.19) which degrades native collagen to a variety of small peptides, liberating NH_2-terminal glycine and a wide spectrum of C-terminal amino acids. This enzyme has a specific peptide composition requirement, Pro (Hyp)–X–Gly–Pro (Hyp)–Y–, scission occurring between X and Gly (refs. 21–23).

Recently there have been reports[24–26] of "collagenases" in cultures of mammalian cells and other tissues detected and measured by their action on the synthetic peptide substrates designed specifically for assays of the bacterial collagenase. Since all the mammalian collagenases isolated to date attack the collagen molecule in a manner similar to the tadpole collagenase making one or at most three cleavages at temperatures below substrate denaturation as judged by electron microscopy it is likely that the peptide bond specificity is similar and unlike that for the bacterial enzyme. If this is true, then the synthetic peptide substrates do not detect true animal collagenolytic activity but may indicate the presence of an unidentified peptidase.

We report here the separation of two distinct enzymes in a crude tadpole-tail-fin tissue culture medium, one of which degrades native collagen but not the two synthetic peptides and the other which cleaves the peptides but not native collagen.

METHODS

The crude enzyme powder was prepared by the tissue culture technique previously described[8]. Briefly, strips of tail fin from premetamorphic bullfrog tadpoles, *(Rana catesbeiana)* previously exposed to antibiotics in the aquarium water for 24 h, were cultured on filter paper discs floating in protein free media suitable for amphibian tissue culture. The culture fluid was replaced daily and the harvested media centrifuged, dialyzed and lyophilized.

Collagenolytic activity was measured by the release of radioactive peptides from acid-extracted [^{14}C]glycine-labeled reconstituted guinea-pig-skin collagen[8], and by decrease in viscosity of calf-skin collagen solution in 0.05 M Tris buffer (pH 7.6) containing 0.2 M NaCl at 20°. Peptidase activity was monitored by methods described elsewhere[27–29]. Pz–Pro–Leu–Gly–Pro–D-Arg was incubated with the enzyme at pH 7.5, 37° for varying time periods. Pz–Pro–Leu, which is insoluble in acidified water, was extracted with ethyl acetate and determined spectrophotometrically at 320 nm. Z–Gly–Pro–Gly–Gly–Pro–Ala was split at the Gly–Gly bond. The resulting Gly–Pro–Ala content was determined with ninhydrin. These methods were modified by increasing the time of incubation of enzyme with substrate to 5, 12 and 24 h at 37°. Identification of the reaction products obtained with synthetic substrates was carried out by separation on paper chromatography (Whatman No. 1) using appropriate markers in *n*-butanol–acetic acid–water (4:1:5, by vol.) solvent mixture (upper phase).

The crude lyophilized enzyme powders from cultures of tail, back skin, and gill of bullfrog tadpole were stored at 15° over anhydrous $CaSO_4$. A solution of powder was made with 0.05 M Tris buffer (pH 7.4) containing $CaCl_2$ and centrifuged for 15 minutes at 27 000 × *g* at 0°. The amber colored supernate was brought to 20%

saturation by addition of solid $(NH_4)_2SO_4$ (special enzyme grade Mann Research Laboratories, New York, N.Y.). The 20–50% $(NH_4)_2SO_4$ pellet was dissolved in 0.05 M Tris buffer (pH 7.4) containing 5 mM $CaCl_2$ and dialyzed against 0.01 M Tris, 1 mM $CaCl_2$ (pH 7.4) at 4° for 16 h with three changes of buffer and lyophilized. The supernate of this last step was dialyzed under the same conditions and lyophilized. All fractions were analyzed for collagenase, peptidase and caseinolytic activity.

The lyophilized redissolved retentate from the $(NH_4)_2SO_4$ fractions were placed on a Sephadex G-200 column (1.5 cm × 70 cm) previously equilibrated with 0.01 M Tris buffer (pH 7.4) containing 1 mM $CaCl_2$, 0.2 M NaCl and eluted with the same buffer. 1-ml fractions were collected every 5 min, the effluent was monitored at 280 nm. The column and fractions were kept at 4°. Protein fractions were assayed for collagenase and peptidase activities. Chromatography of the redissolved $(NH_4)_2SO_4$ precipitate was also accomplished on an Agarose (8%) column under the same conditions.

An alternate approach to purification of the dialyzed $(NH_4)_2SO_4$ fractions employed the polyacrylamide disc electrophoresis system of DAVIS[31] and ORNSTEIN[30]. Here riboflavin 0.0005% (refs. 32, 33) was substituted for persulfate. A Tris–glycine buffer of pH 8.3 was used and the system run at 1 mA per tube at 4°. The gel was sliced longitudinally on a Saran Wrap covered steel plate kept over ice in the cold room at 4° with the aid of a Stadie Riggs blade. One-half was stained for protein content with Amido Schwarz. The other half was sliced transversely with a gel slicer made after the description of CHRAMBACH[34]. Each gel slice was eluted with 0.5 ml of 0.05 M Tris, 5 mM $CaCl_2$ buffer (pH 7.4) at ice bath temperature. The eluents were examined for collagenolytic and peptidase activities.

The gel slab method of RAYMOND[35] employing the DAVIS[31] and ORNSTEIN[30] system of buffers and solutions was used to increase the capacity of the purification procedure outlined above. By this procedure, instead of microgram amounts of enzyme, milligram amounts of protein could be purified. This system was operated at 4° with 10 mA for the first hour and 20 mA for the remaining 5 h. The gel slab (12 cm × 16 cm) was removed and 0.15-cm longitudinal slices taken from the left, right and center of the gel. These slices were stained as before with Amido Schwarz dye for proteins. The remaining slab was cut transversely into 0.5-cm pieces. These pieces were sliced into cubes (0.5 cm × 0.25 cm) and eluted at ice bath temperature with 0.05 M Tris, 5 mM $CaCl_2$, pH 7.4. Eluted fractions which exhibited enzyme activity, either collagenase or peptidase were rerun in the DAVIS[31] and ORNSTEIN[30] system of polyacrylamide gel electrophoresis as a criterion of homogeneity.

RESULTS

$(NH_4)_2SO_4$ fractionation was successful in separating the enzyme activities. Polyacrylamide electrophoretic analysis of the fractions obtained is shown in Fig. 1. The three gel patterns represent the initial enzyme solution and the $(NH_4)_2SO_4$ fractions. Note that most of the collagenase activities is in the 50% saturated $(NH_4)_2SO_4$ precipitate and the peptidase activity is in the supernatant fluid. The distribution of enzyme activities as measured by release of ^{14}C-labeled peptides from native collagen, and the cleavage of synthetic peptides is correlated with the gel slice from which the protein was eluted as illustrated in Fig. 1.

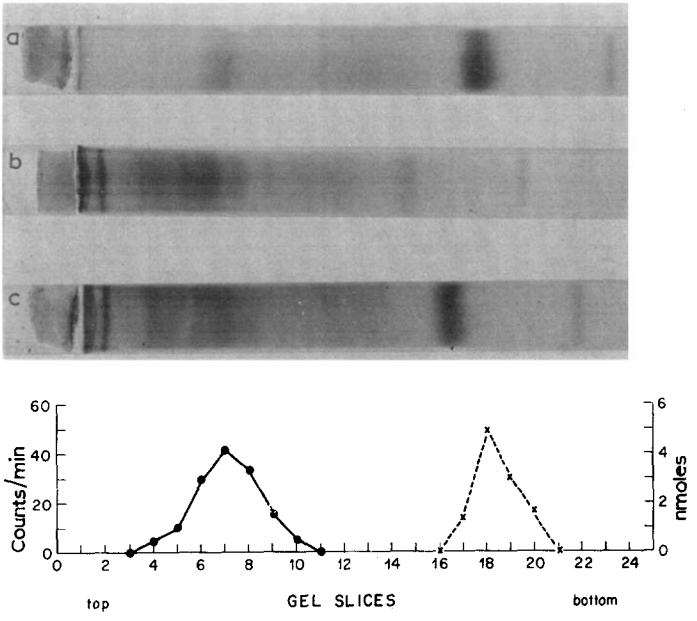

Fig. 1. Correlation of protein bands obtained from polyacrylamide gel electrophoresis of initial enzyme solution with enzyme activity eluted from these bands. (a) $(NH_4)_2SO_4$ supernate; (b) $(NH_4)_2SO_4$ precipitate; (c) initial enzyme solution. Collagenase activity: ●—●, release of [^{14}C]-glycine-containing peptides. Peptidase activity: ×— — —×, cleavage of Pz-Pro-Leu-Gly-Pro-D-Arg. Details in METHODS.

Sephadex column chromatography of the $(NH_4)_2SO_4$ fractions resulted in the separation of collagenase from peptidase as shown in Fig. 2. Agarose chromatography (Fig. 3) resolved the collagenolytic activity into two peaks both of which were clearly

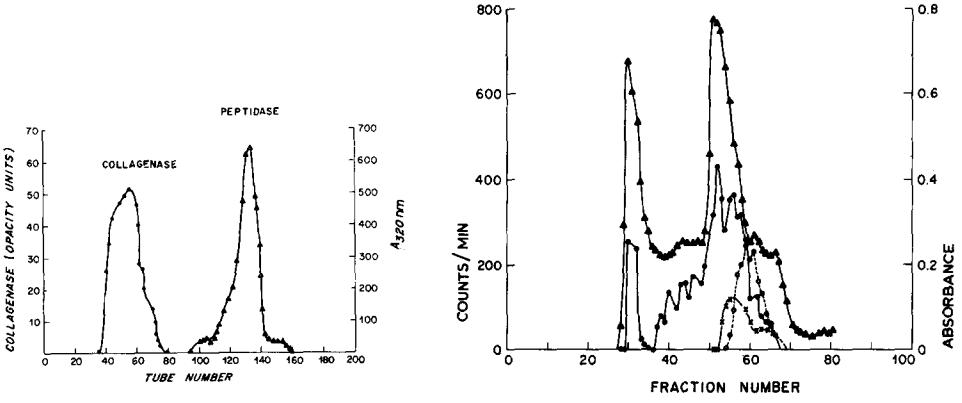

Fig. 2. Pattern of enzyme activity obtained from chromatography on Sephadex G-200 of $(NH_4)_2SO_4$ precipitate and supernate. Details in METHODS. ●—●, collagenase $(NH_4)_2SO_4$ ppt.; △—△, peptidase $(NH_4)_2SO_4$ supernate (Pz-Pro-Leu-Gly-Pro-D-Arg).

Fig. 3. Separation of enzymes. Agarose chromatography. Two peaks of collagenase activity are seen. Peptidase and caseinolytic activity are present on the far shoulder of the second collagenase peak. Details in METHODS. ▲—▲, $A_{280\ nm}$; ●—●, [^{14}C]collagen; ○— — —○, synthetic peptide ($A_{320\ nm}$); ×—×, casein ($A_{280\ nm}$).

TABLE I

COLLAGENASE AND PEPTIDASE ACTIVITY OF TADPOLE FIN

	Collagenase activity (counts/min per mg)*	*Peptidase activity (nM/mg**)*
Crude powder	460	3.9
20–50% $(NH_4)_2SO_4$ ppt.	602	8.5
50% $(NH_4)_2SO_4$ supernatant	42	13.0
Gel slab	21 000	92.0

* Counts/min per mg release of [^{14}C]glycine-containing peptides from collagen.
** nM/mg cleavage of Pz–Pro–Leu–Gly–Pro–D-Arg.

separated from the peptidase and caseinolytic activities which moved close together.

A summary of the distribution of activity and the degree of purification is given in Table I. Final purification by gel slab electrophoresis increased collagenolytic activity 46-fold over that of the starting material and a 24-fold increment in peptidase activity was also obtained. Collagenase thus purified exhibited no caseinolytic activity or peptidase activity whereas the peptidase showed activity against synthetic peptides and slight (1–2%) proteolysis using casein as substrate but none against native collagen. The peptidase cleaved both Z–Gly–Pro–Gly–Gly–Pro–Ala and Pz–Pro–Leu–Gly–Pro–D-Arg. Separation of the reaction products by paper chromatography established that the point of cleavage by the peptidase was the same as that for *Cl. histolyticum* collagenase for which these synthetic peptides were designed. This was verified by use of the markers Gly–Pro–Ala; Z–Gly–Pro–Gly; Pz–Pro–Leu and Gly–Pro–D-Arg. Typical enzyme kinetics were established for the peptidase (Fig. 4). A similar analysis was reported earlier for the tadpole collagenase[8].

DISCUSSION

The isolated tadpole collagenase did not attack the synthetic peptides widely

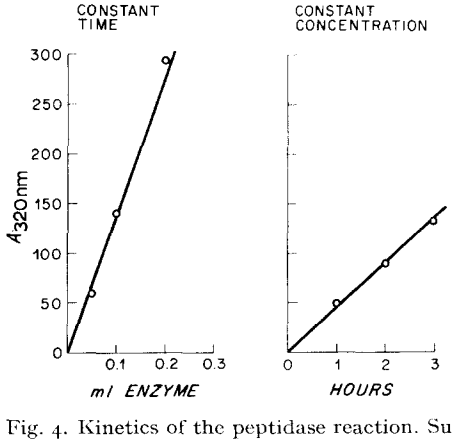

Fig. 4. Kinetics of the peptidase reaction. Substrate: Pz–Pro–Leu–Gly–Pro–D-Arg.

employed in assaying for the presence of bacterial collagenase. The peptidase which could be readily and completely separated from collagenase by differential precipitation, by chromatographic means, and also by electrophoresis, cleaved these peptides but at a considerably lower rate than the bacterial enzyme. The latter exhibited an 800-fold greater activity in units/mg over that of the amphibian peptidase. Published reports[24,25] of peptidase activity from animal tissues (called by these authors "collagenase") employing the synthetic peptides as substrates and using crude or partially purified preparations as the source of enzyme were in the range of activity described for the tadpole peptidase.

It is not unexpected that among the variety of proteases and peptidases still poorly characterized, one or more will cleave the same synthetic substrate which is susceptible to bacterial collagenase. Such observations in no way indicate that these peptidases can cleave native collagen. Since the collagenolytic enzyme identified by degradation of the native substrate can further degrade the initial two high-molecular-weight fragments to smaller polypeptides after their denaturation at physiologic temperature and pH, any other protease or peptidase activity might be expected to act at an advanced stage of degradation. The peptidase activity reported here may serve the function of further degradation of the reaction products resulting from the initial cleavage of collagen by collagenase. Its possible role in the latter phases of a sequential degradation process is not excluded.

Synthetic peptides employed for monitoring collagenolytic activity of *Cl. histolyticum* collagenase should not be used to study collagenolytic activity in animal tissues. Their value is restricted to the *Cl. histolyticum* enzyme, at this time, since their structures are based on this enzyme's known specific requirements and there is no reason to assume that other collagenases have the same substrate specificity.

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