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PURIFICATION OF TADPOLE COLLAGENASE AND CHARACTERIZATION USING COLLAGEN AND SYNTHETIC SUBSTRATES

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Summary

Tadpole collagenase hydrolyzed native and denatured collagen and synthetic peptides with sequences of 2,4-dinitrophenyl-L-prolyl-L-leucylglycyl-L-isoleucyl-L-alanylglycyl-L-arginine amide and 2,4-dinitrophenyl-L-prolyl-L-glutaminyl-glycyl-L-isoleucyl-L-alanylglycyl-L-glutaminyl-D-arginine. The specific enzyme activity against the latter substrate and collagen fibrils is found to be 933 nmol/min per mg protein and 8440 units (μg collagen degraded/min), respectively. Optimum pH for the enzyme is 7.5—8.5.

A collagenase complex with α_2-macroglobulin did not hydrolyze collagen fibrils, but digested the synthetic substrates at the Gly-Ile bond.

The amino acid composition of the enzyme was determined.

Immunoelectrophoresis of the enzyme at pH 8.6 against anti-tadpole collagenase rabbit immunoglobulin G shows a single precipitin line at a position migrating faster than human serum albumin and corresponding to enzyme activity against collagen fibril and synthetic substrates.

Introduction

Ubiquitous distribution in tissues and physiological importance of collagenase are now well accepted [1]. However, the properties of the enzymes show significant discrepancies [2—9] even with enzyme from the same tissue and animal. These may be partly ascribed to the fact that the enzyme levels in

Abbreviations: DNP-peptide I, 2,4-dinitrophenyl-L-prolyl-L-leucylglyceryl-L-isoleucyl-L-alanylglycyl-L-arginine amide; DNP-peptide II, 2,4-dinitrophenyl-L-leucylglycyl-L-isoleucyl-L-alanylglycyl-L-arginine amide; DNP-peptide III, 2,4-dinitrophenyl-L-propyl-L-glutaminylglycyl-L-isoleucyl-L-alanylglycyl-L glutaminyl-D-arginine; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate.

tissues are extremely low. However, the high sensitivity of the assay method, using labeled substrates with radioactive materials, makes it feasible to handle these enzymes, even though they are still in an impure state.

The purpose of this study is to elucidate enzymic and chemical properties of tissue collagenase by preparing pure enzyme from the tadpole skin. The discrepancy between the present results and those reported by Harper et al. [3] is also discussed.

Materials

DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH_2 (DNP-peptide I), DNP-Leu-Gly-Ile-Ala-Gly-Arg-NH_2 (DNP-peptide II) and DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH (DNP-peptide III) were obtained from the Protein Research Foundation, Minoh, Osaka, Japan. 2-(*N*-Morpholino)ethanesulfonic acid (MES) and agarose A-37 was purchased from Nakarai Chemicals, Ltd., Kyoto, agar from Difco Lab., Lot No. 0560-2, Detroit, Mich., U.S.A., tryptamine from Tokyo Kasei Kogyo Co. Ltd., Tokyo, and polyethyleneglycol 1000 from Wako Pure Chemical Industries Ltd., Osaka. All other reagents used were of analytical grade.

Methods

Preparation of crude collagenase

Bullfrog tadpole, *Rana catesbiana*, back-skin explants were cultured in Tyrode solution for 3 days at 37°C, as described previously [10,11]. After removal of debris materials by centrifugation, the cultured media were concentrated by ultrafiltration through an Amicon Diaflo UM 10 membrane and stored at −20°C until use. Approx. $7 \cdot 10^4$ units of crude collagenase were obtained from 1000 bullfrog tadpoles.

Purification of collagenase

Collagenase was purified from the concentrated cultured media by enzyme digestion with a mixture of testicular hyaluronidase, RNAase and DNAase, followed by collagen-polyacrylamide gel chromatography and Sephadex G-75 superfine chromatography, according to a slight modification of the method previously reported [2]. After affinity chromatography on a collagen gel column, the eluate fractions, containing more than 2800 units/A_{280nm} of collagenase activity and comprising of 86% of the total enzyme activity recovered, was concentrated by ultrafiltration through a Diaflo UM 10 membrane in the presence of 0.01% polyethyleneglycol 1000 to prevent leakage of the enzyme. The resultant enzyme solution was heat-shocked at 37°C for 20 min and applied on a series of three Sephadex G-75 superfine columns equilibrated with 0.05 M Tris-HCl buffer (pH 7.6)/1 M NaCl/5 mM $CaCl_2$.

Collagenase assay

Collagenase activity was assayed using ^{14}C-labeled, reconstituted guinea-pig skin collagen fibrils as substrate [11]. 1 unit enzyme activity is the amount of protein necessary to degrade 1 μg collagen per min at pH 7.6 and 37°C.

Peptidase assays

DNP-peptides I, II and III were employed as substrates for the assay of peptidase activity of collagenase preparation [12]. The peptides were dissolved in 0.05 M Tris-HCl buffer (pH 7.6)/0.15 M NaCl/5 mM $CaCl_2$/0.02% bovine serum albumin, at a final concentration of $5 \cdot 10^{-4}$ M. In the case of DNP-peptide I, ethanol was added to the buffer solution at a final concentration of 10%, because of a relatively poor solubility of the peptide in aqueous solution. To 0.1 ml each of the peptides buffer solution without albumin was added to adjust the enzyme volume to 0.1 ml at 37°C. The reaction was started by adding appropriate amounts of enzyme. After a given period of incubation at 37°C, 0.5 ml 1 M HCl was added to stop the reaction and the DNP-peptide fragments released were extracted by vigorous shaking with 1 ml ethyl acetate (for DNP-peptide I and II) or ethyl acetate/*n*-butanol [1 : 0.15 (v/v) for DNP-peptide III] followed by centrifugation at 1000—1500 × *g* at room temperature for 10 min to separate the two layers. The degree of hydrolysis was determined by measuring the absorbances of the organic layers at 365 nm (for DNP-peptide I and III) and 345 nm (for DNP-peptide II). The molecular extinction coefficients at 365 nm of DNP-peptides I and III and at 345 nm of DNP-peptide II in organic solution were estimated to be 1.76, 1.49 and $1.73 \cdot 10^4$, respectively [12].

Amino acid analysis

150—300 μg purified collagenase were hydrolyzed with 0.5 ml 6 M HCl in an evacuated, sealed tube for 24 h at 110°C. In order to determine complete amino acid composition, acid hydrolysis of the enzyme (150—300 μg) with 0.5 ml 4 M methanesulfonic acid/0.2% tryptamine was also performed in an evacuated, sealed tube for 24 h at 115°C [13]. Tryptamine [3-(2-aminoethyl)-indole] was prepared from its hydrochloride salt [14].

After hydrolysis with 6 M HCl, the hydrolyzate was dried and dissolved in 0.2 M citrate buffer (pH 2.2). In the case of sulfonic acid hydrolysis, however, the hydrolyzate was neutralized with 0.2 ml 10 M NaOH, then adjusted to pH 2.2 with 0.7 ml 0.2 M citrate buffer. Amino acid analysis was performed with a JEOL model 6AS amino acid autoanalyzer. Corrections were made for the destruction of serine, cystine and tyrosine that occurred during hydrolysis with 6 M HCl or 4 M methanesulfonic acid, by using the factors of 10, 40, and 15 or 15, 25 and 15%, respectively. Tryptophan content was calculated without correction, assuming tadpole collagenase to be a non-glycoprotein, since the presence of sugars in a protein sample is known to affect the recovery of tryptophan by hydrolysis with acid [13].

Preparation of human α_2-macroglobulin

Human α_2-macroglobulin was prepared according to the method of Iwamoto et al. [15] from outdated human blood which was obtained from the Blood Bank of the University Hospital, Tokyo Medical and Dental University.

Preparation of antisera to tadpole collagenase, serum and human serum albumin

Two rabbits were each injected intramuscularly with 180 μg purified

tadpole-skin collagenase emulsified with Freund's complete adjuvant (1 : 1, v/v) followed by similar booster injections at days 19, 38 and 59, using 212, 440 and 180 μg enzyme without Freund's complete adjuvant, respectively. The antisera titers were monitored by bleeding at intervals between booster injections and the rabbit producing antibody were bled out on day 69. Antisera to tadpole serum and human serum albumin were prepared in the same way. Normal serum was obtained from the same rabbit prior to immunization. Control sera and anti-sera were stored at −20°C until used.

Purification of immunoglobulins from antisera

Antisera (70 ml) to tadpole collagenase was dialyzed against 0.015 M phosphate buffer (pH 8.0) and chromatographed on a DEAE-cellulose (DE-32) column (2.6 × 40 cm) equilibrated with the same buffer, in the cold. Non-adsorbed proteins and the washings with 0.02 M phosphate buffer were combined, concentrated and applied to a Sephadex G-200 superfine column, (2.6 × 100 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 7.6)/0.15 M NaCl/5 mM $CaCl_2$. Elution position of the immunoglobulin was located by the double diffusion method using anti-rabbit immunoglobulin G goat-antisera and purified tadpole collagenase.

Immunoglobulin G to human serum albumin was also prepared from its antisera as described above.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using a 6% gel in Tris-glycine buffer (pH 8.3)/0.1% SDS [16].

Immunoelectrophoresis was performed according to the method of Scheidegger, using Tris-HCl buffer [ionic strength (I) = 0.05], pH 8.6 [17].

Results

Distribution of collagenase in cultured medium in terms of molecular weight

Previous studies on the affinity chromatography of tadpole collagenase from cultured media of back-skin explants [2,11] have shown that the purified enzyme accounting for about 60—70% of total enzyme has a molecular weight of approx. 43 000. The presence of enzyme with different molecular sizes, as claimed by Harper et al. [3], was tested by subjecting concentration culture media to gel chromatography. As shown in Fig. 1, only an enzyme peak distributed around molecular weight of 40 000 was observed. No enzyme activity was detected in the eluate fractions corresponding to higher and lower molecular weights under the conditions tested.

Purity of collagenase preparation

Fig. 2 shows an elution profile of tadpole collagenase on gel chromatography at the final step of purification following affinity chromatography. The protein concentration and enzyme activity against collagen were coincident. Polyacrylamide electrophoresis of the peak fraction (tube number 124 in Fig. 2) showed a single protein band corresponding to enzyme activity, as reported previously [2]. When specific enzyme activity was calculated on a per unit absorbance

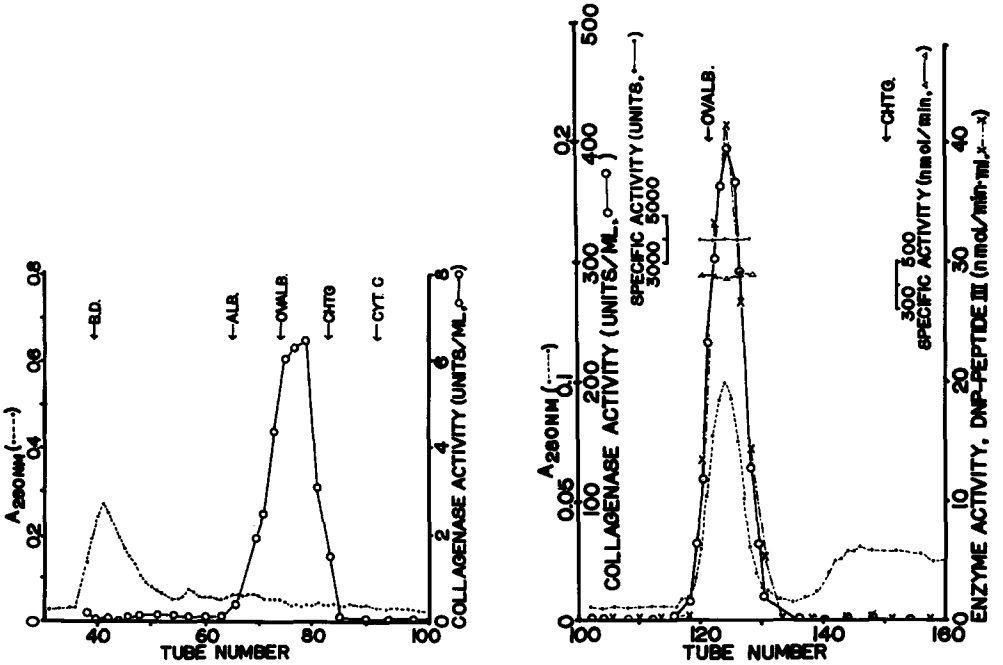

Fig. 1. Sephadex G-200 chromatography of crude collagenase. 5 ml concentrated cultured medium (175 units collagenase activity) were applied on a column (3 × 90 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 7.6)/0.15 M NaCl/5 mM $CaCl_2$ (5-ml fractions; flow rate 25 ml/h). B.D., blue dextran; ALB, bovine serum albumin; OVALB, ovalbumin; CHTG, chymotrypsinogen and CYTC, cytochrome *c*.

Fig. 2. Sephadex G-75 superfine chromatography of tadpole collagenase following affinity chromatography. About 9300 units collagenase (enzyme activity/A_{280nm}: more than 2800 units) was applied to a series of three columns (1.8 × 95 cm each) equilibrated with 0.05 M Tris-HCl buffer (pH 7.6)/1 M NaCl/5 mM $CaCl_2$ and upward elution was performed (3-ml fractions; flow rate 6 ml/h). Substrates employed were ^{14}C-labeled collagen fibrils (○———○), DNP-peptide III (X· — · — ·X). ●------●: absorbance at 280 nm. Specific enzyme activities against [^{14}C]-collagen fibrils (●———●, units/A_{280nm}) and DNP-peptide III (△———△, nmol/min · A_{280nm}).

basis, the values obtained for tubes 120—128 were found to be fairly constant (3900—4000 units).

Recently, we have succeeded in preparing synthetic substrates for serum peptidase and tissue collagenases [12]. It is known that the former enzyme hydrolyzes DNP-peptides I and II but not III, whereas the latter hydrolyzes DNP-peptides I and III, but not II. Both enzymes split their peptide substrates at the same Gly-Ile bonds [12]. Peptidase activity in the eluate was, therefore, assayed using DNP-peptide II and III as substrates.

DNP-peptide III-hydrolyzing activity was found to be only in collagenase fractions. The specific enzyme activity per absorbance unit at 280 nm was essentially constant (Fig. 2). The peptidase activity was inhibited by 10 mM EDTA and $2 \cdot 10^{-3}$ M β-mercaptoethanol but not by 10 mM phenylmethylsulfonylfluoride. No hydrolyzing activity against DNP-peptide II was detected in the eluate.

In order to obtain the relation between absorbancy at 280 nm and protein concentration, the enzyme fractions (tubes 122—126) were pooled, enzyme

activity and absorbance was determined then dialyzed against water, lyophilized and weighed. Collagenase solution at the concentration of 1 A_{280nm} was shown to correspond to 0.466 mg/ml (or 1 mg/ml to 2.146 A_{280nm}), indicating that the specific enzyme activity per mg protein against collagen and DNP-peptide III was 8440 units and 933 nmol/min, respectively. The activity against collagen was more than 2000-fold, compared to that (3.95 units/mg) reported by Harper et al. [3] which was calculated from the results given in Table I of ref. 3 as follows: 1450/1800 × 1/4 × 1/60 × 100 × 1000/85 = 3.95.

Optimum pH of collagenase

It has been difficult to determine the optimum pH for collagenase using collagen as a substrate, since the molecular state in solution of native collagen changes with varying pH, even kept at a given ionic strength and hydrolysis rate of collagen primarily depends on its molecular states. Therefore, DNP-peptide I, which is much more susceptible to the action of collagenase than DNP-peptide III, was employed for this purpose.

The optimum pH of the enzyme was found to be 7.5—8.5. Enzyme activity was completely depressed below pH 6.0 and above pH 9.5 under our conditions.

Effect of α_2-macroglobulin on collagenase activity as observed by using collagen and DNP-peptide I as substrates

Table I shows that tadpole collagenase was inhibited by α_2-macroglobulin, when collagen was employed as a substrate [18], while the enzyme activity was fully preserved when synthetic substrates were employed. The same result was obtained with human synovial collagenase.

Comparison of hydrolysis of native and denatured collagens by collagenase

As shown in Fig. 3, purified tadpole collagenase hydrolyzed denatured collagen at the 3/4 : 1/4 locus first, releasing α^A and α^B, then further hydrolyzed the fragments into smaller peptides (Fig. 3b, 8—30 min gels). When hydrolysis rates against native and denatured collagens were compared, incubation times

TABLE I

EFFECT OF HUMAN α_2-MACROGLOBULIN ON THE HYDROLYSIS OF ^{14}C-LABELED COLLAGEN FIBRILS AND DNP-PEPTIDE I BY PURIFIED TADPOLE AND HUMAN SYNOVIAL COLLAGENASES

0.1-ml aliquots of purified tadpole collagenase were preincubated with an equal volume of 0.05 M Tris-HCl buffer (pH 7.6)/0.15 M NaCl/5 mM $CaCl_2$ or with human α_2-macroglobulin for 30 min at 0°C, then assayed for enzyme activity using ^{14}C-labeled collagen fibrils and DNP-peptide I as substrates. Similar experiments were performed with partially purified human synovial collagenase for comparison.

Experimental	Rate of hydrolysis	
	[^{14}C]Collagen (munits)	DNP-peptide I (nmol/h)
Tadpole collagenase + buffer	972	6.08
Tadpole collagenase + α_2-macroglobulin	76	6.48
Synovial collagenase + buffer	36	0.88
Synovial collagenase + α_2-macroglobulin	8	0.94

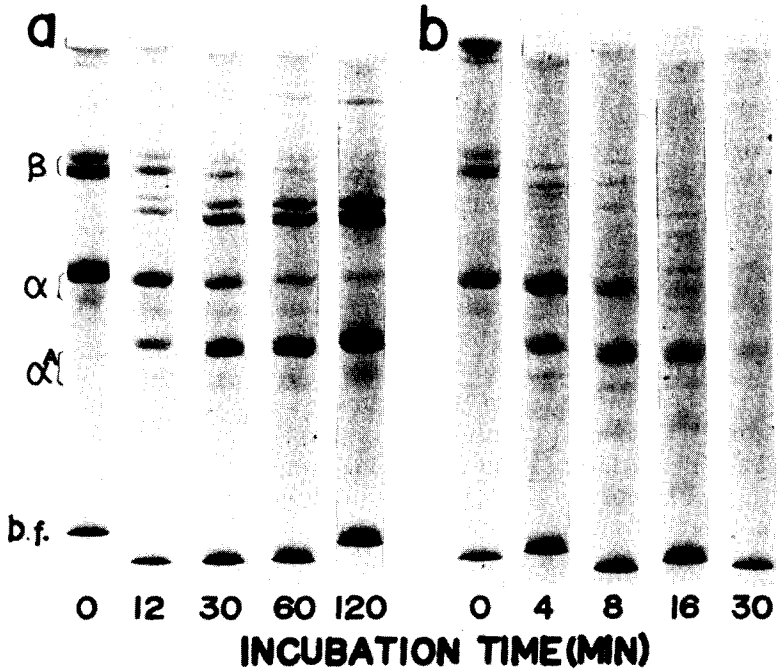

Fig. 3. Comparison of hydrolysis of native and denatured collagens by collagenase. Native (a) and heat-denatured (b) acid-soluble guinea-pig skin collagens, 300 μg each, in 0.3 ml 0.05 M Tris-HCl buffer (pH 7.6)/0.15 M NaCl/5 mM $CaCl_2$/0.5 M D-glucose were incubated with 10 μl enzyme (tube No. 121 in Fig. 1) at 30°C and 40-μl aliquots were withdrawn at incubation times indicated. After stopping the reaction by adding 20 μg EDTA, 20 μg pepsin was added as an internal marker, then SDS-gel electrophoresis was performed. b.f.: buffer front, pepsin and α^B.

required for more than 50% hydrolysis were 30 and 8 min, respectively. The α_1 component of denatured collagen was completely degraded after 16 min, while this fragment from native collagen remained even at 60—120 min incubation, although α_2-component and α_2^A-fragment were poorly stained.

α^B-Fragments were shown to migrate close to pepsin.

Amino acid composition of collagenase

Table II shows the amino acid composition of tadpole collagenase, using an estimated molecular weight of 43 000 [2]. It should be noted that the content of acidic amino acids (aspartate and glutamate) amounted to 24% of the total amino acids, while that of basic amino acids (histidine, lysine and arginine) was about 11%. The enzyme contains cystine and/or cysteine and tryptophan, but not hydroxyproline or hydroxylysine. The contents of hydrophobic (alanine, valine, leucine, isoleucine, tyrosine and phenylalanine) and aromatic amino acids (tyrosine, phenylalanine and tryptophan) were about 36 and 13%, respectively.

Anti-tadpole collagenase antibody

Further characterization of purified and crude tadpole collagenases and comparison of the immunoreactivity of the enzyme with those from various animal

TABLE II

AMINO ACID COMPOSITION OF TADPOLE COLLAGENASE

Amino acid	Hydrolysis by (residues per molecules *)	
	6 M HCl	4 M Methanesulfonic acid
Asp	54	55
Thr	22	22
Ser	22	23
Glu	38	37
Pro	25	24
Gly	32	31
Ala	26	26
1/2Cys	2	4
Val	21	20
Met	4	4
Ile	18	17
Leu	30	30
Tyr	17	19
Phe	27	27
Trp	(5) **	5
His	10	10
Lys	20	19
Arg	13	12
Total	386	385

* Assuming the molecular weight of collagenase as 43 000.

** Taken from the results obtained by 4 M methanesulfonic acid hydrolysis.

sources was achieved using anti-tadpole collagenase rabbit immunoglobulin G. The immunoglobulin preparation did not contain α_2-macroglobulin. Immunodiffusion of purified and crude tadpole collagenases against the immunoglobulin preparation showed a fused single precipitin line (Fig. 4). However, neither enzymes from chick skin, human skin and synovial fluid nor tadpole serum formed precipitin lines against the immunoglobulin. Immunoelectrophoresis also showed a single arc precipitin at a position migrating faster than human serum albumin (Fig. 5), and corresponding to collagenase activity, using either [^{14}C]collagen fibrils or NDP-peptide III as substrates. The migration of collagenase was located by measuring enzyme activity in a series of 5-mm segment gels with one electrophoretic run and by formation of immunoprecipitin line using the immunoglobulin with the other run. These results suggest that the immunoglobulin G is a monospecific antibody against tadpole collagenase. No tadpole serum component was contained in the purified enzyme preparation, as expected (Fig. 5).

Immunoinhibition of collagenase activity

Since mono-specific antibody against tadpole collagenase was obtained, quantitative inhibition of purified enzyme by the immunoglobulin G was examined, using [^{14}C]collagen fibrils and DNP-peptide I as substrates. Enzyme activity was completely inhibited by the immunoglobulin down to 64-fold dilution, then increased with further dilution of the globulin and 50% inhibition was observed with the globulin at a concentration between 512- and 1024-fold dilution. Similar results were obtained with synthetic substrate, although com-

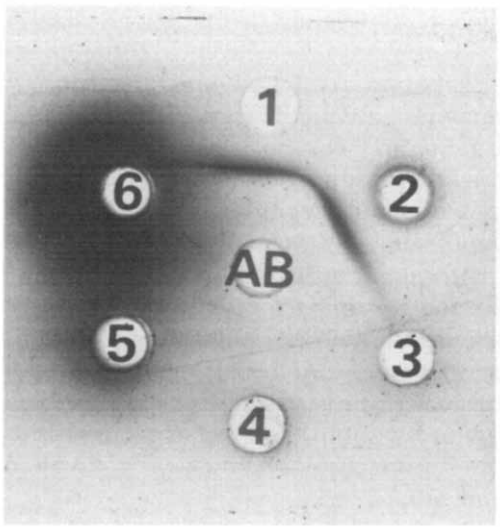

Fig. 4. Double diffusion of collagenase from various sources against anti-tadpole collagenase rabbit immunoglobulin G. After 24 h immunodiffusion at room temperature, the gel plate was washed with 0.15 M NaCl/0.02% NaN_3 for 24 h to remove free proteins, then stained with Amido Black 10B and destained with 5% acetic acid. Wells: 1, 8.4 units (1 μg) purified tadpole collagenase; 2, 6.0 units crude tadpole collagenase; 3, 1.0 units chick skin collagenase; 4, 4.0 units human skin collagenase; 5, 2.4 units human synovial collagenase; 6, 10 μl tadpole serum; AB, 15.2 A_{280nm}/ml anti-tadpole collagenase rabbit immunoglobulin G.

plete inhibition was not observed (94—96% inhibition) even with the globulin at concentrations of 1—64-fold dilutions. Anti-human serum albumin rabbit immunoglobulin G showed no inhibition of collagenase activity, indicating that the above inhibition is specific for anti-collagenase immunoglobulin G.

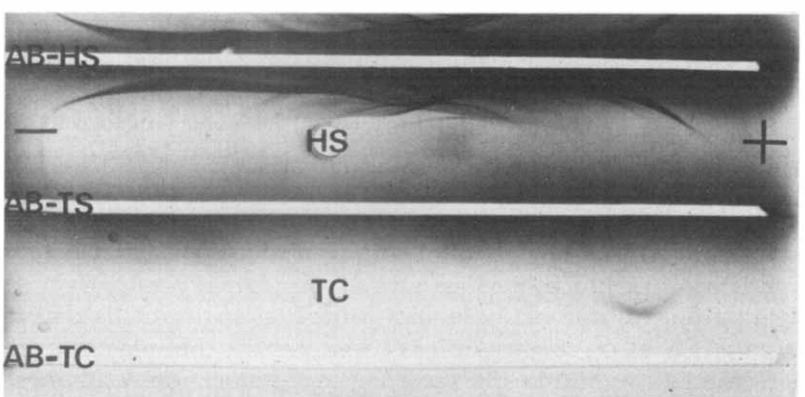

Fig. 5. Immunoelectrophoresis of tadpole collagenase. 10 μl purified collagenase (5.9 units) was electrophoresed on 1.2% agar-agarose gel plate with Tris-HCl buffer (pH 8.6 $I = 0.05$). After 20 h immunodiffusion, the gel plate was washed, stained and destained, as described in Fig. 4. Wells: upper, human serum (HS), 10 μl; lower, tadpole collagenase (TC), 10 μl. Troughs: top, anti-human serum rabbit antiserum (AB-HS); middle, anti-tadpole serum rabbit antiserum (AB-TS) and bottom, anti-tadpole collagenase rabbit immunoglobulin G (AB-TC).

Discussion

The observations described here are consistent with those obtained in the earlier study [10]. When the specific enzyme activity of purified collagenase are compared with that reported by Harper et al. [3], one can tell that specific activity of their highly purified enzyme is less than one-thousandth of our enzyme. In other words, more than 99.9% of their enzyme preparation should be contaminant proteins. Their purified enzyme is an ascending portion of the first protein peak eluted by agarose gel chromatography of the 30% saturation $(NH_4)_2SO_4$ precipitate of lyophilized cultured media. Since their enzyme fraction consisted mainly of contaminant protein(s), as judged by specific enzyme activity, the protein bands on SDS-polyacrylamide gel electrophoresis (Fig. 9 in ref. 3) do not provide very much evidence for collagenase and its zymogen. The use of 0.5 μg of their enzyme for electrophoresis should not show a collagenase protein band, since enzyme content in their preparation should be less than 0.5 ng which is far below the sensitivity of the method employed.

The observations of Harper and coworkers may be explained by the following: (1) Cultured media of tadpole skin explants contain contaminant proteins of large molecular weights (Fig. 1). Since tissue collagenase has a high affinity with macromolecular components in tissues and easily forms complexes at low ionic strength [20,21] and Harper and colleagues prepared lyophilized cultured media as a starting material, the formation of enzyme complexes with tissue components is significant in their preparation. (2) Collagenase is known to be recovered in the precipitated fraction at 30—50% saturation $(NH_4)_2SO_4$ [10]. Since they used a 0—30% saturation $(NH_4)_2SO_4$ precipitate as an enzyme fraction where large molecular weight components of proteins were precipitated and dissolved it in a buffer solution of low ionic strength (0.01 M Tris-HCl buffer/1 mM $CaCl_2$/ no NaCl), they might obtain the enzyme mostly in complex forms with tissue macromolecules. 0.2 M NaCl as an elution buffer for gel filtration is not high enough to dissociate the enzyme from the complexes (see Fig. 1A in ref. 3) [21,22].

McCroskery et al. [23] reported that purified rabbit tumor collagenase digests native collagen but does not significantly attack denatured collagen. However, purified tadpole enzyme having more than 2-fold specific activity (8440 units/mg) compared with that (3583 units/mg) of their enzyme does hydrolyze denatured collagen much faster (4-fold or more) than native collagen at the 3/4 : 1/4 locus (Fig. 3). Further degradation of denatured α-fragments was observed with increasing incubation time. The difference in the substrate specificity of both enzymes might be due to difference in sources of tissues and/or species.

α_2-Macroglobulin inhibited tadpole skin and human synovial collagenases, when collagen was used as a substrate. [18]. However, the enzyme did hydrolyze synthetic substrate even in the presence of globulin (Table I). These results suggest that collagenase in a form of complex with α_2-macroglobulin still possesses its active site unmasked and a small molecular size of substrate such as synthetic peptide can access to the site, as observed with many other proteases [24]. It should be noted that the combined use of collagen and synthetic substrates for collagenase assay in the presence of phenylmethylsulfonyl-

fluoride could facilitate the determination of individual amounts of free and bound enzyme in addition to the sum of enzymes in both forms.

Tadpole collagenase was found to migrate faster than human serum albumin on agar gel electrophoresis at pH 8.6 (Fig. 5). This suggests that the enzyme is an acidic protein. High content of acidic amino acids in the enzyme protein would support the observation, though no quantitation of amide content was carried out in this study. Earlier observations on the behaviors of the enzyme on disc electrophoresis [2] and DEAE-cellulose chromatography [10] are consistent with the results described above. Inhibitory effect of basic proteins such as lysozyme, histone and protamine on collagenase [25] could be explained by the electrostatic interaction between the proteins and the enzyme.

The immunoinhibition of collagenase, as observed by using collagen and DNP-peptide I as well as DNP-peptide III (data now shown) as substrates, suggests that (an) antigenic sites(s) may be located at a locus close to the active site of the enzyme, where such molecular sizes of hepta-octapeptides had no access, though complete inhibition of enzyme activity was not obtained with the synthetic peptides.

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