Biochimica et Biophysica Acta, 482 (1977) 159—172

BBA 68119

RADIAL DIFFUSION ASSAY OF TISSUE COLLAGENASE AND ITS APPLICATION IN EVALUATION OF COLLAGENASE INHIBITORS

J.A. YANKEELOV, Jr. [a,*], W.B. WACKER [b] and M.M. SCHWERI [a]

[a] *Department of Biochemistry and* [b] *Department of Ophthalmology, University of Louisville, Health Sciences Center, Box 1055, Louisville, Ky. 40201 (U.S.A.)*

(Received September 16th, 1976)

Summary

A radial diffusion assay for tissue collagenase (EC 3.4.24.3) has been devised which is simple, sensitive and capable of application to large numbers of samples. The assay employs an agarose matrix containing solubilized lathyritic rat skin collagen as substrate. Fibril formation is induced for 2 h at 37°C subsequent to 41 h digestion at 28°C. The procedure results in sharply defined zones of lysis which may be measured directly or after photography. The characteristics of the procedure are otherwise similar to those reported for other radial diffusion assays. The new method was used to examine the action of 10 compounds which were known or potential inhibitors of tadpole collagenase. The concentration of inhibitor required to produce 50% inhibition is reported for the following compounds: α_2-macroglobulin, 142 μg/ml; *N*-acetylcysteine, $\geqslant$100 mM; cysteine, 8.7 mM; EDTA, 0.46 mM; histidine, $\geqslant$100 mM; 2,3-dimercaptopropanol, 0.5 mM and mercaptoacetic acid, 70 mM. The procedure also has potential for clinical determinations (e.g. tears, synovial fluid) since assay dishes may be prepared in advance and only 15 μl of sample is required.

Introduction

Radial diffusion enzyme assays are simple, sensitive and linear over a wide range of concentration [1]. Assay of tissue explants for collagenase (EC

* To whom requests for reprints should be addressed.

Abbreviations: Tris, tris-(hydroxymethyl)-aminomethane; TC^A, tropocollagen fragment containing amino terminus of the monomer; TC^B, tropocollagen fragment containing carboxy terminus of the monomer; r.d.u., radial diffusion units as defined in text; BIP, α-bromo-β-(5-imidazolyl)-propionic acid; α_2-M, human α_2-macroglobulin; I_{50}, concentration of inhibitor required to produce 50% inhibition of collagenase activity.

3.4.24.3) activity employing unsupported collagen gels [2] may be considered to be a special type of radial diffusion assay, but the absence of a support medium and the inherent mechanical instability of these gels limit the scope of these procedures.

Initial attempts to develop a radial diffusion assay for tissue collagenase were complicated by possible thermal denaturation of the collagen substrate at the usual setting temperature of noble agar used for the support [3]. In addition the single cleavage mode of tissue collagenases [4] results in formation of diffuse boundaries of the zones of digestion on agar-supported, preformed collagen gels [3]. The present report describes a radial diffusion assay system for tissue collagenase which eliminates the above constraints and provides a simple and sensitive assay system which is particularly valuable for screening potential collagenase inhibitors. The new system incorporates collagen into the gel without the threat of thermal denaturation and produces sharp, easily measured boundaries.

Methods

Collagenase. Tadpole collagenase was prepared from tail fin and back skin by culture as described by Nagai et al. [5] and was purified by precipitation with ammonium sulfate and chromatography on Sephadex G-200 [6]. Pooled fractions containing collagenase activity were concentrated 20-fold by ultrafiltration using an Amicon UM 2 filter at 4°C.

α_2-Macroglobulin. This protein was purified from outdated human serum by Cohn fractionation [7] followed by chromatographic purification according to the method of Betlach and McMillan [8]. α_2-Macroglobulin was located by Ouchterlony double-immunodiffusion [9] using goat antiserum to human α_2-macroglobulin (Meloy Laboratories). The concentration of purified protein was determined from $A_{277\text{nm}}$ using an $E^{1\%}_{1\text{cm}}$ of 9.1 [10]. This protein was exhaustively dialyzed against Tris buffer II prior to testing as an inhibitor.

Buffers. Buffers employed in this study were as follows: Tris buffer I: 0.05 M Tris, 0.4 M NaCl, 3 mM $CaCl_2$ (pH 7.6, 20°C). Tris buffer II: 0.01 M Tris, 0.2 M NaCl, 5 mM $CaCl_2$ (pH 7.6, 20°C). Phosphate buffer: ionic strength, 0.4 (pH 7.6, 20°C).

Collagen. Soluble calf skin collagen was prepared according to the method of Bensusan and Hoyt [11]. The lyophilized product was stored at −20°C. Collagen was resolubilized with a wrist action shaker for 100 min at 3–5°C using phosphate buffer. The solubilized collagen was dialyzed (4°C) for 24 h against Tris buffer I. The retentate was centrifuged at 25 000 × *g* for 30 min (2°C). The clear solution was diluted with Tris buffer I to give a final concentration of 2 mg/ml collagen as determined by amino acid analysis.

Lathyritic rat skin collagen was prepared as described by Bornstein and Piez [12] with the following stipulations: (a) Purina lab chow (meal) was mixed with the β-aminopropionitrile on a daily basis to ensure the strength of the lathyrogen and (b) the collagen solution was vacuum filtered (4°C) in portions through 12.5-cm 934 AH Reeve Angel glass fiber filters prior to precipitation of the collagen with NaCl. Considerable turbidity was removed by the filtration. No physical damage to the collagen resulted as determined by analytical

ultracentrifugation. Stock solutions of purified collagen were diluted and equilibrated with Tris buffer I to a concentration of 6.5 mg/ml. Collagen prepared in the above manner could be stored for several months at 0°C. Deterioration of stored samples is evidenced by a loss of boundary sharpness in the assay.

Other materials. Other products used in this study and their corresponding sources were as follows: Trypsin (Type III), Tris-(hydroxymethyl)-aminomethane (Trizma base), L-histidine, *N*-acetyl-L-cysteine, β-aminopropionitrile fumarate, Sigma Chemical Co.; SeaPlaque agarose, Marine Colloids, Inc. (Rockland, Maine); disodium salt of ethylenediaminetetraacetic acid dihydrate, cysteine (free base), Schwarz-Mann; 2,3-dimercapto-1-propanol, thiolacetic acid, 3-mercaptopropionic acid, mercaptoacetic acid, Aldrich Chemical Co.; NaN_3, Matheson Coleman and Bell; DL-α-bromo-β-(5-imidazolyl)propionic acid, Pierce Chemical Co.; 5,5′-dithiobis-(2-nitrobenzoic acid), Calbiochem; Petri dishes (50 × 12 mm), Falcon Plastics; tadpoles (*Rana catebiana*), Lemberger Corp., Oskosh, Wisconsin. Well-cutters (3.0 mm outer diameter), a product of Gelman, Scientific Products. Sephadex G-200, a product of Pharmacia Fine Chemicals, was sieved prior to use. The 70—90 μm fraction was selected for column packing.

Well-opacity screening assay. Chromatographic fractions were screened for collagenase activity by placing 100-μl portions of 2 mg/ml Tris-buffered calf skin collagen in each of the wells of a Falcon Micro Test II plate. The plate is covered and placed in a moist incubator for 45 min at 37°C. The plate containing the formed collagen gels is removed from the incubator, and 25 μl of a chromatographic fraction or test sample is added to the surface of the gel in each of the coded wells. The plate is returned to the 37°C incubator overnight. The wells are viewed from below while illuminated from above with diffuse light. Fractions containing collagenase activity are located by a loss of opacity in the test wells containing enzyme relative to opacity in wells containing only buffer.

Sulfhydryl determinations. Sulfhydryl groups were determined with 5,5′-dithiobis-(2-nitrobenzoic acid) [13].

Photography. The assay dishes were photographed individually using an illuminator described by Hunter [14]. A Honeywell Pentax SP 1000 35 mm single lens reflex camera equipped with a 50 mm SMC-Takumar macro lens and Kodak B and W 35 mm Plus X Pan PX 402 film was used. Exposure was at a lens setting of f/5.6 for 1/15 s. Films were processed with Kodak D-76 developer diluted 1 : 1.

Viscosimetric assay. This assay was similar to that described by Werb and Gordon [15], but was performed at 28°C instead of 35°C. Each assay contained 1.2 ml of Tris-buffered collagen (650 μg/ml) containing 59 mM arginine · HCl and 0.2 ml of enzyme in Tris buffer II containing 415, 4020 and 8150 r.d.u. per ml based on direct assay. Assays were performed using Cannon-Ubbelohde semi-micro viscometers with flow times of 266 and 257 s for water (20°C).

Preparation of assay dishes. SeaPlaque agarose (1.2 g) is suspended in 60 ml of Tris buffer I containing 0.04% NaN_3. The mixture is degassed for 15 min in a suction flask at 20°C and then heated to above 75°C to dissolve the agarose. The hot agarose solution (48 ml) is transferred to a 100 ml capacity water-

jacketed beaker with the circulating water maintained at 37.0° C. The solution is stirred constantly with a Z-shaped stirring blade. After the agarose has equilibrated to 37° C an equal volume of 0.2% collagen * (20° C) is added dropwise to the agarose over a period of 7 min. Stirring is maintained at a rate which does not introduce bubbles. The mixture is allowed to stir for 30 min during which time finely divided collagen fibrils are produced. Using a pipet (prewarmed to 37° C) 6.0-ml aliquots of the mixture are transferred rapidly into each of fifteen 50 × 12 mm Petri dishes (sufficient for 60 assays). Each dish is allowed to stand open for 2 h at room temperature. The cast plates are covered and placed in a cold room at 4° C for at least 40 h. The thermal reversal of the collagen fibril formation produces transparent plates. Plates prepared in this manner may be stored for several weeks. The dishes are equilibrated at room temperature prior to use. Any accumulated liquid is removed from the surface by a suction tube, and 3.0-mm wells are cut. A 4-holed plexiglass template bonded to a drilled out Petri dish lid is convenient for routine positioning of the well cutters.

Assay procedure. Each well of the assay dishes is identified by a waterproof marking on the outside wall. Wells are loaded at room temperature with 15 μl of the test solution using a 25 μl Hamilton syringe. The dishes are covered and secured in an aluminum tray on the surface of a covered constant temperature bath (28° C). After an incubation period of 41 h the dishes are immediately transferred to an incubator at 37° C for an additional 2 h. During this time, the undigested collagen forms fibrils revealing clear zones of lysis set in an opaque white matrix. The resulting sharply defined zones gradually lose contrast over a period of several hours. The plates may be measured directly or photographed as described by Hunter [14]. Photography is recommended for maximal precision.

A permanent plexiglass supporting plate with a disc removed to accommodate the dish facilitates photography. A line is scribed on an exposed portion of this plate to serve as a reference distance. Paper identification collars are included as shown in Results. The film need be processed only to the negative which is measured directly on a microcomparator. A magnification factor is determined from the reference line and measured distances are corrected to actual size.

Evaluation of potential collagenase inhibitors was made by combining 50 μl of Tris-buffered inhibitor (pH 7.6) with an equal volume of enzyme. The inhibitor and enzyme concentrations recorded are those of the final solutions. The enzyme-inhibitor solutions were allowed to equilibrate (20° C) approx. 1 h prior to assay. Since some compounds (e.g. arginine) are known to inhibit fibril formation, control wells were included for each inhibitor without enzyme.

Evaluation of assay data. Two diameter measurements (at right angles) are made for each zone and the average of these measurements is used as a single value in the statistical evaluation. Since boundaries of the zones of digestion are sharp and measurements by the microcomparator can be made to the nearest hundredth of a millimeter, estimation of the diameter is not the limiting factor in the precision of the measurements.

---

* In some instances it may be desirable to refilter the collagen.

Results

Earlier approaches to developing a radial diffusion assay using preformed collagen gels in a supporting agar matrix revealed that tissue collagenase produced zones of lysis with poorly defined boundaries [3]. Bacterial collagenase, which makes multiple cleavages in collagen monomer, produces sharp boundaries under the same conditions. The difference is a manifestation of the single cleavage mode of tissue collagenase [4] resulting in a population distribution of fibrils with various extents of degradation at the advancing enzyme front. Both calf skin and lathyritic rat skin preformed collagen gels exhibit diffuse zones of lysis when exposed to tissue collagenase at 37° C. The zones produced cannot be measured satisfactorily without the aid of high contrast photography [3] and, therefore, the usefulness of the procedure is restricted. Utilization of the unique properties of lathyritic collagen eliminates the above limitation and use of SeaPlaque agarose (low-setting temperature) permits preparation of an agarose-supported collagen gel without the threat of substrate denaturation.

Since lathyritic collagen fibrils produced in the agarose may be completely resolubilized by storage in the cold, digestion of the collagen in free solution may proceed within the agarose matrix during the main digestion period at a temperature below that which induces fibril formation. A subsequent short period of incubation at physiological temperature induces fibril formation and produces sharply defined zones of lysis.

Fig. 1 shows the appearance of a typical assay dish containing the resolubilized collagen in agarose after 41 h of incubation at 28° C and again after an additional 2 h incubation at 37° C. Incubation at 28° C produces no obvious change in the agarose collagen matrix and the plate remains clear. However, sharply defined zones of digestion are visible after incubation for the 2 h at 37° C. The increase in temperature simultaneously promotes fibril formation in the undigested collagen while completing denaturation of the fragments (TC^A and TC^B) of digested collagen. The fragments have melting temperatures several degrees below physiological temperature [6]. A sharp discontinuity appears in the gel at the front of the advancing enzyme. Earlier studies of the time dependence of other radial diffusion assays [1] including that of tadpole collagenase on preformed agar-calf skin collagen gels [3] indicated that for moderate amounts of enzyme, the rate of advance of the zone boundary would be very slow after 40 h. The excellent definition of the zones produced substantiates the assumption that advance of the digestion front during the final 2 h is minimal.

Fig. 2 shows a typical standard curve obtained with the radial diffusion assay employing delayed fibril formation. It is apparent that for zone diameters beyond 6.5 mm, the logarithm of the collagenase activity is a linear function of the diameter of the zone of lysis. This characteristic of linearity is typically observed for other radial diffusion assays [1]. Accordingly, the present system may be described by Eqn. 1.

$$\log_{10}(A) = m(D) + b \tag{1}$$

A is the collagenase activity in radial diffusion units (r.d.u.) and D is the

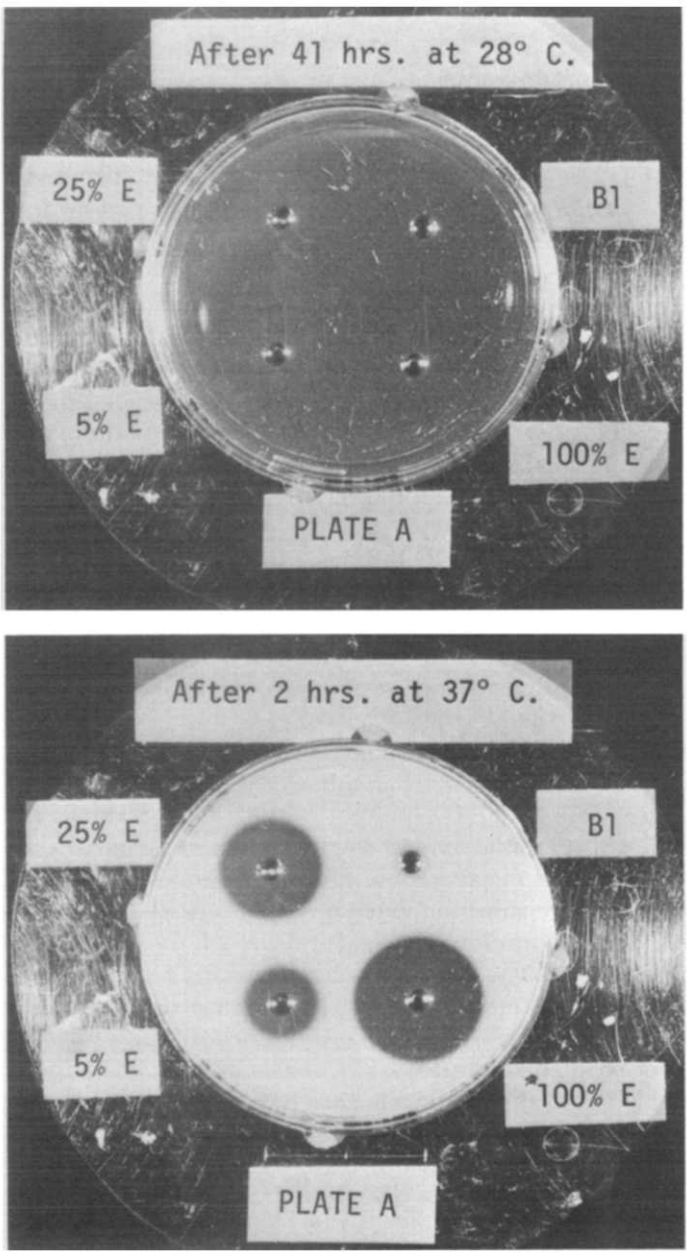

Fig. 1. Behavior of assay dish with wells loaded with 15 µl of the following solutions: B1, Tris buffer II; "100% E", 8.2 r.d.u. collagenase/µl; "25% E", 2.0 r.d.u. collagenase/µl; "5% E", 0.41 r.d.u. collagenase/µl. Upper frame: Appearance after 41 h incubation at 28°C. Lower frame: same dish after an additional incubation for 2 h at 37°C. The fine horizontal line below the dish represents an actual distance of 20.05 mm.

measured zone diameter. The slope and intercept are denoted by m and b, respectively. In this system it is convenient to define a test sample which produces a 10 mm digestion zone as containing 10 r.d.u. of activity. Under the

conditions defined, 10 r.d.u. corresponds to digestion of 240 μg collagen *. Typical slopes of standard curves are close to 0.17 log units per mm. Thus, for many purposes it is only necessary to include one or two enzyme standards to verify the relationship.

Eqn. 2 was obtained from a least squares plot of the data shown in Fig. 2 using zones of digestion greater than and including 6.47 mm (2.4 r.d.u.).

$$\log(A) = 0.1753(D) - 0.753 \quad (2)$$

A more detailed analysis of the data obtained for Fig. 2 is shown in Table I. Each of the values shown is the average of five determinations. Table I shows the measured diameters and calculated activities as well as corresponding standard deviations. The relative standard deviation of each activity measurement is also indicated. The average relative standard deviation for the present method (8.8%) is very similar to that calculated (8.5%) from published values for six other radial diffusion assays [1]. This observation indicates that in situ fibril formation does not alter the essential character of the radial diffusion system.

The buffer blank obtained is peculiar to the particular preparation of lathyritic collagen. In the two preparations examined one was found to produce no blank at all, in the other, the front advanced less than 0.25 mm beyond the well edge. The assay was also found to be specific for collagenase in that trypsin samples of 0.2 mg/ml produced no digestion. Trypsin at very high concentrations (3—6 mg/ml) showed significant digestion, but the boundaries were observed to be quite diffuse compared to those produced by tissue collagenase. In this connection, enzymes which attack only gelatin will not exhibit activity since gelatin does not undergo fibril formation.

Fig. 3 shows collagen digestion measured viscosimetrically at three different levels of collagenase activity. The total number of radial diffusion units of enzyme in each viscometer is shown in the figure. The viscometer with 83 r.d.u. of collagenase contained 30 times the amount of enzyme required to place a measurement in the linear region of the radial diffusion assay or about 135 times that detectable by the new method.

Since Werb and Gordon [15] have recently related the viscosimetric assay to the widely used radiofibril assay for the specific case of mouse peritoneal macrophage collagenase digestion of rabbit skin collagen, an indication of the relative sensitivity of the present assay to the ^{14}C assay may be determined. These authors found that enzyme digesting 12.6 μg collagen/min per ml in the viscosity assay was equivalent to 0.64 unit of activity in the radiofibril assay [15]. If a factor of 20 also approximates the behavior of tadpole collagenase with lathyritic rat skin collagen, it may be conservatively estimated from the viscosity data of Fig. 3 that the present assay system is capable of measuring as little as 0.01 unit of activity defined for the radiofibril assay [16].

Since the diameter of the zone produced in radial diffusion assay increases only in proportion to an exponential increase in enzyme activity, it follows that the most sensitive, as well as most economical range for studying inhibi-

* This estimate assumes that a 2.8 mm diameter plug of agarose-collagen is removed when the well is punched.

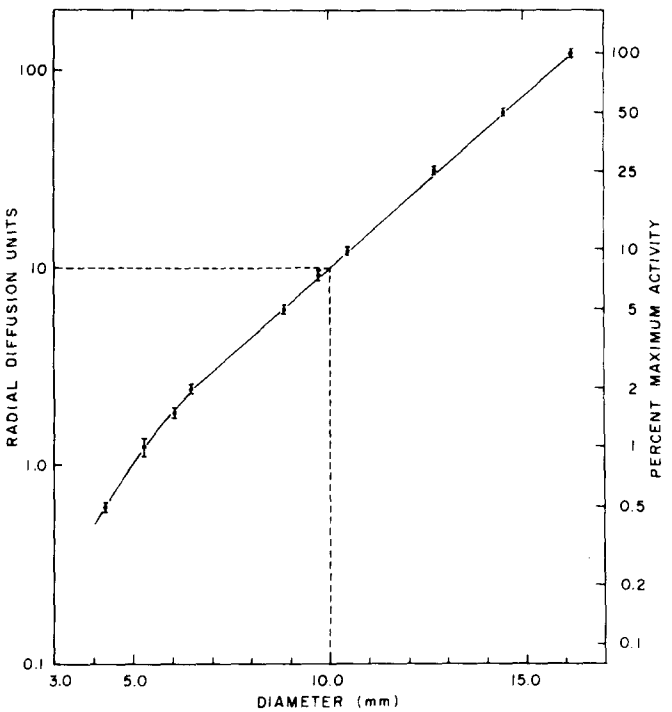

Fig. 2. Standard curve showing relationship of enzymatic activity to the diameter of zone. Each point is the average of five determinations. Error bars represent the S.E. Left ordinate expressed as radial diffusion units as defined in text. Right ordinate expressed as percent of maximum activity.

tion of collagenase is the lowest log unit of enzyme activity which falls in the linear range. Thus, an enzyme sample containing about 2 r.d.u. per μl (12.8 mm zone) after dilution with buffer containing no inhibitor is near optimal for evaluating the concentration of inhibitor required to produce 50% inhibition.

TABLE I

STATISTICAL EVALUATION OF LINEAR REGION OF STANDARD CURVE

Number of determinations in each case was 5.

Zone of lysis		Activity			
Diameter (mm)	S.D.	r.d.u.	S.E.	S.D. [a]	Relative S.D. [b] (%)
6.47	0.28	2.41	0.13	0.28	11.4
8.83	0.29	6.24	0.32	0.72	11.5
9.71	0.28	8.9	0.47	1.05	11.7
10.46	0.14	12.0	0.29	0.65	5.4
12.67	0.25	29.4	1.32	2.95	10.0
14.45	0.15	60.3	1.68	3.75	6.2
16.21	0.14	122	3.13	7.00	5.7

[a] Calculated from the least squares equation fitted to the data: Log (r.d.u.) = 0.1753 (diameter) — 0.753.

[b] Average relative standard deviation (S.D.) from this data is 8.8%. The average relative standard deviation for six other radial diffusion assays [1] is 8.5%.

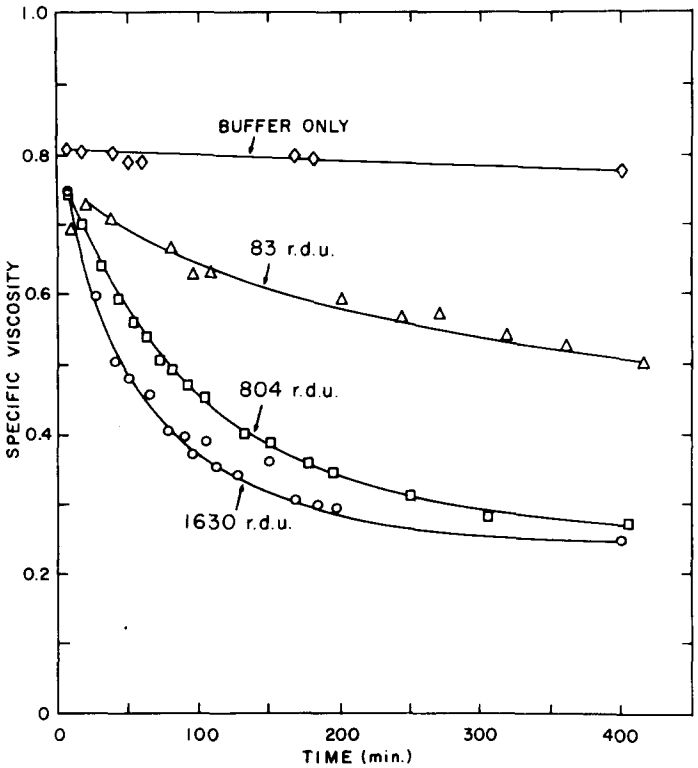

Fig. 3. Viscosimetric determination (28°C) of same collagenase sample referred to in Figs. 1 and 2. Each viscometer contained 1.4 ml solution. The numbers above each curve are the total number of radial diffusion units of collagenase which were present during the determination. The two data points at 400 min were obtained by interpolation of the smooth curve generated from the other data shown and data obtained after 1200 min.

Standards producing about 11 mm digestion or containing 1.0 r.d.u. per μl should be included in the series to precisely define the expected digestion at the 50% activity level.

Application to enzyme chromatography

Fig. 4 shows the radial diffusion assay of collagenase activity in fractions collected from a gel filtration of tadpole collagenase. The fractions containing the collagenase activity were first qualitatively detected by the well-opacity assay (see Methods) using an incubation period of 27 h at 37° C. The profile of activity was determined by radial diffusion assay. Peak activity recorded corresponds to 1127 r.d.u. per ml of column effluent. Activities were estimated by microcomparator on negatives.

Evaluation of potential collagenase inhibitors

Fig. 5 illustrates how collagenase inhibition is easily recognized by inspection with four representative inhibitors.

Fig. 6 shows the results of preliminary examination of selected potential and known tissue collagenase inhibitors. The enzyme samples corresponding to

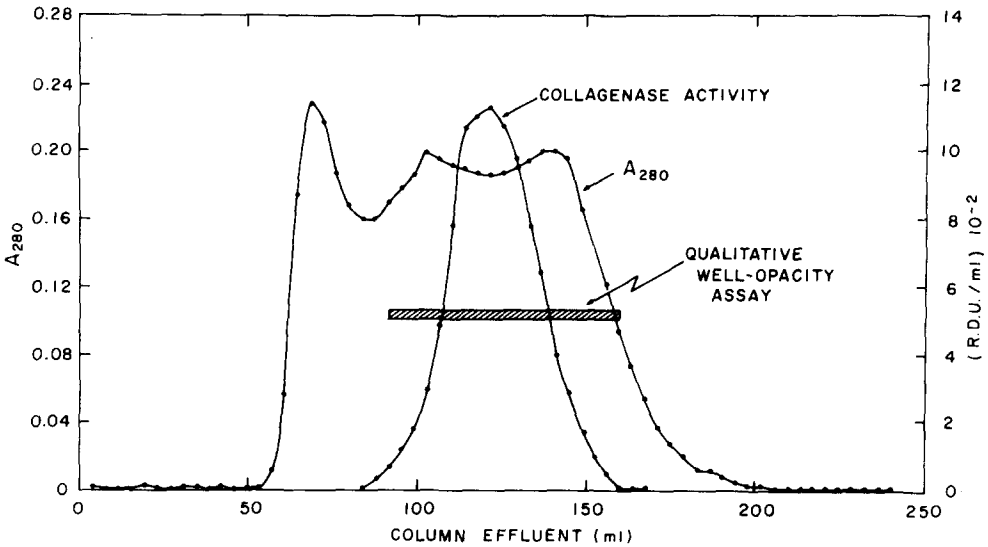

Fig. 4. Application of radial diffusion assay to enzyme chromatography. Ammonium sulfate precipitated day 3 and 4 tail fin collagenase (4.6 ml; A_{280nm} 4.86) in Tris buffer II was applied to a 1.6 × 85 cm column of Sephadex G-200 (particle size: 75–90 μm). Flow rate, 3.5–7.1 ml/h; fraction size, 3.8 ml. Recovery of protein as estimated by A_{280nm} was 83%. Collagenase activity was detected by well-opacity assay (horizontal bar) and quantitated by radial diffusion assay. Recovery of enzymatic activity from the chromatograph was 72%.

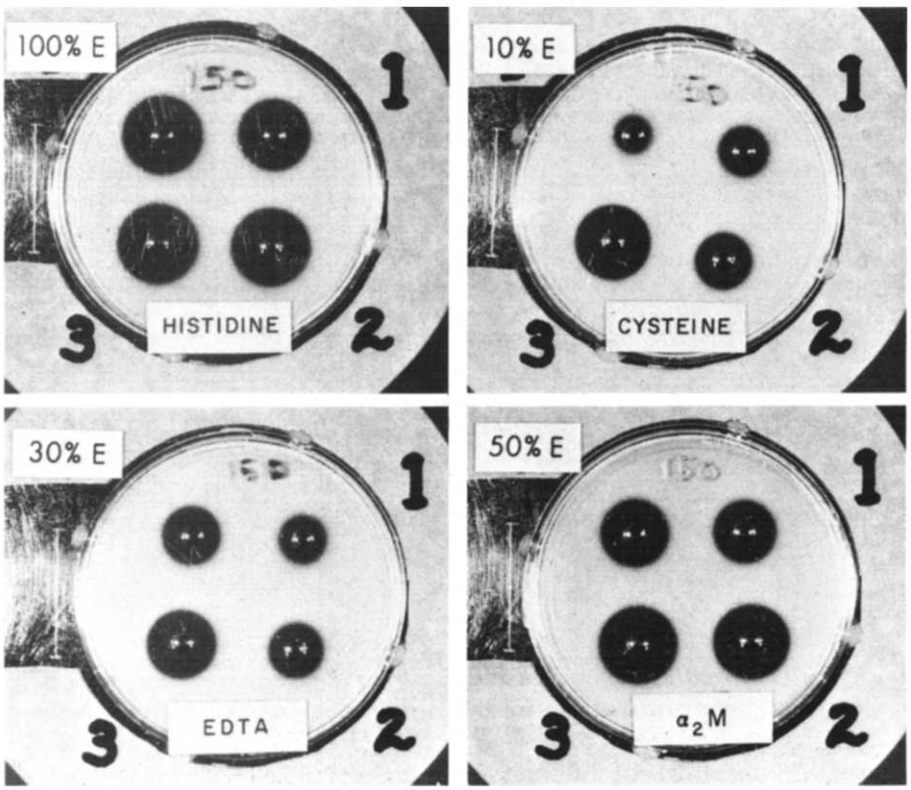

Fig. 5. Screening of potential collagenase inhibitors. Concentrations of inhibitors corresponding to the plates as labeled are as follows. Histidine (mM): 1, 26.5; 2, 2.65; 3, 0.265. Cysteine (mM): 1, 100; 2, 10; 3, 1. α_2-Macroglobulin (μg/ml): 1, 182; 2, 18.2; 3, 1.82. EDTA (mM): 1, 10; 2, 1; 3, 0.1. Vertical line to left of each dish measures 20.05 mm. Remaining wells were loaded with standards as indicated. Details are given in Methods.

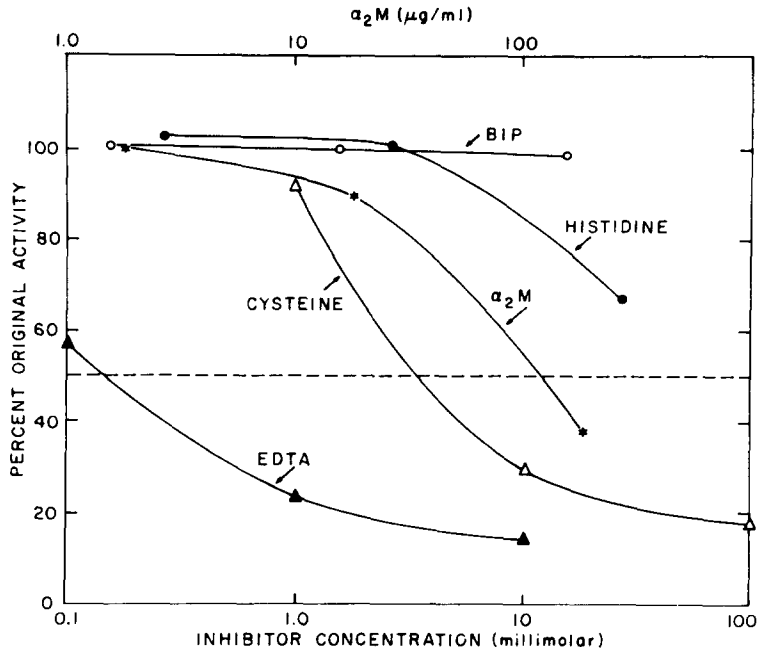

Fig. 6. Screening of potential collagenase inhibitors. Recovery of activity is indicated as a function of increasing concentration of inhibitor. Each point represents a single determination. The lower abscissa indicates the concentration of all compounds with the exception of α_2-macroglobulin which is expressed by the upper abscissa. Additional details are given in Methods.

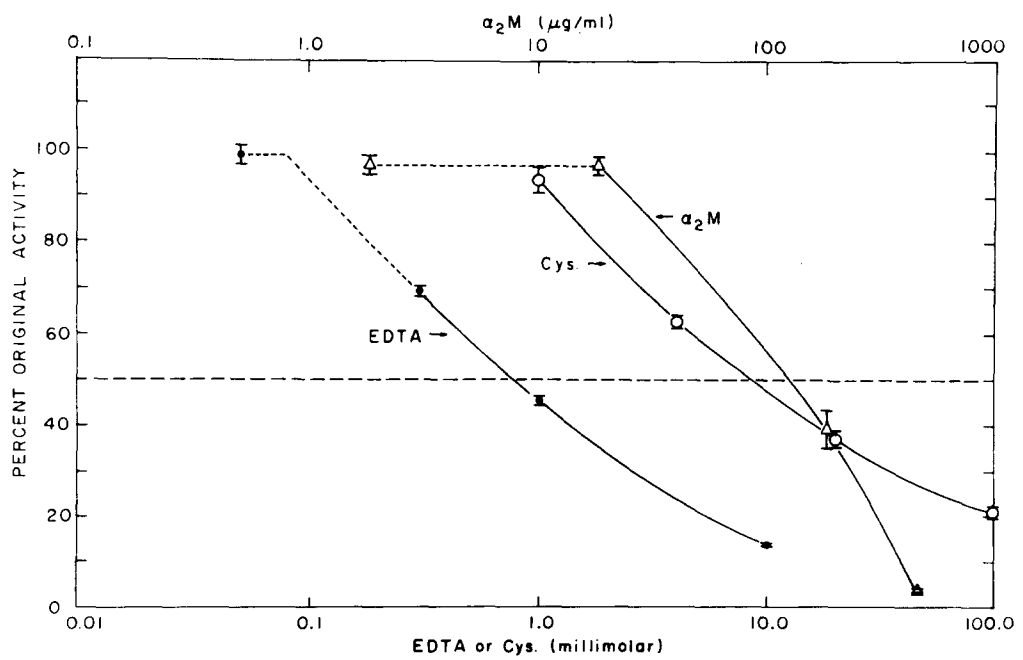

Fig. 7. Determination of I_{50} values. Each point is the average of four determinations. Error bars indicate S.E. Lower abscissa is for EDTA and cysteine. Upper abscissa is for α_2-macroglobulin. Additional details are given in Methods.

100% activity contained 1400 r.d.u. per ml. Each point on the graph represents a measurement from a single diffusion zone. The plot reveals that α-bromo-β-(5-imidazolyl)-propionic acid [17] is non-inhibitory at 0.0156 M or less, but histidine does inhibit measurably at concentrations approaching 0.1 M. Histidine also inhibits bacterial collagenase [18] but inhibition has not been previously reported for tissue enzyme. α_2-Macroglobulin, cysteine and EDTA are known inhibitors of tissue collagenases [19,5]. This preliminary ranking of inhibitors permits estimation of the concentration range required to produce 50% inhibition and, therefore, facilitates more precise evaluations. In addition, the inhibitor trend over concentrations differing by several order of magnitude permits selection of those inhibitors which show a strong and uniform concentration dependence.

The screening experiment shown in Fig. 6 was followed by determination of I_{50} values for each of three inhibitors: α_2-macroglobulin, cysteine and EDTA. The results are shown in Fig. 7. The I_{50} values estimated for these inhibitors are 131 μg/ml, 7.8 mM and 0.73 mM, respectively.

Table II summarizes results obtained with a number of potential and known tissue collagenase inhibitors. The observation that the I_{50} for EDTA with

TABLE II

SUMMARY OF SELECTED COMPOUNDS EXAMINED FOR INHIBITOR BEHAVIOR WITH TADPOLE COLLAGENASE

Compound	Number of determinations	Concentration range [a]	Inhibition [b]	Individual I_{50} determinations	Average I_{50} ± S.E.
α_2-Macroglobulin	3	1.82—455 μg/ml	+	118, 176, 131 μg/ml	142 ± 17 μg/ml ($2 \cdot 10^{-7}$ M)
N-Acetylcysteine [c]	2	0.5—100 mM	+	⩾100 mM	—
Cysteine [c]	4	1—100 mM	+	3.0, 15.0, 9.0, 7.8 mM	8.7 ± 2.5 mM
EDTA	6	0.05—10 mM	+	0.13, 0.27, 0.38, 0.66, 0.73, 0.61 mM	0.46 ± 0.11 mM
Histidine	3	0.3—100 mM	+, —, +	⩾100 mM	—
α-Bromo-β-(5-imidazolyl)-propionic acid	2	0.17—17 mM	—	—	—
2,3-Dimercapto-propanol [c]	3	0.001—42 mM	+	⩽0.4, 0.9, 0.2 mM	0.5 mM
Mercaptoacetic acid [c]	1	1—100 mM	+	70 mM	70 mM
Mercaptopropionic acid [c]	1	1—100 mM	+	>100 mM	—
Thiolacetic acid [c]	1	0.5—50 mM	—	—	—

[a] Concentration range indicates the total range covered by the set of experiments.

[b] (—) indicates an experiment which showed no measureable inhibition.

[c] The sulfhydryl titres per mol were determined on weighed samples of these compounds and were as follows: *N*-acetylcysteine, 1.03; cysteine, 0.96; 2,3-dimercaptopropanol, 1.98; mercaptoacetic acid, 0.98; mercaptopropionic acid, 1.01; thiolacetic acid, 0.41.

tadpole collagenase is a factor of 19 lower than that required for cysteine is similar to the response of corneal collagenase to these inhibitors [20]. However, the data on the corneal enzyme indicates cysteine and *N*-acetylcysteine are comparable inhibitors for that enzyme [20]. The present study shows acetylcysteine is a substantially less effective inhibitor than cysteine in the case of the tadpole enzyme. Dimercaptopropanol was expected to be a potent inhibitor of tissue collagenase based on its ability to chelate Zn [21,22]. It has been reported to be a strong inhibitor of bacterial collagenase [23]. The inhibitory behavior of histidine and sulfhydryl compounds with tadpole collagenase suggests that incorporation of both a sulfhydryl group and an imidazole group in the same molecule might provide enhanced inhibition. This possibility is under study.

Discussion

The radial diffusion assay described here is based on use of lathyritic collagen as substrate. Use of lathyritic collagen permits a supply of substrate of increased chemical definition that may be fully resolubilized in the agarose support. Digestion occurs in an agarose-stabilized liquid phase. Several methods were tried for casting collagen-agarose gels including bypassing the initial formation of collagen fibrils. The method found to produce gels of greatest uniformity is the one described in the Experimental Section above. Enough collagen can be prepared from 10 to 15 lathyritic rats to perform several thousand assays. The semilogarithmic response of the assay is well suited to purification studies [1].

The radial diffusion method described here for ranking tissue collagenase inhibitors is rapid and requires small amounts of both enzyme and inhibitor. Inhibitors of greatest interest are readily identified by inspection. The zones may be measured directly for semi-quantitative work. The zones formed gradually fade over a period of hours permitting ample time for photography or delineation of the zone boundaries. Highest precision is obtained when measurements are made by microcomparator on negatives after photography.

Assays of enzyme/inhibitor mixtures may be complicated by the fact that inhibitor as well as enzyme are undergoing independent diffusion during the period of digestion. Diffusion rates of inhibitors may to some extent affect their apparent efficacy. Therefore, some differences may be expected from the I_{50} measured by other assays. We anticipate that these differences are not likely to be large or important in ranking inhibitors. The ease with which small amounts of potential inhibitors can be screened and ranked is a prime consideration.

This assay requires only 15 μl of sample and large numbers of assay plates may be prepared in advance and stored. Consequently, the procedure has potential for clinical testing of tissue fluids such as tears, synovial fluid or tumor extracts.

Acknowledgements

The authors are appreciative of the advice and cooperation of members of the departments of Biochemistry, Microbiology and Immunology, and

Ophthalmology in various phases of this work. Thanks are due to Dr. Harlie Parish for the determination of sulfhydryl titres. The support of this research by Grant R01 EY00969 of the National Eye Institute is gratefully acknowledged.

References

1 Schill, W.B. and Schumacher, G.F.B. (1972) Anal. Biochem. 46, 502—533
2 Lapiere, C.M. and Gross, J. (1963) in Mechanisms of Hard Tissue Destruction, (Sognnaes, R.F., ed.), pp. 663—694, American Association for the Advancement of Science Publication No. 75
3 Yankeelov, Jr., J.A. and Schweri, M.M. (1975) Fed. Proc. 34, 698 (Abstr.)
4 Gross, J., Harper, E., Harris, Jr., E.D., McCroskery, P.A., Highberger, J.H., Corbett, C. and Kang, A.H. (1974) Biochem. Biophys. Res. Commun. 61, 605—612
5 Nagai, Y., Lapiere, C.M. and Gross, J. (1966) Biochemistry 5, 3123—3130
6 Sakai, T. and Gross, J. (1967) Biochemistry 6, 518—527
7 Schmid, K., Rosa, E.C. and MacNair, M.B. (1956) J. Biol. Chem. 219, 769—780
8 Betlach, C.J. and McMillan, D.E. (1972) Anal. Biochem. 49, 103—108
9 Munoz, J. (1971) in Methods in Immunology and Immunochemistry (Williams, C.A. and Chase, M.W., eds.), Vol. 3, pp. 146—160, Academic Press, New York
10 Dunn, J.T. and Spiro, R.G. (1967) J. Biol. Chem. 242, 5549—5555
11 Bensusan, H.B. and Hoyt, B.L. (1958) J. Am. Chem. Soc. 80, 719—724
12 Bornstein, P. and Piez, K.A. (1966) Biochemistry 5, 3460—3473
13 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70—77
14 Hunter, J.R. (1959) Nature 183, 1283—1284
15 Werb, Z. and Gordon, S. (1975) J. Exp. Med. 142, 346—360
16 Werb, Z. and Reynolds, J.J. (1974) J. Exp. Med. 140, 1482—1497
17 Yankeelov, Jr., J.A. and Jolley, C.J. (1972) Biochemistry 11, 159—163
18 Head, D.L. and Yankeelov, Jr., J.A. (1976) Int. J. Peptide Protein Res. 8, 155—165
19 Barrett, A.J. and Starkey, P.M. (1973) Biochem. J. 133, 709—724
20 Berman, M. and Dohlman, C. (1975) Arch. Ophthalmol. (Paris) 35, 95—108
21 Stability Constants, Special Publications 17 and 25 (1964 and 1971) The Chemical Society, London
22 Berman, M.B. and Manabe, R. (1973) Ann. Ophthalmol. 5, 1193—1209
23 Seifter, S. and Harper, E. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. 3, 3rd edn., pp. 649—697