Purification and Properties of a Specific Collagenase from Rabbit Synovial Fibroblasts

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1. A specific collagenase from the culture medium of rabbit synovial fibroblasts was purified by gel filtration and ion-exchange chromatography. 2. The enzyme was homogeneous on polyacrylamide-gel electrophoresis and showed only traces of contaminants when tested in gels with a non-specific antiserum. 3. The rabbit fibroblast collagenase could hydrolyse collagen both in solution and in fibrillar form. Viscometry showed that at 35°C the purified enzyme could hydrolyse >50 nmol of collagen/min per mg of enzyme. 4. The purified collagenase cleaved collagen in solution at either 24° or 35°C into the characteristic $\frac{1}{4}$ - and $\frac{3}{4}$ -length fragments. However, as compared with the impure enzyme, the purified enzyme at 35°C had a much decreased capacity to further degrade the initial specific cleavage products. 5. The specific rabbit collagenase had a mol.wt. of approx. 32 000 as estimated by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, and 35 000 by gel filtration.

Collagen is the major protein of connective tissues, yet it is only in recent years that enzymes have been characterized that can degrade this macromolecule specifically (for reviews, see Harris & Krane, 1974*a,b,c*). However, the precise role of specific collagenases in degrading collagen during the remodelling of connective tissues *in vivo* is still unclear, largely because it has been difficult to demonstrate these enzymes in tissues. Most of our present knowledge on the action of collagenase comes from studies in which the enzyme has been isolated from the culture medium of tissues growing *in vitro*. One way in which the role of collagenases in the physiological and pathological degradation of collagen can be clarified is by the use of a specific antiserum, both to demonstrate the enzyme in tissues and to inhibit the enzyme specifically in biological test systems. Such a research programme necessitates the purification and characterization of a collagenase for the production of the specific antiserum.

Several reports have appeared on the purification of collagenases from human tissues (Bauer *et al.*, 1971, 1972; Woolley *et al.*, 1973; Ohlsson & Olsson, 1973), but we chose to study rabbit collagenase initially, because of the relative ease in setting up biological model experiments under controlled conditions. Additionally, we found that cell cultures of rabbit fibroblasts derived from rabbit synovium secrete large amounts of a specific collagenase into their serum-free culture medium (Werb & Burleigh, 1974; Werb & Reynolds, 1974), and provide a convenient source of the enzyme. The present paper demonstrates that rabbit collagenase from the medium of

rabbit fibroblasts growing in culture has been purified to a high specific activity, comparable with any reports in the literature, and also characterizes its action on collagen in solution and in fibrillar form. The paper (Werb & Reynolds, 1975) following the present one describes the production of a specific antiserum to rabbit collagenase and its use in immunoinhibition studies.

Materials and Methods

Materials

Sartorius membrane filters (SM 12133) were obtained from Sartorius G.m.b.H., 34 Göttingen, West Germany and PM-10 membranes were purchased from Amicon Ltd., High Wycombe, Bucks., U.K.

Sephadex G-100, Sephadex G-25, Blue Dextran 2000, QAE-Sephadex A-50 and Sepharose 6B, were purchased from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Biorex 70 was from Bio-Rad Laboratories, St. Albans, Herts., U.K. Freund's complete and incomplete adjuvants, were from Difco Laboratories, Detroit, Mich., U.S.A.

Rabbit anti-(human α_2 -macroglobulin) antiserum prepared by Dakopatts, Copenhagen, Denmark, was purchased from Mercia Diagnostics Ltd., Watford, Herts., U.K.

Albumin (crystalline bovine serum), agarose, insoluble collagen from bovine achilles tendon, carbonic anhydrase (bovine), lysozyme (egg-white, 3×crystallized), albumin (egg-white, grade V) were

purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., and Azocoll from Calbiochem, London W14 1AS, U.K.

Azocasein was the gift of Dr. P. M. Starkey, Strangeways Research Laboratory, Cambridge, U.K.

All other materials were obtained from commercial suppliers.

Methods

Rabbit synovial fibroblasts were derived from explants of normal rabbit synovium and maintained in culture as described previously (Werb & Burleigh, 1974). Conditioned medium was the source of collagenase, and was prepared by exposing confluent monolayers of fibroblasts to serum-free Dulbecco-Eagle's medium (Gibco Biocult Ltd., Paisley PA3 4EP, Renfrewshire, U.K.) for 3 days, and repeating this cycle twice. Only fibroblasts having intrinsic secretory rates of >1 unit of collagenase activity/24h per 1×10^6 cells were used. Medium was pooled in batches of 1–2 litres; then 1/10 vol. of 500mM-Tris-HCl buffer, pH 7.6, was added and enough solid CaCl_2 to make the solution 5mM. Pooled medium was concentrated by ultrafiltration in a Chemlab 400ml ultrafiltration apparatus fitted either with Amicon PM-10 or with Sartorius SM 12133 ultrafiltration membranes.

Collagenase activity. This was determined as a routine by measuring the release of ^{14}C -labelled peptides from reconstituted collagen fibrils. Soluble rabbit skin collagen was prepared by injecting rabbits with [^{14}C]glycine, essentially by methods used previously for rat skin collagen preparation (Kang *et al.*, 1966; Werb & Burleigh, 1974). The collagen used had a specific radioactivity of 7092 d.p.m./mg. One unit of collagenase activity is defined as the solubilization of $1 \mu\text{g}$ (3.3 pmol) of reconstituted rabbit collagen fibrils/min at 37°C ; Z. Werb, M. C. Burleigh and J. J. Reynolds (unpublished work) have shown that this unit is identical with that defined by Werb & Burleigh (1974) who used rat collagen at 35°C .

Assays of collagenase with collagen in solution. The rate of cleavage of collagen molecules by collagenase was measured viscometrically with collagen in solution at 35°C (McCroskery *et al.*, 1973). At this temperature the specific products of collagenase digestion denature spontaneously to gelatin which has negligible viscosity, and the rate of cleavage of collagen can be calculated from the initial linear rate of change of viscosity and the initial collagen concentration. A concentration of collagen was chosen at which there was a linear relationship between the enzyme concentration and the initial rate of change of viscosity. The assay mixture (2ml) contained 600 μg of rabbit collagen, 200mM-NaCl, 10mM- CaCl_2 , 50mM-Tris-HCl buffer, pH 7.6, and 50mM-

arginine. The arginine prevented the formation of collagen fibrils at this temperature. Viscosities were measured in Ostwald type U-tube microviscometers (Scientific Supplies, London EC1R 5EB, U.K.) having flow-times for water of either 60 or 25s (type UM-3 or UM-4 respectively), and were expressed as a percentage of the initial specific viscosity, η_{sp} . Assays were also made at 24°C under these conditions and the reaction products were analysed, after denaturation by heating in sodium dodecyl sulphate, by electrophoresis in polyacrylamide gels by using the sodium dodecyl sulphate-discontinuous buffer system of Neville (1971), as described previously (Werb & Reynolds, 1974).

Neutral proteinase activity. This was assayed with azocasein as substrate (Werb *et al.*, 1974).

Cathepsin D activity. This was measured by using [^3H]acetyl haemoglobin as substrate (Barrett, 1972).

Protein determination. Protein was measured as E_{280} units, assuming $E_{280}^{\text{mg/ml}} = 1.0$.

Polyacrylamide-gel electrophoresis. Electrophoresis was performed by using the Tris-glycine buffer system of Davis (1964) except that sample gels were not used. Gels (5mm diam.) were pre-run overnight at 1 mA/tube with the gel buffer as the upper reservoir buffer to remove contaminants from the gels which inhibited enzymic activity; the constant current used was 2mA/tube, and the gels were run for 200min with an initial potential gradient of 10 V/cm. Electrophoresis of samples used for assaying enzymic activity was at 4°C . Samples were cut into 2mm-thick slices and each slice was soaked overnight at 4°C in 250 μl of 50mM-Tris-HCl buffer, pH 7.6, containing 200mM-NaCl and 5mM- CaCl_2 . Portions (100 μl) were assayed for enzymic activity by using the radioactive collagen fibrils. The gels were stained for protein with Coomassie Brilliant Blue R250 as described by Barrett (1973). Electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate utilized the discontinuous buffer system described by Neville (1971).

Molecular weights for markers on Sephadex G-100 and sodium dodecyl sulphate-polyacrylamide gels were taken from Smith (1970).

Column chromatography. All buffers contained 0.01% sodium azide as preservative. Collagen-Sepharose adsorbent was prepared by the method of Bauer *et al.* (1971).

Immunological methods. Antisera to rabbit fibroblast collagenase were raised in sheep, as described elsewhere (Werb & Reynolds, 1975). Immunelectrophoresis at pH 8.6 was made in 1% (w/v) agarose plates with a buffer consisting of 75mM-Tris, 25mM-diethyl barbituric acid and 5mM- CaCl_2 . Samples were placed in 5 μl (2mm diam.) wells and electrophoresed at 10 V/cm until Bromphenol Blue bound to albumin reached the end of the trough (4cm).

Double immunodiffusion in gels was carried out by the method of Ouchterlony (1967).

Results

Purification of rabbit fibroblast collagenase

A three- or four-stage procedure consisting of ultrafiltration, gel filtration and ion-exchange chromatography was used to give a rapid, reliable purification to high specific activity. Purified enzyme preparations retained activity for up to 6 months at 4°C.

Two procedures were used, having the same first two stages but differing thereafter, and these are outlined in Scheme 1. The summarized data for two preparations by Procedure I and two preparations by Procedure II are shown in Table 1.

Ultrafiltration. Conditioned culture medium concentrated to between 1/100 and 1/500 of the original volume by ultrafiltration retained a high proportion

Scheme 1. Flow diagram summarizing the two procedures for purification of collagenase from the culture medium of rabbit fibroblasts

Experimental details are given in the text and quantitative results are presented in Table 1.

Table 1. Purification of rabbit fibroblast collagenase

The yields of protein and the enzymic activity are expressed per 1000ml of conditioned culture medium, and are mean values based on two separate preparations for either procedure I or II. Collagenase activities are expressed as units and protein is based on E_{280} (see under 'Methods'). Purification achieved during the concentration of the culture medium is not taken into account in the purification factor (see the Results section for an explanation). n.d., Not determined.

Stage	Protein (E_{280} units)	Activity (units)	Specific activity (units/ E_{280} units)	Purification factor	Yield (%)
Procedure I					
0. Culture medium	n.d.	1860	n.d.	—	100
1. Concentrate	60	1550	26	1	83
2. Sephadex G-100	10.5	980	93	3.6	52
3. QAE-Sephadex	2	762	381	14.7	40
4. Sephadex G-75	0.22	380	1730	66.5	19
Procedure II					
0. Culture medium	n.d.	1242	n.d.	—	100
1. Concentrate	31.6	1151	36	1	92
2. Sephadex G-100	6.2	480	77	2.1	38
3. Biorex 70	0.21	137	652	18.1	11
4. Biorex 70 (stepwise elution)	0.20	110	550	15.3	9

Fig. 1. *Sephadex G-100 gel chromatography of rabbit collagenase*

—, Distribution of collagenase activity; ····, protein measured as E_{280} ; ---, cathepsin D; ----, neutral proteinase measured with azocasein as substrate in the effluent from a column (70cm×2.5cm) of Sephadex G-100. Concentrated culture medium (10ml, 920 units) was applied to the column and 5ml fractions were collected at 40ml/h (see the Materials and Methods section for details). The fractions combined for further purification are indicated by the horizontal bar. Total column volume was 275ml.

of the initial enzymic activity if precautions were taken to wash any residual collagenase from a coating that formed over the surface of the membrane (50mM-Tris-HCl buffer containing up to 0.7M-NaCl was used). The collagenase apparently had a high affinity for insoluble particulate materials and precipitates formed during filtration were also rinsed in the above-mentioned buffer. The concentrates were quite viscous, probably because of the hyaluronic acid secreted by the fibroblasts. Purification of the collagenase by up to 15-fold was achieved during concentration because proteins were removed by non-specific precipitation. Thus starting concentrates (stage 1) had specific activities ranging from 10–50 units/mg. This purification was not taken into account in calculating the purification factor during subsequent procedures (Table 1).

Gel filtration. The crude concentrate contained, in addition to the collagenase, neutral proteinase activity demonstrable with azocasein as substrate. The collagenase was partly separated from neutral proteinase, cathepsin D and cathepsin B1-like activities by the following procedure. The concentrate was filtered through a column (2.5cm×70cm) of Sephadex G-100 equilibrated with 50mM-Tris-HCl buffer, pH 7.6, containing 200mM-NaCl and 5mM-CaCl₂ (Fig. 1). The collagenase and neutral-proteinase activities frequently appeared in double peaks if very highly concentrated samples were used, and some activity was also found in the void volume (Fig. 1). These peaks retained the same elution positions when passed through additional Sephadex G-100 columns and it seems likely that these cationic enzymes stick

non-specifically to other materials in the mixture. More dilute crude enzyme did not give such marked peak-splitting, and samples of collagenase purified by ion-exchange column chromatography first, followed by gel filtration, gave single peaks.

Ion exchange with QAE-Sephadex. Samples of collagenase partially purified by gel filtration were adjusted to a conductivity of 2.5mMho at 20°C by dialysis against the Tris-HCl, pH 7.6 (50mM in Cl⁻) containing 5mM-Ca²⁺ in the ultrafiltration apparatus, and applied to a column (1.5cm×25cm) of QAE-A50 Sephadex equilibrated with Tris-HCl buffer, pH 7.6, (50mM in Cl⁻ ions) containing 5mM-Ca²⁺. The collagenase was not retained by the column under these conditions (Fig. 2) and a gradient of 0–0.5M-NaCl eluted the rest of the protein. The collagenase activity was recovered in high yield (80%) by this procedure and was separated from much of the neutral proteinase activity. Attempts to chromatograph the collagenase on QAE-Sephadex or DEAE-Sephadex under conditions where the enzyme was retained (pH 7.5, 25mM-Cl⁻) resulted in total loss of enzymic activity. The enzyme from the QAE-Sephadex step was further purified by a second gel filtration on a column (1.5cm×90cm) of Sephadex G-75 in 50mM-Tris-HCl buffer, pH 7.6, containing 400mM-NaCl and 5mM-CaCl₂. Even under these conditions of increased salt concentration, a large proportion of the enzymic activity and protein could not be recovered, and there were considerable losses of enzymic activity in the concentration step required to decrease the volume of the sample to be applied to the column. Specific activities of purified collagenase by this

Fig. 2. QAE-Sephadex chromatography of rabbit fibroblast collagenase

Collagenase partially purified by gel filtration on Sephadex G-100 was applied (4mg in 6ml) to a column (25cm $\times$ 1.5cm) of QAE-Sephadex equilibrated at pH 7.6 with a buffer containing Tris, 5mM-Ca²⁺ and 50mM-Cl⁻. The fractions (3ml) were collected at 56ml/h. The column was eluted with this buffer until the E_{280} was at the baseline value then a linear gradient consisting of 150ml of the equilibration buffer and 150ml of the buffer containing 0.5M-NaCl was applied, indicated by the arrow. —, Distribution of collagenase activity and ····, protein measured as E_{280} in effluent from a column of QAE-Sephadex. The distribution of neutral proteinase, as measured with gelatin as substrate (---) residual cathepsin D activity (-·-·-) and the salt gradient as measured by conductivity of the fractions at 20°C (-·-·-) are also shown. Gelatinase units represent the hydrolysis of 1 μ g of gelatin to peptides of mol.wt. <5000/min at 37°C. The fractions combined for further purification are indicated by the horizontal bar (stage I-3 enzyme).

procedure of up to 1800 units/mg were obtained. The mol.wt. of the collagenase was estimated by gel filtration to be 35000 (Andrews, 1965).

Biorex 70. Collagenase partially purified by gel filtration was adjusted to pH 6.8 with 0.1M-acetic acid, and a conductivity of 5mMho. No enzymic activity was lost by this procedure, whereas large losses occurred when the preparation was equilibrated to those ionic conditions by dialysis, presumably through sticking of enzyme to the surface of the dialysis tubing. The sample was applied to a column (1.5cm $\times$ 20cm) of Biorex 70 equilibrated with a buffer consisting of 40mM-Tris, 5mM-CaCl₂ adjusted to pH 6.8 and 5mMho by the addition of acetic acid and NaCl. The column was then eluted by a linear gradient consisting of equal volumes of this buffer and of 50mM-Tris-HCl buffer containing 5mM-CaCl₂ and 1M-NaCl. As shown in Fig. 3, the neutral proteinase activity largely was not retained by the resin, and the collagenase was eluted at approx. 200mM-NaCl (12mMho). Not all the collagenase activity applied to the column was recovered by this procedure, probably owing to some inactivation of the enzyme, but preparations with specific activities of 900 units/mg, as measured in the assay with reconstituted fibrils, were obtained. When the specific activity was measured by the change in viscosity of collagen in solution at 35°C this preparation degraded more

that 50nmol of collagen/min per mg of enzyme protein; this is illustrated in Fig. 4.

Attempts at further purification of collagenase purified to stage II-3 (Scheme 1), by gel filtration and ion-exchange chromatography, were unsuccessful, but such preparations could be concentrated by diluting the sample to a conductivity of 8mMho with water and applying the sample (up to 50ml) to a column containing 2ml of Biorex 70 (equilibrated as above), followed by stepwise elution with 2ml volumes of 50mM-Tris-HCl buffer, pH 7.6, containing 300mM-NaCl, or 500mM-NaCl, or 1M-NaCl.

Collagen affinity chromatography

The suitability of a collagen affinity adsorbent for collagenase as a purification procedure was assessed immunologically. Collagenase partially purified by gel filtration through Sephadex G-100 (450 units in 2ml), was applied to a 5ml column of collagen linked to Sepharose (Bauer *et al.*, 1971), equilibrated with 50mM-Tris-HCl buffer, pH 7.6, containing 200mM-NaCl and 5mM-CaCl₂. The enzymic activity was adsorbed under these conditions, but only 15% of the original activity was recovered by eluting the column either at low pH [50mM-sodium acetate buffer, pH 4.5, containing 200mM-NaCl and 5mM-CaCl₂ (Nagai & Hori, 1972)] or at high ionic strength

Fig. 3. Biorex 70 chromatography of rabbit fibroblast collagenase

Collagenase partially purified by gel filtration (40ml) was applied to a column (16cm×1.5cm) of Biorex 70 (see the Materials and Methods section). Fractions (3ml) were collected at 70ml/h. The distribution of collagenase (—), protein as measured by E_{280} (·····), neutral proteinase as measured by the degradation of azocasein (— · — ·) in the effluent are shown. The salt gradient used for the elution is shown by the measurement of conductivity (---). The horizontal bar shows the fractions combined for use as purified enzyme or for concentration on an additional Biorex column.

Fig. 4. Degradation of collagen in solution at 35°C by purified collagenase

Collagen purified to stage II-4 was examined for its capacity to hydrolyse collagen in solution by following the change in the specific viscosity, $\eta_{sp.}$, of the solution. Δ , 0.08 μ g of enzyme; $\circ$, 0.8 μ g of enzyme; $\bullet$, control mixture containing buffer instead of enzyme.

[50mM-Tris-HCl buffer, pH7.6, containing 1M-NaCl and 5mM-CaCl₂ (Bauer *et al.*, 1971)]. In addition, the eluted collagenase, although slightly increased in specific activity still gave three to four lines in double immunodiffusion in gels against a non-specific antiserum to rabbit collagenase (X311/4; Werb & Reynolds, 1975). Hence this method was not suitable for purification of rabbit collagenase for either enzymological or immunochemical uses. Another affinity adsorbent tried was insoluble collagen fibrils. Bovine achilles tendon (1g) was mixed with 1g of dry Sephadex G-25 in the buffer used for the collagen-Sepharose, and packed into a 1 cm-diameter column; collagenase was applied to this mixed bed column as for collagen-Sepharose. All the collagenase activity was retained by the column, but as in the above affinity columns, less than 25% of the activity was recovered on subsequent elution, and this material was not immunologically homogeneous.

Assessment of purity of the collagenase preparations

During the purification steps a non-specific sheep antiserum to the crude rabbit collagenase preparation was useful; fractions for further purification could be

EXPLANATION OF PLATE I

Immunodiffusion plate and disc gels

(a) Immunodiffusion of fractions from Sephadex G-100 chromatography of rabbit fibroblast medium concentrates. Centre wells all contain a non-specific antiserum raised against rabbit collagenase (X311/4). R, Well containing rabbit serum; M, well containing medium concentrate that was loaded on column; all other wells contain samples of fractions as numbered (see the text). Collagenase peak was fraction 23-25. (b) Disc-gel electrophoresis of rabbit fibroblast collagenase at pH 8.6. (i), Concentrated culture medium (stage 1); (ii), purified collagenase (stage I-3). Migration was downwards. (c) Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of rabbit fibroblast collagenase. (i), Concentrated culture medium (stage 1); (ii), purified collagenase (stage II-4). Standards: (iii), bovine serum albumin, 68000; (iv), ovalbumin, 42000; (v), carbonic anhydrase, 29000; (vi), lysozyme, 14300. Migration was downwards and the arrow shows the collagenase band.

EXPLANATION OF PLATE 2

Disc gels and immunelectrophoresis plates

(a) Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of the denatured products of digestion of collagen with purified rabbit collagenase. (i), Collagen; (ii), collagen treated with collagenase (stage II-4) at 25°C; (iii), collagen treated with collagenase (stage II-4) at 35°C. Migration was downwards. (b) Immunelectrophoresis of rabbit fibroblast collagenase. (i), Well 1, rabbit immunoglobulin G; trough I, goat antiserum to rabbit serum proteins; well 2, purified rabbit collagenase (stage II-3); trough II, nonspecific antiserum to rabbit fibroblast collagenase (X311/4). (ii) Well 1, purified rabbit fibroblast collagenase (stage II-3); well 2, concentrated culture medium (stage 1), trough contained specific antiserum to rabbit collagenase (A3/2). (iii) Concentrated culture medium electrophoresed in the agarose gel and stained for protein.

Fig. 5. Gel electrophoresis of rabbit collagenase

Distribution of collagenolytic activity after electrophoresis of rabbit fibroblast collagenase in polyacrylamide gels at pH8.6. Gels were sliced into 2mm segments and the enzymic activity was determined. ▲, 20 μ l of concentrated culture medium (stage 1); ●, 50 μ l of purified collagenase (stage I-3).

pooled on the basis both of specific enzymic activity and of immunological purity. This procedure is illustrated in Plate 1(a) for a Sephadex G-100 chromatogram. By using a non-specific antiserum (X311/4) the various components are distributed differently through the chromatogram. The collagenase peak (fractions 23–25) had several lines evident. Even the collagenase of highest purity attained contained immunologically detectable contaminants.

Electrophoresis of the collagenase from rabbit fibroblasts on polyacrylamide gels

Collagenase activity was not reliably recovered from polyacrylamide gels at pH8.6. Under the best conditions the enzyme migrated into the gels with activity being recoverable about one-third of the way into the gel (Plate 1b and Fig. 5), with both crude and purified enzymes behaving similarly. Small differences in the pH and ionic strength of buffers during electrophoresis seemed to influence this behaviour, because in some cases the activity was found adherent to the gel from the top down to about 35% of the distance to the buffer front. No enzymic activity could be recovered from gels electrophoresed at pH3.5.

The purity of the collagenase preparations was examined by electrophoresis in gels containing sodium dodecyl sulphate as shown in Plate 1(c); the purified

collagenase, which had the mobility near that of carbonic anhydrase (mol.wt. 29000), still had minor contaminating protein bands, although these are not apparent in Plate 1. By sodium dodecyl sulphate–polyacrylamide-gel electrophoresis the mol.wt. of the collagenase was estimated to be 32000.

Collagenolytic activity of purified collagenase

The action on collagen in solution of collagenase purified by procedure II was assessed by comparing the products of reaction of collagenase on rabbit collagen in solution at 24° and 35°C. At 24°C the reaction mixtures were incubated until the η_{sp} had dropped to 44% of the initial value, then for a further 12h. At 35°C the reaction mixtures were incubated until η_{sp} had dropped to 10% of the initial value then for a further 10h. These conditions assured that reactions had gone almost to completion. As shown in Plate 2(a), typical reaction products of collagenase (Werb & Burleigh, 1974; Werb & Reynolds, 1974) were seen at 24°C; doublets arising from the α 1 and α 2 chains, and from the cross-linked β chains were cleaved to α^A and β^A fragments running below the α and β bands respectively, and to the smaller α^B fragments running near the buffer front. On some occasions the α^B fragments ran as only a single band on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. After incubation with the purified collagenase at 35°C, the typical α^A , β^A and α^B fragments were still seen, although some intermediate bands were also visible. When reaction mixtures at 35°C were incubated for shorter periods of time there was little evidence for multiple bands. Hence we conclude that highly purified collagenase has a decreased capacity to degrade collagen further than the $\frac{1}{4}$ - and $\frac{3}{4}$ -length fragments, in contrast with our previous observations with partially purified collagenase (Werb & Burleigh, 1974).

Immunoelectrophoresis of rabbit fibroblast collagenase

In a further attempt to characterize the preparations of purified and crude collagenase they were examined by immunoelectrophoresis at pH8.6 (Plate 2b). The collagenase barely migrated under these conditions and then slightly more towards the cathode, like immunoglobulin G. Purified collagenase gave only a single line with the specific antiserum [Plate 2b(ii)] but the crude enzyme gave a single line that was more cathodally moved than the purified collagenase. It seems likely that the presence of impurities such as hyaluronic acid in the crude concentrated medium causes anomalous migration of the enzyme. From these observations, and from the behaviour of collagenase on disc gel electrophoresis and ion-exchange chromatography, we conclude that

the collagenase must be a basic protein and have an isoelectric point (pI) near 8.

Discussion

Our previous work demonstrated that cell cultures of rabbit fibroblasts derived from rabbit synovium secrete large amounts of a specific collagenase into their serum-free culture medium (Werb & Burleigh, 1974; Werb & Reynolds, 1974). Such conditioned culture medium has provided us with a convenient source of crude enzyme for purification, because compared with all other sources, the enzyme is already partially purified away from tissue proteins and serum components. This point must be borne in mind when considering the purification factors quoted in Table 1; as discussed below, it is not easy at present to compare results of different workers because of differences in definition of specific activities, but we consider that our collagenase preparation is as pure as any so far reported.

Two procedures have been developed for a relatively simple preparation of collagenase of high specific activity. Both procedures (Table 1) start by concentrating with an ultrafiltration step the serum-free medium of rabbit fibroblasts secreting collagenase. The concentrated culture medium is then passed through a Sephadex G-100 column; this step separates away a large amount of protein but does not clearly separate other proteinases from the collagenase. With care both the first two steps give a reasonable recovery of the enzyme. In procedure I (see Scheme 1 and Table 1) the collagenase fraction from the Sephadex G-100 column is chromatographed on a QAE-Sephadex column, a procedure used previously for human synovial collagenase (Woolley *et al.*, 1973), and then passed through a Sephadex G-75 column. In procedure II the collagenase fraction from the Sephadex G-100 column is twice passed through a Biorex 70 ion-exchange column, the first time being a chromatography step, and the second time a concentration step. Biorex 70 has been used in the purification of human granulocyte collagenase (Ohlsson & Olsson, 1973). Enzyme prepared by procedure II does not have as high a specific activity on collagen fibrils as the preparation obtained by procedure I, but does have a lower capacity to give multiple products when reacted with collagen at 35°C. Moreover, on immunological evidence procedure II gives more highly purified collagenase, although it still contains minor contaminants when tested at high protein concentrations against non-specific antiserum (Werb & Reynolds, 1975). However, the yield by procedure II was not as high, with a major loss of activity occurring at the chromatography step with Biorex 70. We have found that human synovial collagenase sticks tightly to Biorex 70 and often gives partially inactive enzyme on elution (Z. Werb & J. J. Reynolds, unpublished work). It

therefore seems likely that the rabbit collagenase preparations made by this method contain a significant proportion of molecules that are enzymically inactive but still immunologically reactive. It would be interesting to see if procedure-II preparations had a higher specific activity in the presence of metal ions such as Zn^{2+} . Stepwise elution of rabbit collagenase from Biorex 70 does not cause any loss of activity and this method is very convenient for concentrating small amounts of enzyme.

Attempts at other purification steps were largely unsuccessful. Chromatography on DEAE-cellulose gave no recovery of enzymic activity; this was also true of electrophoresis in gels, which was also hampered by the variable migration of collagenase. Affinity chromatography on collagen-Sepharose (Bauer *et al.*, 1971) or insoluble collagen gave little purification, and such preparations were impure by immunological tests.

The mol.wt. estimated for rabbit collagenase is 32000–35000, which is one of the lowest so far reported for a specific collagenase (Harris & Krane, 1974*a,b,c*), and we found no evidence for any enzyme of higher molecular weight in our preparations.

It is not easy to compare the activity of purified rabbit collagenase with literature reports of other collagenases because there may be large species differences in activity, and also because many reports have not given sufficient details to calculate activities in terms of amount of collagen hydrolysed/min per mg of enzyme. Viscometric assays showed that enzyme prepared by procedure II had a specific activity of >15 mg (50 nmol) of collagen hydrolysed/min per mg of protein at 35°C, and we consider that the specific activity of the rabbit collagenase reported in this paper is comparable with the activity of purified tadpole collagenase (Nagai & Hori, 1972) and rat skin collagenase (Tokoro *et al.*, 1972), and is far higher than preparations of human collagenase (Bauer *et al.*, 1971). A collagenase from a rabbit tumour has been purified (E. D. Harris, personal communication) and has a similar specific activity to our collagenase. Moreover the tumour collagenase has a similar molecular weight to our enzyme and has little capacity to degrade collagen at 35°C to multiple products. It seems likely that any capacity to degrade collagen past the stage of $\frac{1}{4}$ - and $\frac{3}{4}$ -length fragments means that the collagenase still has traces of neutral proteinase activity (Werb & Reynolds, 1974), as was the case for the partially purified enzyme (Werb & Burleigh, 1974).

The preparation of rabbit fibroblast collagenase in reasonable yield in a highly purified form, free of contaminating proteinases, has now enabled us to prepare and characterize a specific antiserum to this tissue proteinase for use in biological and biochemical studies. Part of this work is reported in the following paper (Werb & Reynolds, 1975).

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References

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Barrett, A. J. (1972) in *Lysosomes: a Laboratory Handbook* (Dingle, J. T., ed.), pp. 46–135, North-Holland Publishing Co., Amsterdam
- Barrett, A. J. (1973) *Biochem. J.* **131**, 809–822
- Bauer, E. A., Jeffrey, J. J. & Eisen, A. Z. (1971) *Biochem. Biophys. Res. Commun.* **44**, 813–818
- Bauer, E. A., Eisen, A. Z. & Jeffrey, J. J. (1972) *J. Biol. Chem.* **247**, 6679–6685
- Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
- Harris, E. D. & Krane, S. M. (1974a) *N. Engl. J. Med.* **291**, 557–563
- Harris, E. D. & Krane, S. M. (1974b) *N. Engl. J. Med.* **291**, 605–609
- Harris, E. D. & Krane, S. M. (1974c) *N. Engl. J. Med.* **291**, 652–661
- Kang, A. H., Nagai, Y., Piez, K. A. & Gross, J. (1966) *Biochemistry* **5**, 509–515
- McCroskery, P. A., Wood, S. & Harris, E. D. (1973) *Science* **182**, 70–71
- Nagai, Y. & Hori, H. (1972) *J. Biochem. (Tokyo)* **72**, 1147–1153
- Neville, D. M. (1971) *J. Biol. Chem.* **246**, 6328–6334
- Ohlsson, K. & Olsson, I. (1973) *Eur. J. Biochem.* **36**, 473–481
- Ouchterlony, O. (1967) in *Handbook of Experimental Immunology* (Weir, D. M., ed.), pp. 655–706, Blackwells Scientific Publications, Oxford and Edinburgh
- Smith, M. H. (1970) in *Handbook of Biochemistry* (Sober, H. A., ed.), pp. C10–C23, Chemical Rubber Co., Cleveland
- Tokoro, Y., Eisen, A. Z. & Jeffrey, J. J. (1972) *Biochim. Biophys. Acta* **258**, 289–302
- Werb, Z. & Burleigh, M. C. (1974) *Biochem. J.* **137**, 373–385
- Werb, Z. & Reynolds, J. J. (1974) *J. Exp. Med.* **140**, 1482–1497
- Werb, Z. & Reynolds, J. J. (1975) *Biochem. J.* **151**, 655–663
- Werb, Z., Burleigh, M. C., Barrett, A. J. & Starkey, P. M. (1974) *Biochem. J.* **139**, 359–368
- Woolley, D. E., Glanville, R. W. & Evanson, J. M. (1973) *Biochem. Biophys. Res. Commun.* **51**, 729–734