Purification of Pig Synovial Collagenase to High Specific Activity

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1. Pig synovium in tissue culture secretes a specific collagenase in a latent form. 2. The latent enzyme was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and activated with 4-aminophenylmercuric acetate, and the active enzyme was purified by chromatography on Ultrogel AcA44, DEAE-cellulose, heparin–Sephacrose and a zinc–chelate medium to a specific activity of 53 400 units/mg. of protein. 3. The enzyme was shown to be essentially homogeneous by polyacrylamide-gel electrophoresis. 4. The purified collagenase digested collagen to give the characteristic three-quarter and one-quarter pieces.

Mammalian collagenases (EC 3.4.24.3) cleave all three polypeptide chains of the collagen molecule at a single specific site to give fragments that are three-quarters and one-quarter of the size of the molecule. Although mammalian collagenases cannot be directly extracted from most tissues, they are synthesized and secreted when tissues are placed in culture. Mammalian collagenases have been detected and purified from many tissues and there is now good evidence that these enzymes are involved in the catabolism of collagen in normal and pathological connective tissues (see Harris & Cartwright, 1977).

Collagenase is usually found in tissue culture medium in a latent form. The nature of this latency has been the subject of considerable controversy and two explanations have been put forward. Firstly, it is proposed that latent collagenase is a proenzyme, as it can be activated by proteolytic enzymes (Oronsky *et al.*, 1973; Vaes & Eeckhout, 1975; Bauer *et al.*, 1975; Birkedal-Hanson *et al.*, 1976; Horwitz & Crystal, 1976; Woessner, 1977; Werb *et al.*, 1977). Secondly it is suggested that the latent enzyme is active collagenase complexed with an inhibitor, as activation can also be accomplished by using either chaotropic reagents (Nagai *et al.*, 1975; Shinkai & Nagai, 1977; Sakamoto *et al.*, 1978a) or thiol-binding reagents (Sellers *et al.*, 1977; Berman *et al.*, 1977; Vater *et al.*, 1978; Morales *et al.*, 1978).

In many of these studies only partially purified enzyme was used. In order to understand the properties of the enzyme and its role in connective-tissue turnover and disease it is important to purify both the latent and active forms of collagenase. Stricklin *et al.* (1977, 1978) have purified latent and active forms of collagenase from human skin fibroblast culture medium and compared their chemical properties. The latent collagenase could be activated by trypsin.

In the present study we have cultured pig synovial tissue (Fell & Jubbs, 1977) and detected a latent collagenase in the culture medium. This latent

collagenase can be activated by treatment with either trypsin or 4-aminophenylmercuric acetate. We have activated pooled culture medium with 4-aminophenylmercuric acetate and describe a new purification procedure for the active enzyme.

Materials

Chemicals were obtained from the following suppliers: SDS, acrylamide and methylenebisacrylamide from BDH, Poole, Dorset, U.K.; fluorescamine from F. Hoffman–La Roche and Co., Basel, Switzerland; 4-aminophenylmercuric acetate and butane-1,4-diol diglycidyl ether from Aldrich Chemical Co., Gillingham, Kent, U.K.; Coomassie Brilliant Blue R250 from Raymond A. Lamb, London NW10 6JL, U.K.; *N*-succinimidyl-3-(4-hydroxyphenyl)propionate from Pierce and Warriner, Chester CH1 4EF, U.K.; DEAE-cellulose (DE-52) from Whatman Biochemicals, Maidstone, Kent, U.K.; Sepharose 6B from Pharmacia, Hounslow, Middx., U.K.; Ultrogel AcA44 from LKB Instruments, Croydon, Surrey, U.K.; carrier-free $^{125}\text{I}_2$ (100–240 mCi/ml, 1 MS 30) from The Radiochemical Centre, Amersham, Bucks., U.K.; ultrafiltration membranes (PM10) from Amicon, High Wycombe, Bucks., U.K.; heparin from Sigma, Poole, Dorset, U.K.; $(\text{NH}_4)_2\text{SO}_4$ (specially prepared for enzyme purification) and Brij 35 from Fisons, Loughborough, Leics., U.K. All other chemicals and biochemicals were commercially available analytical grade reagents.

Methods

Synovial tissue was removed from the metacarpophalangeal joints of 5–7-month-old pigs (70–95 kg). Synovial villi with some subsynovial tissue from 16 joints were cut off, washed in Dulbecco's modified Eagle's medium (Vogt & Dulbecco, 1960) and cultured at 37°C in 500 ml bottles containing 150 ml of the above medium in an atmosphere of $\text{CO}_2/\text{O}_2/\text{N}_2$ (1:4:15). The medium was changed daily for 2 days

Abbreviation used: SDS, sodium dodecyl sulphate.

and then every 2–3 days until the collagenase activity in the medium fell below 0.5 unit/ml (usually 18 days). The collections of medium from pig synovial cultures from day 3 onwards were combined, filtered through Whatman 54 filter paper and diluted with 1:10 volume of 1M-Tris/HCl buffer, pH 7.6, containing 100mM-CaCl₂. The medium was stored frozen at –20°C until required for enzyme preparation.

Column chromatography

All buffers contained Brij 35 (0.05%) and toluene (0.03%) and all chromatographic procedures were carried out at 4°C. Heparin–Sephacrose was prepared by the method of Sakamoto *et al.* (1975). The zinc-chelate column was prepared by the method of Porath *et al.* (1975). Epoxy-activated Sepharose 6B was prepared by the method of Sundberg & Porath (1974).

Protein determination

Protein in column eluates was monitored by measuring the A_{280} . It was also measured by using fluorescamine (Weigle *et al.*, 1972). A sample containing 1–5 µg of protein in 200 µl of 25 mM-sodium cacodylate buffer, pH 7.2, containing 10mM-CaCl₂, 0.05% Brij and 1M-NaCl was diluted to 1.0ml with 0.2M-borate buffer, pH 9.2. Then 50 µl of fluorescamine (3 mg/ml in dry acetonitrile) was added while the sample was mixed on a vortex-mixer. The fluorescence was measured on a Locarte spectrofluorimeter; the excitation wavelength was 396 nm and the emission wavelength 475 nm. Bovine serum albumin was used as a standard.

Enzyme assays

[¹⁴C]Acetylated collagen was used to measure collagenase activity in the diffuse-fibril assay (Cawston & Barrett, 1979). One unit of collagenase digests 1 µg of reconstituted collagen fibrils/min at 37°C. Neutral proteinase activity was measured with azocasein as substrate. A sample was made up to 375 µl with 100mM-Tris/HCl buffer, pH 7.6, containing 15mM-CaCl₂, and 125 µl of azocasein (6%, w/v) was added. After incubation at 37°C for 20 h, 2.5 ml of trichloroacetic acid (3%, w/v) was added and each tube left for 20 min. After filtration the A_{366} was measured. Azocasein was prepared by the method of Charney & Tomarelli (1947), except that sulphanilamide was replaced by sodium sulphanilate.

Iodination of proteins

N-Succinimidyl-3-(4-hydroxyphenyl)propionate was iodinated with Na¹²⁵I, extracted into benzene and dried down by the method of Bolton & Hunter (1973). Protein samples (80 µl) with sodium borate

buffer, pH 8.5 (20 µl) were added to tubes containing 20–100 µCi of dried iodinated ester and agitated for 15 min at 0°C.

SDS/polyacrylamide-gel electrophoresis

Protein samples were mixed with 50mM-Tris/HCl, pH 6.8, containing 2.5% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 8% (v/v) glycerol and 0.01% Bromophenol Blue, and heated at 90°C for 2 min. Polyacrylamide slab gels (14 cm × 16 cm × 0.1 cm) were poured, the lower gel containing 6–10% (w/v) total acrylamides and 2.5% (w/w) bisacrylamide and the spacer gel containing 4.5% (w/v) total acrylamides and 2.5% (w/w) bisacrylamide. The gels were polymerized in the presence of 0.03% ammonium persulphate and 0.025% *NNN'*-tetramethyl-1,2-diaminoethane. The buffer system described by Laemmli & Favre (1973) was used for all gels, and the gels were poured and run in the apparatus described by Reid & Bielecky (1968). Electrophoresis of protein samples was performed at room temperature in the presence of 0.1% SDS at a constant voltage of 150 V until the tracking dye migrated to within 1 cm of the bottom of the gel (4.5 h). The molecular-weight markers used were: phosphorylase A, 100 000; human transferrin, 78 000; bovine serum albumin, 68 000; ovalbumin, 43 000; carbonic anhydrase, 29 000; soya-bean trypsin inhibitor, 22 000; lysozyme, 14 300.

Gel slabs were stained for 15 min in a solution of 250 mg of Coomassie Brilliant Blue R250 in 75 ml of methanol/186 ml of water, containing 30 g of trichloroacetic acid and 9 g of sulphosalicylic acid at 60°C, and destained in a solution of 250 ml of ethanol/80 ml of acetic acid/670 ml of water at room temperature.

Polyacrylamide-gel electrophoresis at alkaline pH

Polyacrylamide slab gels (14 cm × 16 cm × 0.1 cm) were poured to form a gel containing 5% (w/v) total acrylamides and 2.5% (w/w) bisacrylamide. The buffer system was that described by Laemmli & Favre (1973) and Triton X-100 (0.1%) was added to all buffers. The polymerization, fixing and staining was as described for SDS-containing gels.

Radioautography

The polyacrylamide gels were dried on to 3 MM Whatman filter paper *in vacuo* at 75°C for 45 min and radioautographed by using Fuji RX Medical X-ray film. Polyacrylamide gels were dried, sliced and counted for radioactivity in a Packard PGD Auto-gamma counter.

Results

Scheme 1 summarizes the five stages of purification we developed for pig synovial collagenase. This pro-

cedure gives a rapid and reliable method for the purification of pig collagenase with high yields of enzymic activity.

Scheme 1. Purification of pig synovial collagenase

Concentration of medium

Pooled medium was thawed and taken to 20% saturation with $(\text{NH}_4)_2\text{SO}_4$, left overnight at 2°C and filtered through a Whatman 54 filter paper. $(\text{NH}_4)_2\text{SO}_4$ was added to the filtrate to 60% saturation and stored at 2°C overnight. The medium was centrifuged at 22000g for 1 h at 0°C. The pellet was suspended in 25 mM-sodium cacodylate buffer at pH 7.2 containing 1 M-NaCl, 10 mM- CaCl_2 , 0.05% Brij and 0.03% toluene (column buffer), dialysed against this buffer overnight and centrifuged at 40000g for 1 h at 4°C, and the pellet discarded.

4-Aminophenylmercuric acetate activation of latent collagenase

The collagenase in the concentrated medium was all latent; it required 4-aminophenylmercuric acetate in the assay to demonstrate any collagenolytic activity. The latent enzyme was activated by making the supernatant 0.7 mM-4-aminophenylmercuric acetate by adding 10 mM-4-aminophenylmercuric acetate stock solution. The mixture was incubated at 37°C for 4 h and then cooled to 4°C. All of the detectable collagenase in the concentrated medium was activated by this procedure.

Gel filtration on Ultrogel AcA44

A large column (4.4 cm × 86 cm) of Ultrogel AcA44 was equilibrated with column buffer, and the concentrated medium was applied and filtered by reverse flow.

Fig. 1. Gel filtration of pig synovial collagenase

Concentrated medium (57 ml) was activated and applied to a column (4.4 cm × 86 cm) of Ultrogel AcA44 eluted with 25 mM-sodium cacodylate, pH 7.2, containing 1 M-NaCl, 10 mM- CaCl_2 , 0.05% Brij and 0.03% toluene, at a flow rate of 25 ml/h. Fractions (8 ml) were collected, and the eluate was monitored for protein (A_{280} ; ····), collagenase (—) and neutral proteinase (A_{366} ; ----). A 100 μ l portion of column eluate was added to the azocasein assay.

Fig. 1 illustrates the elution profile we obtained. The collagenase was eluted as a sharp peak free of the majority of contaminating proteins, as shown by the A_{280} of the fractions. Activation of the collagenase before gel filtration improved the separation achieved, as the active enzyme is eluted after the latent enzyme. Two peaks of neutral proteinase (azocasein substrate) activity were detected, and one of these peaks was eluted with the collagenase. The fractions containing collagenase were combined and concentrated to 5–10 ml in an Amicon ultrafiltration cell fitted with a PM 10 membrane.

DEAE-cellulose chromatography

The collagenase, partially purified by gel filtration, was dialysed overnight against 25 mM-sodium cacodylate, pH 7.6, containing 15 mM- CaCl_2 , 0.05% Brij 35 and 0.03% toluene, and then applied to a column (1.0 cm $\times$ 9.0 cm) of DEAE-cellulose equilibrated with this buffer. The elution profile is shown in Fig. 2. Most of the collagenase and neutral proteinase activity passed unretarded through the column. A small fraction of the collagenase was retarded by the DEAE-cellulose under these conditions. The proteins adsorbed on the column were eluted with a 0–0.4 M-NaCl gradient and a further small amount of protein could be eluted by washing the column with 1 M-NaCl (results not shown).

DEAE-cellulose chromatography did not give good purification, but did remove proteins that contaminated the final preparation if the procedure was not included. The fractions containing collagenase that passed straight through the column were pooled.

Heparin-Sephrose chromatography

The pooled collagenase fractions from the DEAE-cellulose column were dialysed overnight against 25 mM-sodium cacodylate containing 10 mM- CaCl_2 , 0.05% Brij and 0.03% toluene, and then applied to a column (1.0 cm $\times$ 5.0 cm) of heparin-Sephrose equilibrated with this buffer. The sample was washed on to the column with the same buffer and then a salt gradient (0–0.4 M-NaCl) applied to the column. Fig. 3 illustrates the purification we obtained with this method. Most of the protein passed through the column, but the collagenase was bound and eluted at approx. 0.2 M-NaCl. A small amount of neutral-proteinase activity was eluted with the collagenase. The fractions containing collagenase were combined.

Zinc-chelate affinity chromatography

The chelate column (0.6 cm $\times$ 12 cm) was charged with ZnCl_2 as described by Porath *et al.* (1975) and then equilibrated with 25 mM-sodium borate buffer, pH 8.0, containing 0.15 M-NaCl. The combined

Fig. 2. DEAE-cellulose chromatography of pig synovial collagenase

Pooled fractions from Ultrogel AcA44 gel filtration in 25 mM-sodium cacodylate, pH 7.6, containing 15 mM- CaCl_2 , 0.05% Brij 35 and 0.03% toluene were applied to a column (1.0 cm $\times$ 9.0 cm) of DEAE-cellulose equilibrated with the same buffer. The adsorbed protein was eluted with a linear salt gradient (0–0.4 M-NaCl), and the column eluate monitored for protein (A_{280} ; $\cdots$), collagenase (—) and neutral proteinase (A_{366} ; ----). A 10 μ l portion of column eluate was added to the azocasein assay.

Fig. 3. *Heparin-Sepharose chromatography of pig synovial collagenase*

Pooled fractions from DEAE-cellulose chromatography in 25 mM-sodium cacodylate, pH 7.6, containing 10 mM- CaCl_2 , 0.05% Brij 35 and 0.03% toluene were applied to a column (1.0 cm $\times$ 5.0 cm) of heparin-Sepharose and eluted with this buffer. Adsorbed protein was removed by washing the column with a linear salt gradient (0–0.4 M- NaCl). $\cdots$, A_{280} ; —, collagenase; ----, neutral proteinase (A_{366}); $\square$, conductivity. A 10 μl portion of column eluate was added to the azocasein assay.

Fig. 4. *Zinc-chelate affinity chromatography of pig synovial collagenase*

Fractions containing collagenase from heparin-Sepharose chromatography in 25 mM-sodium borate buffer, pH 8.0, containing 0.15 M- NaCl , were loaded on to a column (0.6 cm $\times$ 12.0 cm) of iminodiacetic acid-Sepharose saturated with ZnCl_2 . The column was eluted sequentially with the following buffers: (1) 25 mM-sodium cacodylate, pH 6.5; (2) 25 mM-sodium cacodylate, pH 6.5, plus 0.8 M- NaCl ; (3) 50 mM-sodium acetate, pH 4.7, plus 0.8 M- NaCl ; (4) 0.05 M-EDTA plus 0.5 M- NaCl adjusted to pH 7.0. All buffers contained 0.05% Brij 35, 0.03% toluene and 1 mM- CaCl_2 . The column eluate was monitored for protein (A_{280} ; $\cdots$), collagenase (—) and neutral proteinase (A_{366} ; ----). A 10 μl portion of column eluate was added to the azocasein assay.

collagenase fractions from the heparin-Sepharose column were dialysed overnight against this buffer and then applied to the column. The column was

eluted in a stepwise manner with the following buffer: (1) 25mM-sodium cacodylate, pH6.5; (2) 25mM-sodium cacodylate, pH6.5, plus 0.8M-NaCl;

Table 1. *Purification of pig synovial collagenase*

Samples were taken from each collagenase pool and assayed for collagenase activity and protein. The protein determination for the dilute-medium sample was unreliable and the purification factor was calculated by taking the concentrated medium as stage 1. The purified collagenase also digested azocasein, and 0.2 μ g of collagenase gave an absorbance at 366 nm of 0.5 after incubation with azocasein. For further experimental details, see the Methods section.

	Collagenase (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (factor)
Dilute medium	18340	(524)	35	100	—
Concentrated medium	19250	85	227	105	1
Gel filtration (AcA44)	26800	9.1	2940	146	12
DEAE-cellulose	15320	4.7	3290	84	14
Heparin-Sepharose	15350	0.70	21680	84	96
Zinc-chelate-Sepharose	12820	0.24	53400	70	235

Fig. 5. *SDS/polyacrylamide-slab-gel electrophoresis of samples from each stage of the collagenase purification: comparison of radioautography (a) with Coomassie Brilliant Blue staining (b)*

Samples containing 2–10 μ g of protein from each purification step (1–5 in Scheme 1) were labelled with 125 I, reduced and applied to SDS/polyacrylamide gels. After electrophoresis, the gels were stained, dried and radioautographed. The molecular-weight markers (M) are as described in the Methods section and BPB indicates the position of the tracking dye, Bromophenol Blue.

(3) 50mM-sodium acetate buffer, pH4.7, plus 0.8M-NaCl; (4) 50mM-EDTA plus 0.5M-NaCl. All of the above buffers contained 0.05% Brij 35, 0.03% toluene and 1mM-CaCl₂. Fig. 4 illustrates the elution profile of this column, and the active collagenase was eluted from the column at pH4.7 and 0.8M-NaCl (elution buffer 3). A small amount of neutral-proteinase activity was also eluted.

Purification of pig synovial collagenase

Samples were taken from each collagenase pool throughout the procedure and assayed for collagenase activity and protein content in order to assess the overall purification of the enzyme. Table 1 demonstrates the results that we obtained. It was difficult to obtain a reliable estimate for the protein content of the starting material as a non-dialysable contaminant was present which interfered with the fluorescamine protein assay. To overcome this, the purification factor was calculated by taking the concentrated medium as stage 1, and a purification factor of 235 was obtained. The final specific activity of the isolated collagenase was 53400 units/mg of enzyme protein, and 70% of the initial collagenase activity was recovered in the preparation. The total number of units of collagenase rose during the purification. Gel filtration of the activated concentrated medium revealed more active enzyme (146%) than could be detected by assay of the concentrated medium, and the reason for this is not clear. It is possible that some of the 4-aminophenylmercuric acetate in the assay of concentrated medium was removed by thiol-

containing proteins in the medium and insufficient free 4-aminophenylmercuric acetate was present to activate all the latent enzyme.

Assessment of collagenase purity

The purity of the final preparation and the fractionation achieved at each stage of purification was examined. Samples from the collagenase pools from each stage of the purification procedure (1-5 in Scheme 1) were radioiodinated as described in the Methods section and then applied to an SDS/polyacrylamide gel.

Fig. 5 is a photograph of the gel after staining and drying. Fig. 5(a) is a radioautograph of the dried gel and Fig. 5(b) is a photograph of the stained gel, and they show the proteins present at each stage of the purification and the purity of the final protein peak from the zinc-chelate column (gel sample 5). The purified collagenase migrated as a single band when stained with Coomassie Brilliant Blue. However, when the dried gel was radioautographed, traces of other protein bands of lower molecular weight were visible (Fig. 5a). The dried SDS/polyacrylamide gel containing the iodinated collagenase was cut into 2mm strips and counted for radioactivity in a Packard PGD Autogamma counter, and 94% of the radioactivity was found to be present in the major band. The SDS-containing gel shows that the collagenase had a mol.wt. of approx. 44000. The values on Fig. 5 correspond to the molecular weights of standard proteins. It is noteworthy that the collagenase appears to be relatively pure when

Fig. 6. *Electrophoresis of pig synovial collagenase at alkaline pH and elution of enzymic activity*

Two samples of purified pig synovial collagenase (2.5 µg) were applied to a polyacrylamide gel, pH8.7, containing 0.1% Triton X-100. After electrophoresis a portion of the gel containing one sample was stained with Coomassie Brilliant Blue and that containing the other sample was sliced, eluted with buffer, and assayed for collagenase. A broad peak of collagenase activity was eluted from the gel in the region of the stained protein band.

Fig. 7. Specific cleavage of collagen by pig synovial collagenase

A portion of the collagenase peak eluted from the polyacrylamide gel (Fig. 6) was mixed with 10 μ g of acid-soluble rat skin collagen in Tris/HCl, pH 7.6, 200 mM-NaCl and 1 M-glucose and incubated at 25°C for 18 h. After electrophoresis on an SDS/polyacrylamide gel (8%, w/v) under reducing conditions and staining with Coomassie Brilliant Blue the characteristic three-quarter and one-quarter fragments

stained (Fig. 5b, gel sample 4), but iodination of the protein reveals many contaminating protein bands at this stage of purification. Fig. 5 also demonstrates the purification achieved with the final zinc-chelate column.

Polyacrylamide-gel electrophoresis

It is necessary to establish that the major protein band seen after SDS/polyacrylamide-gel electrophoresis of the purified enzyme is collagenase. As it is very difficult to recover activity after denaturation and electrophoresis in SDS, two samples from the final enzyme preparation were loaded on to a polyacrylamide gel at alkaline pH, as described in

Fig. 8. SDS/polyacrylamide-gel electrophoresis of collagenase eluted from alkaline polyacrylamide gel

A portion of the collagenase peak eluted from the polyacrylamide gel (Fig. 6) was iodinated with $^{125}\text{I}_2$ and applied to a 10% (w/v) polyacrylamide gel containing 0.1% SDS with unlabelled pig synovial collagenase and molecular-weight standards. After electrophoresis the gel was stained (b), dried and radioautographed (a). The collagenase eluted from the polyacrylamide gel corresponded to the pig synovial collagenase standard. M, Molecular-weight standards; 1, ^{125}I -labelled collagenase eluted from the polyacrylamide gel; 2, purified pig synovial collagenase (0.5 μ g).

were produced by the pig synovial collagenase from the α - and β -chains of collagen. Sample 1, pig synovial collagenase + type-I collagen; sample 2, type-I collagen alone.

the Methods section, with the standards bovine serum albumin and ovalbumin. After electrophoresis the gel was cut vertically to remove one enzyme sample and the rest of the gel was fixed and stained. The portion of the gel removed was sliced into 2 mm slices and eluted overnight at 2°C with 25 mM-sodium cacodylate, pH 7.6, containing 10 mM-CaCl₂, 0.05 % Brij and 0.03 % toluene. Fig. 6 shows that the collagenase preparation migrated as a single band in the polyacrylamide gel and that collagenase activity could be eluted from the region of the gel corresponding to the staining band. A small amount of neutral-proteinase activity was also recovered from the eluted slices in the same region of the gel. The remaining eluate from the slice number 20 underneath the peak was divided into two portions.

One portion was mixed with acid-soluble rat skin type-I collagen at 25°C and left overnight. Fig. 7 shows that the enzyme gave the characteristic three-quarter and one-quarter pieces after denaturation of the mixture in SDS and electrophoresis. The other portion of the eluate was labelled with ¹²⁵I₂, denatured in SDS and loaded on to an SDS/polyacrylamide gel. The dried gel was radioautographed, and Fig. 8 shows that the single protein band moved with the same mobility as the final preparation. This establishes that the major protein band seen in the polyacrylamide gel corresponds to the major protein band seen in the the SDS-containing gel.

Discussion

The purification of collagenase from tissue-culture medium is difficult, as so little enzyme protein is present and, in conventional procedures, large losses occur. Also a large proportion of the isolated protein is often used up in demonstrating the homogeneity of the final preparation, as the techniques commonly employed to measure and detect the protein are relatively insensitive.

In the present procedure we have tried to overcome these problems. Pig synovial tissue was used, as it is readily available and can be easily cultured on a large scale. The inclusion of the detergent Brij 35 dramatically improved the recovery of the enzyme during purification. Dilute solutions of enzyme could be kept for long periods of time at 4°C in the presence of this detergent. Without detergent the enzyme was unstable during purification and sometimes rapidly lost activity, as reported for other collagenases (Werb & Reynolds, 1975; Woolley *et al.*, 1978; McCroskery *et al.*, 1975). The protein assay with fluorescamine was useful for measuring low concentrations of proteins; concentrations as low as 1–2 µg/ml could be measured by this method, and the detergent Brij 35 did not interfere. This assay does exclude the use of some buffers (e.g. Tris), and the cacodylate and borate buffers employed during the purification were chosen for this reason. The iodination of the protein

pools before electrophoresis and subsequent radioautography has proved particularly useful when trying to assess the purification achieved at each stage of the procedure. It is also a useful method for the accurate detection of the contamination of the purified enzyme by minor proteins. The conditions used for the iodination are very mild and little denaturation of the small amounts of protein occurred.

The heparin-Sepharose column proved to be useful for the purification of pig collagenase. This medium, originally described by Sakamoto *et al.* (1975), binds collagenase and presumably acts as a cation-exchanger. The recoveries of enzyme achieved by using heparin-Sepharose were greater than those achieved by using CM-cellulose, although both methods gave very similar separations. Sakamoto *et al.* (1978b) have suggested that the only collagenase to bind to this matrix is the mouse bone enzyme cultured in the presence of heparin. We have found that the pig synovial enzyme and also rabbit bone and uterus enzyme (T. E. Cawston & J. A. Tyler, unpublished work) will bind to heparin-Sepharose under the conditions described.

Considerable purification of the pig collagenase was achieved by zinc-chelate affinity chromatography. The active enzyme binds tightly to the column and is eluted at low pH and high ionic strength. The exposed histidine and/or thiol groups of proteins are thought to bind to the chelated zinc attached to the column (Porath *et al.*, 1975) and collagenase binds to the column via these amino acids and not because it is a zinc metalloprotein. When no zinc was present on the column, the collagenase did not bind. Other metal ions have been tried, and some activity was recovered with Ni²⁺ attached, but no activity could be recovered from the copper-chelate column. The zinc-chelate column gave the best purification and recovery of activity.

A large number of collagenases have been purified from a variety of tissues and species, but reported final specific activities have ranged from 31 to 3500 units/mg (see McCroskery *et al.*, 1975; Werb & Reynolds, 1975; Woolley *et al.*, 1975, 1978; Stricklin *et al.*, 1977; Sakamoto *et al.*, 1978b; Gillet *et al.*, 1977; Nagai & Hori, 1972; Ohya & Hashimoto, 1977). Some differences can be explained because different assay conditions have been used. In the present study the specific activity of the final enzyme preparation is very high when compared with the previously isolated collagenases. The reason for this is not clear. The diffuse-fibril assay is more sensitive than the conventional fibril assay, and the specific activity was found to be 26300 units/mg of protein by using the latter assay. It seems unlikely that the specific activity of the pig enzyme should be greatly different from that of other species. In our early attempts to isolate this enzyme with no detergent, the specific activity of the enzyme did not increase

throughout the procedures, even though it was apparent that large amounts of protein were separated from the enzyme at each stage. It is possible that these preparations contained a proportion of denatured enzyme protein. Little was known of the latency of this enzyme when many of the collagenases were isolated, and only the enzyme that was already active in the culture medium or became active during purification could be detected. It is likely that large amounts of latent collagenase were present, but went undetected, as we have found that latent collagenase behaves in a similar way to active collagenase during purification. This could account for the lower specific activities previously reported.

A small amount of neutral-proteinase activity was associated with the collagenase, but 500-fold more enzyme preparation had to be used in the azocasein assay than the collagenase assay to demonstrate this activity. Considerable controversy exists with regard to the non-specific proteolytic activity of purified collagenase preparations. Some workers (Werb & Reynolds, 1975; Woolley *et al.*, 1975, 1978; McCroskery *et al.*, 1975; Ohyama & Hashimoto, 1977) have not detected caseinolytic activity in purified preparations of collagenase, but in some cases the techniques used were insensitive. Others (Nagai & Hori, 1972; Gillet *et al.*, 1977; Sakamoto *et al.*, 1978b) have detected caseinolytic activities, even though the enzyme was purified to high specific activity. In the present study the isolated enzyme was at least 94% pure, and low caseinolytic activity was associated with the purified enzyme. It is not possible to exclude the possibility that this neutral metallo-proteinase activity is a contaminating proteinase. This activity is eluted from the alkaline polyacrylamide gel with the collagenase, and the stabilities of both activities after incubation at 45 and 50°C for increasing periods of time follows the same profile. We suggest that it is possible that this comparatively low proteolytic activity is an intrinsic property of the collagenase molecule.

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