Properties of pig synovial collagenase

Jenny A. TYLER and Tim E. CAWSTON

Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

(Received 10 December 1979)

1. Properties of a purified chemically activated form of pig synovial collagenase were examined and compared with a spontaneously active form of the enzyme. 2. The active enzyme has a specific activity of 53 000 units ($\mu\text{g}/\text{min}$)/mg, a mol.wt. of 44 000 (by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in 2-mercaptoethanol) and pI 5.2 (by isoelectric focusing in polyacrylamide gels). 3. The activity has the characteristics of a metalloproteinase that degrades types I and III soluble or insoluble collagens in preference to type II, at an optimum pH of 6.5–8.5. 4. There is no detectable difference in these properties between the chemically activated and spontaneously active form of collagenase.

The activity of collagenase *in vivo* is controlled by several complex mechanisms. The enzyme is often secreted in a latent form (Harper *et al.*, 1971; Vaes, 1972); it is inhibited by the serum proteins α_2 -macroglobulin (Birkedal-Hansen *et al.*, 1975) and β_1 -anti-collagenase (Woolley *et al.*, 1976) and by tissue inhibitors (Shinkai & Nagai, 1977; McCroskery *et al.*, 1975; Sellers *et al.*, 1977; Welgus *et al.*, 1978; Vater *et al.*, 1979).

The specific cleavage of collagen by mammalian collagenase to give one-quarter and three-quarter fragments is thought to be a carefully controlled rate-limiting step in normal collagen turnover (for review see Harris & Cartwright, 1977). Pathological conditions characterized by excessive degradation of joint tissue presumably result from increased collagenase activity, but little is known about which control processes have broken down or why. A thorough understanding of the properties of collagenase is required before any attempt can be made to prevent irreversible loss of collagen in connective-tissue disease.

In a recent paper (Cawston & Tyler, 1979) we reported the isolation of a chemically activated form of pig synovial collagenase from culture medium. We now describe the properties of this enzyme and those of a spontaneously active collagenase purified from the same source.

Materials

Chemicals were obtained from the following suppliers: fluorescamine from F. Hoffman–La Roche and Co. (Basle, Switzerland); Coomassie Brilliant Blue R250 from Raymond A. Lamb

Abbreviation used: SDS, sodium dodecyl sulphate.

(London NW10 6JL, U.K.); acrylamide, methylenebisacrylamide and SDS from BDH Chemicals (Poole, Dorset, U.K.); 4-aminophenylmercuric acetate from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); ampholyte solutions for isoelectric focusing from LKB Produkter (Stockholm, Sweden); papain type III from Sigma Chemical Co. (Poole, Dorset, U.K.); 1,9-dimethyl-Methylene Blue from Serva Feinbiochemica (Heidelberg, Germany). All other chemicals were analytical grade.

Pig tendon and articular cartilage were obtained within 2 h of killing from animals at the local abattoir, and stored at -20°C until used. Pepsin-extracted salt-soluble bovine collagens types I, II and III were generously given by Dr. A. J. Bailey (A.R.C. Meat Research Institute, Langford, Bristol, U.K.). α_2 -Macroglobulin was generously given by Dr. A. J. Barrett (Strangeways Research Laboratory, Cambridge, U.K.).

Methods

Throughout the present paper 'collagenase' refers to the enzyme produced by pig synovium that has been activated with 4-aminophenylmercuric acetate and isolated from tissue-culture medium. For details of methods see Cawston & Tyler (1979).

Spontaneously active enzyme was prepared by the same procedure, except that activation with 4-aminophenylmercuric acetate and the DEAE-cellulose chromatography were omitted. This collagenase preparation became fully active during purification (hence spontaneously active). Table 1 gives the details of the purification. All buffers contain Brij 35 (0.05%), toluene (0.03%) and CaCl_2 (10 mM) unless otherwise stated.

Table 1. *Purification of spontaneously activated pig synovial collagenase*
For experimental details see the text.

	Collagenase (units)	Protein (mg)	Specific activity (units/mg)	% of active enzyme	Purification factor (fold)
Dilute medium	42 840	703.7	60.8	0	
Concentrated medium	86 490	110.6	781	0	1
Gel filtration (Ultrogel AcA44)		17.39		75	
Heparin-Sepharose	58 000	2.75	21 120	100	27
Zn ²⁺ -chelate-Sepharose	51 300	0.96	53 230	100	68

Protein determination

Protein in solution was determined with fluorescamine, with bovine serum albumin as a standard (Weigle *et al.*, 1972).

Enzyme assays

Collagenase. (a) Collagen fibrils. [¹⁴C]Acetylated acid-soluble rat skin collagen (1 mg/ml) was used in the diffuse-fibril assay (Cawston & Barrett, 1979) for routine determination of collagenase activity. One unit of collagenase digests 1 µg of reconstituted collagen in 1 min at 37°C, pH 7.6.

[¹⁴C]Acetylated collagen (1 mg/ml) was dialysed against the following buffers at 2°C in the presence of 200 mM-NaCl: 25 mM-sodium cacodylate buffer, pH 5.5, 6.0, 6.5, 7.0 and 7.5; 50 mM-Tris/HCl buffer, pH 7.0, 7.5, 8.0 and 8.5; 25 mM-sodium borate pH 8.0, 8.5, 9.0, 9.5 and 10.0. To assess activity at different pH values, fibrils were pre-formed by incubation at the required pH at 37°C overnight. Collagenase was dialysed against the same buffers at 2°C in the presence of 5 mM-CaCl₂, and assayed by the fibril assay (Nagai *et al.*, 1966).

To assess collagenase stability at different pH values, an enzyme solution was dialysed against the above buffers at 2°C in the presence of 5 mM-CaCl₂.

After 4 days at 2°C, collagenase was diluted into 25 mM-cacodylate buffer, pH 7.5, and activity was determined by the diffuse-fibril assay.

(b) Soluble collagen. Salt-soluble collagen (2 mg/ml) was mixed with collagenase or trypsin in 50 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl, 10 mM-CaCl₂ and 250 mM-glucose at 25°C. The reaction was terminated with 1 mM-1,10-phenanthroline or 5 mM-di-isopropyl phosphorofluoridate. Collagen and degradation products were treated with 2% (v/v) 2-mercaptoethanol and 2.5% (w/v) SDS at 90°C for 5 min and analysed by SDS/polyacrylamide-gel electrophoresis.

(c) Insoluble collagen. Collagenase (1 µg) in 100 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl was incubated with approx. 10 mg of articular cartilage at 37°C for 48 h. The reaction was terminated with 1 mM-1,10-phenanthroline and the mixture was centrifuged (1400 g) for 15 min. The

supernatant and a papain digest of the pellet (Dingle *et al.*, 1973) were assayed for collagen (as hydroxyproline; Burleigh *et al.*, 1974).

Proteinase

For this assay, 50 µl of 100 mM-Tris/HCl buffer, pH 7.6, containing 15 mM-CaCl₂ and 100 mM-NaCl was mixed with 50 µl of [¹⁴C]acetylated casein (2 mg/ml) and made up to 250 µl with enzyme or 50 mM-Tris/HCl buffer, pH 7.6. After incubation at 37°C, 50 µl of 18% (w/v) trichloroacetic acid was added and the mixture was left at 2°C for 30 min to precipitate undigested casein. This was removed by centrifugation (1400 g) for 15 min and the activity was measured from soluble ¹⁴C radioactivity in the supernatant.

Polyacrylamide-gel electrophoresis

Polyacrylamide gels that contained either SDS or Triton X-100 (0.1%) were run in the discontinuous buffer system described previously (Cawston & Tyler, 1979): separating gel, 5–10% (w/v) acrylamide in 370 mM-Tris/HCl buffer, pH 8.8; stacking gel, 4.5% (w/v) acrylamide in 125 mM-Tris/HCl buffer, pH 6.8; reservoir buffer, 25 mM-Tris/HCl/0.19 M-glycine buffer, pH 8.2. All gels were polymerized in the presence of 0.03% ammonium persulphate and 0.025% NNN'-N'-tetramethyl-1,2-diaminoethane, and contained 2.5% (w/w) bisacrylamide. Protein samples were prepared in 62.5 mM-Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 0.01% Bromophenol Blue and either SDS or Triton X-100 (2%, v/v).

Gel slabs were stained at 60°C for 15 min in aq. 25% (v/v) methanol containing 3% (w/v) sulphosalicylic acid, 10% (w/v) trichloroacetic acid and 0.01% Coomassie Brilliant Blue R250, and destained at room temperature with 8% (v/v) acetic acid/aq. 25% (v/v) ethanol.

Isoelectric focusing in polyacrylamide gels

For this 6% (w/v) acrylamide gels containing 2.5% (w/v) bisacrylamide and 3% Ampholine (LKB) with a pH range of 3–10 or 5–7 were polymerized in glass tubes (0.5 cm × 8 cm) in the

presence of 0.03% ammonium persulphate, 0.025% *NNN'*-tetramethyl-1,2-diaminoethane and 0.1% Triton/X-100. The upper (cathode) reservoir contained 0.1% NaOH and the lower (anode) reservoir contained 0.2% H_3PO_4 . Muslin was secured over the bottom of the tubes to prevent the gels falling out. Gels were prefocused at 200V for 1 h (eight tubes). Protein samples were underlayered on to the gels in 20% (v/v) glycerol/0.1% Bromophenol Blue, and run at 200V for 14 h. The gels were sliced and eluted at 2°C overnight with 50mM-cacodylate buffer (pH 7.5)/10mM- CaCl_2 for collagenase assays, or with distilled water to determine the pH gradient.

Results

Isolation of spontaneously active collagenase

Serum-free medium, freshly obtained from pig synovium in tissue culture, contains latent collagenase. Activity can be revealed by treating the medium with organic mercurials such as aminophenylmercuric acetate (0.66mM) or proteinases (trypsin, 5µg/ml). Latent collagenase will also gradually become active if synovial medium or dilute column fractions are stored at 4°C. Our early attempts to devise a purification scheme for pig collagenase were hampered by the presence of variable proportions of latent and active species in different preparations; methods were therefore worked out by using aminophenylmercuric acetate-activated medium as the starting material (Cawston & Tyler, 1979). We were concerned that treatment with this mercurial may alter certain properties of collagenase, and have now isolated a spontaneously activated enzyme by using similar purification steps except that aminophenylmercuric acetate treatment and the DEAE-cellulose chromatography were omitted.

Pooled medium containing latent enzyme was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, and purified by chromatography on Ultrogel AcA44, heparin-Sepharose and iminodiacetic acid-Sepharose saturated with ZnCl_2 as described previously (Cawston & Tyler, 1979). The collagenase pool from each purification step was assayed for protein content and for enzyme activity in the presence and absence of aminophenylmercuric acetate (0.66mM) to determine the proportion of active enzyme. The results are shown in Table 1. The amount of protein in the dilute medium is an estimate, as the sample contained a component that interfered with the fluorescamine assay method. The purification factor was therefore calculated starting from the concentrated medium. The total apparent number of collagenase units increased during the purification, suggesting that the aminophenylmercuric acetate did not activate all the latent enzyme present in the crude sample.

Latent collagenase was eluted from the column of Ultrogel AcA44 as a single peak corresponding to a mol.wt. of 39 500; however, the final preparation of active collagenase was eluted from Ultrogel AcA44 in a position corresponding to a mol.wt. of about 31 500. All the enzyme was active before the heparin-Sepharose column stage. The mechanism of this activation or that effected by aminophenylmercuric acetate is not understood.

Optimal activity and stability of collagenase

Mammalian neutral collagenases, by definition, cleave collagen to give characteristic one-quarter (αA) and three-quarter (αB) pieces, which are readily identified by denaturing the degraded collagen and subjecting the mixture to electrophoresis in the presence of SDS. Fig 1 shows the αA and αB products from soluble collagen types I, II and III after incubation with pig synovial collagenase (240:1, w/w) at 25°C at pH 7.4 for 18 h.

The collagenase gave a linear dose-response relation when increasing amounts of enzyme were added to a fixed concentration of acid-soluble type-I [^{14}C]acetylated collagen in the diffuse-fibril assay method. The breakdown of collagen was also linear with time (Cawston & Barrett, 1979). The pH-dependence of collagenase activity was measured

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of digestion products of soluble collagens*

Samples (60µg) of soluble collagen types I (1), II (2) and III (3) were incubated at 26°C at pH 7.4 for 18 h with buffer (a) or with 0.125µg of collagenase (b). The separating gel contains 8% (w/v) acrylamide. For further details see the Methods section. The undigested α - and β -components of collagen are cleaved to characteristic three-quarter (αA and βA) and one-quarter (αB) fragments.

(Fig. 2). Optimum activity was over a fairly limited neutral range (pH 7.5–8.5), but 50% of the activity at optimum pH was obtained from pH 6.5 to 9.0.

Fig. 2. Activity of collagenase at different pH values. Collagenase was incubated with acid-soluble type-I collagen at 37°C for 18 h in various buffers at the pH values shown. O, 25 mM-Sodium cacodylate; ●, 50 mM-Tris/HCl; ▲, 25 mM-sodium borate. For further details see the text.

Less than 20% of this activity was seen below pH 5.5 or above pH 9.0. Despite a narrow range of optimal activity, collagenase is stable over a wide pH range, from pH 6.5 to 9.

The effect of various reagents on the activity of pig synovial collagenase was examined and the results are shown in Table 2. The enzyme was completely inhibited by the metal-chelating agents 1,10-phenanthroline and EDTA, and to a smaller extent by other metal chelators. Thiol compounds also inhibited collagenase, with dithiothreitol being the most effective. No inhibition was found with di-isopropyl phosphorofluoridate or pepstatin, and 20% inhibition was observed when 4-aminophenyl-mercuric acetate was added.

These findings support the view that the pig synovial enzyme is a metalloproteinase. Little inhibition was obtained with zinc-EDTA, and the results are consistent with the enzyme containing intrinsic Zn^{2+} and requiring Ca^{2+} for activity, as reported by Berman & Manabe (1973) and Seltzer *et al.* (1976, 1977). Insufficient enzyme protein was available for detection of Zn^{2+} in the purified preparation.

It was noteworthy that low concentrations of Zn^{2+} inhibit collagenase; 5 μM the pig collagenase was inhibited by 43%. Similar results have been

Table 2. Inhibition of pig synovial collagenase activity

Collagenase was preincubated at pH 7.5 at 25°C for 15 min with the inhibitors shown before assay. The concentration given is that in the final reaction mixture. For further details see the text. Inhibition is expressed as the percentage decrease in activity in the presence of an inhibitor compared with that of the enzyme alone.

Inhibitor	Concn.	Inhibition (%)
1,10-Phenanthroline	2 mM	99
EDTA	10 mM	95
	1 mM	77
8-Hydroxyquinoline	10 mM	97
Iminodiacetic acid	10 mM	49
Histidine	1 mM	55
Zn^{2+} -EDTA	10 mM	32
	1 mM	13
L-Cysteine	10 mM	88
	1 mM	29
2-Mercaptoethanol	10 mM	83
	1 mM	72
Dithiothreitol	1 mM	90
	0.1 mM	25
Glutathione	10 mM	64
DL-Penicillamine	10 mM	70
Aminophenylmercuric acetate	1 mM	20
Di-isopropyl phosphorofluoridate	5 mM	0
Pepstatin	10 $\mu g/ml$	0
Zn^{2+}	0.5 mM	85
	5 μM	43
Serum (sheep)	5% (v/v)	99
α_2 -Macroglobulin	100 $\mu g/ml$	99
	75 $\mu g/ml$	97
	50 $\mu g/ml$	59
	25 $\mu g/ml$	29

Fig. 3. *Elution of enzyme activity after electrophoresis* Samples containing $2\mu\text{g}$ of collagenase (a) and $2\mu\text{g}$ of spontaneously activated collagenase (b) were applied to a 7% (w/v) polyacrylamide gel, pH 8.7, containing 0.1% Triton X-100. After electrophoresis, two 1.5 cm-wide strips under the samples were removed, sliced horizontally, eluted with buffer and assayed for collagenase (●) and proteinase (○). For further details see the text.

reported by Seltzer *et al.* (1977) for human skin collagenase.

The inhibition by 1,10-phenanthroline appears to be reversible at 4°C by dialysis of the sample to remove the chelator, and a proportion of the original activity is recovered. No activity can be recovered if the enzyme is treated with 1,10-phenanthroline at 37°C .

Whole sheep serum (5%, v/v) completely inhibited collagenase, as did human α_2 -macroglobulin, where the decrease in activity was proportional to the amount of inhibitor present from 25 to $100\mu\text{g}/\text{ml}$.

Electrophoretic properties and charge

Collagenase and spontaneously active collagenase when run in gel electrophoresis at pH 8.8 migrated the same distance into the gel (Fig. 3). Enzyme mobility at an acrylamide concentration of 7% (w/v) is mainly due to charge, suggesting that pig collagenase would be negatively charged at this pH.

Fig. 4. *Isoelectric focusing of collagenase* Samples containing $2\mu\text{g}$ of collagenase were focused in a pH 5–7 gradient in the presence of Triton X-100 (0.1%). For further details see the text. Collagenase activity (●) and the pH value (○) of each gel slice is shown.

Isoelectric focusing of several collagenase samples in polyacrylamide gels (one of which is shown in Fig. 4) confirmed this, giving $\text{pI } 5.23 (\pm 0.18 \text{ s.e.m.; } n = 6)$. Less than 5% of the collagenase activity was recovered from the focusing gels.

Estimation of molecular size by gel chromatography

A column ($1.5 \text{ cm} \times 80 \text{ cm}$) of Ultrogel AcA44 equilibrated at 2°C with 25 mM-sodium cacodylate buffer, pH 7.5, containing 1 M-NaCl, 10 mM- CaCl_2 , 0.05% Brij and 0.03% toluene was used to estimate the molecular size of collagenase. The column was calibrated with bovine serum albumin (mol.wt. 68 000), ovalbumin (mol.wt. 43 000), chymotrypsinogen A (mol.wt. 25 000), soya-bean trypsin inhibitor (mol.wt. 22 000) and cytochrome c (mol.wt. 13 000). A single peak of activity in the mol.-wt. range 29 000–31 000 was obtained.

Electrophoresis in the presence of SDS

Collagenase and spontaneously active collagenase when treated with 2.5% (w/v) SDS and 4% (v/v) 2-mercaptoethanol at 90°C for 5 min and run under reducing conditions in polyacrylamide gels containing 0.1% (w/v) SDS gave a band corresponding to a mol.wt. of 44 000 (Fig. 5, gel 1a). Under non-reducing conditions in the presence of 1 mM-iodoacetic acid (Fig. 5, gel 1b) or with buffer alone (Fig. 5, gel 1c), collagenase appears to run as a doublet, with mol.wts. of 37 450 and 36 800. The standard proteins used to estimate molecular weight [phosphorylase A (mol.wt. 100 000), human transferrin (mol.wt. 78 000), bovine serum albumin

Fig. 5. *SDS/polyacrylamide-gel electrophoresis of reduced and non-reduced samples of collagenase* Collagenase (1.5 µg; 1), spontaneously activated collagenase (1 µg; 2) and molecular-weight markers (M) were treated with 2% (w/v) 2-mercaptoethanol (a), 1 mM-iodoacetic acid (b) or 10 mM-Tris/HCl buffer (c) at 90°C for 5 min. The separating gel contained 10% (w/v) acrylamide. For further details see the text.

(mol.wt. 68 000), catalase (mol.wt. 58 000), carbonic anhydrase (mol.wt. 29 000) and soya-bean trypsin inhibitor (mol.wt. 22 000)] confirm that reducing or non-reducing conditions were maintained during electrophoresis, as subunits of phosphorylase A (mol.wt. 100 000) and transferrin (mol.wt. 78 000) clearly seen in the reduced sample (Fig. 5, gel Ma) are absent from the unreduced samples (Fig. 5, Mb, Mc).

Substrate specificity

Collagenase degraded all three types of soluble collagen to one-quarter and three-quarter pieces at 25°C overnight (Fig. 1), but more than 50% of the type-II collagen remained as intact α -chains at the same ratio (240:1, w/w) of collagen to enzyme as for types I and III collagen, which were completely converted. A further study of the susceptibility of type-II collagen was made by using higher enzyme concentrations. Fig. 6 shows degradation of type-I and type-II collagens by collagenase at ratios (w/w) of 480:1 (gel 2b), 240:1 (gel 2c) and 80:1 (gel 2d). Trypsin (2 µg/ml) digestion of the collagen was the same as with buffer alone (gels 1a and 2a). This preferential degradation was also observed with insoluble collagen. Collagenase solubilized 50% of

Fig. 6. *SDS/polyacrylamide-gel electrophoresis to compare digestion of type-I collagen with that of type-II collagen*

Samples (60 µg) of soluble collagen types I (1) and II (2) were incubated at 26°C pH 7.5 for 18 h with assay buffer alone (a) or 0.125 µg (b), 0.25 µg (c) or 0.75 µg (d) collagenase. The separating gel contains 8% (w/v) acrylamide. For further details see the text.

the collagen from pig tendon, but only 18% from articular cartilage when incubated at 37°C for 48 h.

Discussion

The properties of pig synovial collagenase are shown in Table 3. The enzyme has a specific activity of 53 000 units/mg, a mol.wt. of 44 000 (by SDS/polyacrylamide-gel electrophoresis in 2-mercaptoethanol) and pI 5.2. Like other mammalian collagenases, the activity has the characteristics of a metalloenzyme that degrades collagen at an optimum pH of 7.5–8.5.

The spontaneously active collagenase has a very similar specific activity (53 200 units/mg) to the aminophenylmercuric acetate-treated enzyme (53 400 units/mg). It has a similar charge (Fig. 3b) and size by gel filtration and behaves in the same manner when run in reduced and non-reduced conditions on SDS gel electrophoresis (Fig. 5, gels 2a, 2b and 2c).

There is no detectable difference in these properties between the chemically activated and spontaneously active form.

A comparison of the general properties of collagenase is extremely difficult, as several groups

Table 3. *Properties of pig synovial collagenase*

Metalloenzyme requires Ca^{2+} for activity	
Stability	pH 6.0–9.5
Optimal activity	pH 7.5–8.5
Molecular weight	
Gel filtration	31 500
SDS/polyacrylamide-gel electrophoresis	
2-Mercaptoethanol absent	37 000
2-Mercaptoethanol present	44 000
Isoelectric point	5.2
Substrates	Type-I and type-III collagens degraded more readily than type II
Specific activity	
Collagenolytic	53 000 units/mg
Caseinolytic	106 units/mg

have isolated the enzyme to various degrees of purity with final specific activities ranging from 35 to 53 000 units/mg of protein (Nagai & Hori, 1972; McCroskery *et al.*, 1975; Werb & Reynolds, 1975; Woolley *et al.*, 1975b, 1978; Stricklin *et al.*, 1977; Gillet *et al.*, 1977; Ohyama & Hashimoto, 1977; Sakamoto *et al.*, 1978; Cawston & Tyler, 1979). These differences may reflect the various species and tissues used, but could also be due to the presence of denatured or undetected latent enzyme in some of the preparations.

The values for molecular size of active collagenase estimated by gel filtration range from 23 000 to 130 000, but many of these were obtained with partially purified material, and it is known that anomalous results are obtained with crude preparations, partly owing to the existence of several forms of the enzyme (Nagai & Hori, 1972; Stricklin *et al.*, 1978; J. A. Tyler & T. E. Cawston, unpublished work), but also because of interactions with other molecules, such as collagen (Fiedler-Nagy *et al.*, 1977) and heparin (Sakamoto *et al.*, 1978). Even pure collagenase can give several apparent values in different gel-filtration media: 42 000 from Sephadex G-100 and 30 000 and 25 000 from Ultrogel AcA44 (Stricklin *et al.*, 1977).

Molecular-weight estimations based on reduction with 2-mercaptoethanol and electrophoresis in SDS appear to be more consistent, with a range from 30 000 to 50 000. Our value of 44 000 for pig synovial collagenase compares with 32 000 for the rabbit collagenase (Werb & Reynolds, 1975) and 33 000 for the human collagenase (Woolley *et al.*, 1975b). Reported estimates from other tissues include mouse bone, 47 000 (Sakamoto *et al.*, 1978), human skin, 60 000 (Woolley *et al.*, 1978), 50 000 and 45 000 (Stricklin *et al.*, 1977) and rabbit

tumour, 34 000 (McCroskery *et al.*, 1975). We found that pig collagenase that was not reduced had a lower apparent molecular weight in SDS/polyacrylamide gels (37 000), but do not know the reason for this anomalous behaviour. The same result was obtained in the presence of 8 M-urea. The presence of carbohydrate or a high charge density affects the binding of SDS and therefore the mobility of certain polypeptides, e.g. membrane glycoproteins (Banker & Cotman, 1972) or histone F1 (Panyim & Chalkley, 1971). It has been reported that unreduced cross-linked protein aggregates migrate faster than expected compared with similar reduced samples (Dunker & Kenyon, 1975) and that anomalous mobility may depend on the exact conditions used in the preparation of gel samples with respect to temperature and SDS concentration (Shirama *et al.*, 1974).

Pig collagenase migrated about 10 cm into a 15 cm basic polyacrylamide gel [5% (w/v) acrylamide] (Cawston & Tyler, 1979) and 8.5 cm into a 7% (w/v) acrylamide gel at pH 8.8. Collagenase from rabbit synovial fibroblasts also migrates towards the anode at pH 8.6 (Werb & Reynolds, 1975), as do human skin and synovial collagenase at pH 8.3 (Woolley *et al.*, 1973). However, human synovial enzyme appears to have a greater mobility in 7.5% (w/v) acid gels, pH 4.5 (Woolley *et al.*, 1975b), and human skin collagenase was said to be a very basic protein (Stricklin *et al.*, 1977) because of its highly cationic behaviour on ion-exchange chromatography. Collagenase activity was eluted from polyacrylamide gels after isoelectric focusing in a region corresponding to pH 5.2 for pig synovial enzyme, pH 6.8–7.0 for rabbit tumour enzyme (McCroskery *et al.*, 1975) and pH 6.7 for human skin procollagenase (Stricklin *et al.*, 1978). Different

electrophoretic properties of collagenase from different species are probably due to minor changes in amino acid content, but may also reflect different stages of activation, e.g. latent pig synovial collagenase is more basic than the active form (J. A. Tyler & T. E. Cawston, unpublished work).

Although all reported mammalian collagenases initiate degradation of collagen by cleaving a single bond in each chain in the molecule to give one-quarter and three-quarter triple-helical fragments (Gross, 1974), the rate of attack and choice of substrate varies. Type-II collagen is more resistant to attack by skin and synovial enzymes than are types-I and -III (Woolley *et al.*, 1975a; McCroskery *et al.*, 1975), and the skin enzyme does not appear to degrade type-IV collagen (Woolley *et al.*, 1978; Timpl *et al.*, 1979). These variations in the rate of collagen breakdown are said to be due to differences in the detailed structure of the substrates (Robertson & Miller, 1972; Miller *et al.*, 1976). A tumour enzyme has been described that degrades type-IV collagen but not types I, II, III or V (Liotta *et al.*, 1979), and in at least one case degradation of type-III collagen in preference to type I by human polymorphonuclear leucocytes appears to be an 'enzyme specificity rather than a result of a difference in the substrates' (Horwitz *et al.*, 1977). Clearly, a greater understanding of the factors that determine specificity is required in order to predict the rates of turnover for each collagen type exposed to collagenase.

A neutral metalloproteinase assayed by degradation of [^{14}C]acetylated casein is present in our final collagenase preparations. Other workers have reported similar findings (Gillet *et al.*, 1977; Stricklin *et al.*, 1977; Sakamoto *et al.*, 1978). This activity is uncovered from a latent form by aminophenyl-mercuric acetate and has the same charge (Fig. 3) and size (by gel filtration) as collagenase, but degrades casein much less readily than the same weight of collagen. This may simply reflect lower activity on suboptimal substrates observed with many proteinases, but may be an important factor in the activation process of latent collagenase, and requires investigation.

We thank Mrs. Beryl King for expert technical assistance and Mr. Richard Laws for the tissue culture. We are grateful to Dr. A. J. Barrett and Dr. J. T. Dingle for their advice and encouragement. This work was supported by a grant from the Medical Research Council.

References

- Banker, G. A. & Cotman, C. W. (1972) *J. Biol. Chem.* **247**, 5856–5861
- Berman, M. B. & Manabe, R. (1973) *Ann. Ophthalmol.* **5**, 1193–1209
- Birkedal-Hansen, H., Cobb, C. M., Taylor, R. E. & Fullmer, H. (1975) *Arch. Oral Biol.* **20**, 681–685
- Burleigh, M. C., Barrett, A. J. & Lazarus, G. S. (1974) *Biochem. J.* **137**, 387–398
- Cawston, T. E. & Barrett, A. J. (1979) *Anal Biochem.* **99**, 340–345
- Cawston, T. E. & Tyler, J. A. (1979) *Biochem. J.* **183**, 647–656
- Dingle, J. T., Horsfield, P., Fell, H. B. & Barratt, M. E. J. (1973) *Ann. Rheum. Dis* **34**, 303–311
- Dunker, K. & Kenyon, A. J. (1975) *Biochem. J.* **153**, 191–197
- Fiedler-Nagy, C., Coffey, J. W. & Salvador, R. A. (1977) *Eur. J. Biochem.* **76**, 291–297
- Gillet, C., Eeckhout, Y. & Vaes, G. (1977) *FEBS Lett.* **74**, 126–128
- Gross, J. (1974) *Harvey Lect.* **68**, 351–432
- Harper, E., Bloch, K. J. & Gross, J. (1971) *Biochemistry* **10**, 3035–3041
- Harris, E. D. & Cartwright, E. C. (1977) *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J. ed.), pp. 249–278, North-Holland, Amsterdam
- Horwitz, A. L., Hance, A. J. & Crystal, R. G. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 897–901
- Liotta, G. A., Shigeto, A., Robey, P. G. & Martin, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2268–2272
- McCroskery, P. A., Richards, J. F. & Harris, E. (1975) *Biochem. J.* **152**, 131–142
- Miller, E. J., Harris, E. D., Chung, E., Finch, J. E., McCroskery, P. A. & Butler, W. T. (1976) *Biochemistry* **15**, 787–792
- Nagai, Y. & Hori, J. (1972) *Biochim. Biophys. Acta* **263**, 564–573
- Nagai, Y., Lapiere, C. M. & Gross, J. (1966) *Biochemistry* **5**, 3123–3130
- Ohyama, H. & Hashimoto, K. (1977) *J. Biochem. (Tokyo)* **82**, 175–183
- Panyim, S. & Chalkley, R. (1971) *J. Biol. Chem.* **246**, 7557–7560
- Robertson, P. B. & Miller, E. J. (1972) *Biochim. Biophys. Acta* **289**, 247–250
- Sakamoto, S., Sakamoto, M., Matsumoto, A., Goldhaber, P. & Glimcher, M. J. (1978) *FEBS Lett.* **88**, 53–58
- Sellers, A., Cartwright, E., Murphy, G. & Reynolds, J. J. (1977) *Biochem. J.* **163**, 303–307
- Seltzer, J. L., Welgus, H. G., Jeffrey, J. J. & Eisen, A. Z. (1976) *Arch. Biochem. Biophys.* **173**, 355–361
- Seltzer, J. L., Jeffrey, J. J. & Eisen, A. Z. (1977) *Biochim. Biophys. Acta* **485**, 179–187
- Shirama, K., Tsujii, K. & Takagai, T. (1974) *J. Biochem. (Tokyo)* **75**, 309–319
- Shinkai, H. & Nagai, Y. (1977) *J. Biochem. (Tokyo)* **81**, 1261–1268
- Stricklin, G. P., Bauer, E. A., Jeffrey, J. J. & Eisen, A. Z. (1977) *Biochemistry* **16**, 1607–1615
- Stricklin, G. P., Eisen, A. Z., Bauer, E. A. & Jeffrey, J. J. (1978) *Biochemistry* **17**, 2331–2337
- Timpl, R., Martin, G. R. & Brucker, P. (1979) in *Biochemistry and Pathology of Basement Membrane* (Robert, L., ed.), pp. 130–141, S. Karger, Basel
- Vaes, G. (1972) *Biochem. J.* **125**, 275–289
- Vater, C. A., Mainardi, C. L. & Harris, E. D. (1979) *J. Biol. Chem.* **254**, 3045–3053

- Weigle, M., Debernardo, S. L., Tangi, J. P. & Leimgruber, W. (1972) *J. Am. Chem. Soc.* **94**, 5927–5928
- Welgus, H. G., Stricklin, G. P., Eisen, A. Z., Bauer, E. A. & Jeffrey, J. J. (1978) *Clin. Res.* **26**, 489A
- Werb, Z. & Reynolds, J. J. (1975) *Biochem. J.* **151**, 645–653
- Woolley, D. E., Glanville, R. W. & Evanson, J. W. (1973) *Biochem. Biophys. Res. Commun.* **51**, 729–734
- Woolley, D. E., Lindber, K. A., Glanville, R. W. & Evanson, J. M. (1975a) *Eur. J. Biochem.* **50**, 437–444
- Woolley, D. E., Glanville, R. W., Crossley, M. J. & Evanson, J. M. (1975b) *Eur. J. Biochem.* **54**, 611–622
- Woolley, D. E., Roberts, D. R. & Evanson, J. M. (1976) *Nature (London)* **261**, 325–327
- Woolley, D. E., Glanville, R. W., Roberts, D. R. & Evanson, J. M. (1978) *Biochem. J.* **169**, 265–276