INHIBITION OF HUMAN COLLAGENASE ACTIVITY BY A SMALL MOLECULAR WEIGHT SERUM PROTEIN

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SUMMARY

Fractionation of human serum proteins by gel filtration in Sephadex G-200 revealed two regions of collagenase inhibition which corresponded to α_2-macroglobulin and a smaller serum component which eluted after α_1-antitrypsin. The smaller collagenase inhibitor, having a molecular weight of 40,000 was separated from α_1-antitrypsin by chromatography in Sephadex DEAE A.50. It was found to inhibit human collagenases derived from skin, rheumatoid synovium, gastric mucosa and granulocytes, but not the neutral proteases trypsin and papain. Purified preparations of α_1-antitrypsin inhibiting trypsin and papain had no effect on the collagenase activities. The small collagenase inhibitor may have importance as a regulatory factor in the control of collagenase activity in vivo.

INTRODUCTION

A variety of neutral collagenases have now been characterised from both human and animal tissues (see review, Harris & Krane, 1974), and the role of serum antiproteases in the control of collagenase activity has often been discussed[2-8]. It has long been known that whole serum has the ability to inhibit collagenases and there is general agreement that this action is largely attributable to the α_2-M component[2,5,6,8]. However, Eisen and co-workers reported that α_1-AT also was an inhibitor of human skin collagenase[4,9,10] and rheumatoid synovial collagenase[4,11], and similar results have been reported for the enzymes derived from human granulocytes[12] and platelets[13]. In contrast, α_1-AT was reported as non-inhibitory when examined against human corneal[7], mouse bone collagenase[5] and rabbit synovial collagenase[6].

As α_1-AT and α_2-M have molecular weight values respectively of

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Abbreviations: α_2-M, α_2-macroglobulin; α_1-AT, α_1-antitrypsin.

approximately 45,000 and 725,000 the in vivo permeability barrier to the latter might suggest that the smaller protein is of greater importance in regulating collagenase activity physiologically. As the role of α_1-AT as a collagenase inhibitor appeared uncertain, we examined the inhibitory properties of the small molecular weight serum proteins against four human collagenases derived from skin[14], rheumatoid synovium[2], gastric mucosa[15] and granulocytes[12]. This report describes the finding of a small serum protein capable of inhibiting all four collagenases, its separation from α_1-AT, and a comparative study of the action of both proteins on collagenase and neutral protease activities.

METHODS

Enzyme preparations. Human collagenases derived from skin, rheumatoid synovium and gastric mucosa were obtained using tissue culture methods described previously[2]. Explants of each tissue were maintained in culture flasks containing serum-free Dulbecco's modified Eagle's medium in an atmosphere of 95% O_2 : 5% Co_2 for 7 days at 37°C. The culture medium was changed every 24 h and collagenase activity released into the medium was concentrated by ultrafiltration using Amicon PM-10 membranes at 4°C.

Polymorphonuclear leucocytes collected by centrifugation of rheumatoid synovial fluids and homogenised in 50mM Tris-HCl, pH 8.0 containing 0.17M NaCl and 10mM $CaCl_2$at 4°C provided the source of granulocyte collagenase.

Trypsin (Type III) and papain (mercuripapain) were purchased from Sigma (London) Chemical Co. Ltd.

Assays. Collagenase activity was assayed by measuring the release of soluble radioactive products from a pellet of thermally reconstituted ^{14}C-glycine-labelled collagen fibrils by a modification of the method of Nagai et al[16].

Protease activity was measured by the method of Kunitz[17] in which trypsin or papain degraded the substrate casein for 6 h at 37°C. The reactions were terminated by the addition of trichloroacetic acid and the absorbance of the supernatant at 280nm was measured.

Serum protein fractionation. Human serum samples obtained from normal adult males were fractionated by gel filtration at 4°C in a column (90 x 2cm) of Sephadex G-200. The proteins were eluted with 20mM Tris-HCl buffer, pH. 8.0, containing 0.17M NaCl and 10mM $CaCl_2$, at a flow rate of 12ml/h. 2ml fractions were collected and examined for inhibitory activity by adding 100ul to the collagenase assay prior to the addition of enzyme. The content of α_2-M and α_1-AT of each eluant fraction was determined immunologically by single radial immunodiffusion using immuno-kits from Hyland Laboratories Inc., and ICL Scientific, respectively.

Further separation of the small molecular weight serum proteins was achieved by ion exchange chromatography at 4°C in a column (35 x 1.6cm) of Sephadex DEAE A-50 equilibrated with 50mM Tris-HCl buffer, pH 8.1 containing 10mM $CaCl_2$. Elution with this buffer was followed by a salt gradient elution at a flow rate of 12ml/h. 2ml fractions were collected and examined for their inhibitory capacity in both collagenase and protease assays.

Molecular Weight determination. The molecular weight of the small collagenase inhibitor was determined by gel filtration at 4°C in a column (50 x 0.9cm) of Sephadex G-75 superfine. The eluting buffer was that described for the Sephadex G-200 column and 0.66ml fractions were collected at a flow rate of 5ml/h. The elution positions of the protein standards transferrin, ovalbumin, carbonic anhydrase and myoglobin provided a size calibration of the column. The void volume, Vo, and the salt exclusion volume, V_t, were determined with solutions of Blue Dextran and potassium ferricyanide respectively.

RESULTS AND DISCUSSION

After fractionation of human serum proteins by gel filtration in Sephadex G-200 the capacity of each eluant fraction to inhibit human skin collagenase activity was assessed (Fig.1). Two regions of enzyme inhibition were observed and these corresponded to fractions containing α_2-M, measured immunologically, and to a smaller serum component which eluted after α_1-AT. It was observed that those fractions containing maximal α_1-AT content did not produce maximal inhibition of collagenase activity, and as the α_1-AT protein peak and region of collagenase inhibition were not coextensive these fractions were pooled and concentrated by ultrafiltration.

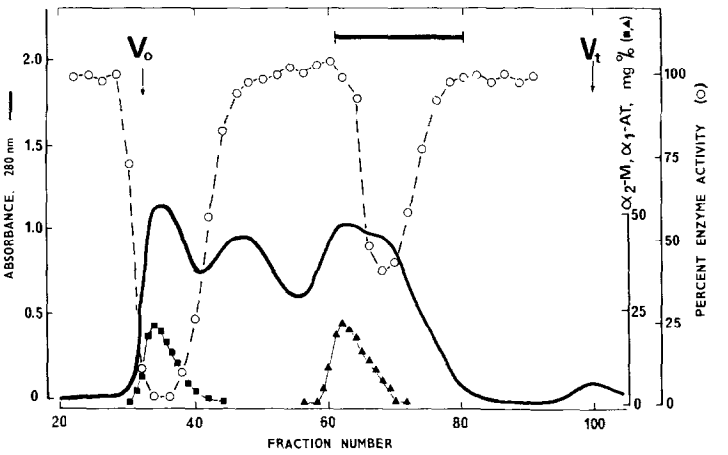


Fig.1. Gel filtration of human serum on Sephadex G-200 and measurement of collagenase activity. 3ml of serum was eluted with buffer at a flow rate of 12ml/h and 2ml fractions were collected. Each was examined for inhibitory activity by adding 100µl to the collagenase assay prior to the addition of enzyme. Enzyme activity was plotted as a percentage of the control value containing no serum protein (○). α_2-M (■) and α_1-AT (▲) content of the eluant fractions was determined immunologically. ——— , absorbance at 280nm; ⊢——⊣ , fractions pooled for concentration.

The resulting sample was dialysed free of salt and applied to a column of Sephadex DEAE, A-50. Elution of the ion exchange resin column using a linear salt gradient is shown in Fig.2. When each eluant fraction was examined in the collagenase assay system, inhibition was found to be separated from the α_1-AT peak, measured immunologically and by its inhibition of trypsin activity. The collagenase inhibitor, which eluted in a position along the salt gradient between 0.02-0.04M NaCl, did not inhibit trypsin activity in the protease assay system. Similarly, the α_1-AT protein peak, which eluted between 0.08-0.1M NaCl, did not inhibit collagenase activity.

Those fractions containing the collagenase inhibitor and the α_1-AT protein peak were concentrated separately by ultrafiltration and their action on four

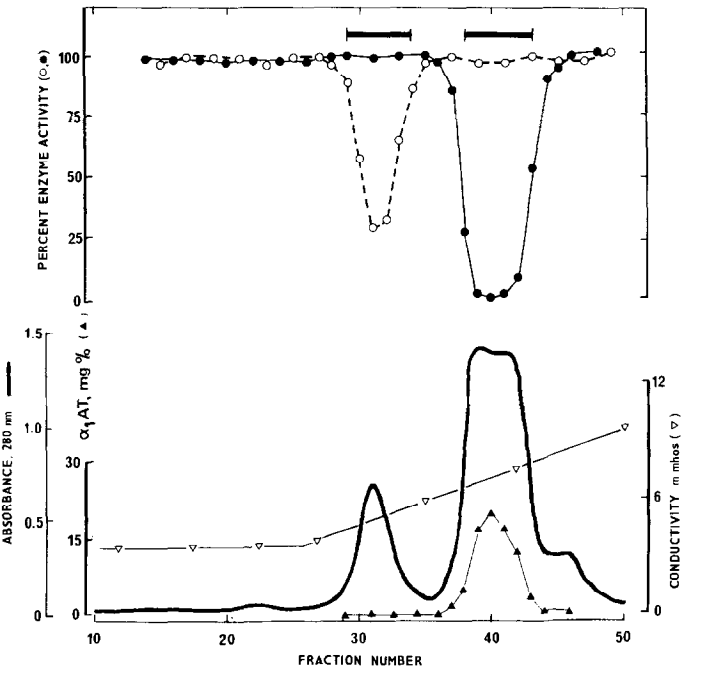
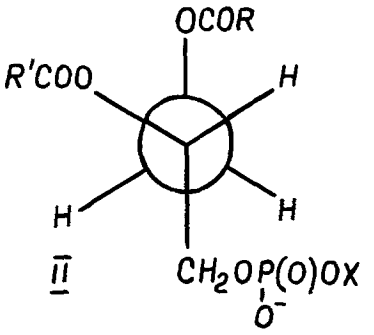


Fig.2. Separation of the small collagenase inhibitor from α_1-AT by ion exchange chromatography on Sephadex DEAE A-50. 2ml of the concentrated protein solution obtained from Fig.1 was applied to the resin and examined for collagenase inhibition (○), trypsin inhibition (●), and α_1-AT content (▲). ——, absorbance at 280nm; (▽), conductivity; |——|, fractions pooled for concentration.



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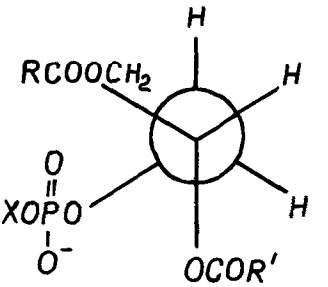

III

choline and the other glycerophospholipids makes it likely, that they should contain high populations of the same rotamers. If this be so, application of the carboxyl sector rule [17] leads to the conclusion that those glycerophospholipids which are derivatives of 3-sn-phosphatidic acid should reveal a positive Cotton effect (see Scheme).

Circular dichroism measurements carried out with a number

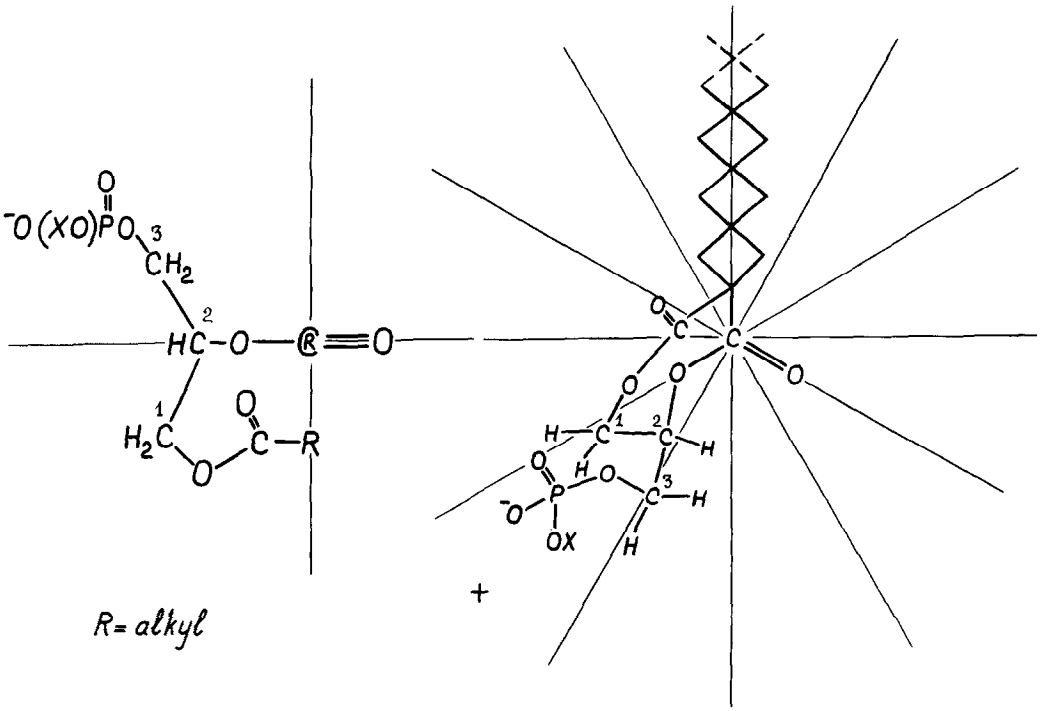

TABLE 1

INHIBITION OF COLLAGENASE AND PROTEASE ACTIVITIES BY THREE SERUM PROTEINS

ENZYME	BUFFER CONTROL (cpm)	40,000 SERUM INHIBITOR (cpm)	PERCENT INHIBITION	α_1-AT (cpm)	PERCENT INHIBITION	α_2-M (cpm)	PERCENT INHIBITION
SKIN COLLAGENASE	980	320	67	1005	0	40	96
RHEUMATOID SYNOVIAL COLLAGENASE	968	374	63	980	0	28	97
GASTRIC MUCOSAL COLLAGENASE	770	20	97	780	0	20	97
GRANULOCYTE COLLAGENASE	780	560	28	775	0	99	87
	(E_{280nm})	(E_{280nm})		(E_{280nm})		(E_{280nm})	
TRYPSIN	0.60	0.59	2	0	100	0.05	92
PAPAIN	0.98	1.01	0	0	100	0.03	97

Further characterization of the small inhibitor is in progress. Its specificity for collagenases is of particular interest as its molecular size, which is very similar to that found for many collagenases themselves, would probably permit access to tissue locations from which α_2-M might be excluded. The importance of the inhibitor in collagen metabolism has yet to be established, but its measurement in serum from normal subjects and patients with various connective tissue diseases might help to elucidate whether it functions physiologically or in pathological states as a regulatory factor in collagen catabolism.

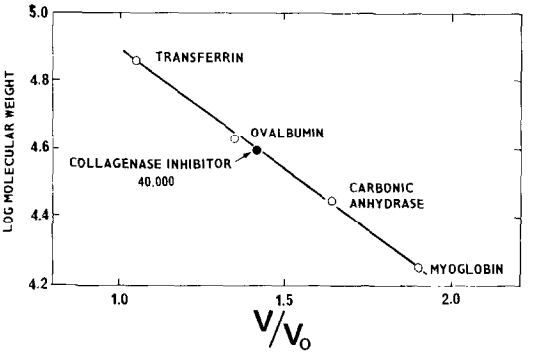

Fig.3. Molecular weight determination of the small serum inhibitor by gel filtration on Sephadex G-75 superfine. The protein standards transferrin, ovalbumin, carbonic anhydrase and myoglobin were used to calibrate the column. A molecular weight of 40,000 was calculated for the collagenase inhibitor from a plot of elution volume/void volume versus log molecular weight of the protein standards.

Table 1 footnote

The collagenase inhibitory activity of α_1-AT, α_2-M and the 40,000 molecular weight serum protein was examined by adding equal amounts (100µl) of the protein concentrates to ^{14}C-labelled collagen fibril assays containing 50mM Tris-HCl pH 8.0, 0.2M NaCl, 10mM $CaCl_2$, 1800cpm of ^{14}C-radioactivity and 50µl of each collagenase solution. Control and experimental assays were run for 16 h at 37°C.

The protease-inhibiting activity of the three serum proteins was examined by adding similar quantities to trypsin and papain assays at pH 7.0 using casein as substrate[17]. After termination of the reactions by addition of trichloroacetic acid the absorbance of the supernatants was measured at 280nm (E_{280nm}).

The final concentration of α_1-AT and α_2-M in the assays was 30 and 45µg/ml respectively. Each result represents the mean of at least four assays after subtracting control values and is expressed as percentage inhibition of the enzyme control.

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