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INHIBITION PROPERTIES OF SEPHAROSE-BOUND TRYPSIN AND A PROTEASE ON THE SURFACE OF EHRLICH ASCITES TUMOUR CELLS

F S STEVEN, M M GRIFFIN, S ITZHAKI and A AL-HABIB

Department of Medical Biochemistry, Stopford Building, University of Manchester, Manchester M13 9PT (U K)

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Ehrlich ascites cells have been shown to possess a protease with β-naphthylamidase activity located on the surface of these cells This enzyme is protected from the inhibitory action of protein inhibitors of trypsin (EC 3 4 21 4) in free solution, but is inhibited by high concentrations of active site-directed inhibitors of trypsin We believe the protection against inhibition is provided by the location of this protease on the cell surface We employed a model system of trypsin coupled to Sepharose to demonstrate the protective action of an inert surface, resulting in a marked reduction in inhibition of trypsin-Sepharose, compared to trypsin in free solution, when exposed to both high and low molecular weight inhibitors This cell surface protease has been shown to play a role in activation of the zymogen of collagenase exported by tumour cells This role may have important implications for tumour cell invasion of the intercellular matrix

Introduction

Sonicates of tumour cells have been shown to contain a zymogen of a protease contained in a granule fraction [1,2] Ehrlich ascites cells grown in mice are well known to be very resistant to osmotic shock and have very tough plasma membranes It was therefore of interest to determine whether intact tumour cells exhibited similar protease activity This study describes four linked observations (i) the presence and quantitation of this protease with caseinolytic, β-naphthylamidase and esterase activity against substrates for trypsin on the surface of these cells, (ii) unexpected inhibition properties of the cell surface protease when exposed to molecules which inhibit trypsin in free solution, (iii) a model system, made by coupling trypsin to Sepharose-4B, which behaved similarly to the protease on the cell surface in the presence of trypsin inhibitors and (iv) the role of this protease on the surface of tumour cells in the activation of latent collagenase exported by these cells when grown on collagen gels

We describe the cell surface enzyme as a 'protease' on the basis of its ability to (i) cleave α-*N*-benzoyl-DL-arginine-β-naphthylamide [3], *N*-carbobenzoxy-L-tyrosine nitrophenyl ester [4], *N*-α-benzoyl-arginine ethyl ester and casein, (ii) be inhibited by low molecular weight active site-directed inhibitors of trypsin and trypsin-like enzymes and (iii) exhibit similar inhibition characteristics to trypsin-Sepharose We believe this cell surface protease to be very similar in its properties to bound trypsin but for the time being it will be simply referred to as a cell surface protease

Abbreviations Bz-Arg-β-naphthylamide, α-*N*-benzoyl-DL-arginine-β-naphthylamide, TLCK, *N*-α-*p*-tosyl-L-lysine-chloromethyl-ketone HCl, NPGB, 4-nitrophenyl-4-guanidino-benzoate HCl, MUGB, 4-methyl-umbelliferyl-*p*-guanidino-benzoate HCl, PMSF, phenylmethylsulphonyl fluoride, CTN, *N*-carbobenzoxy-L-tyrosine-nitrophenyl ester, Bz-Arg-OEt, *N*-α-benzoyl-L-arginine ethyl ester HCl

Materials

Ehrlich ascites cells were grown intraperitoneally in mice [5] and collected after 8 days The pooled

cells were separated from the surrounding ascitic plasma by low speed centrifugation The cells were washed six times in 0 9% (w/v) NaCl to provide a final suspension containing 0 5 10^8 cells/ml The washing of the cells was necessary to remove extracellular inhibitors of trypsin and also plasma proteins since we wished to use these cells for assays of proteolytic activity with casein as substrate (see later) The same results were obtained with cells washed in NaCl or phosphate-buffered saline

Crystalline trypsin (EC 3 4 21 4), ovomucoid, soybean trypsin inhibitor, *N*-α-*p*-tosyl-L-lysine-chloromethyl-ketone HCl (TLCK), α-*N*-benzoyl-DL-arginine-β-naphthylamide HCl (Bz-Arg-β-naphthylamide), 4-methyl-umbelliferyl-*p*-guanidinobenzoate HCl (MUGB), phenylmethylsulphonyl fluoride (PMSF) and *N*-carbobenzoxy-L-tyrosine nitrophenyl ester (CTN) were obtained from Sigma, *N*-α-benzoyl-L-arginine ethyl ester HCl (Bz-Arg-OEt) was purchased from Aldrich Chemical Company and Trasylol (10 000 KIE/ml) was kindly supplied as a gift from Bayer 4-Nitrophenyl-4-guanidino-benzoate (NPGB) was obtained from B D H and tropocollagen was prepared from rat-tail tendons [6] Leupeptin was purchased from Scientific Marketing Associates, London Pooled normal human serum was provided by a local hospital

Activated AH-Sepharose-4B was purchased from Pharmacia and trypsin-Sepharose prepared according to the manufacturer's instructions The activity of the trypsin-Sepharose was assayed with Bz-Arg-β-naphthylamide and calibrated as the equivalent weight crystalline trypsin

Methods

Trypsin β-naphthylamidase activity was assayed fluorimetrically [3] with Bz-Arg-β-naphthylamide as substrate after 1 h at 40°C Trypsin esterase activity was measured with CTN as described by Martin et al [4] Trypsin caseinolytic activity was assayed with 12 mg casein (pH 8 0) in a total volume of 3 ml for 2 h at 37°C followed by trichloroacetic acid precipitation and fluram [7] assay of the solubilised peptides [8] In those experiments where inhibitors were included in the digest, the degree of inhibition in each tube was presented as the residual enzymic activity expressed as a percentage of the control enzymic activity in which no inhibitor was added In each experiment a series of screw-cap plastic tubes containing 1 μg trypsin (or an equivalent quantity of trypsin-Sepharose or tumour cells) plus incremental additions of a potential inhibitor were pre-incubated at 37°C for 10 min, prior to adding the substrate to give a final volume of 3 ml The resultant enzymic activity was determined after 1 h with Bz-Arg-β-naphthylamide, 0 5 h with Bz-Arg-OEt and 2 h with casein as substrate, respectively

Lactic acid dehydrogenase activity [9], leaked by Ehrlich ascites cells during 1 h incubation in 0 1 M Tris-HCl buffer, pH 8 0, was assayed to measure the degree of cell rupture during this incubation period Collagenase activity of the washed tumour cells and cells exposed to inhibitors incorporated in preformed collagen gels was demonstrated in the classic procedure of Gross and Lapiere [10], after 18 h and longer periods of incubation at 37°C Incubation of tumour cells at 15°C on collagen gels was used to obtain electrophoretic evidence of the selective cleavage of tropocollagen molecules into 0 75 and 0 25 length fragments [11]

Results and Discussion

Detection of protease

Preliminary experiments with intact Ehrlich ascites tumour cells demonstrated the presence of protease activity capable of cleaving Bz-Arg-β-naphthylamide [3], CTN [4] and casein on the cell surface

The β-naphthylamidase activity of a suspension of washed tumour cells was shown to be directly proportional to the quantity of cells added to the test system (over the range 0–3 10^7 cells/tube) Similarly, the β-naphthylamidase activities of both trypsin and a Sepharose-trypsin suspension were shown to be directly proportional to the quantities of these enzymes added to the test system Independent β-naphthylamidase assays conducted at 10 min intervals over 0–1 h with each of these three enzyme systems, indicated that the production of β-naphthylamine was linear over this period of time for each enzyme system

The assay of tumour cell caseinolytic activity is presented in Fig 1, in which two control series of analyses were carried out simultaneously with the cell surface protease assay Fluram-positive material dif-

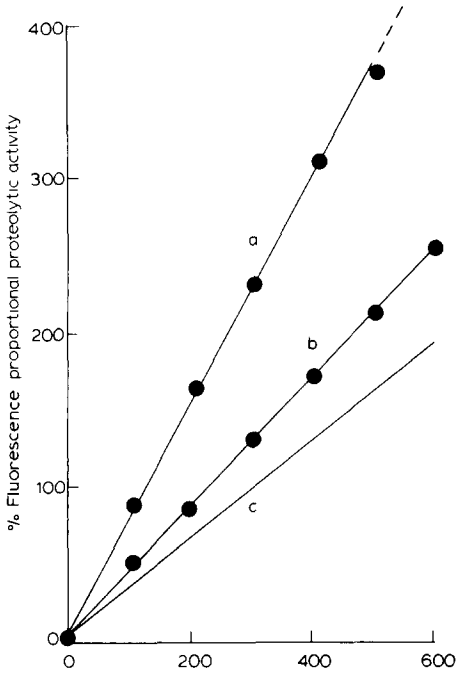


Fig 1 The assay of cell proteolytic activity with casein as substrate 0–600 μl cells (0 5 10^8 cells/ml) were incubated with 12 mg casein in 3 ml pH 8 0 solution for 2 h at 37°C, followed by precipitation of the undegraded protein with trichloroacetic acid and fluram assay of the solubilised peptides, line a Line b was obtained with two series of controls, (i) the cells alone in the absence of casein and (ii) cells plus casein plus 100 μM NPGB to inhibit the cell surface protease Line b represents non-enzymic products produced by the cells The line c represents a–b, i e , the enzyme digestion products

fuses from the cells during the incubation period and this must be accounted for by differential analysis Two methods provide the necessary data When a control series of tubes containing the cells and no added substrate is run alongside the test system, then line b in Fig 1 is obtained for this control series and represents the diffusion of fluram-positive material from the cells A second check on this non-enzymic product formation is obtained by running a second control series of tubes in which cells and substrate are incubated in the presence of 100 μM NPGB (the latter completely inhibits the cell-surface protease, see later) The residual fluram-positive materials are derived from non-enzymic activity (e g , diffusion from the cells) and this again is represented by the line b in Fig 1 The line a in Fig 1 represents the total product formation i e , enzymic degradation of casein plus non-enzymic products diffusing from the cells The difference between lines a and b represents the net protease activity, line c in Fig 1

Location of protease activity

It could be suggested that the enzyme which we are studying is located within these cells rather than on their surfaces A number of observations oppose this suggestion (a) When the cells were deliberately ruptured by sonication the observed β-naphthylamidase activity did not increase (b) Previous studies of the granule and post-granule supernatant fractions [1] of these cells demonstrated the presence of a zymogen which required trypsin or chymotrypsin activation, whereas the enzyme described in this study required no prior activation (c) The cytosol also possessed a potent inhibitor which would be expected to inactivate this intracellular enzyme in free solution [1] (d) The possibility that β-naphthylamidase was exported from the cell during the period of 1 h at 37°C, employed for enzyme assay in these studies, was checked by separating the extracellular fluid from the cells by centrifugation after 1 h at 37°C and assaying the β-naphthylamidase activity of the extracellular fluid and the precipitated cells independently No β-naphthylamidase activity was found in the extracellular fluid fraction, all the activity remained with the precipitated cells (e) Later in this paper it will be shown (Fig 2) that the tumour cells are surrounded by trypsin inhibitors present in the ascitic plasma and inhibitors are also exported by the tumour cells during the course of 1 h at 37°C, thus any β-naphthylamidase exported by these cells would not be capable of measurement by direct assay (f) In order to overcome the rather long digestion period for Bz-Arg-β-naphthylamide assays we employed continuous recording spectrometry over 0–4 min using CTN [4] as substrate, and demonstrated linear esterase activity of these cells in this period of time, which would be difficult to achieve if the enzyme was continuously being exported from the cells into the extracellular fluid, or if the substrate had to pass into the cells, be cleaved and re-exported as digestion products (g) The pellet (containing membrane and cell organelles) centrifuged down from the sonicated tumour cells was washed in 0 9% (w/v) NaCl and

shown to possess protease activity

From these observations we concluded that the protease which is located on the surface of the tumour cells does not result from nonspecific leakage of enzymes during the preparation of the cells or the digestion period at 37°C This conclusion was reinforced by the absence of lactic acid dehydrogenase [9] in the extracellular fluid when the cells were cultured in the Bz-Arg-β-naphthylamide buffer for 1 h at 37°C

Estimation of trypsin equivalence of Ehrlich ascites cells

It was possible to compare the β-naphthylamidase and caseinolytic activity of the cell surface protease directly with trypsin in free solution, which had previously been standardized by active site titration with NPGB [12], by the burst analysis technique When such a comparison was made with Bz-Arg-β-naphthylamide as substrate, 1 μg trypsin was equivalent to 3 3 10^7 cells, and with casein as substrate 1 μg trypsin was equivalent to 5.4 10^7 cells These figures must be considered approximations for two very different assays, however it can be concluded that these results are of a similar order of magnitude and indicate that the cell surfaces are well endowed with an enzyme capable of proteolysis and β-naphthylamidase activity We believe this activity is associated with a single protease since all the active site-directed reagents and active site titrants for trypsin inhibit both activities of this cell surface enzyme

Failure of protein inhibitors of trypsin to inhibit the cell surface protease

Incremental addition of 0–20 μg soybean trypsin inhibitor, Trasylol, ovomucoid and 200 μl pooled human serum failed to inhibit the β-naphthylamidase and caseinolytic activity of the cell surface protease of Ehrlich ascites cells The ascitic plasma which originally surrounded the tumour cells contained an inhibitor of trypsin in free solution, Fig 2, curve a, but this ascitic plasma had no inhibitory effect on the tumour cell surface protease (Fig 2, line b)

The evidence presented above could be interpreted in two different ways (i) simply that the surface enzyme is not trypsin-like and therefore would not be inhibited by inhibitors of trypsin and (ii) that the surface to which the enzyme is bound changes the properties of a trypsin-like enzyme in such a way that its inhibition kinetics have been modified by comparison with trypsin in free solution The data which we present below indicates that the cell surface enzyme behaves like trypsin bound to Sepharose beads and shows that it is perfectly possible for authentic trypsin molecules to behave in the manner described for the cell surface protease For these reasons we feel justified in believing the cell surface enzyme to be 'trypsin-like' although we refer to it simply as a protease

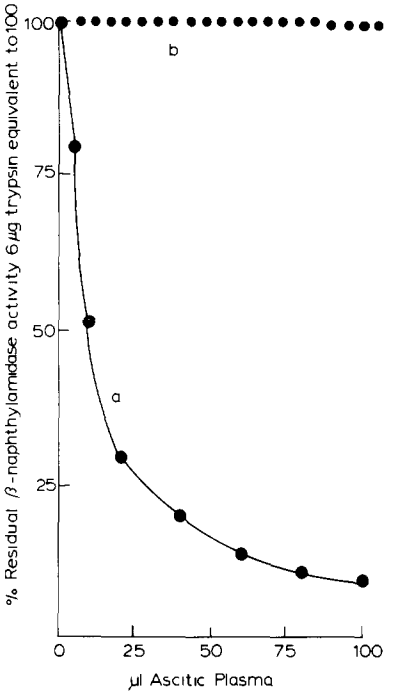


Fig 2 The inhibition of free trypsin β-naphthylamidase activity by ascitic plasma Curve a shows the inhibition of 6 μg trypsin incubated for 1 h with incremental additions of ascitic fluid The dotted line b shows the lack of inhibition exhibited by 200 μl cells in the presence of ascitic fluid

In view of the data in Fig 2 and the observed protease activity of the Ehrlich ascites cells (Fig 1) it would really have been surprising if this enzyme was inhibited by the trypsin inhibitors present in the ascitic plasma, serum, or by ovomucoid, soybean trypsin inhibitor and Trasylol The cell surfaces must have been in close contact with protein inhibitors of proteolytic enzymes (in free solution) and would be

likely to be presaturated with these when the cells were obtained from the tumour-bearing mice

Inhibition of tumour cell surface protease with active site directed agents for trypsin

In our assay systems we have used 1 μg trypsin/3 ml i e , 1 3 10^{-8} M, or an equivalent quantity of tumour cells We determined the kinetics of inhibition of the tumour cell surface protease in the presence of incremental additions of NPGB [12], MUGB [13], TLCK and 4-amino-benzamidine (Fig 3) All these reagents were capable of causing inhibition of the cell surface protease, however, the concentrations of these reagents required for this inhibition were much greater than would have been expected for the inhibition of trypsin in free solution (see later) The

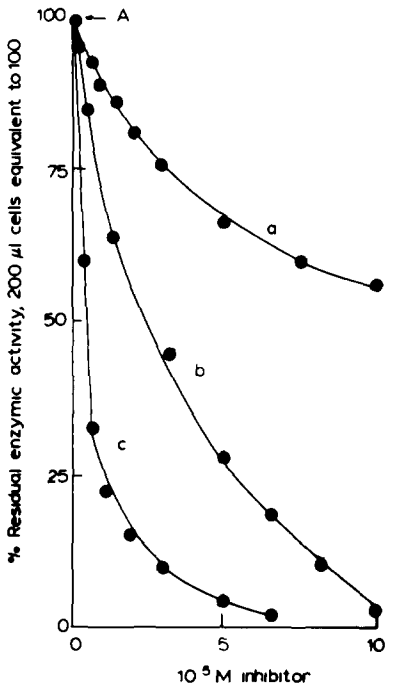


Fig 3 Inhibition of Ehrlich ascites cell surface protease by low molecular weight inhibitors The initial activity of the cell surface enzyme is represented by 100 at the point indicated by the arrow A Curve a represents incremental addition of 4-aminobenzamidine with corresponding inhibition of β-naphthylamidase activity Curve b is a composite curve for the effects of incremental additions of either NPGB or MUGB on the caseinolytic activity, these agents had identical inhibitory action Curve c represents TLCK inhibition of the β-naphthylamidase activity All results were presented as percentages of the control at A

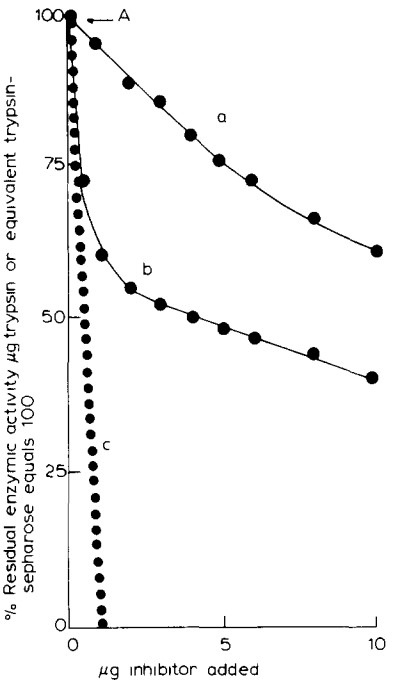
cell surface protease was also inhibited by PMSF which inhibits serine enzymes, including trypsin, and similar inhibition was observed with leupeptin, a peptide specific for trypsin-like serine enzymes (see later) The fact that all these reagents are site-specific for trypsin (or trypsin-like enzymes) demonstrates the validity of our belief for this class of enzymic activity being used to describe the cell surface protease

Inhibition of trypsin-Sepharose by protein inhibitors

Serum contains seven known inhibitors [14], each of which reacts stoichiometrically with trypsin in free solution, similarly ovomucoid and soybean trypsin inhibitor react stoichiometrically with trypsin in free solution by interaction of the enzyme active site with a specific site-directed locus on the inhibitor We were able to demonstrate stoichiometric interaction of trypsin in free solution with ovomucoid and soybean trypsin inhibitor (in this case 1 3 10^{-8} M being required to inhibit 1 μg trypsin in a 3 ml test system) On the contrary, non-stoichiometric inhibition of trypsin-Sepharose was observed with these proteins (Figs 4 and 5) Much higher concentrations of these protein inhibitors were required to cause partial inhibition of an equivalent quantity of trypsin-Sepharose For example, 1 5 10^{-7} M soybean trypsin inhibitor (10 μg/3 ml) caused 60% inhibition of trypsin-Sepharose and 1 2 · 10^{-7} M ovomucoid (10 μg/3 ml) caused 40% inhibition of trypsin-Sepharose (Fig 4)

Clearly the presence of trypsin linked to the surface of Sepharose beads has markedly changed the inhibition characteristics of the original trypsin in such a manner that it is much less readily inhibited by protein inhibitors of trypsin in free solution In fact the Sepharose-bound trypsin is now behaving rather like the tumour cell surface protease (described above) in its inhibition by proteins, although Sepharose offers less protection than the tumour cell surface

We can see the same type of change when free trypsin and trypsin-Sepharose were pretreated with incremental addition of pooled human serum (Fig 5) None of the seven inhibitors of free trypsin was capable of causing inhibition of trypsin-Sepharose when 30 μl serum were added to the test system, whereas 1 μl serum inhibited 1 μg trypsin in free solution



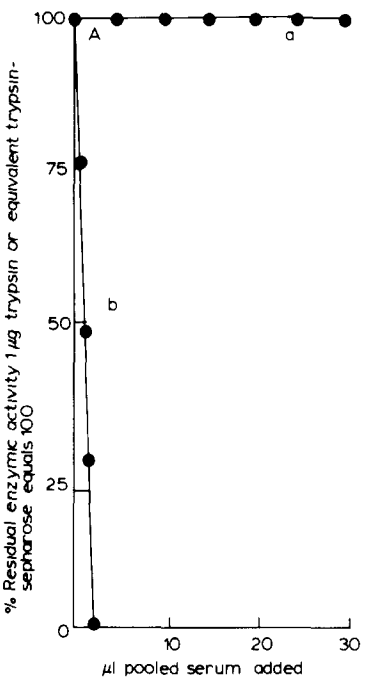


Fig 4 Inhibition of trypsin-Sepharose and trypsin in free solution by soybean trypsin inhibitor and ovomucoid, assayed with Bz-Arg-β-naphthylamide The initial activity of 1 μg trypsin or the equivalent quantity of trypsin-Sepharose is represented by 100 at the point indicated by the arrow A Curve a ovomucoid and curve b soybean trypsin inhibitor on trypsin-Sepharose The dotted line c represents the inhibition of trypsin in free solution by either soybean trypsin inhibitor or ovomucoid, which have almost identical effects

Fig 5 Inhibition of trypsin in free solution but not trypsin-Sepharose by pooled serum, assayed with Bz-Arg-β-naphthylamide Conditions were as for Fig 4, line a trypsin-Sepharose and line b trypsin in free solution

Inhibition of trypsin and trypsin-Sepharose with active site directed agents

Active site titrants for trypsin, NPGB and MUGB, both react stoichiometrically with respect to the product formed in the burst analysis technique [12, 13] We found that a 10-fold increase in concentration was required to produce complete inhibition of trypsin in free solution and an approx 100-fold increase in concentration was required to inhibit surface-bound trypsin in the form of trypsin-Sepharose (data not shown)

Again it can be concluded that the incorporation of trypsin in the surface of the Sepharose beads has altered the inhibition kinetics of the surface-bound trypsin, making this less readily inhibited by active site titrants for trypsin The plots of these data were similar to those shown in Fig 3 where the tumour cell surface protease was under study

Inhibition of trypsin, trypsin-Sepharose and tumour cell surface protease with TLCK

The active site directed agent TLCK-inhibited trypsin in free solution, trypsin-Sepharose and the tumour cell surface protease almost equally well (Fig 3) although it was slightly more effective against trypsin in free solution

Esterase inhibition assayed with Bz-Arg-OEt

The inhibition of trypsin, trypsin-Sepharose and the protease on the surface of Ehrlich ascites tumour cells each assayed with Bz-Arg-OEt, followed the same patterns as described above for Bz-Arg-β-naphthylamide and casein assays It can be concluded that the esterase, β-naphthylamidase and proteolytic activities of these enzyme system were similarly inhibited by these inhibitors and that the state of the enzyme, i e , in free solution, bound to Sepharose or bound to

the cell-surface, defined the type of inhibition kinetics obtained

Inhibition of tumour cell surface protease with leupeptin and PMSF

The aldehydic peptide, leupeptin, is a specific inhibitor of trypsin and trypsin-like serine proteases The inhibition of the cell surface protease by leupeptin and by PMSF required approx 10-times as much of these inhibitors as was required to produce an equivalent degree of inhibition of trypsin in free solution (data not shown) The evidence demonstrated the involvement of a serine residue in the active site of the cell surface protease which was also inhibited by the trypsin-directed agent, leupeptin

Role of tumour cell surface protease in collagenolysis

The evidence presented above can be summarised as follows (i) the protease on the surface of Ehrlich ascites tumour cells is much less readily inhibited by active site directed agents than is free trypsin, (ii) protein inhibitors of trypsin caused little or no measurable inhibition of the cell protease

We sought a physiologically important enzyme system which might demonstrate a physiological role for the tumour cell surface in zymogen activation Tumour cells placed on reconstituted collagen gels [6,15] in petri dishes and kept at 37°C produced massive zones of fibril lysis around the sites of application within 18 h (Fig 6) Inclusion of soybean trypsin inhibitor, Trasylol or ovomucoid in the collagen gel failed to inhibit fibril lysis although the inclusion of 5 mM EDTA in the collagen gel caused complete inhibition of fibril lysis, as would be expected from the knowledge that mammalian collagenase requires Ca^{2+} for activity [16] The inclusion of 100 μM TLCK, NPGB or MUGB in the collagen gels prior to placing the cells on the gel surface resulted in no fibril lysis These agents are directed towards trypsin-like enzymes and have no action on manifest collagenase, these agents must have had their inhibitory effect on the process of fibril lysis at the stage of zymogen activation, i e , activation of a precursor of collagenase exported by the tumour cells In order to demonstrate this export of a zymogen of collagenase we employed collagen gels containing 100 μM TLCK plus 0 5 μg/ml chymotrypsin and compared

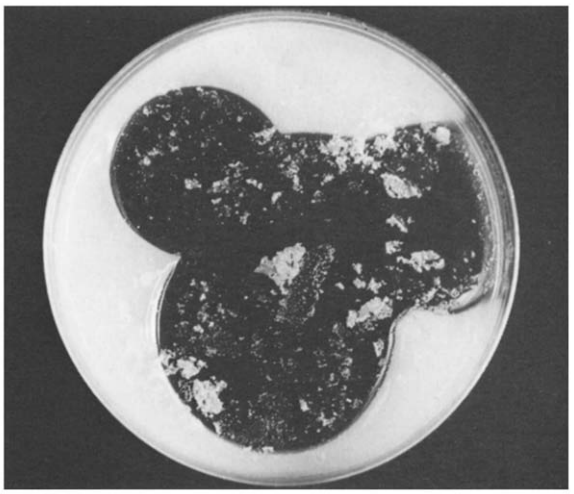


Fig 6 Lysis of collagen fibrils by tumour cells Tumour cells were dropped on the surface of reconstituted collagen fibrils and placed at 37°C over a black surface After 18 h fibril lysis is shown as black zones at the sites of cell application, surrounded by white areas of undegraded collagen fibrils

cells on these gels to similar cells placed on gels containing TLCK only The controls, with TLCK-inhibited cells, produced no fibril lysis but the gels supplemented with chymotrypsin exhibited good fibril lysis (similar to Fig 6) at the sites of cell deposition within 18–24 h Since chymotrypsin is not inhibited by TLCK and has no ability to cause collagen fibril lysis, the observed regain in collagenolytic activity in the TLCK plus chymotrypsin gels can only have been caused by chymotrypsin activation of a zymogen of collagenase exported by the tumour cells The evidence of Fig 6 indicates that the exported zymogen of collagenase is normally activated by intact tumour cells resulting in fibril lysis surrounding the site of application The failure of protein inhibitors to prevent collagen fibril lysis whilst low molecular weight-active site inhibitors of trypsin-like enzymes prevented collagen fibril lysis, coupled with the kinetic data already presented, indicates that the tumour cell surface protease plays a crucial role in this activation of the zymogen of collagenase

It could be argued that the tumour cells were not viable for 18 h at 37°C and the collagenolytic activity was of lysosomal origin, i e , cathepsin Normally cathepsins are not exported as zymogens requiring

tryptic or chymotryptic activation We also used sonicated cells which did not induce collagen fibril lysis in 18 h, which suggests that live cells with intact membranes were required to produce active collagenase outside the cells The time required to demonstrate collagen fibril lysis is controlled by the slow rate of collagen degradation rather than the rate of export of zymogen and its activation to manifest collagenase We were able to demonstrate good fibril lysis when tumour cells were placed on collagen gels for 1 h prior to being washed off the surface with 0.9% (w/v) NaCl the washed gel developed zones of collagen fibril lysis in 18 h under the sites on which the cells were originally placed This evidence indicates that live cells produce manifest collagenase within the first hour of contact with the collagen gels

Product of collagen fibril lysis

When the collagen gels were maintained at 15°C we were able to demonstrate the presence of 0 75 and 0 25 length α-chains, characteristic of mammalian collagenase activity (Fig 7), on polyacrylamide gel electrophoresis [17]

We conclude that Ehrlich ascites cells possess a protease located on the cell surface This serine enzyme can be inhibited by low molecular weight inhibitors of trypsin but not by a number of protein inhibitors of trypsin in free solution This protection from inhibition can be simulated to some extent by trypsin coupled to the surface of Sepharose in a model system The cell-surface protease plays a role in the activation of the zymogen of collagenase (the latter being exported from the tumour cells) to manifest collagenase The extracellular collagenase plus the cell surface protease produce collagenolysis of the surrounding collagen fibrils in the immediate neighbourhood of tumour cells placed on collagen gels

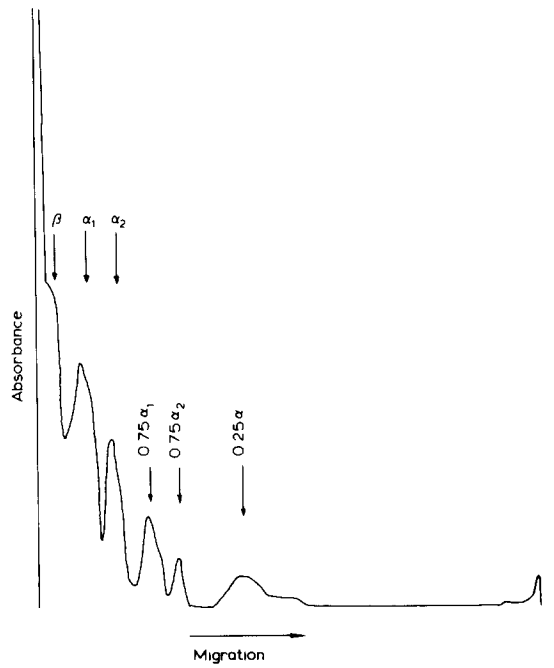


Fig 7 Gel electrophoretic separation of collagenase degradation of collagen fibrils formed at 15°C Collagen fibrils exposed to Ehrlich ascites cells at 15°C for 18 h, on denaturation yielded a mixture of undegraded β- and α-chains plus 0 75 and 0 25 length fragments The positions of β, α_1 and α_2 bands were defined with standards derived from tropocollagen

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