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EVIDENCE FOR INHIBITORS OF THE CELL SURFACE PROTEASE GUANIDINOBENZOATASE

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Guanidinobenzoatase is a protease present on the surface of tumour cells. The present study describes the isolation of a protein inhibitor of guanidinobenzoatase obtained from extracts of liver and pancreas and purified by affinity techniques. Pancreatic acinar cells have been shown to possess a latent form of guanidinobenzoatase and this latency is due to complex formation with the inhibitor. A fluorescent marker has been employed to demonstrate the presence or absence of the inhibitor on sections of pancreatic tissue. The inhibitor has been shown to be exchangeable with liver and pancreatic inhibitors obtained from different species. It is postulated that these inhibitors may play a role in enzyme control.

KEY WORDS: Protease, inhibitor, guanidinobenzoatase, cell-surface.

INTRODUCTION

Active site titration of trypsin with nitrophenyl-*p*-guanidinobenzoate[1] and 4-methylumbelliferyl-*p*-guanidinobenzoate[2] has become one of the standard procedures for the assay of this enzyme. In each case one molecule of active trypsin is inhibited by one molecule of titrant with the production of one molecule of product.[1,2] These active site titrants were employed to inhibit a trypsin-like enzyme on the surface of Ehrlich ascites tumour cells;[3] this study led to the discovery of another protease, guanidinobenzoatase, which cleaved guanidinobenzoate from these active site titrants without being inhibited in the process.[4] The enzyme guanidinobenzoatase was shown to be a seryl protease selective for arginyl residues and capable of degrading fibronectin.[4,5]

Guanidinobenzoatase has now been purified by simple affinity techniques.[5] Kinetic studies have led to the design of fluorescent probes which act as competitive inhibitors of guanidinobenzoatase.[5] These probes have been employed to locate cells possessing guanidinobenzoatase in frozen sections or wax embedded sections of human pathological tissues.[5] Cells capable of migration were shown to possess guanidinobenzoatase and these included tumour cells.

Bovine and human pancreatic acinar cells have now been shown to possess a latent form of guanidinobenzoatase due to the presence of inhibitors. We now present the evidence of these inhibitors and their purification on simple affinity systems. In order to describe the assay and location of these inhibitors it is necessary to give a brief account of the isolation of guanidinobenzoatase and its location on cells, since these techniques play an important role in isolating inhibitors and demonstrating their presence on the pancreatic acinar cell surface. We also present evidence that purified guanidinobenzoatase cleaves the peptide GlyArgGlyAsp which is known to

be concerned with the attachment of cells to fibronectin.[6] Thus the study of naturally occurring inhibitors of guanidinobenzoatase could have application to the control of cellular invasion.

MATERIALS

The source of guanidinobenzoatase was mouse Ehrlich ascites plasma.[4] 4-Methylumbelliferyl-*p*-guanidinobenzoate, agmatine sulphate, phenylmethylsulphonyl fluoride and 9-aminoacridine were obtained from Sigma, St. Louis, Mo., U.S.A. The synthetic peptide GlyArgGlyAsp[6] was purchased from Bachem, Bubendorf, Switzerland.

Fresh bovine pancreas was obtained from the local slaughterhouse; part of the tissue was immediately placed in either liquid nitrogen or in 4% w/w formaldehyde in isotonic saline. The frozen pancreas was sectioned at 4 μm and the formaldehyde fixed tissue was wax embedded, sectioned at 4 μm, and finally dewaxed through xylene, cyclohexanone and ethanol. Samples of hamster pancreas and normal human pancreas were prepared as frozen sections.

Fresh bovine pancreas and lamb's liver were used as the source of protein inhibitors of guanidinobenzoatase.

CNBr Activated CH-Sepharose-4B and Sepharose-2B were purchased from Pharmacia, Uppsala, Sweden.

METHODS

Assay of Guanidinobenzoatase and its Inhibition

4-methylumbelliferyl-*p*-guanidinobenzoate was employed to assay guanidinobenzoate as previously described[4] by the production of fluorescent 4-methylumbelliferone. Inhibition experiments were carried out with 10 μl ascitic plasma, preincubated at 37° C for 10 min with the potential inhibitor, prior to adding the substrate.[4] Lineweaver-Burk plots were used to define the type of inhibition.

Location of Cell Surface Guanidinobenzoatase

The fluorescent probe, 9-aminoacridine, was used to locate cells possessing guanidinobenzoatase,[5] this involved immersing the slides for 2 min in 10^{-3} M 9-aminoacridine followed by three 2 min washes in isotonic saline. The slides were then viewed under a Leitz Orthoplan fluorescent microscope with filters in position 2 and 2. Under these conditions unstained cells and connective tissue appeared blue whilst cells possessing guanidinobenzoatase appeared yellow.[5]

Isolation of Guanidinobenzoatase

Agmatine, the decarboxylation product of arginine, was coupled to activated CH-Sepharose-4B by its α-amino group according to the procedure recommended by its manufacturers. The isolation required a three step procedure. The ascitic plasma contained an α-1-4-glucosidase which was first adsorbed onto Sepharose-2B. The

unbound proteins were washed from the Sepharose-2B and subjected to cellulose acetate electrophoresis. The GBase band was eluted and equilibrated with agmatine CH-Sepharose-4B for 2 h at 37° C. Guanidinobenzoatase was selectively bound to the agmatine-sepharose. The unbound proteins were eluted with isotonic NaCl and the sepharose finally washed with water. The enzyme was eluted from the support with 0.1 M Na_2SO_4 pH 6.0, dialysed and freeze dried.[5]

Assay of Guanidinobenzoatase Bound to Agmatine-CH-Sepharose-4B

The activity of guanidinobenzoatase on the agmatine-CH-Sepharose-4B beads was checked by incubating a small aliquot of wet beads with the substrate in the enzyme assay. After 1 h, the reaction mixture was centrifuged and the methylumbelliferone produced determined by fluorimetry.[5]

Cleavage of the Synthetic Peptide GlyArgGlyAsp

0.7 mg of the synthetic peptide was dissolved in 1 ml distilled water. This was added to an aliquot (0.1 ml) of guanidinobenzoatase bound to CH-Sepharose-4B and shaken for 8 h at 37°C. The digestion products were washed from the Sepharose with distilled water concentrated by rotary evaporation and made up to 1 ml. The cleavage products were compared to the original peptide by ascending chromatography on Schleicher and Schull F1440 cellulose thin layer sheets with *n*-butanol : acetic acid : water (12 : 3 : 5) as solvent and by paper electrophoresis at pH 6.4 (50 ml acetic acid: 2 ml pyridine: 448 ml water).

Isolation of Inhibitor from Lamb's Liver

Fresh lamb's liver (20 g) was homogenised in 10 volumes of distilled water containing 10^{-4} M phenylmethylsulphonyl fluoride using an ultra-turrax for 30 seconds. The homogenate was passed over a column of Sepharose-2B to remove the low molecular weight components (including phenylmethylsulphonyl fluoride) from the protein fraction which eluted first. This protein fraction was tested for ability to, (a) degrade 4-methylumbelliferyl-*p*-guanidinobenzoate and (b) inhibit a known activity of guanidinobenzoatase present in 10 μl mouse ascitic plasma. This protein fraction will be referred to as the liver extract, a similar extract was also prepared from bovine pancreas and will be referred to as the pancreatic extract.

The isolation of the inhibitors from these extracts required the use of guanidinobenzoatase coupled to CH-Sepharose-4B, to act as an affinity ligand for selective binding of the inhibitor. Rather than elute the enzyme from the guanidinobenzoatase-agmatine-CH-Sepharose-4B it was found to be more convenient to transfer the enzyme straight to fresh activated CH-Sepharose-4B and thus retain the maximal enzymic activity. This was achieved by simply adding equal volumes of damp, fresh activated CH-Sepharose-4B to the enzymic bound support and adding 3 ml 0.1 M phosophate buffer pH 6.0 containing 6×10^{-5} M 4-methylumbelliferyl-*p*-guanidinobenzoate to displace the enzyme from the agmatine-Sepharose. After 1 h at 37° C, the excess reagent and products were washed from the Sepharose with distilled water. The damp Sepharose with bound guanidinobenzoatase was then equilibrated at 37° C for 2 h with the liver extract (containing inhibitor), then the unbound proteins were eluted with isotonic NaCl followed by distilled water. The bound protein was eluted with

0.3 M guanidine hydrochloride, exhaustively dialysed, then freeze dried, prior to gel electrophoresis[7] and assay for ability to inhibit guanidinobenzoatase.

RESULTS

9-Aminoacridine Staining of Pancreatic Acinar Cells

It was noted that formaldehyde-fixed pancreatic tissue which had been wax embedded, sectioned and dewaxed through organic solvents bound 9-aminoacridine on the surfaces of acinar cells (Figure 2), whereas the acinar cells in frozen sections of the same tissue did not accept 9-aminoacridine (Figure 1). This observation was made with sections obtained from human, mouse, hamster and bovine pancreas. The role of formaldehyde in the exposure of cell-bound guanidinobenzoatase was examined by placing a series of frozen sections of bovine pancreas in ten troughs of isotonic NaCl with incremental additions of formaldehyde (0.4% w/w) for 20 min. The treated sections were then stained with 9-aminoacridine and binding of the fluorescent probe was observed to take place only after the concentration of formaldehyde had exceeded 0.8% w/w. In a similar series of experiments we employed incremental additions of guanidine hydrochloride in isotonic saline (pH 7.0), the binding of 9-aminoacridine to acinar cells was observed only after the concentration of guanidine hydrochloride exceeded 0.3 M.

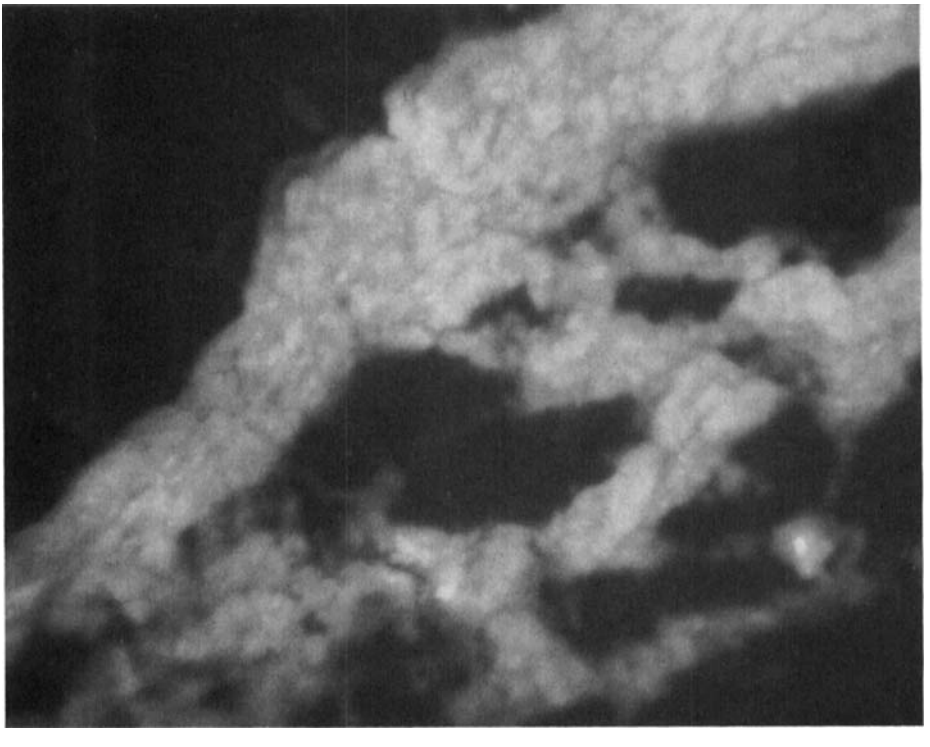

FIGURE 1 Frozen section of bovine pancreas stained with 9-amino acridine. Magnification × 300. The acinar cells have not bound 9-amino acridine and appear blue because the inhibitor is still bound to the surface guanidinobenzoatase. (See Colour Plate 1).

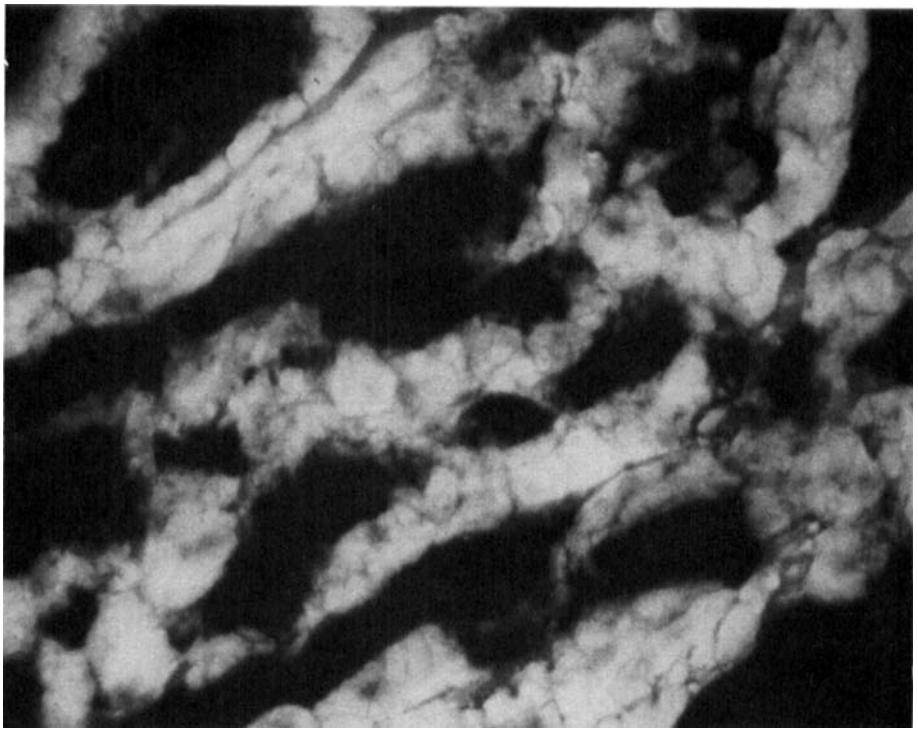

FIGURE 2 Formaldehyde treated frozen sections of bovine pancreas stained with 9-amino acridine. Magnification × 300. The acinar cells now bind 9-amino acridine and appear yellow because the guanidinobenzoatase on the cell surfaces is no longer inhibited. (See Colour Plate 11).

Inhibition of Guanidinobenzoatase by the Lamb's Liver Extract

The liver extract contained an enzyme which cleaved 4-methylumbelliferyl-*p*-guanidinobenzoate but which was not guanidinobenzoatase since it had a different K_m and this extract contained an inhibitor of guanidinobenzoatase. Allowances had to be made for the product formed by the former enzyme (probably an esterase) in calculating the inhibition of mouse guanidinobenzoatase by the liver extract. In Figure 3 this inhibition is presented for a typical liver extract; similar results were obtained from extracts of bovine pancreas. Lineweaver–Burk plots (Figure 4) confirmed the presence of a non-competitive inhibitor for guanidinobenzoatase in liver extract. Similar results were found for pancreatic extracts.

Isolation of Guanidinobenzoatase and its Inhibitor by Affinity Systems

Guanidinobenzoatase which had been selectively bound to agmatine-CH-Sepharose-4B and eluted with Na_2SO_4 was subjected to polyacrylamide gel electrophoresis (Figure 5) and exhibited a single band of protein with approximate molecular weight of 71 kD, Figure (6A). When the guanidinobenzoatase was transferred to fresh activated CH-Sepharose-4B and the inhibitor present in the lamb's liver homogenate bound to this support, the eluted inhibitor under electrophoresis exhibited a major band of

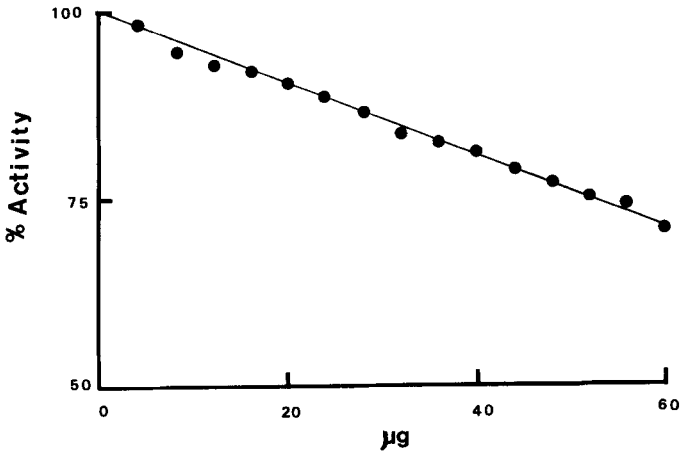

FIGURE 3 Inhibition of purified guanidinobenzoatase by purified liver inhibitor. Each tube contained 200 µg of affinity purified guanidinobenzoatase and incremented additions of affinity purified inhibitor. The residual enzymic activity was assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate.

approximate molecular weight 67 kD (Figure 5). The apparent molecular weights were determined in the unreduced state (Figure 6B) on 12.5% gels.

Inhibition of Guanidinobenzoatase by Purified Inhibitor

The lamb's liver inhibitor purified on these affinity systems inhibited pure mouse guanidinobenzoatase (data similar to Figure 4) and also inhibited the uptake of 9-aminoacridine on pancreatic acinar cells, pretreated with formaldehyde to expose the enzyme, (data similar to Figure 1). The inhibitor was displaced from pancreatic acinar cells by subsequent treatment with either 4% w/w formalin or 0.3 M guanidine hydrochloride in NaCl pH 7.0; this allowed the enzyme to accept 9-aminoacridine (data similar to Figure 2).

Exchange of Inhibitors on Cell Bound Guanidinobenzoatase

The frozen sections of bovine pancreas provided a test system to demonstrate inhibition of cell bound guanidinobenzoatase. The frozen sections do not bind 9-aminoacridine, are termed "negative" (e.g. Figure 1) and possess inhibitor. After formaldehyde treatment the acinar cells stain yellow and are now termed "positive" (e.g. Figure 2) exhibiting bound enzyme. We employed one section in a series of exchanges, as indicated in Table I, and monitored each exchange with 9-aminoacridine staining. Similar results were obtained with human and hamster pancreatic sections.

Cleavage of GlyArgGlyAsp

Pure guanidinobenzoatase bound to agmatine-CH-Sepharose-4B degraded the tetrapeptide GlyArgGlyAsp into two peptide fragments. These had R_F values of 0.04 and 0.1 in *n*-butanol : acetic acid : water (12 : 3 : 5).

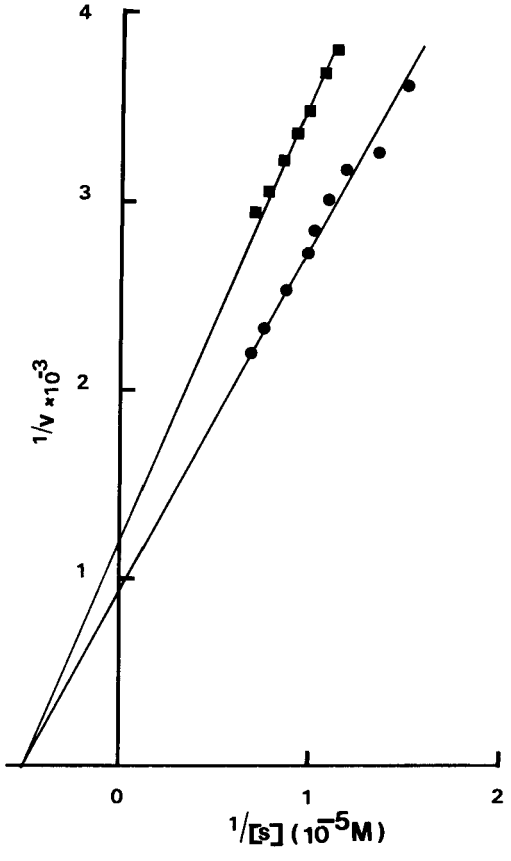

FIGURE 4 Non-competitive inhibition of guanidinobenzoatase by liver extract assayed with 4-methyl-umbelliferyl-*p*-guanidinobenzoate. Each tube contained 10 μl ascitic plasma with incremental additions of the substrate in the absence of inhibitor (a) and the presence of inhibitor (b). 50 μl extract equivalent to 200 μg liver protein.

TABLE I
Exchange of inhibitors on pancreatic cell surface

Tissue	Treatment	Staining	Results	Status
Frozen	1. None	Blue	Negative	E-I
Section	2. HCHO	Yellow	Positive	E
of Pancreas	3. Liver Extract	Blue	Negative	E-I
	4. HCHO	Yellow	Positive	E
	5. Liver Extract	Blue	Negative	E-I
	6. Guanidine Hydrochloride	Yellow	Positive	E
	7. Pancreatic Extract	Blue	Negative	E-I
	8. HCHO	Yellow	Positive	E

E indicates active enzyme; E-I indicates inactive inhibited enzyme.

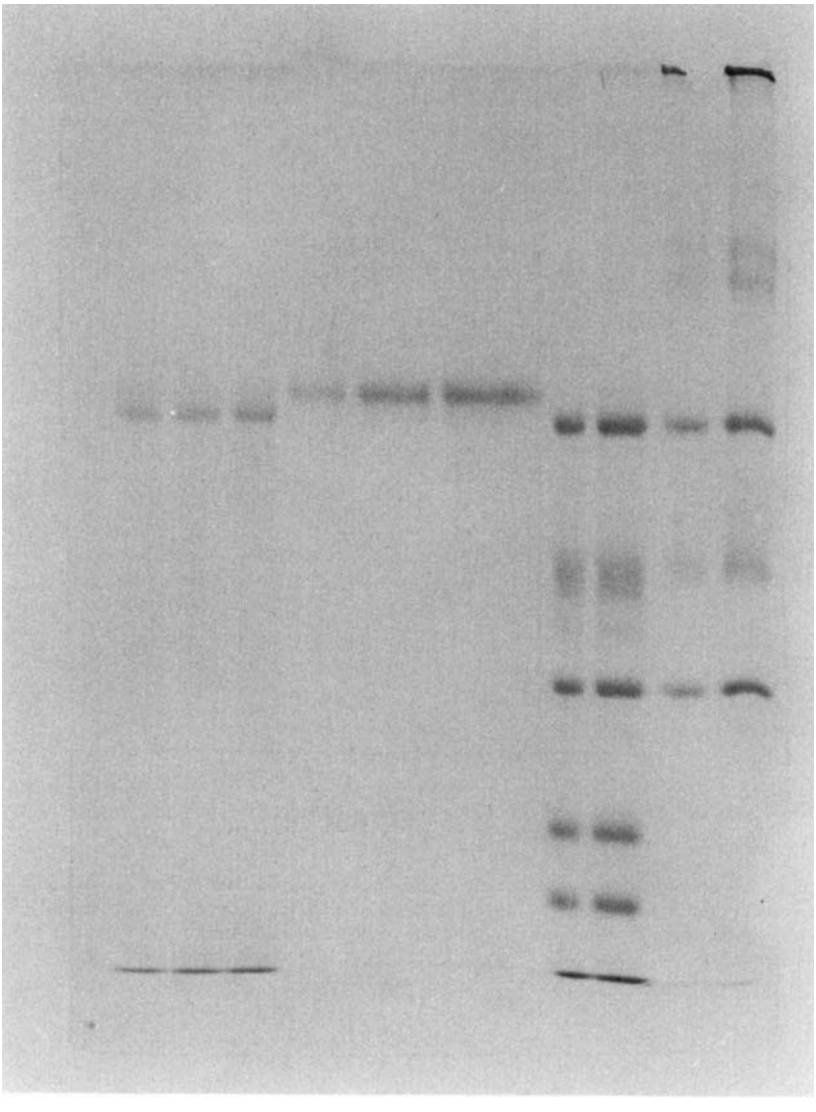

FIGURE 5 Polyacrylamide gel electrophoresis of purified guanidinobenzoatase and purified liver extract inhibitor. Lanes 1, 2, 3; 40 μl, 50 μl and 60 μl liver inhibitor. Lanes 4, 5, 6; 20 μl, 40 μl and 60 μl guanidinobenzoatase (4 mg/ml). Lanes 7, 8; 5 μl, 10 μl low molecular weight standards. Lanes 9, 10; 5 μl, 10 μl high molecular weight standards.

Paper electrophoresis of these digestion products for 1 h pH 6.4 (50 ml pyridine: 2 ml glacial acetic acid: 448 ml water) resulted in the two peptides moving in approximately equal distances in opposite directions (Figure 7) whilst the tetrapeptide remained at the origin. The net charge of the tetrapeptide would be zero whilst the expected glycylarginyl peptide should have a net positive charge and the aspartylglycyl peptide should have a net negative charge at this pH. Both the tetrapeptide and the positively

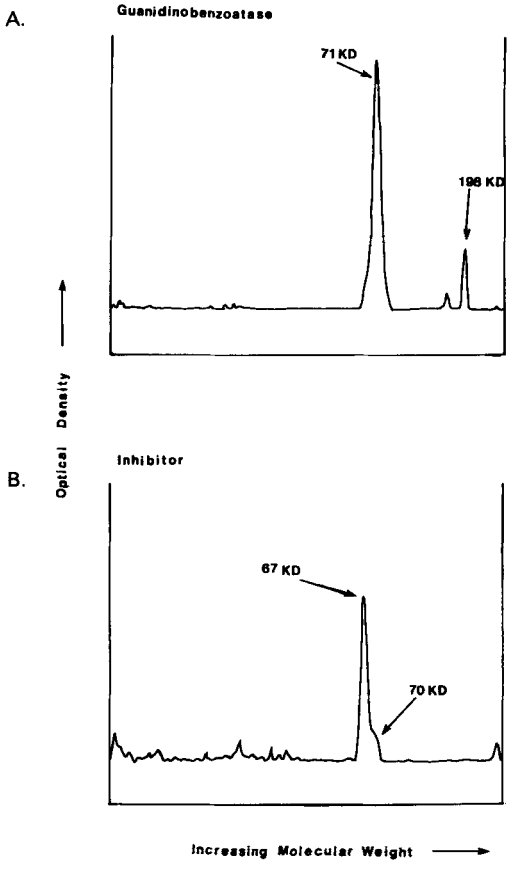

FIGURE 6 A. Electrophoretic migration of purified guanidinobenzoatase in the unreduced state in 12.5% polyacrylamide gels.[7] B. Electrophoretic migration of purified inhibitor of guanidinobenzoatase in the unreduced state in 12.5% polyacrylamide gels.[7]

charged peptide were shown to give the typical arginine reaction with the Sakaguchi reagent.

DISCUSSION

Acinar cells in fresh frozen sections of pancreas do not stain with 9-aminoacridine, yet these cells stain strongly after exposure to fomaldehyde, (Figures 1 and 2). This evidence would suggest some form of activation of guanidinobenzoatase by formaldehyde or more likely that the formaldehyde displaced an inhibitor from an enzyme-inhibitor complex. Incremental addition of formaldehyde or guanidine hydrochloride both indicate a gradual activation of guanidinobenzoatase which would argue in favour of the gradual displacement of an inhibitor. If an inhibitor has been displaced in the above sequence of events it should be possible to replace this inhibitor on the cell-bound enzyme in formaldehyde-fixed pancreatic sections. Extracts of various

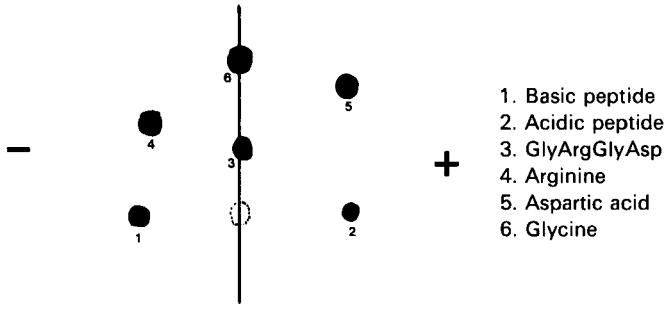

FIGURE 7 Cleavage of the synthetic peptide GlyArgGlyAsp by purified guanidinobenzoatase demonstrated by paper electrophoresis.

tissues were prepared and the inhibitory activity towards guanidinobenzoatase was found in liver and pancreas. We included phenylmethylsulphonyl fluoride in these extracts to inhibit seryl proteases and observed that the liver extract contained an enzyme capable of degrading guanidinobenzoate. This enzyme was obviously *not* guanidinobenzoatase which is inhibited by both the phenylmethylsulphonyl fluoride and the extracted protein inhibitor of guanidinobenzoatase (see Figure 3). The liver extract contained a non-competitive inhibitor of guanidinobenzoatase assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate (Figure 4). This extract was able to inhibit guanidinobenzoatase on the surface of formaldehyde-treated pancreatic acinar cells in such a way that the active centre failed to stain with 9-aminoacridine. (see text and Table I). This inhibition was reversed by a subsequent treatment with formaldehyde or with guanidine hydrochloride as demonstrated by the cell-bound enzyme's ability to be stained with 9-aminoacridine. In the series of experiments outlined in Table I it can be seen that the pancreatic acinar cell's enzyme is initially masked by pancreatic inhibitor, but that this can be exchanged for the liver inhibitor from a different species (lamb), and finally replaced with bovine pancreatic inhibitor.

The isolation of mouse guanidinobenzoatase employed an electrophoretic partial purification of the enzyme. This was introduced in an attempt to reduce enzymic digestion of this enzyme during isolation since it was observed that two components were obtained when this step was omitted. The three step procedure yielded a single protein (Figures 5 and 6A) with enzymic activity. The guanidinobenzoatase-CH-Sepharose-4B complex prepared by exchange of enzyme from the agmatine-Sepharose to fresh sepharose was employed to selectively bind the liver inhibitor. The inhibitor could be displaced with guanidine hydrochloride as a single protein band seen in unreduced polyacrylamide gel electrophoresis (Figures 5 and 6B). The purified inhibitor was capable of blocking the approach of 9-aminoacridine to the active centre of guanidinobenzoatase in free solution (data similar to Figure 4) and on formaldehyde-prepared acinar cell surfaces (data similar to Figure 1). The ability of this liver protein to inhibit the cell-bound protease is in marked contrast to the approach problems observed with protein inhibitors and the cell bound neutral protease of Ehrlich ascites tumour cells.[8] In the latter case low molecular weight inhibitors and peptide fragments of protein inhibitors were able to reach the active centre whereas the intact protein inhibitors could not do so.[8,9]

Concentrated human urine contained inhibitors of guanidinobenzoatase both in free solution and on the surface of cells. We reasoned that since human serum has no

inhibitory action against guanidinobenzoatase, any peptidyl inhibitors of this enzyme present in the urine must be derived from within the bladder rather than filtration from the blood. We sought a urinary protein which might yield suitable peptides. The most convenient way of obtaining such a protein is to collect the protein precipitating when urine is cooled in an ice bath. Dialysis, papain digestion and chromatographic separation into a number of peptide fractions yielded two which were capable of inhibiting guanidinobenzoatase in free solution and the enzyme on formalin-prepared pancreatic acinar cells. Again these peptide inhibitors were displaced from the active centre of guanidinobenzoatase by formaldehyde treatment.

It must be concluded from these studies that there exists a number of protein and peptide inhibitors of guanidinobenzoatase. These inhibitors may play a physiological role in the control of this enzyme which is capable of degrading the essential tetrapeptide at the binding site of fibronectin for cell attachment.[6] We do not know the role of the latent guanidinobenzoatase activity of pancreatic acinar cells, it would seem to be different from the role of guanidinobenzoatase on the surface of cells capable of migration. Whatever the role of this latent enzyme, it offers an excellent demonstration technique for inhibitors of cell-bound guanidinobenzoatase. It is possible that these inhibitors could have a role in preventing cell migration, in view of the occurrence of this enzyme on cells capable of migration and the enzyme's ability to cleave fibronectin at the specific peptide required for cell binding.

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