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A LATENT THIOL PROTEINASE FROM ASCITIC FLUID OF PATIENTS WITH NEOPLASIA

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Pepsin treatment of ascitic fluid from patients with neoplasia generated a cysteine (thiol) proteinase activity which resembles cathepsin B (EC 3.4.22.1) in its requirements for thiol activators, susceptibility to inhibitors and specificity for synthetic substrates. As judged by gel filtration, pepsin reduced the molecular size of the latent enzyme from an M_r of 41 000 to 33 000 after activation. Both forms are larger than human liver cathepsin B. In addition to its presence in ascitic fluid, the pepsin-activated species was found in the medium of ascites cells maintained in culture. The latent enzyme may be an enzyme-inhibitor complex or an inactive precursor of a cathepsin B-like proteinase.

Introduction

Invasion of surrounding tissue and metastatic spread by tumour cells are two of the most important aspects of cancer pathology. Penetration of cells through the extracellular matrix, an obligatory step for these processes, is thought to be mediated by the release of proteolytic enzymes from the tumour [1]. It has recently been demonstrated that human malignant breast tumours maintained in organ culture secrete a stable, cathepsin B-like thiol proteinase, and a role for this enzyme in invasive processes has been suggested [2–5]. As characterization and purification of this proteinase have been impeded by the small amounts of material available, we have searched for other possible sources of this enzyme.

A common complication in the progress of many cancers, for example cancer of the ovary, is the spread of tumour to the peritoneum and the subsequent development of ascites. In this condition, tumour cells multiply in the peritoneal cavity and large volumes of fluid accumulate there. As this fluid becomes the medium in which the malignant cells grow, a build up of secreted products from these cells would be expected. Samples of ascitic fluid were therefore assayed for stable thiol proteinase activity. While very low thiol proteinase activity was detected in untreated fluid, a much larger amount was present in a latent form. Some of the characteristics of this latent thiol proteinase are described.

Materials and Methods

Ascitic fluid

Sterile fluid was obtained, in the course of treatment, by aspiration from patients with ascites due to ovarian carcinomata. Only fluid shown to be free of hepatitis (Australia antigen negative) was used. Similar results were obtained with fluids from various patients.

Abbreviations: Bz-Arg-Na, α-*N*-benzoyl-DL-arginine 2-naphthylamide; CBz, α-*N*-benzyloxycarbonyl; 4-MeONa, 4-methoxy-β-naphthylamide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Mes, 2-(*N*-morpholino)-ethanesulphonic acid; Pipes, piperazine-*N*,*N'*-bis(2-ethanesulphonic acid).

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Culture of ascites cells

Cells from 300 ml ascitic fluid from a patient with ovarian carcinoma were centrifuged ($400 \times g_{av}$), washed for 10 min and resuspended in Hank's balanced salt solution. Red blood cells were removed by centrifugation over a Ficoll-Hypaque gradient (density 1.077 g/ml, Ref. 6). Using this technique the largest population of tumour cells was found at the interface between the Ficoll-Hypaque and the overlaid solution. These cells were collected and suspended in Dulbecco's modified Eagle's medium containing penicillin and streptomycin (5 μg/ml each) and 10% foetal calf serum. Cells were plated in 3.5-mm Petri dishes at a density of $2 \cdot 10^5$ cells/ml. Non-adherent cells were washed off the culture dishes after 48 h incubation at 37°C in a 95% air/5% CO_2 atmosphere; fresh medium was added and the cells were cultured in serum-containing medium for an additional 6 days. At this time the culture dishes contained an almost confluent layer of well-attached cells displaying an epithelial morphology.

To determine release of the latent thiol proteinase, serum-free medium was added to the culture dishes and replaced every 3 days over a 9-day period. Media were assayed for the latent enzyme by pepsin treatment as described below. At the end of the experiment, cells from each dish were harvested and DNA content was determined by the fluorescence method of Kissane and Robbins [7].

Materials

Bz-Arg-Na, β-naphthylamine, phenylmethylsulphonyl fluoride, iodoacetic acid and proteins used as molecular weight standards were from Sigma (St. Louis, MO, U.S.A.), CBz-Arg-Arg-4MeONa, CBz-Ala-Arg-Arg-4MeONa, CBz-Val-Lys-Lys-Arg-4MeONa and 1-methoxy-3-naphthylamine from Enzyme Systems Products (Indianapolis, U.S.A.) and pepsin from Worthington (Millipore Corporation, Freehold, NJ). Leupeptin, antipain and pepstatin were obtained through the generosity of the U.S.-Japan Medical Science Program. Purified human α_2-macroglobulin was a gift from Dr. A.J. Barrett (Strangeways Laboratory, Cambridge, U.K.). Purified human liver cathepsins B and D were prepared essentially according to Barrett's procedure [8].

Preparation of activated enzyme

A 20-ml sample of ascitic fluid was activated by digestion at 40°C for 1 h following the addition of 180 ml 0.1 M sodium formate pH 3.0/0.56 mg/ml pepsin. Pepstatin (5 mg in 10 ml 95% ethanol) was added to inactivate the pepsin, the solution cooled to room temperature and its pH raised to 5.5 by addition of 2.0 M Tris-HCl, pH 9.0. The mixture was centrifuged ($12\,000 \times g_{av}$ for 15 min) and concentrated to 30 ml by ultrafiltration using a Millipore PSAC membrane, then dialyzed against 50 mM sodium acetate (pH 5.5)/200 mM NaCl/1 mM EDTA. This preparation, in which most of the protein had been digested to small fragments, which were removed by ultrafiltration and dialysis, was used for the characterization of the cathepsin B-like activity.

Enzyme assay

Cathepsin B-like activity was assayed by the colorimetric method of Barrett [9], except that the more specific substrate CBz-Arg-Arg-4-MeONa (0.5 mM) was used [10] and the incubation volume was 0.8 ml.

A. Assay of latent enzyme from ascitic fluid. Samples (5 μl) were mixed with 45 μl 0.1 M sodium formate (pH 3.0)/0.56 mg/ml pepsin and incubated for 1 h at 40°C. The volume was then made up to 200 μl by addition of Brij-35 (0.05%) and 0.6 ml assay buffer (0.1 M sodium potassium phosphate (pH 6.0)/1 mM EDTA/2.7 mM cysteine) was added. With this buffer concentration the assay pH remained close to pH 6.0. Substrate (20 mM in 5% dimethyl formamide, 20 μl) was added and the incubation usually continued for 1 h at 40°C, after which the reaction was terminated and colour developed by addition of 0.8 ml diazotized Fast Garnet GBC/mersalyl acid/Brij-35 reagent [9].

B. Assay of active enzyme. The assay was as above except that the pre-incubation with 0.1 M sodium formate containing pepsin was omitted.

Characterization studies

For studies with the inhibitors iodoacetic acid and phenylmethylsulphonyl fluoride, the inhibitor was pre-incubated with the enzyme in 67 mM sodium potassium phosphate (pH 6.0)/1 mM EDTA/0.05% Brij-35 in the absence of cysteine for 15 min, in a total volume of 0.2 ml. The assay was then started by addition of 67 mM sodium potassium phosphate (pH 6.0)/1 mM EDTA/2.7 mM cysteine (0.6 ml)/substrate. The normal assay procedure was then fol-

lowed. Phenylmethylsulphonyl fluoride was dissolved in ethanol, which was present at a final concentration of 5% during the pre-incubation. Control experiments demonstrated that this amount of ethanol had no detectable effect on enzyme activity.

Brij-35 (0.05%) was routinely used as an enzyme diluent; however, when thiol activator was omitted from the assay mixture, no enzyme activity was found. If Brij-35 was not used, a small amount of activity was observed in the absence of thiol. The inactivation was no doubt due to the presence of oxidizing contaminants in the detergent [11]. In assays containing thiol activator the small quantity of detergent had no effect on enzyme activity.

Assay of latent enzyme in culture media

Activation of the latent enzyme was carried out by incubating 150 μl medium with 30 μl of a solution of pepsin (0.5 mg/ml) in 0.8 M sodium acetate, pH 3.8, for 1 h at 40°C. Aliquots (50 μl) of the activated sample were then assayed at 40°C in 50 mM sodium Pipes (pH 6.5)/1 mM EDTA/2 mM cysteine, using 0.5 mM CBz-Arg-Arg-4-MeONa as substrate, for 3–4 h as above.

Stable cathepsin B-like activity in the cell culture medium was assayed as desbribed previously [3] using CBz-Arg-Arg-4-MeONa.

Endopeptidase demonstration

Pepsin-activated thiol proteinase which had been partially purified by gel filtration on an Ultrogel AcA-54 column as above, was incubated with 4 mg human α_2-macroglobulin, in the presence of 2 mM cysteine, at 4°C for 90 min. The mixture was then chromatographed as above and the fractions assayed using CBz-Arg-Arg-4-MeONa.

Results

Generation of cathepsin B-like activity. Assay of ascitic fluid with a good substrate for cathepsin B, CBz-Arg-Arg-4-MeONa [10], gave essentially no activity. On the assumption that proteinases in this medium could be present in a latent form, various disruptive treatments were carried out. Digestion of samples of ascitic fluid with pepsin at low pH generated substantial amounts of cathepsin B-like activity. On investigation of conditions for the production of maximal activity, it was found that pepsin digestion proceeded optimally at a pH between 3.0 an 3.5. This seems to represent a compromise between loss of pepsin activity at increasing pH and the loss of cathepsin B-like activity at a pH lower than 3.0. The generation of activity was dependent on the amount of pepsin used and the time of digestion (Fig. 1). At optimal time and pepsin concentration a maximal activity is reached. This maximal activity remains constant over a range of pepsin concentrations and digestion times, but after an excess of either, the resultant cathepsin B-like activity is reduced, indicating degradation of the activated enzyme. In addition to pepsin, it was demonstrated that similar amounts of the lysosomal aspartic proteinase, cathepsin D, also generated cathepsin B-like activity from ascitic fluid.

Molecular size before and after pepsin treatment. A sample of untreated ascitic fluid was analyzed by gel filtration through a calibrated Ultrogel AcA-54 column. After pepsin treatment of the fractions the cathepsin B-like activity was found in a single peak (M_r approx. 41 000) eluting slightly later than ovalbumin (Fig. 2a). When a sample of ascitic fluid was first digested with pepsin, then applied to the column the peak of activity eluted more slowly (Fig. 2b) indicating a decrease in molecular weight (to M_r approx. 33 000) due to pepsin digestion. The activated species is however larger than human liver

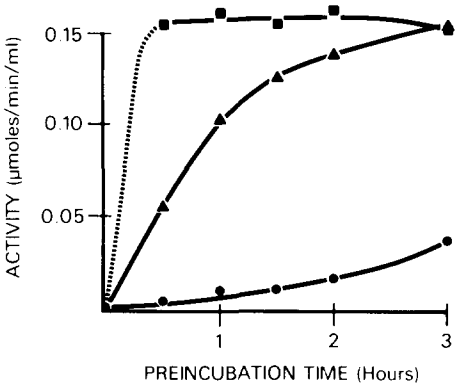

Fig. 1. Pepsin generation of cathepsin B-like activity. Ascitic fluid samples were digested with pepsin, 0.02 mg/ml (●——●); 0.1 mg/ml (▲——▲) or 1 mg/ml (■——■) at pH 3.0 for the indicated period then assayed. Activties are expressed per ml ascitic fluid.

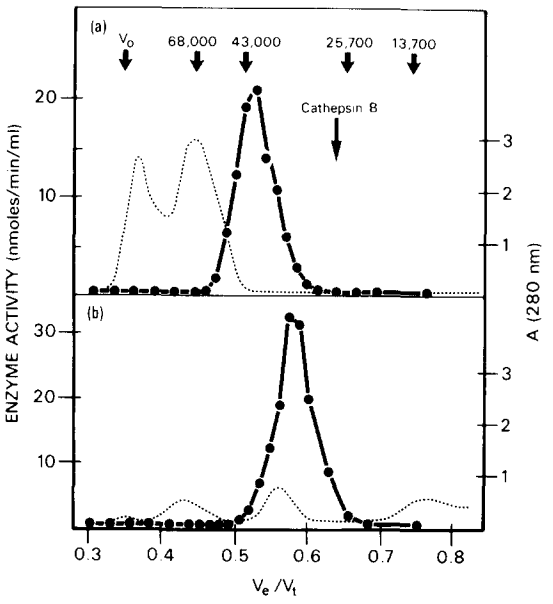

Fig. 2. Effect of pepsin treatment on molecular size. An Ultrogel AcA-54 column (1.5 × 85 cm) was equilibrated in 50 mM sodium acetate (pH 5.5)/200 mM NaCl/1 mM EDTA and eluted with the same buffer at 11 ml/h. Enzyme activity (•———•) was measured against CBz-Arg-Arg-4-MeONa. A_{280}, (· · · · · ·) of the column eluant was recorded using an LKB Uvicord III column monitor. Arrows indicate the positions of the standards, Blue Dextran 2000 (V_0), bovine serum albumin, ovalbumin, chymotrypsinogen and ribonuclease, using molecular weights from Weber and Osborn [12]. Potassium ferricyanide was used to estimate V_t. The elution position of human liver cathepsin B was determined in a separate run. (a) Untreated ascitic fluid was chromatographed and the fractions were pepsin-digested, then assayed for activity. (b) Ascitic fluid was digested with pepsin, concentrated and then chromatographed and the fractions assayed for activity without further pepsin digestion. Data are presented for the same starting volume of ascitic fluid as in (a).

cathepsin B (Fig. 2). Also illustrated in the figure is the extensive destruction of the bulk protein in the ascitic fluid by pepsin during the activation step. Much of the protein is broken down to small peptides which are lost during dialysis.

Demonstration of endopeptidase character of the enzyme. The high molecular weight (725 000 Ref. 13) serum proteinase inhibitor, α_2-macroglobulin, binds endopeptidases [14]. Once complexed, proteinases are inactive against high molecular weight substrates; they do however still show activity against substrates of low molecular weight such as those used in this study. The pepsin-activated enzyme was found to complex to α_2-macroglobulin. This was demonstrated by incubating a sample of activated enzyme with α_2-macroglobulin followed by chromatography of the mixture through an Ultrogel AcA-54 column. Activity against CBz-Arg-Arg-4-MeONa which eluted in the included volume before being mixed with α_2-macroglobulin (Fig. 2b) was found to elute at the void volume, indicating the formation of a proteinase-inhibitor complex.

Thiol requirements. Sulphydryl reagents were required for optimal activity of the pepsin-activated enzyme. For routine assays 2 mM cysteine was used. Dithiothreitol, reduced glutathione and 2-mercaptoethanol, all at a thiol concentration of 2 mM, gave 106, 89 and 61% activity, respectively, relative to the activity with cysteine set at 100%. Assay without sulphydryl reagent (in the absence of Brij-35) gave 13% of this value.

Inhibitors. Inhibition studies also characterized th pepsin-generated enzyme as a cysteine proteinase. Preincubation with 1 mM iodoacetate caused complete inactivation, while preincubation with 1 mM phenylmethylsulphonyl fluoride had no effect. The presence of *p*-chloromercuribenzoate (0.1 mM) abolished the small amount of activity measured when cysteine was omitted from the assay, but if cysteine was introduced after incubation with the mercurial, full regeneration of activity was observed. Leupeptin (1 μg/ml) and antipain (1 μg/ml), both good inhibitors of cathepsin B [15], gave 95 and 80% inhibition, respectively. Pepstatin (1 μg/ml), a good inhibitor of aspartic proteinases [15], had no effect and omission of EDTA from the assay mixture resulted in a loss of activity. These results show that inhibitors of serine, aspartic- and metallo-proteinases are without effect on pepsin-generated ascites enzyme whilst compounds acting on thiol groups are good inhibitors. The enzyme therefore seems to be a cysteine proteinase, and its inhibition by leupeptin and antipain suggests a similarity to cathepsin B.

Substrate specificities. The latent thiol proteinase was discovered by its activity, after pepsin treatment, on the synthetic substrate CBz-Arg-Arg-4-MeONa. Like cathepsin B [10], the enzyme from ascitic fluid was much more active against substrates containing two or more basic amino acids next to the C-terminal chromophore (Table I) than against substrates with a

TABLE I

SUBSTRATE SPECIFICITY OF THE PEPSIN-GENERATED THIOL PROTEINASE

Relative activity was measured at pH 6.0 (standard assay). Substrate concentration was 0.5 mM which is optimal, except for Bz-Arg-Na where 2 mM was used. This is the maximum possible concentration but is not saturating for the enzyme. Activities are relative to that obtained with CBz-Arg-Arg-4MeONa set at 100. K_m was assayed at pH 6.5 (optimum pH) in 50 mM sodium-Mes, 1 mM EDTA/2 mM cysteine, using a substrate range of 0.025–1 mM. The data were fitted to the equation $v = Vs/(K_m + S + S^2/K_i)$ using a non-linear least-squares computer programme derived according to Cleland [18]. The derived kinetic constants are reported ± S.E., determinations of kinetic constants for the enzyme with Bz-Arg-Na were not attempted.

Substrate	Relative activity	K_m (mM)	K_i (mM)
CBz-Arg-Arg-4MeONa	100	0.15 ± 0.01	1.8 ± 0.3
CBz-Ala-Arg-Arg-4MeONa	90	0.19 ± 0.01	3.6 ± 0.6
CBz-Val-Lys-Arg-4MeONa	180	<0.05	~1.5
Bz-Arg-Na	4.4	–	–

single basic amino acid, although activity against Bz-Arg-Na was detectable. Substrate saturation profiles at pH 6.5 showed that, as has been reported for cathepsin B [10] and the cathepsin B-like proteinase from human tumours [4], a fall-off in activity occurs at high substrate concentrations, indicating substrate inhibition. As seen before for cathepsin B and the tumour thiol proteinase [4] and reported by Knight for cathepsin B [16], the pepsin-generated ascites enzyme has a higher affinity for CBz-Arg-Arg-4-MeONa than for CBz-Ala-Arg-Arg-4-MeONa (Table I). The activated ascites enzyme is also similar to cathepsin B in its very high affinity for the synthetic substrate CBz-Val-Lys-Lys-Arg-4-MeONa (Table I), which has been reported to be a very good substrate for porcine liver and parathyroid gland cathepsin B [17]. Using the colorimetric assay for the estimation of 1-methoxy-3-naphthylamine it was not possible to obtain an accurate K_m for this substrate, hence only an approximate value is reported.

pH Optimum. The pH optimum of the enzyme for CBz-Arg-Arg-4-MeONa, CBz-Ala-Arg-Arg-4-MeONa and CBz-Val-Lys-Lys-Arg-4-MeONa was found to be pH 6.5 in each case (Fig. 3). These results are similar to those reported for the action of bovine spleen cathepsin B on CBz-Arg-Arg-4-MeONa [10] and porcine parathyroid gland cathepsin B on CBz-Val-Lys-Lys-Arg-4-MeONa [17]. The pH profile for the ascites enzyme is markedly skewed, with a rapid fall-off of the activity towards the alkaline region. This is indicative of the instability of the enzyme above pH 7.0 and suggests that the true pH optimum may in fact be above pH 6.5.

pH Stability. Upon activation by pepsin, the thiol proteinase from ascitic fluid is increasingly unstable as the pH increases above neutrality (Fig. 4). This

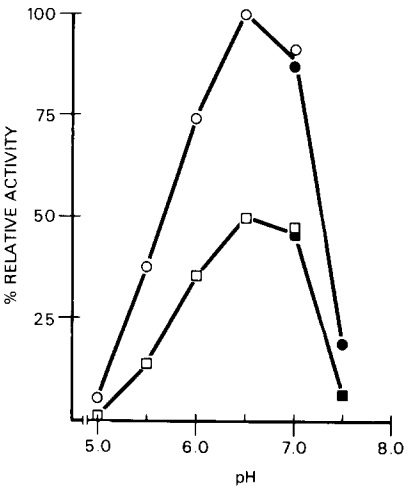

Fig. 3. pH activity profile. The pepsin-activated ascites enzyme was assayed using CBz-Val-Lys-Lys-Arg-4-MeONa (0.5 mM, circles), or CBz-Arg-Arg-4-MeONa (0.5 mM, squares). Assay buffers were 50 mM sodium MES/1 mM EDTA/2 mM cysteine for pH values from 5.0 to 7.0 (open symbols) and 50 mM sodium-Hepes/1 mM EDTA/2 mM cysteine for pH values 7.0 and 7.5 (closed symbols). Assay duration was 1 h. Results are expressed relative to the maximum activity obtained with CBz-Val-Lys-Lys-Arg-4-MeONa set to 100%.

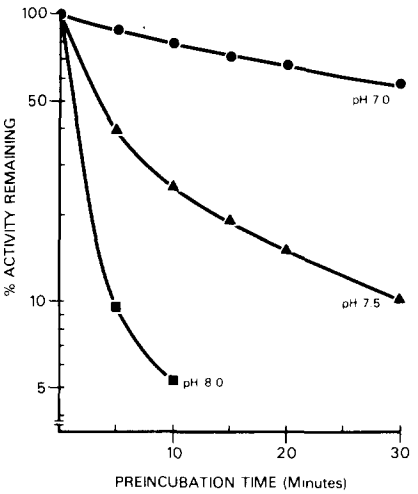

Fig. 4. pH stability of the pepsin-generated enzyme. Samples of activated enzyme were pre-incubated for the indicated period at 40°C in 90 mM sodium-Hepes at the indicated pH in a total volume of 0.2 ml. Assay buffer and substrate were then added and colour developed after a 1-h incubation.

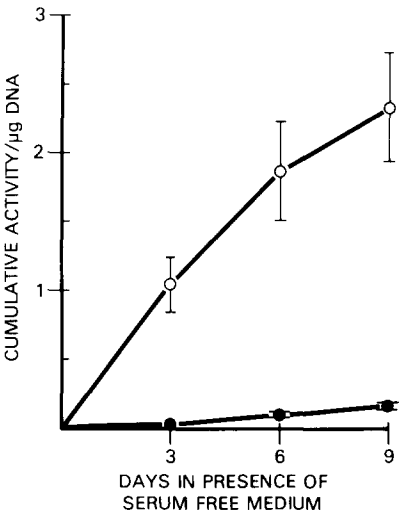

Fig. 5. Release of latent thiol proteinase activity from cultured human ascites cells. Ascites cells were isolated from the ascitic fluid and cultured in growth medium containing foetal calf serum (10%). At day 0 of the secretion study, serum-containing medium was replaced with serum-free medium and changed every 3 days. Closed circles represent thiol proteinase activity before pepsin treatment of media sampled, open circles are the activities generated by pepsin treatment. Values represent the mean obtained from six culture dishes. Cumulative activity is expressed in µmol substrate hydrolyzed per 4-h assay.

instability was not as marked as that observed for purified cathepsin B from human liver, which was previously found to lose 80% of its activity after incubation at pH 7.0 for 15 min [4]. The loss of activity at increasing pH is not due to oxidation of the essential thiol group in the proteinase as similar pH sensitivity was observed when the pH treatment was carried out in the presence of 2 mM cysteine. Before pepsin treatment the latent enzyme is relatively stable. Preincubation at 40°C for 15 min at pH 7.5 and 8.0 caused a loss of 17 and 24%, respectively, of the total activity possibly after subsequent pepsin treatment.

Source of the enzyme. Ascitic fluid is a transudate of plasma which is trapped in the peritoneal space when malignant cells block the lymphatic vessels normally draining this cavity [19] and due to the release from these cells of chemical mediators which increase the permeability of small blood vessels in the peritoneum [20]. Therefore sera from normal individuals and carcinoma patients were pepsin digested and assayed for cathepsin B-like activity. Little was found, indicating that the latent enzyme is not derived from plasma. As another possible source ascites cells from fresh fluid were cultured and the medium assayed for the latent enzyme (Fig. 5). Relative to a small amount of non-latent stable thiol proteinase activity, a large amount of pepsin generatable activity was released by these cells into the culture medium. This release was maintained at a constant level during the culture period investigated, indicating that the ascites cell population is the major source of the latent enzyme.

Discussion

The activity generated by pepsin digestion of the latent enzyme has been characterized as a cysteine (thiol) proteinase with enzymatic properties very similar to those of cathepsin B and the thiol proteinase secreted from human mammary carcinoma. The activated species is intermediate in molecular size between cathepsin B and the tumour thiol proteinase. Enzyme latency in ascitic fluid could be due to an enzyme-inhibitor complex or a precursor form of cathepsin B. Alternatively, it might be argued that pepsin digestion has altered the active site of a func-

tional proteinase, rendering it active against substrates that it could not previously cleave. As ascites is a transudate of plasma and therefore would be expected to contain the known inhibitors of thiol proteinases, α_2-macroglobulin [21] and the α-thiol proteinase inhibitor [22,23], it is unlikely that the fluid would contain an active thiol proteinase. Indeed it was demonstrated (unpublished data) that ascitic fluid has the capacity to inhibit cathepsin B.

The latency of the untreated enzyme could therefore be due to the presence of an inhibitor which is selectively removed by pepsin digestion. The small molecular size of the latent enzyme (approx. 40 000) argues against this species being a complex with either of the plasma thiol proteinase inhibitors both of which are relatively large (α_2-macroglobulin M_r 725 000 [13]; α-thiol proteinase inhibitor minimal M_r 60 000 [23]). Epidermis [24], cartilage [25] and many other tissues [26] are known to contain thiol proteinase of about 13 000 molecular weight. It is possible that the latency of the enzyme in ascites is due to the presence of a cathepsin B-like thiol proteinase complexed with such an inhibitor, and that this complex can be dissociated by pepsin. It was of interest therefore to find that cells cultured from ascitic fluid release latent enzyme, implying that either enzyme and inhibitor are released from the same cell population or that the product released from the cells is an inactive precursor of a cathepsin B-like proteinase. The situation seems similar to that of the release of collagenase [27] and neutral metallo-proteinase [28,29] in culture, where enzyme in the medium is usually in a latent form. The resolution of these problems would be greatly facilitated if pure latent enzyme were available. Efforts to this end are presently underway.

In addition to pepsin, the aspartic (acid) tissue proteinase cathepsin D was found to be an activator of the latent ascites enzyme, indicating that the generation of active thiol proteinase may be a physiological occurrance. Furthermore, Esumi et al. [30] have isolated an aspartic proteinase from ascitic fluid of a patient with ovarian cancer. Under certain conditions therefore the latent enzyme may be activated in the peritoneum.

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