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Identification of the aspartic proteinases from human erythrocyte membranes and gastric mucosa (slow-moving proteinase) as catalytically equivalent to cathepsin E

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Three aspartic proteinases with similar M_r values (approx. 80000) but from distinct sources (human gastric mucosa, human erythrocyte membranes and rat spleen) were shown to have immunological cross-reactivity and comparable mobilities when subjected to polyacrylamide-gel electrophoresis under non-denaturing conditions. Kinetic parameters ($k_{cat.}$, K_m and K_i) were determined for the interactions of the three enzymes with two synthetic chromogenic substrates and five inhibitors (naturally occurring and synthetic). On this basis it would appear that all of the enzymes should be considered equivalent to cathepsin E. pH–activity measurements indicated that the aspartic proteinase that originated from the erythrocyte membranes retained activity at a higher pH value than either of its readily soluble counterparts.

INTRODUCTION

Aspartic proteinases are produced by a number of cells and tissues within the human body (for a review see ref. [1]). Many are secretory proteins (e.g. pepsin, gastricsin and renin) that are deliberately released into extracellular spaces, whereas some effect their function primarily within the cell of origin (e.g. cathepsin D). Of the intracellular enzymes, cathespin D has been studied in a variety of species and is by far the best characterized proteinase. It is a glycoprotein with an (intact) M_r of 42000, but consists of one (rat) or two chains (human) depending on the species of origin. By contrast, relatively little information is available on cathepsin E. Until recently, it has been localized in polymorphonuclear leucocytes and macrophages [2] and had been isolated only from rabbit bone marrow [3] and rat spleen [4]. By contrast, spleen of bovine or human origin appears to contain little if any of this enzyme, and indeed there has been considerable uncertainty as to whether this proteinase is a genuine enzyme in its own right or is derived from dimerization of cathepsin D [5].

Very recently, a number of reports have indicated considerable similarity between the slow-moving (aspartic) proteinase (SMP) from human [6] and rat gastric mucosae [7] and cathepsin E from spleen [8] or neutrophils [9,10]. Whereas these proteins are all readily soluble, the aspartic proteinase in erythrocytes is membrane-bound [11]. This enzyme (EMAP) from human erythrocytes has also been suggested to have physicochemical and immunological similarities to human SMP [12] and rat cathepsin E [13].

However, none of these recent investigations has detailed the functional aspects of the proteinase molecules, i.e. the expression of the catalytic activities. With the ready availability of synthetic substrates [14,15] and inhibitors [16] of aspartic proteinases in our laboratories, it was thus considered of interest to examine whether these proteinases possess similar enzymic activities. Since the most detailed original report identifying and characterizing cathepsin E employed rat spleen as the tissue of origin [4] and since human spleen does not appear to contain the enzyme in significant amounts, the proteinases from erythrocyte membranes and gastric mucosa were examined in relation to rat (spleen) cathepsin E.

MATERIALS AND METHODS

The slow-moving proteinase from human gastric mucosa was purified to homogeneity as detailed previously [6], and the proteinase from human erythrocyte membranes and cathepsin E from rat spleen were purified to single-band components as reported in ref. [13].

The hydrolysis of the two chromogenic substrates

Lys-Pro-Ile-Glu-Phe-Phe(-4-NO_2)-Arg-Leu

and Pro-Pro-Thr-Ile-Phe-Phe(-4-NO_2)-Arg-Leu

at their Phe–Phe(-4-NO_2) bonds was monitored spectrophotometrically at 300 nm at a variety of pH values as described previously [14,15]. Ionic strength was maintained constant at 0.1 by the addition of NaCl as necessary [14], and kinetic parameters were obtained with appropriate concentrations of substrate. For each determination initial rates (v) were measured with at least six values of initial substrate concentration [S]. In all cases Michaelis–Menten kinetics were observed [17] and the best values for the kinetic parameters K_m and $V_{max.}$

Abbreviations used: SMP, slow-moving proteinase; EMAP, erythrocyte membrane aspartic proteinase; Boc, t-butyloxycarbonyl; Phe(-4-NO_2), nitrophenylalanine.

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were determined by computerized least-squares fitting of the measured initial velocities to those predicted by the Michaelis–Menten equation in a similar manner to that described previously [14,15]. Values of $k_{cat.}$ were obtained from the equation $V_{max.} = k_{cat.}[E]$.

The active concentration of each of the three enzyme preparations was determined by titration against a solution of isovaleryl-pepstatin whose concentration had been pre-determined by amino acid analysis. Kinetic constants (K_i) for the interaction of a number of inhibitors were determined as described previously [6,18].

The protein inhibitor (M_r approx. 17000) from *Ascaris lumbricoides* was kindly given by Dr. T. Hofmann, University of Toronto, Toronto, Ont., Canada, and Dr. R. Peanasky, University of South Dakota, Vermillion, SD, U.S.A. Synthetic inhibitors used were:

H-256 = Pro-Thr-Glu-Phe^{R}Phe-Arg-Glu
H-77 = D-His-Pro-Phe-His-Leu^{R}Leu-Val-Tyr
H-261 = Boc-His-Pro-Phe-His-Leu^{OH}Val-Ile-His
L-363 564 = Boc-His-Pro-Phe-His------Sta------Leu-Phe-NH_2

where R represents the reduced isostere ($-CH_2-NH-$) as replacement of the $-CO-NH-$ of the peptide bond in the substrate [19,20], OH represents the $-CHOH-CH_2-$ analogue [20] and Sta represents statine (4-amino-3-hydroxy-6-methylheptanoic acid) [16,20].

H-256, H-77 and H-261 were generously provided by Professor M. Szelke, Ferring Research Institute, University of Southampton, Southampton, U.K. L-363 564 was kindly supplied by Dr. J. Boger and Dr. J. Jacobs, Merck, Sharp and Dohme Research Laboratories, West Point, PA, U.S.A.

Cathepsin D from rat liver and an affinity-purified antibody against rat cathepsin D [21] were kindly given by Dr. G. Bonelli, University of Turin, Turin, Italy.

Cathepsin D from human spleen and (separate) antisera to cathepsin D, SMP and EMAP were prepared and zymograms, SDS/polyacrylamide-gel electrophoresis and Western blots (with these polyclonal antisera) were carried out as described in detail previously [6,13].

RESULTS AND DISCUSSION

Previous reports [6,12,13] have indicated that the three proteinases from erythrocyte membranes (EMAP), gastric mucosa (SMP) and spleen (cathepsin E) all have intact M_r values (by gel filtration and/or SDS/polyacrylamide-gel electrophoresis under non-reducing conditions) of approx. 80000. When subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions, however, all three enzymes are composed of subunits of M_r approx. 42000, and, as reported previously [6,13], cathepsin E and EMAP both migrate as single bands whereas SMP, purified to homogeneity as described previously [6], consists of two subunits with similar but distinct M_r values (Fig. 1*a*). Immunostaining with an antiserum to EMAP (Fig. 1*b*) indicated cross-reactivity of the two subunits of SMP as well as with cathepsin E (even of rat origin). Similar results were obtained with antiserum to SMP (not shown). By contrast, human cathepsin D showed no cross-reactivity with antiserum to EMAP (Fig. 1*b*) or SMP [6].

Electrophoresis of the native enzymes at pH 7.5 with subsequent staining for proteolytic activity (against haemoglobin as substrate at pH 3.5; Fig. 2) indicated that SMP, EMAP and cathepsin E had all migrated with comparable mobilities and considerably in advance of the (diffuse) zones observed for human or rat cathepsin D (owing to the known isoenzyme forms of this proteinase).

These results are in accord with previous findings [6,12,13] that SMP, EMAP and cathepsin E are all distinct in character from cathepsin D.

Kinetic parameters (K_m and $k_{cat.}$) for the hydrolysis of two chromogenic peptide substrates were measured at pH 3.5 for EMAP, SMP and cathepsin E (Table 1). Comparable values were obtained in all cases. The availability of these convenient chromogenic substrates facilitated examination of the interaction of a number of inhibitors, both naturally occurring and synthetic, with the three enzymes. Isovaleryl-pepstatin inhibited all of the enzymes with sub-nanomolar K_i values, but this finding is of little diagnostic value since, almost by definition, all aspartic proteinases are (fairly) strongly inhibited by this compound.

By contrast, the protein inhibitor from *Ascaris lumbricoides* (M_r 17000 [22]) has been reported previously to be a potent inhibitor of cathepsin E [23] and SMP [6]

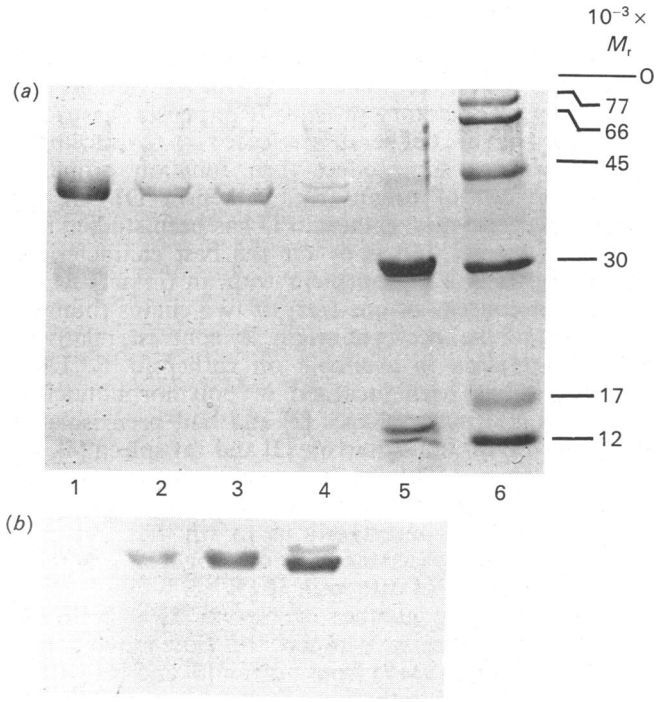

Fig. 1. SDS/polyacrylamide-gel electrophoresis under reducing conditions of various proteinases of human or rat origin

Samples (approx. 1 μg of protein) were loaded on to 15% polyacrylamide gels in buffer containing 100 mM-dithiothreitol. O, Origin. Lane 1, rat cathepsin D; lane 2, rat cathepsin E; lane 3, human EMAP; lane 4, human SMP; lane 5, human cathepsin D; lane 6, M_r standards (ovotransferrin, M_r 77000; albumin, M_r 66000; ovalbumin, M_r 45000; carbonic anhydrase, M_r 30000; myoglobin, M_r 17000; cytochrome *c*, M_r 12000). After electrophoresis, the gels were stained with Coomassie Blue (*a*) or electroblotted on to nitrocellulose (*b*) and immunostained with rabbit anti-EMAP serum [13] followed by peroxidase-conjugated sheep anti-(rabbit IgG) antibody.

Table 1. Kinetic constants for the hydrolysis of two synthetic chromogenic substrates by EMAP, by SMP and by spleen cathepsin E

Substrate 1 is Pro-Pro-Thr-Ile-Phe-Phe(-4-NO_2)-Arg-Leu and substrate 2 is Lys-Pro-Ile-Glu-Phe-Phe(-4-NO_2)-Arg-Leu. All reactions were carried out at pH 3.5 in 0.1 M-sodium formate buffer at 37 °C. The progress of each reaction was monitored by observing the decrease in A_{300}. The values given are estimates ± S.E.

	Kinetic constant	EMAP	SMP	Cathepsin E
Substrate 1	K_m (mM)	0.03±0.006	0.04±0.007	0.03±0.003
	$k_{cat.}$ (s^{-1})	130±10	110±9	75±3
Substrate 2	K_m (mM)	0.06±0.003	0.06±0.007	0.07±0.008
	$k_{cat.}$ (s^{-1})	155±5	150±9	170±9

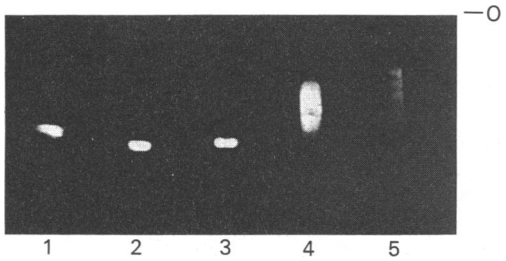

Fig. 2. Zymogram of various proteinases of human or rat origin

Samples were subjected to polyacrylamide-gel electrophoresis at pH 7.5 under non-denaturing conditions [21]. Staining for proteolytic activity was carried out by immersion of the gel for 15 min in a 2% (w/v) solution of haemoglobin in 0.1 M-sodium citrate buffer, pH 3.5. After rinsing, the gel was stained with Coomassie Blue in order to show the clear zones of enzymic activity. O, Origin. Lane 1, human SMP; lane 2, human EMAP; lane 3, rat cathepsin E; lane 4, rat cathepsin D; lane 5, human cathepsin D.

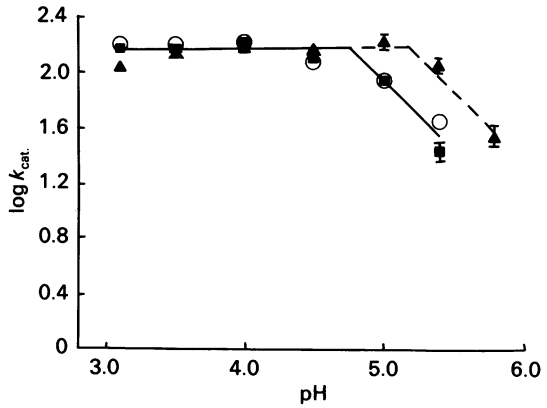

Fig. 3. pH-dependence of the log $k_{cat.}$ value for the hydrolysis of Lys-Pro-Ile-Glu-Phe-Phe(-4-NO_2)-Arg-Leu by EMAP, SMP and cathepsin E

Cleavage of the Phe–Phe(-4-NO_2) bond by cathepsin E (○), EMAP (▲) and SMP (■) was monitored spectrophotometrically at 300 nm. Error bars are added to indicate the estimated error obtained from computer fits of the plots of initial velocity versus substrate concentration. Where no error bars are shown the error obtained was within the size of the symbol used. The (angled) theoretical lines are drawn with a slope of −1.

Table 2. Inhibition constants (K_i) for the interaction of three aspartic proteinases with various inhibitors

All measurements were carried out at 37 °C in 0.1 M-sodium formate buffer, pH 3.5. The substrte used was Pro-Pro-Thr-Ile-Phe-Phe(-4-NO_2)-Arg-Leu. All inhibitions were competitive in nature and the estimated precision of the values obtained was in the range ±5–10%.

	K_i (nM)		
Inhibitor	EMAP	SMP	Cathepsin E
Ascaris protein	7	10	8
L-363 564	12	8	9
H-261	13	6	8
H-256	8	7	6
H-77	60	50	50

but to have no effect on cathepsin D [6,23]. This was confirmed by our present findings (Table 2) in that cathepsin E, EMAP and SMP were all inhibited strongly by this protein. The synthetic inhibitor L-363 564 (containing statine, which acts as a dipeptide analogue) was also a potent inhibitor of all three enzymes. Similarly, the compounds H-256 (which is an analogue of the synthetic substrates used in this series), H-77 and H-261 (which are analogues of the renin substrate angiotensinogen), devised and synthesized by Szelke and colleagues [19,20], were all effective inhibitors of the three enzymes and the potency of each inhibitor was comparable towards each of the three enzymes.

Thus there would seem little doubt that EMAP, SMP and cathepsin E [despite the fact that it was necessary (for the reasons explained above) to use cathepsin E from rat tissue] are all very similar proteolytic enzymes indeed, so that the former two should probably now be renamed as cathepsin E. Final confirmation of the identity of the enzymes must await determination of their (gene) sequences.

However, in order to extend the kinetic evaluation further, pH–activity profiles were obtained for the three enzymes. [The work with the inhibitors (Table 2) was carried out at pH 3.5 in order to maintain continuity with previous investigations.] Whereas the values obtained for K_m did not vary substantially over the pH range 3.1–5.8, $k_{cat.}$ values were found to diminish as the

pH rose above pH 5 (Fig. 3). This is entirely in keeping with findings reported previously (e.g. [15]) for other aspartic proteinases, and the apparent pK_a has been suggested to arise from dissociation of a proton from the catalytic complex (of two aspartic acid residues and a water molecule [15,16]). Irrespective of the precise value of the apparent pK_a, the EMAP enzyme was clearly more active at pH 5 than were the two other enzymes. Indeed, at pH 5.8 this enzyme, which originated as a membrane-associated proteinase, still retained approx. 25% of its catalytic activity, whereas the other two enzymes were completely inactive. It was not feasible to collect data at higher pH values because of the intrinsic instability manifested in the family of 'acid' proteinases to which the three enzymes belong.

Thus, on the basis of their physicochemical, immunological and catalytic properties, these three enzymes would appear to be very similar, despite their different origins. They can also be readily distinguished from the better-known enzyme cathepsin D (of human or rat origin) by a number of criteria: (*a*) intact M_r values of the native enzymes, 80000 compared with 42000; (*b*) inhibition (or lack of) by *Ascaris* protein; (*c*) immunological non-identity; cathepsin E of rat origin readily reacts against antiserum to human EMAP or SMP but displays no cross-reactivity with an antibody to rat cathepsin D (results not shown).

Cathepsin D has long been known to exist in a number of isoenzyme forms, and a similar phenomenon may explain the subtle distinctions that were observed in the properties of EMAP, SMP and cathepsin E. All three are glycoproteins [6,13], and small changes in glycosylation or other post-translational modifications *in vivo* or during purification may account for the small difference in pH–activity profiles and for the resolution of SMP into two slightly different subunits on electrophoresis in reduced SDS/polyacrylamide gels whereas EMAP and cathepsin E seem to consist of only a single subunit. Further work is needed to clarify these details and to investigate whether the intact enzymes contain two catalytically active subunits (each of M_r approx. 40000) disulphide-linked to one another. {Proteolytic activity is observed in the presence of reducing agents ([13]; J. Kay & I. M. Samloff, unpublished work).} If correct, such a novel feature would distinguish these 'double-headed' proteinases categorically from the other monomeric aspartic proteinases [1], and indeed from most other proteolytic enzymes with the exception of the much larger multicatalytic cysteine proteinases [24].

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