ISOLATION AND CHARACTERIZATION OF CATHEPSIN D FROM HUMAN GASTRIC MUCOSA

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The molecular weight of the enzyme, purified by ion-exchange chromatography and affinity chromatography, was determined by gel filtration on Sephadex G-100 as 49 000. After treatment with 2-mercaptoethanol, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate resolved the enzyme into two chains, of molecular weights 33 000 and 18 000. This shows that in the native state the enzyme is composed of one light and one heavy chain. Isoelectric focusing in polyacrylamide gel gave four bands, the isoelectric points being 5·5, 6·1, 6·5 and 7·1. The optimum protein substrate (pH optimum 3·2—3·6) was haemoglobin. The best synthetic substrate was methyl ester of pyroglutamyl-histidyl-phenylalanyl-phenylalanyl-alanyl--leucine. The protease was inhibited by the inhibitor of cathepsin D from the potato tubers. It is concluded that the enzyme is cathepsin D from gastric mucosa.

In addition to the pepsinogens of groups I and II (PgI and PgII), *i.e.* precursors of pepsin A and pepsin C (refs[1,2]),* the human gastric mucosa contains another carboxylic protease[4], here called non-pepsin protease[5]. It is the first protease produced by gastric mucosa of the foetus. In the neonates it represents the main portion of proteolytic activity[6,7], in adults it is found as a minor fraction[1,4,8].

Direct proof of the existence of its precursor form is lacking. Its immunological relationship to the neonatal gastric proteases of other mammals has not been demonstrated either[7]. The non--pepsin protease differs from PgI and PgII by greater stability on acidification and neutralization[4,8,9], higher content of basic amino acids[10] and a higher molecular weight[10,11]. However, there are different views as to its origin; the enzyme is regarded as a cathepsin E-like protease[5], cathepsin D (ref.[11]) or another similar enzyme[9,10,12].

The non-pepsin protease is invariably, and often exlusively, present in gastric carcinomas, where PgI and PgII frequently could not be detected[5,6,13]. In this respect it reminds the oncofoetal forms of some proteins. However, extracts from a gastric adenocarcinoma were found to contain further carboxylic proteases, different from the non-pepsin protease and from PgI and PgII, whose occurrence is typical of a tumorous tissue only[5,13].

* Enzymes: pepsin A (E.C.3.4.23.1); pepsin C (E.C.3.4.23.3); cathepsin D (E.C.3.4.23.5); cathepsin E (E.C.3.4.23.—). Abbreviations: Ac-, acetyl; AcOH, acetic acid; Z-, benzyloxycarbonyl. The symbols of amino acids adhere to the recommendation by IUPAC-IUB Commisions on Biochemical Nomenclature[3]. Unless otherwise stated, all amino acids except glycine had the L-configuration; Tyr(I_2), 3,5-diiodotyrosine, $^{\square}$Glu, residue of pyroglutamic acid, PDI, inhibitor of cathepsin D isolated from potatoes.

Studying the carboxylic proteases of human gastric mucosa, we discovered a new protease, hereafter referred to as "protease 1" (ref.[14]), whose properties resemble these "tumour-specific" proteases. The enzyme was now isolated in a pure state and characterised. We have investigated its activity to new synthetic substrates, *viz.* methyl esters of pyroglutamyl-histidyl-phenylalanyl-phenylalanyl-glycyl-glycine (*I*), pyroglutamyl-histidyl-phenylalanyl-phenylalanyl-alanyl-alanine (*II*) and pyroglutamyl-histidyl-phenylalanyl-phenylalanyl-alanyl-leucine (*III*). Recent results indicate that proteases of the same electrophoretic mobility are present in gastric mucosa of other mammals[7].

EXPERIMENTAL

Materials

Substrates. Z-Glu-Tyr, Ac-Phe-Tyr(I_2) and Z-His-Phe-Phe-OMe were afforded by Dr E. Kasafírek, Research Institute for Pharmacy and Biochemistry, Prague. The syntheses and analytical data of ᴰGlu-His-Phe-Phe-Gly-Gly-OMe, ᴰGlu-His-Phe-Phe-Ala-Ala-OMe, ᴰGlu-His-Phe-Phe--Ala-Leu-OMe, Phe-Gly-Gly-OMe.AcOH, Phe-Ala-Ala-OMe.AcOH, Phe-Ala-Leu-OMe.AcOH and Z-Phe-Phe will be described elsewhere.

Inhibitors. Pepstatin A, Protein Research Foundation, Osaka, Japan; cathepsin D inhibitor from potato tubers (PDI) (ref.[15]) was a gift of Dr V. Tomášek, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague; diazoacetyl-D,L-norleucine methyl ester[16] (DAN) and diazoacetylglycine ethyl ester[17] (DAG) were synthetized according to the described procedures.

Other substances and their sources. Bovine serum albumin, ovalbumin, chymotrypsinogen A, α-chymotrypsin, myoglobin and bovine haemoglobin: Serva, Heidelberg, F.R.G.; casein (Hammarsten): E. Merck, Darmstadt, F.R.G.; porcine pepsin (Lot PM 8EB): Worthington Biochemical Corporation, Freehold, N.J., U.S.A.; Noble agar: Difco, Detroit, U.S.A.; agarose (pure): Koch--Light Laboratories Ltd, Colnbrook, England; ampholytes: LKB Producter AB, Bromma, Sweden; Sephadex G-100, Sepharose 4B and Blue Dextran: Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose (DE11): Whatman Biochemicals, Maidstone, Great Britain. Concanavalin-A was afforded by Dr K. Filka, Chemical Institute, Charles University, Prague. The other chemicals were A.G. reagents.

Methods

Affiinity-chromatographic matrices. Aminohexyl-Sepharose 4B, casein-Sepharose 4B and PDI--Sepharose 4B were prepared by reaction of CNBr-activated[18] Sepharose 4B with 1,6-diaminohexane, casein and PDI, respectively. The amine was attached at pH 9·5, casein and PDI at pH 6·8. Concanavalin-A-Sepharose 4B was prepared as described by Lloyd[19]. Pepstatin-Sepharose 4B and Z-Phe-Phe-Sepharose 4B were obtained by condensation of hydroxybenzotriazole esters of pepstatin A and Z-Phe-Phe with aminohexyl-Sepharose 4B in a mixture of anhydrous dioxan and dimethylformamide (2 : 1), ref.[18].

Hydrolysis of haemoglobin. The activities of protease 1 and the non-pepsin protease were measured according to Anson[20]. To 2 ml of a 2% solution of haemoglobin in a 0·2M formate

(Na^+) buffer, pH 3·5, was added 0·1 ml of an enzyme solution. After 20 min at 37°C the reaction was stopped by adding 3 ml of 7% trichloroacetic acid. After 10 minutes standing the precipitate was filtered off on a filter paper Whatman 3 MM. The blank was prepared in the same way except that the trichloroacetic acid was added before the addition of the enzyme. The activities of a gastric extract and a mixture of PgI and PgII were measured in the same way at pH 2·0. A unit of proteolytic activity is here defined as such an amount of an enzyme as increases the absorbance at 280 nm above that of the blank by 1·0 in 60 min.

Hydrolysis of synthetic substrates. The initial rate of cleavage (5—15%) of substrates *I*, *II* and *III* was measured by determining the concentration of the liberated C-terminal hydrolytic products, *i.e.* the tripeptide methyl ester, by the ninhydrin method[21] (Scheme 1). The synthetic methyl esters of the C-terminal tripeptides from substrates *I*, *II* and *III* were used for determining the calibration curves. The values of k_{cat} and K_m, defined in ref.[22], were determined by the Lineweaver–Burk method for 6 to 9 concentrations of a substrate. The calculation of k_{cat} refers to a postulated molecular weight of the protease 49 000. The results were analysed by the least-square method.

Inhibition studies. Inhibition of protease 1 by PDI (ref.[15]) at pH 3·5, DAN (ref.[16]), DAG (ref.[17]) and other substances[23] was investigated according to the procedures described.

Concentration of the proteins was determined[24] with bovine serum albumin as standard.

Electrophoretic techniques. Electrophoresis was conducted in 1·8%gels of agar or agarose, which were 0·06M in Tris-barbitone, pH 8·3 (ref.[25]). The relative mobilities (r_m) of the individual proteases were determined with the aid of Blue Dextran and bromophenol blue as standards[7]. The purity and molecular weights of the proteins were determined by electrophoresis[26] in 10% gels of polyacrylamide (0·6 × 9 cm) containing sodium dodecyl sulphate (SDS). The samples of the proteins were prepared according to the "Standard Procedure". The calibration standards for determining the molecular weights were bovine serum albumin, ovalbumin, chymotrypsinogen A and myoglobin.

Isoelectric focusing. Isoelectric focusing in a 5·5% gel[27] of polyacrylamide (0·6 × 9 cm) containing 2% Ampholine 3—10 was used to verify the purity of protease 1. The gels were stained with Coomassie Brillant Blue G-250. In parallel experiments some of the gels were cut in 2 mm sections, which were eluted with a 0·1M acetate (Na^+) buffer, pH 5·5. The proteolytic activity was measured in the eluates[20].

Gel filtration. The molecular weight of protease 1 was determined by gel filtration[28] on a column of Sephadex G-100 (1·5 × 50 cm), equilibrated with 0·01M-Tris-HCl—0·2M-NaCl, pH 7·5. The calibration standards were bovine serum albumin, ovalbumin and α-chymotrypsin.

RESULTS

Isolation of Protease 1 from Human Gastric Mucosa

Step 1: extraction of gastric mucosa. Excised stomach tissues were freed from blood and mucus by washing with 0·9% NaCl. The gastric mucosa was excoriated with a scalpel. All further operations were carried out at 0—4°C. 25 g of the tissue was homogenized in a mechanical homogenizer with 100 ml of a 0·1 M phosphate (Na^+) buffer, pH 7·4. The homogenate was centrifuged at 20 000 *g* for 30 min. The supernatant, to which Triton X-100 had been added to a concentration of 0·1%, was

dialysed against a 0·02 M phosphate (Na^+) buffer, pH 6·5, then centrifuged at 20 000 *g* for 30 min.

Step 2: chromatography on DEAE-cellulose. The supernatant from Step 1 was applied on a column (2·7 × 100 cm) of DEAE-cellulose equilibrated with the 0·02 M phosphate (Na^+) buffer, pH 6·5. The column was washed with 1 l of the starting buffer, then with a linear gradient of 0–0·6 M-NaCl (0·75 l + 0·75 l) in the same buffer (Fig. 1). Electrophoresis revealed that the starting buffer eluted only protease 1 representing 0·1–0·5% of the total proteolytic activity eluted from the support. This enzyme was purified further. The non-pepsin protease was eluted with 0·1–0·2 M-NaCl and represented 3–5% of the total proteolytic activity. It was well separated from the mixture of PgI and PgII. The fractions containing protease 1 were pooled, dialysed against a 0·1 M acetate (Na^+) buffer, pH 5·6, and centrifuged at 20 000 *g* for 30 min.

Step 3: chromatography on casein-Sepharose 4B. The supernatant from Step 2 was applied on a column (2·5 × 15 cm) of casein-Sepharose 4B equilibrated with the 0·1 M acetate (Na^+) buffer, pH 5·6. After washing the column with the starting buffer and the same buffer containing 1 M-NaCl, protease 1 was eluted with a 0·01 M--Tris-HCl buffer, pH 8·5 (Fig. 2).

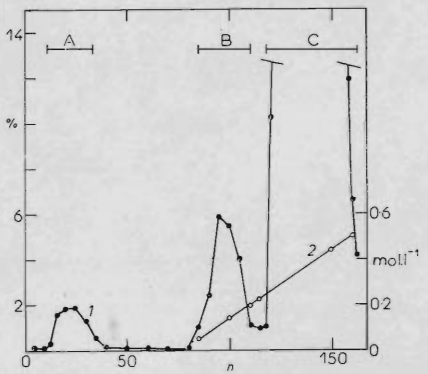


FIG. 1

Chromatography of a gastric mucosa extract on DEAE-cellulose; A protease 1, B non-pepsin protease, C mixture of PgI and PgII, *1* proteolytic activity (%), *2* concentration of NaCl (mol/l^{-1}) *n* number of fraction; fraction volume 15 ml

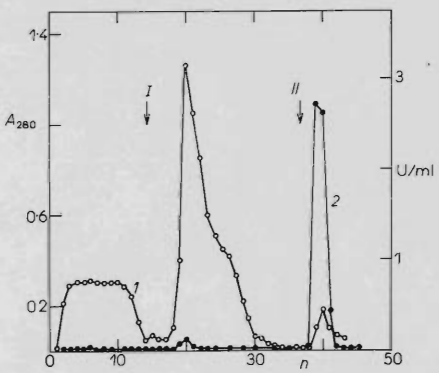


FIG. 2

Chromatography of protease 1 from step 2 on casein-sepharose 4B *I* elution with the starting buffer containing 1M-NaCl, *II* elution with 0·01M-Tris-HCl, pH 8·5, *1* absorbance at 280 nm, *2* proteolytic activity (*U*/ml), *n* number of fraction; fraction volume 15 ml

Step 4: chromatography on Z-Phe-Phe-Sepharose 4B. The protease 1 from Step 3 was applied on a column (1 × 5 cm) of Z-Phe-Phe-Sepharose 4B equilibrated with the 0·01 M-Tris-HCl buffer, pH 8·5. The column was washed with the starting buffer and the starting buffer containing ethylene glycol (20%, v/v), then protease 1 was eluted with the starting buffer containing 30%(v/v) dioxan (Fig. 3). The fractions containing protease 1 were pooled and dialysed against a 0·001 M phosphate (Na^+) buffer, pH 7·0.

Step 5: chromatography on pepstatin-Sepharose 4B. To a solution of the protaese 1 from Step 4 were added 2 ml of sedimented pepstatin-Sepharose 4B. The suspension was brought to pH 3·5–4·0 with 1 M acetic acid, and, 5 minutes later, it was applied on a column (1 × 3 cm) of pepstatin-Sepharose 4B, equilibrated with a 0·1 M acetate (Na^+) buffer, pH 4·0, containing 0·2 M-NaCl. The column was washed with the starting buffer and protease 1 was eluted by increasing pH of the eluting buffer to 8·5 (the results are not given). The enzyme was dialysed against a 0·001 M-phosphate (Na^+) buffer, pH 7·2, then stored at −20°C until used. The purification of protease 1 is outlined in Table I.

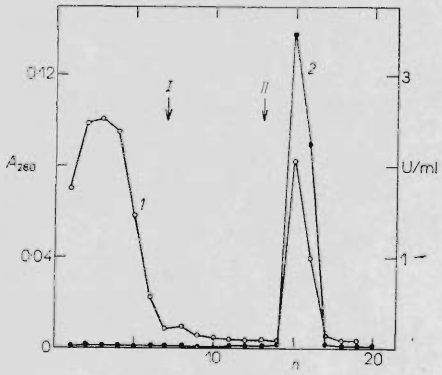


FIG. 3

Chromatography of protease 1 from step 3 on Z-Phe-Phe-Sepharose 4B; *I* elution with the starting buffer containing 20% ethylene glycol, *II* elution with the starting buffer containing 30% dioxan. *1* absorbance at 280 nm, 2 proteolytic activity (U/ml), *n* number of fractions; fraction volume 8 ml

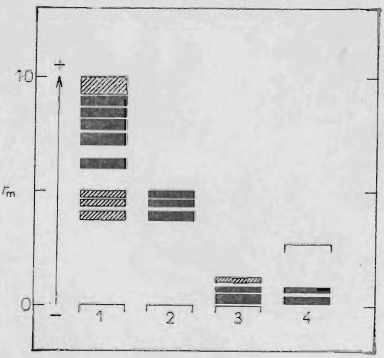


FIG. 4

Schematic presentation of electrophoresis in agarose and in agar gels 1, 2 and 3 electrophoresis in agarose, gel 4 electrophoresis in agar; the relative mobilities of the zones (r_m) refer to bromophenol blue ($r_m = 1$) and Blue Dextran ($r_m = 0$) as standards; marked zones are filled up in black, low-intensity zones are delineated by hatching; *1* extract from gastric mucosa, *2* concentrated non-pepsin protease from Step 2, *3* and *4* concentrated protease 1 from Step 2, ⌐¬ sample application (10 µl)

Properties of Protease 1

Electrophoretic mobility. Agar electrophoresis resolved proteases of the human gastric mucosa extract into several fractions. The non-pepsin protease was always found as low-intensity zones of lower anodic mobilities, compared with those of PgI and PgII (refs[1,2,4–9]). In this study the enzyme was found in three zones of activity (r_m 0·4–0·5, Fig. 4). PgI and PgII were separated into 5 to 6 activity zones of higher mobility (r_m 0·6–0·9). These data are in full accordance with the reported ones[7]. When using the standard conditions no protease 1 was found on the zymograms. It was only after 20 hours' incubation of the gels at 37°C in a moist chamber that it was detected as a zone of low intensity and mobility (r_m 0–0·1; the results are not demonstrated). The isolated and concentrated protease 1 from Step 2 was usually resolved into 2 to 4 zones of activity. In agar, as a result of the higher electroendoosmotic flow, the enzyme migrated to the cathode (Fig. 4).

Purity degree of protease 1. Specific activity of the protease 1 from Step 5 was not increased by subsequent chromatography on PDI-Sepharose 4B or Sephadex G-100 (see lower). Isoelectric focusing in polyacrylamide gel of the enzyme from Step 5 gave four bands that could be stained for protein; pI 5·5, 6·1, 6·5 and 7·1. All the four were proteolytically active (the results are not demonstrated). In polyacrylamide gel electrophoresis in the presence of SDS the same preparation of protease 1 resolved into two polypeptide chains, of molecular weights 33 000 (intense zone) and 18 000 (low-intensity zone, the results are not demonstrated). Consequently, several criteria show that the enzyme was isolated in a fairly pure state.

TABLE I

Purification of protease 1 from human gastric mucosa; 25 g of the mucosa was used

Purification step	Protein mg	Total activity units	Specific activity units . mg^{-1}	Yield %	Purification degree fold
Extract	1 875	[a]	—	—	—
DEAE-Cellulose	463·1	246·4	0·532	100	1
Casein-Sepharose 4B	7·78	80·1	16·75	32·5	31·48
Z-Phe-Phe-Sepharose 4B	0·52	58·2	111·5	23·6	209·6
Pepstatin-Sepharose 4B	0·16	35·1	219·4	14·2	412·2

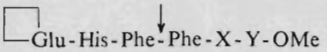
[a] Activity of protease 1 in the extract was not determined.

Molecular weight. In the gel filtration on Sephadex G-100 protease 1 was eluted as one symmetrical peak of activity, corresponding to a molecular weight of 49 000. Judging by electrophoresis in polyacrylamide gel in the presence of SDS, the native enzyme seems to be composed of one light and one heavy chain.

Chromatography on Concanavalin-A-Sepharose 4B (ref.[19]). The protease 1 from Step 5 (1 ml) was applied on a column (1 × 5 cm) of Concanavalin A-Sepharose 4. The enzyme got strongly bound to the support and was specifically eluted from it by 0·3 M α-methyl-D-glucopyranoside in the starting buffer. Protease 1 is probably a glycoprotein.

pH *Optimum.* The optimum pH for cleavage of haemoglobin and *III* by protease 1 was 3·2–3·6.

Hydrolysis of proteins and synthetic substrates. In comparison hydrolyses of haemoglobin, casein and serum albumin, the activities of the enzyme were in the ratio 100 : 12·4 : 1·1. At a concentration of 0·1 μM, protease 1 effected no apparent hydrolysis of typical synthetic substrates of pepsin A and pepsin C in an incubation time of 2 h (Table II). By contrast, peptides *I*, *II* and *III* were hydrolysed by the enzyme in the Phe-Phe bond (Scheme 1). Since the hydrolysis of peptide 1 by protease 1 was



Peptide	X	Y
I	Gly	Gly
II	Ala	Ala
III	Ala	Leu

SCHEME 1

very slow and the needed amounts of the enzyme were not readily accessible, the kinetic parameters are not given. The peptides *II* and *III*, however, are sufficiently sensitive substrates of protease 1 and can be used for routine determination of its activity. The course of their hydrolysis fits the Michaelis–Menten kinetics (Table II).

Inhibition studies. The following compounds, at a concentration of 1 mM, did not markedly affect hydrolysis of haemoglobin by 0·05 μM protease 1 (95–110% of the control value): NaCl, $MgCl_2$, $ZnCl_2$, $CaCl_2$, $Cu(CH_3CO_2)_2$, $Pb(CH_3CO_2)_2$, $MnCl_2$, $CoCl_2$, $EDTANa_2$, 2-mercaptoethanol, reduced glutathione, iodoacetic acid. The time courses of inactivation of protease 1 by DAN and DAG are shown in Fig. 5. The enzyme, unlike porcine pepsin, was much less sensitive to the inactivating effect

of DAN. Hydrolysis of haemoglobin (Fig. 6) and substrate *III* (not shown) by protease 1 was inhibited by PDI. By contrast, the non-pepsin protease was not appreciably inhibited by PDI.

Chromatography on PDI-*Sepharose* 4B. The non-pepsin protease or protease 1 (in either case the fraction from Step 2, 2 ml) dialysed against a 0·1 M acetate (Na^+) buffer, pH 4·0, and 0·5 M-NaCl were applied onto a column (1 × 5 cm) of PDI-Sepharose 4B, equilibrated in the same buffer. The column was washed first with the starting buffer (20 ml), then with the 0·01 M-Tris-HCl buffer, pH 8·5. The non-pepsin protease and other proteins were eluted quantitatively with the starting buffer, while protease 1 remained bound in the column. It was then eluted with an alkaline buffer in c.70% yield. Its specific activity was about 50 times higher than the starting value.

DISCUSSION

In addition to the non-pepsin protease, human gastric mucosa has been found to contain another minor protease, called protease 1. The enzymes differ markedly in

TABLE II

Kinetics of hydrolysis of synthetic substrates by protease 1. Protease 1 from Step 5 was used. The hydrolysis was conducted in a 0·2M formate (Na^+) buffer, pH 3·3, with 3% of dimethylformamide at 40°C

Enzyme μM	Substrate mM	k_{cat} s^{-1}	K_m mM	k_{cat}/K_m s^{-1} mM^{-1}
Protease 1 0·1	Z-Glu-Tyr 0·3	—[a]		
Protease 1 0·1	Ac-Phe-Tyr(I_2) 0·2	—[a]		
Protease 1 0·1	Z-His-Phe-Phe-OMe 0·55	—[a]		
Protease 1 0·1	*I* 0·3—5·0	—	—	<0·01
Protease 1 0·08	*II* 0·35—2·9	0·4	1·6	0·25
Protease 1 0·07	*III* 0·35—2·7	2·1	1·1	1·91
Porcine pepsin[b] 0·005	*III* 0·4—2·1	81	0·5	162

[a] No hydrolysis was detected; [b] 0·2M formate (Na^+) buffer, pH 4·0, 37°C (ref.[14]).

their electrophoretic and chromatographic properties, molecular weights and sensitivity to the inhibition by PDI. The non-pepsin protease is considered by some authors to be cathepsin D or a similar enzyme[10,11]. However, it is known to have some properties typical of cathepsin E (ref.[5]) or pepsinogens[5,6,10]. Our observation that this enzyme is not inhibited by PDI is consistent with these findings. The origin of this protease remains obscure.

Protease 1 is a carboxylic protease, judging from inhibition by pepstatin and DAN (ref.[29]). The identity of its properties with those of the 40 to 50 kilodalton form of cathepsin D, isolated from other mammalian tissues[30–35], is striking if we compare *a*) their molecular weights and chain compositions, *b*) isoelectric points of the individual isoenzymes, *c*) relative hydrolytic rates of haemoglobin and serum albumin, *d*) sensitivities to PDI. It can therefore be classified as cathepsin D of gastric mucosa.

Cathepsin D was invariably present in the gastric mucosa of 45 patients as a minor protein. Its content in the wet tissue is about a tenth of that in, *e.g.*, spleen[32–35] Its proteolytic activity, compared to that of human pepsinogens, is negligible. This probably, is why it usually escaped detection in agar electrophoresis, in which moreover, it migrates to the cathode. Its more basic nature, compared to the non-pepsin protease, is also apparent from the pattern of chromatography on DEAE

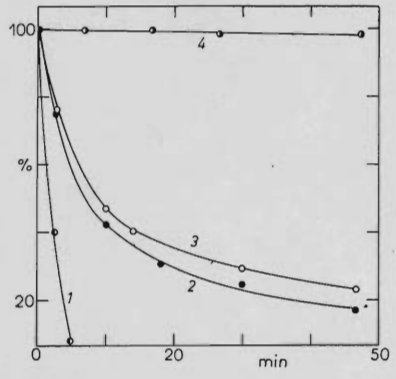


FIG. 5

Inhibition of Protease 1 and porcine pepsin by DAN and DAG, *1* 30 nM pepsin, 1 mM DAN, 1 mM Cu^{2+}; *2* 30 nM protease 1, 1 mM DAN, 1 mM Cu^{2+}; *3* 30 nM protease 1, 1 mM DAG, 1 mM Cu^{2+}; *4* 30 nM protease 1, 1 mM DAN, without Cu^{2+}. The enzymic activities are given in %

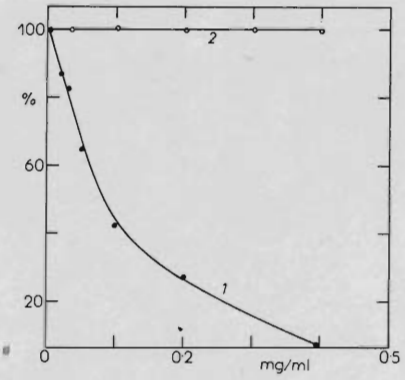


FIG. 6

Inhibition of protease 1 and non-pepsin protease by PDI, Fractions from Step 2 were used; *1* protease 1, *2* non-pepsin protease; haemoglobin as substrate; enzymic activity in %

cellulose, in which it was, like cathepsins D from other tissues[23], eluted with the starting buffer. In a number of similar experiments[5,9,10,13,36–39] no protease of this character was ever found in extracts of human gastric mucosa. It was therefore considered[5] that cathepsin D might not be produced by the gastric mucosa.

Since chromatography of the fraction from Step 2 on pepstatin-Sepharose 4B failed to afford protease 1 of sufficient purity, further two chromatographic steps were introduced. It is worth while to mention that chromatography of the enzyme on Concavavalin A-Sepharose 4B or PDI-Sepharose 4B may be a good supplement to the purification scheme. The procedure described makes it possible to purify cathepsin D without the acid-induced precipitation[30] and to reduce substantially the possible limited proteolysis of the enzyme[40]. Nevertheless, we observed isoelectric heterogeneity. Besides, the one-chain form of cathepsin D, found in various amounts in other mammalian tissues[30,31,35,41], was not detected.

The low-molecular-weight substrates of pepsin were not cleaved by the gastric cathepsin D. For this reason we prepared hexapeptides *I*–*III*, structurally pertaining to the group of pepsin substrates hydrolysed in the phenylalanyl-phenylalanyl--bond[22], which we expected to be sensitive to the hydrolysis by the enzyme. As was observed with porcine pepsin A and bovine cathepsin D (refs[22,42]), in human cathepsin D, too, the incorporation of hydrophobic amino acids into the C-terminal part of these substrates resulted in higher values of k_{cat} and k_{cat}/K_m. This suggests that the secondary enzyme–substrate interactions play a decisive role in the catalysis. In view of the general similarity in specificity between pepsin A and cathepsin D, it is probable that the incorporation of a pyroglutamyl residue into the N-terminal part of the substrates *I*–*III* appreciably increases their sensitifity to hydrolysis. It is well known that the structurally similar prolyl residue in the same position considerably increaess the probability of peptic hydrolysis of proteins in a bond corresponding, by position, to the Phe-Phe bond in the peptides *I*–*III* (ref.[43,44]). Still, the k_{cat} values of pepsin are about 40 times higher than those of cathepsin D. In this respect the gastric and splenic cathepsins D differ from the uterine cathepsin D, which hydrolyses a number of peptides at the same rate as pepsin A does[42]. The dependence of the initial rate on the initial concentration of the peptide *II* or *III* did not deviate from hyperbolicity. This can be regarded as further evidence of close similarity in enzymic properties between the individual isoelectric forms of cathepsin D.

The enzyme studied resembles in many respects the "tumour-specific" proteases (cathepsin D-like enzymes), reproducibly found in gastric adenocarcinomas only[13]. Although further study is necessary, the higher production of cathepsin D by tumour cells can be correlated with the finding of "higher activities" of lysosomal hydrolases in various tumours[45].

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