Substrate and inhibitor studies with human gastric aspartic proteinases

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The separation of pepsin isoenzymes 1, 2, 3 and 5 (gastricsin) in human gastric juice was effected by chromatography on Mono Q ion-exchanger, and slow-moving proteinase was purified to homogeneity by using a modified procedure incorporating a novel affinity-chromatography step. The pH-activity profiles of these enzymes with mucus glycoprotein and basement-membrane substrates were determined; the profiles for pepsin 2 were noticeably different, and, in general, the pH optima for the hydrolysis of basement membrane were more acidic. Pepsin 1 expressed larger specificity constants (k_{cat}/K_m) than pepsin 3 with a series of synthetic peptide substrates, reflecting greater binding (smaller K_m) by pepsin 1. Inhibitor studies at pH 1.7 and 4.5 with a series of P_2 -substituted lactoyl-pepstatis implied that valine at position P_2 was optimal for inhibiting pepsins 1, 2 and 3 but detrimental for pepsin 5, whereas lysine at position P_2 was tolerated well by pepsin 5 but not by pepsins 1, 2 and 3. The potency of lactoyl-pepstatin with lysine at position P_2 did not increase as a function of pH. P_2 -substituted lactoyl-pepstatis failed to show any inhibitory selectivity among pepsins 1, 2 and 3.

INTRODUCTION

Peptic ulcers are thought to arise from an imbalance in defensive (mucus, bicarbonate) and aggressive (acid, pepsin) factors, although environmental and genetic factors may also play a role in the pathogenesis in some patients (Richardson, 1985). The human gastric mucosa produces two types of pepsinogens, I (pepsinogens 1–5) and II (pepsinogens 6 and 7, progastricsins), and also slow-moving proteinase (cathepsin E). These immunologically distinct species can be separated by gel electrophoresis. The relative abundance of pepsinogens I, pepsinogens II and cathepsin E in the fundic-gland mucosa is in the order given (Samloff, 1989). Pepsin 3 and pepsin 5 (gastricsin), derived from pepsinogens I and II respectively, constitute the major active isoenzymes of gastric juice from asymptomatic volunteers; however, the normally minor (< 4% of total pepsin activity) isoenzyme pepsin 1 is increased considerably (16–23% of total pepsin activity) in the gastric juice of peptic-ulcer patients (Pearson *et al.*, 1986). This correlation suggests a possible pathophysiological role for pepsin 1 in peptic ulcer disease. Furthermore pepsin 1 is reported to be more collagenolytic (Etherington *et al.*, 1980) and mucolytic (especially at pH 4) than the major pepsin, pepsin 3 (Pearson *et al.*, 1986), and therefore is often termed the peptic-ulcer-associated pepsin.

Most substrate and inhibitor studies reported have used pig pepsin (Foltmann, 1981; Rich & Bernatowicz, 1982; Rich & Salituro, 1983; Holladay *et al.*, 1987; Pohl & Dunn, 1988), some have used human pepsins 3 and 5 (Valler *et al.*, 1985; Dunn *et al.*, 1986) and only a few have concerned pepsin 1 (Etherington *et al.*, 1980; Roberts & Taylor, 1982; Pearson *et al.*, 1986, 1989). The present paper documents some substrate and inhibitor studies with certain human pepsin isoenzymes, particularly pepsins 1 and 3.

MATERIALS AND METHODS

Materials

Bovine haemoglobin and activated 6-aminohexanoic

acid-Sepharose 4B were obtained from Sigma Chemical Co., Poole, Dorset, U.K., Ultrogel A2 was from LKB Instruments, South Croydon, Surrey, U.K., and concanavalin A-Sepharose 4B and Mono Q were from Pharmacia, Milton Keynes, Bucks., U.K. Matrigel was purchased from Flow Laboratories, Rickmansworth, Herts., U.K., and peptide inhibitors and substrates were either prepared in-house or custom-synthesized. Flat-bottom 96-well tissue-culture clusters were obtained from Costar, Cambridge, MA, U.S.A., and MicroFLUOR 'W' plates were purchased from Dynatech Laboratories, Alexandria, VA, U.S.A. Human gastric juice and tissue were obtained from Charing Cross Hospital, London, U.K., and Northwick Park Hospital, Harrow, Middx., U.K.

Purification of pig gastric mucus glycoprotein

Mucus glycoprotein was purified essentially as described by Scawen & Allen (1977) and Bell *et al.* (1985). Mucus gel was solubilized by homogenization in 0.2 M-NaCl, pH 7.0. After CsCl-density-gradient centrifugation (1.42 g/ml initial density; Beckman L8-70M ultracentrifuge, SW 28 rotor, 100000 *g* for 48 h at 4 °C) and gel filtration (Ultrogel A2) the glycoprotein was reductively methylated (Jentoft & Dearborn, 1979) to diminish background fluorescence.

Preparation of tritiated haemoglobin substrate

The labelled substrate was prepared as described by Hille *et al.* (1970).

Measurement of pepsin activity

With haemoglobin substrate. [^3H]Acetyl-haemoglobin (0.16 mg; sp. radioactivity 8.2 kBq/mg) was incubated (at 37 °C for 10 min) with enzyme and buffer (pH 1.7 or 4.5) in a final volume of 100 μl . Assay buffers (1.05) were HCl/KCl buffer, pH 1.7, and sodium acetate buffer, pH 4.5. Reactions were stopped with trichloroacetic acid (3%, w/v; 500 μl), the mixtures were centrifuged (at 10000 *g* for 3 min), duplicate portions (200 μl) were removed and the radioactivities of the trichloroacetic acid-soluble peptides were counted.

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With cross-linked Matrigel substrate. Matrigel is a preparation of natural solubilized tissue basement membrane containing laminin, collagen type IV, heparan sulphate proteoglycan and entactin. Matrigel (10 ml) was diluted (5-fold) with, then dialysed (4 °C) against, acetic acid (10 mM). This was dispensed (15 µg; 50 µl per well) into a 96-well titre plate (Costar), fixed (1 h) with 4% (w/v) paraformaldehyde and then washed (three times) with water and dried. This cross-linked Matrigel was used in our studies and is referred to as basement membrane in the text below. Incubation mixtures comprised basement membrane (15 µg), pepsins (0.6–9.0 pmol) and a range of buffers (I 0.1) covering pH 1–2 (HCl/KCl buffer) and pH 3–5 (sodium formate buffer), all in a final volume of 100 µl per well. The plates were covered with Titertek plate sealer and incubated (at 37 °C for 1 h) with constant shaking. The reaction was stopped by adding 100 µl of 0.2 M-sodium borate/0.2 M-KCl buffer, pH 8.5 (boric acid titrated with NaOH), and the pH was adjusted to pH 8.5–9.0 with 2 M-NaOH. Portions (100 µl) from each well were transferred to a fresh plate (MicroFLUOR 'W') and 50 µl of fluorescamine (0.03% solution in acetonitrile) was added to each. The fluorescence was measured in a Perkin-Elmer LS-5B luminescence spectrometer at an excitation wavelength of 390 nm and an emission wavelength of 475 nm, and after subtraction of the relevant blanks pepsin activity was expressed as fluorescence units/h per pmol of enzyme.

With mucus glycoprotein substrate. The buffers (I 0.13) and the fluorescamine solution used with mucus glycoprotein were the same as those used with basement membrane above. Purified pig gastric mucus glycoprotein (50 µg) was incubated (at 37 °C for 2 h) with pepsins (1–50 pmol) in a final volume of 60 µl with constant shaking. The sequential addition of stopping buffer (50 µl), NaOH and fluorescamine (50 µl) preceded the measurement of fluorescence.

With synthetic substrates. Synthetic substrates [Lys-Pro-Xaa-Yaa-Phe-Phe(4-NO₂)-Arg-Leu, where Xaa-Yaa is Ile-Glu, Val-Glu or Val-Val] were dissolved and incubated (at 37 °C) in 0.1 M-sodium formate buffer, pH 3.1 (0.5 ml). The incubation mixtures contained pepsins (0.6–1.5 pmol) and covered a range of substrate concentrations (12.5–400 µM) enabling the construction of double-reciprocal plots from calculated initial linear slopes (decrease in A_{300}/min). Kinetic parameters were derived essentially as described by Dunn *et al.* (1987).

Molar concentrations of pepsins and slow-moving proteinase were determined by titrating their activities against the synthetic substrates, with an accurately determined (amino acid analysis) concentration of pepstatin A. The Val-Val-containing substrate was used with pepsin 5 and the Ile-Glu-containing substrate with the other enzymes.

Protein determination

Protein was measured by the method of Bradford (1976), with BSA as standard.

Purification of pepsins

Human gastric juice, when heavily contaminated with bile pigment, was initially exposed to batch ion-exchange chromatography on Whatman DE52 DEAE-cellulose at 4 °C (equilibration buffer, 0.1 M-sodium acetate buffer, pH 5.3; elution buffer, equilibration buffer containing 0.9 M-NaCl). The salt wash, after dialysis against 0.1 M-sodium acetate buffer, pH 3.8, and filtration (pore size 0.2 µm), was chromatographed by f.p.l.c. on Mono Q HR10/10 ion-exchanger (flow rate 4 ml/min). Pepsins were eluted by using a segmented gradient (368 ml) from 0 M- to 1.0 M-NaCl in 0.1 M-sodium acetate buffer, pH 3.8.

Protein elution was monitored at 280 nm and pepsin activity was monitored by the degradation of [³H]acetyl-haemoglobin substrate. Conc. NaHCO₃ was used to adjust fractions to pH 5.0 before they were concentrated (Amicon PM10 membrane) and then dialysed against 0.1 M-sodium acetate buffer, pH 5.3.

Purification of slow-moving proteinase

Slow-moving proteinase was isolated from human gastric mucosa essentially as described by Samloff *et al.* (1987), the final immunoaffinity step being replaced by another affinity-chromatography stage involving the ligand Val-D-Leu-Pro-Phe-Val-D-Leu coupled to activated 6-aminohexanoic acid-Sephacryl 4B. The sample from the concanavalin A-Sephacryl step was adjusted to pH 3.5 before being loaded on to the affinity column (0.5 cm × 2.0 cm) equilibrated in 0.1 M-sodium acetate buffer, pH 3.5. Unbound protein was removed by washing with equilibration buffer, and the slow-moving proteinase was then eluted with 50 mM-sodium phosphate buffer, pH 7.5. This preparation appeared to be homogeneous and free from pepsin when electrophoresed on SDS/polyacrylamide and agarose gels. Slow-moving proteinase (5 µg) migrated as a single species (M_r 90 000) under non-reducing conditions and as two similar species (M_r 42 000–45 000) under reducing conditions on SDS/PAGE and staining with Coomassie Blue. No other bands were evident. On agarose-gel electrophoresis of slow-moving proteinase (0.1 µg) and zymogram staining only one area of haemoglobin degradation was detected; the low mobility of this activity relative to pig pepsin standard identified the species as slow-moving proteinase and confirmed the absence of any significant contamination by other pepsin activities.

RESULTS

Preparation of enzymes

The human pepsins used in this study were separated by ion-exchange chromatography; the elution profile from Mono Q is depicted in Fig. 1. Pepsin 5 was eluted as a discrete peak and pepsin 3 was resolved into three peaks of activity well separated from pepsin 2. Pepsin 1, being a heterogeneous population, was not resolved as a discrete peak and occasionally minor contamination of the latter by pepsin 3 was detected; however, this was easily removed by rechromatographing under identical conditions. The separation of pepsins and their identities were confirmed by agar-gel electrophoresis at pH 5.0 (Roberts & Taylor, 1978). There was also evidence for a pepsin species that was not only eluted from Mono Q, but also migrated on agar-gel electrophoresis, with a mobility between those of pepsins 3 and

Fig. 1. Elution profile of pepsins from Mono Q

The sample (52 ml) from DE52 DEAE-cellulose chromatography was loaded on to a MonoQ column (HR10/10) equilibrated in 0.1 M-sodium acetate buffer, pH 3.8, and the pepsin isoenzymes were eluted with a segmented gradient (0–1.0 M-NaCl) in the same buffer. Bars denote the pooled fractions of each of the separated pepsin isoenzymes (P1–P5). —, A_{280} ; ----, NaCl gradient.

Fig. 2. pH-activity profiles of the pepsin isoenzymes and slow-moving proteinase with basement membrane and mucus glycoprotein substrates

Enzyme specific activity ($\Delta F/h$ per pmol of enzyme) was measured as a function of pH with basement membrane (a) and mucus glycoprotein (b) as substrates. ●, Pepsin 1; ■, pepsin 2; △, pepsin 3; ▲, pepsin 5; ○, slow-moving proteinase.

5. Slow-moving proteinase, as described above, was homogeneous and in the presence of dithiothreitol migrated on SDS/PAGE as two bands of M_r 42000–45000, in contrast with the single 90000- M_r band observed under non-reducing conditions.

pH-activity profiles

The pH-activity profiles of the pepsin isoenzymes and slow-moving proteinase with basement membrane and mucus glycoprotein as substrates are shown in Fig. 2. A study of the linearity of the reaction rates exhibited by individual pepsins validated the fixed-time activity measurements being expressed as rates. With basement membrane pepsin 1 activity decreased as the pH increased, pepsin 2 activity followed a similar profile but reached a plateau around pH 2, and pepsin 3 activity exhibited a definite pH optimum at pH 2–3. The turnover of basement membrane by slow-moving proteinase was barely detectable. Pepsin 5 also showed poor activity but with a definite pH optimum at pH 2.0–2.5. Similar profiles, but with the turnover rate decreased by about 75%, were observed when type IV collagen was used as substrate (results not shown). With mucus glycoprotein substrate pepsin 1 exhibited the greatest turnover of substrate with maximum activity occurring at pH 2.0–2.5 followed by a distinct shoulder at pH 3.0–3.5. The activity then decreased linearly towards zero at higher pH values. Unlike the

profile with basement membrane, pepsin 3 had a broad pH optimum with marginally greater activity evident around pH 4. The limited turnover of mucus glycoprotein by pepsins 2 and 5 showed no discrete pH optima, and slow-moving proteinase, which was slightly more active than the latter, exhibited a distinct pH optimum around pH 4.

Synthetic substrates

The kinetic constants for the hydrolysis of three synthetic substrates with varied P_2 and P_3 substituents by pepsins 1 and 3 are shown in Table 1. The specificity constants (k_{cat}/K_m) implied that all three were good substrates for both pepsins, especially pepsin 1, whose hydrolytic efficiency derived particularly from greater binding (lower K_m). The substitutions in the P_2 and P_3 positions did not markedly alter the kinetic parameters measured for either pepsin; however, the k_{cat} and K_m values observed for pepsin 3 were considerably greater than those for pepsin 1.

Lactoyl-peptatins

The effect of substitution of the valine residue at position P_2 of lactoyl-peptatin on inhibitory potency against the human pepsin isoenzymes is shown in Table 2. At pH 1.7 pepsins 1 and 3 exhibited very similar profiles with the inhibitors substituted at position P_2 . Valine's isopropyl side chain is obviously the preferred substituent in this position, and the basic side chain of

Table 1. Kinetic constants for the hydrolysis of Lys-Pro-Xaa-Yaa-Phe-Phe(4-NO₂)-Arg-Leu by pepsins 1 and 3

Synthetic substrates (12.5–400 μM) were incubated (37 °C) with pepsins (0.6–1.5 pmol) in 0.1 M-sodium formate buffer, pH 3.1 (0.5 ml), and the decrease in A_{300}/min was measured.

Substrate -Xaa-Yaa-	Pepsin 1			Pepsin 3		
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)
-Ile-Glu-	602	0.06	10 033	1938	0.44	4405
-Val-Val-	552	0.06	9200	1919	0.50	3838
-Val-Glu-	528	0.06	8800	1659	0.33	5027

Table 2. Inhibition of human pepsin isoenzymes with P₂-substituted lactoyl-pepstatis at pH 1.7 and 4.5

Haemoglobin substrate was incubated in the presence of (a) HCl/KCl buffer, pH 1.7 (*I* 0.05), and pepsins (0.3–5.7 pmol) or (b) sodium acetate buffer, pH 4.5 (*I* 0.05), and pepsins (1.3–14.2 pmol). Samples were incubated for 10 min (pH 1.7) or 20 min (pH 4.5) at 37 °C. Inhibitor concentrations ranged from 1 nM to 0.1 mM. Abbreviation: Sta, statine (4-amino-3-hydroxy-6-methylheptanoic acid).

						IC ₅₀ (μM)			
						Pepsin 1		Pepsin 2	
						pH 1.7	pH 4.5	pH 1.7	pH 4.5
P3	P2	P1	P1'	P2'					
						pH 1.7	pH 4.5	pH 1.7	pH 4.5
Lac-Val-Sta-Ala-Sta-NH ₂						0.047	0.56	0.043	0.69
—Phe—						0.31	2.6	0.098	0.98
—Ala—						2.7	43.5	0.73	7.3
—Lys—						22.0	> 68.0	3.7	12.9
—Glu—						0.44	30.0	0.1	2.0
								0.027	0.25
								0.13	1.14
								0.87	15.2
								5.4	35.4
								0.25	5.5
								18.5	62.9
								4.7	12.2
								3.2	10.9
								0.88	1.7
								0.32	3.1

lysine is not well tolerated. The rank order of potency against pepsin 5 is almost the reverse of that exhibited by inhibitors with pepsins 1 and 3, lysine, but not valine, being tolerated reasonably well at the P₂ position.

When these assays were repeated at pH 4.5 a change in profile was expected, particularly with the inhibitors containing lysine and glutamic acid. Generally speaking, however, the rank orders of potency for pepsins 1, 3 and 5 were the same at both pH values, although at pH 4.5 the absolute potencies were considerably diminished. This pH-dependent decrease in potency was also evident with pepstatin A (results not shown).

DISCUSSION

Separation of the pepsin components of human gastric juice has been achieved previously by repetitive chromatography on DEAE-cellulose (Roberts & Taylor, 1978) and more recently by using high-performance ion-exchange chromatography (Newton *et al.*, 1987; Peek *et al.*, 1988). The pepsins used in the present study were separated in one step by f.p.l.c. on Mono Q anion-exchanger. This rapid processing of the gastric juice resulted in improved reproducibility and better yields than those observed from DEAE-cellulose chromatography. On storage frozen (–20 °C) no loss of pepsin activity was observed over a period of several months. In contrast, slow-moving proteinase activity was less stable on storage, and therefore was always used immediately after purification. The affinity ligand used in this purification was an effective alternative to the final immuno-affinity column step used by Samloff *et al.* (1987). A similar ligand had been used to separate human pepsin and gastricsin activity from gastric juice (Kučerová *et al.*, 1986), although this involved using organic solvents (20% dioxan) to effect desorption. A decrease in the hydrophobicity of the ligand (removal of one phenylalanine residue) precluded the requirement for solvent elution of slow-moving proteinase from the affinity column used in this study.

The pH-activity profiles with basement membrane and mucus glycoprotein substrates for the various pepsin isoenzymes have many similarities; however, there are also major differences, e.g. low activity and the absence of a pH optimum for pepsin 2 with mucus glycoprotein as substrate. Generally, the pH optima for the hydrolysis of basement membrane are more acidic, and perhaps some of the differences can be explained by the high carbohydrate content of mucus glycoprotein and in particular the presence of dissociable sialic acids and ester sulphates, which, having pK_a values below 3, will be fully ionized at all but the lowest pH values. The slight increase in activity exhibited by the

pepsins with mucus glycoprotein in the range pH 3–5 suggests that the ionization of a carboxylic function, in an acidic amino acid, may be favouring the interaction between substrate and enzymes. The pH-activity profile for pepsin 1 with mucus glycoprotein is very similar to that reported by Pearson *et al.* (1986); however, our pepsin 3 profile differed significantly in that we observed considerable activity at pH 4. This was also noted with the use of a universal buffer system (results not shown). It would appear, therefore, that the pepsins are capable of recognizing and hydrolysing the substrates differentially, which confirms the observation by Pearson *et al.* (1986) that gastric juice from duodenal-ulcer patients produced a pH-activity profile with haemoglobin that differed significantly from that with gastric mucus glycoprotein.

The pepsin isoenzymes can also perceive chromophoric substrates differently. Human pepsins 1 and 3 displayed specificity constants with two substrates in this study that were several orders of magnitude greater than those for human gastricsin (Dunn *et al.*, 1986). The specificity constants for pepsin 1 were greater than those for pepsin 3. This was reflected mostly by the improved binding (defined by K_m) to the substrates. This suggests that pepsins 1 and 3 can bind substrates differently, and by extrapolation to inhibitors these should be capable of differential inhibition if a good correlation between inhibitor K_i and substrate K_m exists. Attempts to design optimal enzyme inhibitors on the basis solely of a knowledge of optimal substrates should, however, proceed with caution, as structural features for good substrates do not always parallel those for good inhibitors (Holladay *et al.*, 1987).

It is apparent that the non-conservative changes made in the P₂ position of lactoyl-pepstatin did not induce selectivity between pepsins 1 and 3; however, selectivity (≥ 390-fold) between pepsin 1 or pepsin 3 and pepsin 5 was evident, confirming the results obtained by Valler *et al.* (1985). On substitution of the isopropyl side chain of valine, this selectivity at both pH 1.7 and 4.5 decreased. The substitution of lysine for valine in the P₂ position of lactoyl-pepstatin was deleterious to pepsins 1 and 3 but was tolerated by pepsin 5. This suggested that the positive basic function of the lysine side chain in the P₂ position is unfavourable, destabilizing binding to pepsins 1 and 3 but not to pepsin 5. Similar effects of a positive charge at position P₂ were reported by Maibaum & Rich (1988), who suggested that the poor potency of inhibitors containing histidine in the P₂ position, against pig pepsin was because binding is destabilized below pH 4.5 as a result of the protonation of histidine's imidazole side chain. The lysine-containing inhibitor was significantly more potent against pepsin 5 than against pepsin 1 or pepsin 3,

particularly at pH 4.5, suggesting that ionizable side chains within the microenvironment of the S_2 pocket in pepsins 1 and 3 are behaving differently from those in pepsin 5. Another explanation for the difference in potencies at pH 4.5 could be that other subtle, favourable, ionic interactions along the active-site cleft of pepsin 5 may exist, thereby improving binding; however, these interactions may be absent from the active-site cleft of pepsins 1 and 3. The improved binding to pig pepsin by substrates containing lysine at position P_2 observed when the pH of incubation is raised from pH 2.8 to pH 5.8 was shown to be due to the dissociation of an enzyme group with $pK_a > 5.8$ (Pohl & Dunn, 1988). Our data suggest that inhibitors with lysine at position P_2 do not show improved binding to human pepsins over a similar pH range; however, as alluded to above, structural features for good substrates do not always parallel those for good inhibitors.

Gastricsin and slow-moving proteinase are immunologically distinct from the pepsinogen-I-derived pepsins. We have not only shown differences in their ability to degrade mucus glycoprotein and basement membrane substrates but have also shown that gastricsin displays different substrate-specificity constants and inhibitor profiles from pepsins 1 and 3. Even within the pepsinogen-derived family of pepsins it is evident that pepsins 1 and 3 can bind substrates differently, although we have been unable to show any inhibitor selectivity between pepsins 1 and 3 by using P_2 -substituted lactoyl-pepstatis. Indeed, we have also studied systematic substitutions in:

where Boc represents t-butyloxycarbonyl and $\text{Cha} \begin{array}{c} \text{OH} \\ | \\ \text{---} \end{array} \text{Phe}$ represents a cyclohexylalanyl-phenylalanyl hydroxyethylene dipeptide isostere, and found that introducing residues of variable length, branching, hydrophobicity and basicity, particularly into positions P_2 – P_2' did not induce any significant inhibitory selectivity between pepsins 1 and 3 (A. Baxter, C. J. Campbell, C. J. Grinham, R. M. Keane, B. C. Lawton & J. E. Pendlebury, unpublished work).

The design of potent pepsin-1-selective inhibitors for the treatment of peptic ulcer disease would be a possible, but testing, challenge.

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