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Comparative Pepstatin Inhibition Studies on Individual Human Pepsins and Pepsinogens 1,3 and 5(gastricsin) and Pig Pepsin A

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Human gastric juice contains 3 major proteolytic components (pepsins1,3 and 5 or gastricsin). Pepsin 1 is increased in peptic ulcer and it's properties are relatively poorly understood. Studies with pepstatin the highly specific aspartic-protease inhibitor have therefore been carried out on individual active and proenzymes to assess any enzymic similarities. Human pepsin 1 was inhibited with high affinity similar to pepsin 3, whereas pepsin 5(gastricsin) was at least 40 times less sensitive. Inhibition of human pepsinogens 1,3 and 5 and pig pepsinogen A showed similar trends to the active enzymes. Studies using Sephadex gel filtration showed that pepstatin does not bind to pepsinogens and inhibition arises from pepstatin binding the pepsins released upon activation. Pepstatin inhibition was shown to be relatively independent of pH between 1.5 and 3.8 although at higher pH inhibition was less effective. The evidence suggests that pepsin 1 is similar to pepsin 3 and pepstatin inhibits by a one to one molecular binding to the active site. The explanation for the reduced affinity of pepstatin to pepsin 5(gastricsin) needs further study by co-crystallisation X-ray analysis.

Keywords: Pepstatin; Pepsins 1,3,5(gastricsin); Pepsinogens; Inhibition

INTRODUCTION

Pepstatin, isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alany1-4-amino-3-hydroxy-6-methylheptanoic acid, M_r 686, (isolated from a strain of Streptomyces)[1] has been shown to inhibit human pepsin (EC 3.4.23.1) and "gastricsin" (EC 3.4.23.3)[2] hepatic cathepsin D (EC 3.4.23.5) and the aspartic proteinases (containing cathepsin E) of rabbit bone marrow.[3] Pepstatin also protects the stomach of pylorus-ligated rats from ulceration[1] and when given orally to two patients with "stomach-ulcers", reduced the total pepsin activity of human gastric juice, collected 60 min later, to below 10% of the original activity.[2]

The pepsin proteolytic content of human gastric juice comprises up to 8 different components. The most electophoretically mobile pepsin 1[4] has been found in increased frequency and amount in the histamine-stimulated gastric juices of patients with gastric and duodenal ulcer[5] and may have an aetiological role in these disorders. The effect of pepstatin upon pepsin 1 and upon the human pepsinogens has therefore been investigated and compared with the effect upon pepsin 3 (human "pepsin" EC 3.4.23.1.) and pepsin 5 ("gastricsin", EC 3.4.23.3). The opportunity has also been taken to study the kinetics of the inhibition of the human pepsins by pepstatin, and the effect of pepstatin upon the human pepsins after they have been inhibited by carbenoxolone. The latter is known to promote the healing of human gastric ulceration[6] and to inhibit the human pepsins *in vitro*[7] and *in vivo*.[8]

EXPERIMENTAL

Materials

Gastric juice was collected by pernasal intragastric tube from patients undergoing augmented histamine

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tests[9] or pentagastrin stimulation tests, using $6\,\mu g\,kg^{-1}$ body weight intramuscularly. Pepsins 1 and 3 were prepared from human gastric juice by repetitive chromatography on diethylaminoethyl cellulose columns.[10] Pepsin 5(gastricsin) was prepared from human gastric juice by a similar method previously outlined.[10,11] Pepsinogens were prepared from human gastric mucosal extracts as described previously.[12] Pepsins and pepsinogens were examined for homogeneity by agar gel eletrophoresis at pH 5.0.[12] The pepsins were shown to be both protein and enzymically single components whereas the pepsinogen preparations possibly contained small amounts of inert protein i.e. presence of non-pepsinogen protein/enzymic material.

Crystalline pig pepsin and bovine haemoglobin substrate powder were obtained from Armour laboratories, Eastbourne, UK. The pig pepsin was at least 90% pure and was used in the calculations of enzyme to inhibitor ratios assuming it to be100% pure. Pig pepsinogen, as a lyophilised powder, was obtained from the Sigma Chemical Co. Poole UK. Pepstatin, sodium salt, was kindly donated by Dr H. Umezewa and K. Goto of the Banyu Pharmaceutical Co. Ltd., Tokyo, Japan and carbenoxolone, disodium salt, by the late Dr S. Gottfried of Biorex Laboratories, London, UK. Measurements of pH were carried out with a Vibron pH meter, model 39A, Electronic Instruments Ltd., Richmond, Surrey, UK.

Methods

Inhibition of Peptic Activity

Pepstatin was prepared as a homogenised stock suspension containing $7.06 \times 10^{-2}\,mmol\,l^{-1}$ in 0.05 M sodium acetate/acetic acid/buffer at pH 4.0. Serial dilutions of the stock solution were then made using the pH 4.0 buffer. Pepstatin dissolved at concentrations below $1.4 \times 10^{-2}\,mmol\,l^{-1}$ at pH 4.0.

Since pepstatin inhibits the proteolytic activity of aspartic proteinases without any pre-incubation the method adopted for the inhibition experiments was as follows:- to 0.05 ml of an appropriate pepstatin solution were added 1.9 ml of 0.2 M glycine/0.1 M NaCl buffered to pH 2.0 with 0.2 M HCl and containing $3.3\,g\,l^{-1}$ bovine haemoglobin. Hb concentrations of $5.0\,g\,l^{-1}$, $3.0\,g\,l^{-1}$, $0.75\,g\,l^{-1}$ and $0.2\,g\,l^{-1}$ were also used for the kinetic constant determinations. The solutions were equilibrated at 37° for 5–10 min and 0.05 ml of enzyme solution, suitably diluted in 0.001 M HCl for pepsins or 0.05 M sodium phosphate buffer at pH 7.4 for pepsinogens, was then added to start the proteolytic assay. Controls were set up using the acetate buffer (pH 4.0) without pepstatin. Incubations in triplicate were carried out for 30 min at 37° over which time the enzyme reaction was linear. Pig pepsin A was used in these experiments as a control.

The proteolytic activity was then determined by the method in reference[13] as modified.[4] The enzyme substrate incubation was stopped by the addition of 2.0 ml of 3% trichloroacetic acid at 4°C and the precipitated haemoglobin separated by centrifugation at 3000 rpm for 10 min. The soluble peptide fragments in the supernatant were then analysed by the reduction of Folin Ciocalteu's reagent and the resultant blue colour measured at 700 nm related to the amount of enzyme activity.

Note that the addition of the zymogens to the pH 2.0 assay reagent gave a rapid conversion of the pepsinogens into pepsins.[14] The concentration of pepstatin referred to in the text corresponds to the concentration in the final reaction mixture.

The concentration of the individual human pepsins and pepsinogens was estimated, as pig pepsin equivalents, by reference to a pepsin standard curve calibrated for proteolytic activity at pH 2.0 against known concentrations of pig pepsin($0–50\,\mu g\,l^{-1}$). In each experiment approximately the same amount of enzyme proteolytic activity was used.

For the experiments with carbenoxolone and pepstatin the individual human pepsins 1,3 and 5 were pre-incubated with carbenoxolone suspensions at concentrations of $0.81\,mmol\,l^{-1}$ and $3.24\,mmol\,l^{-1}$ in a volume of 0.1 ml at 37° for 30 min.[7] The residual proteolytic activity was then determined as above and the sensitivity to pepstatin was ascertained by incorporation of pepstatin into the buffered substrate solutions in the concentrations stated in the text.

Determination of pH-dependent Inhibition

Solutions of 0.2 M glycine containing 0.1 M NaCl and 0.2 M HCl, each containing $3.3\,g\,l^{-1}$ bovine haemoglobin, were mixed to give a range of solutions from pH 1.6 to pH 4.3. Pepstatin solution 0.05 ml in 0.05 M sodium acetate/acetic acid buffer at pH 4.0, was added to give approximately 50% inhibition of the activity at pH 2.0 (the actual concentrations used are given the legend to Figures 4 and 5). A control pH activity curve was set up by the addition of pH 4.0 buffer without pepstatin. Duplicate readings on solutions containing no enzyme were measured at each pH value as these blank readings differed according to the pH of each test solution.

Demonstration of Pepstatin Binding by Sephadex Chromatography

A mixture of pepstatin and pepsin in 0.05 M KCl/HCl buffer at pH 2.0 was pre-incubated for

10 min at 37° and then passed down a Sephadex G-50 column (2 × 55 cm) equilibrated with the buffer alone, at 15 ml h^{-1} at 4°. The eluate was collected in 1.5 ml fractions using a mini Escargot fraction collector (Anachem Ltd., Luton, UK) over 12–24 h. The proteolytic activity of each fraction was then measured on 0.1 ml sample volumes. Pepsinogens were pre-incubated with pepstatin in 0.05 M sodium phosphate pH 7.4 buffer for 10 min at 37° before application to a column of Sephadex G-50 (2 × 55 cm) at 4° equilibrated with the buffer alone. Fractions, 1.5 ml, were collected over 24 h and the proteolytic activity measured on 0.1 ml aliquots.

Control separations were carried out for pepsins and- pepsinogens, without the addition of pepstatin. The Sephadex G-50 columns were calibrated before use with a mixture of dextran blue, human serum albumin and horse myoglobin.

RESULTS

Pepstatin readily inhibits both human pepsins 1 and 3, each enzyme is inhibited to a similar extent (Figure 1a) and pepsin 5 is approximately 40 times less sensitive. The IC_{50} concentrations of pepstatin resulting in 50% inhibition of approximately equal initial amounts of proteolytic activity are for human pepsin 1, 10.8×10^{-9}M, pepsin 3, 5×10^{-9}M and human pepsin 5, 332×10^{-9}M. The inactivation of the pepsinogens by pepstatin shows the same relative trend (Figure 1b). The pepstatin IC_{50}'s of each pepsinogen fraction were:- pepsinogen 1, 100×10^{-9}M, pepsinogen 3, 1.25×10^{-9}M and pepsinogen 5, 645×10^{-9}M. Pepsinogen 5 is thus inactivated at a pepstatin concentration of approximately twice that at which pepsin 5 is inhibited. Pepsinogen 1 however, requires approximately a 10-fold higher

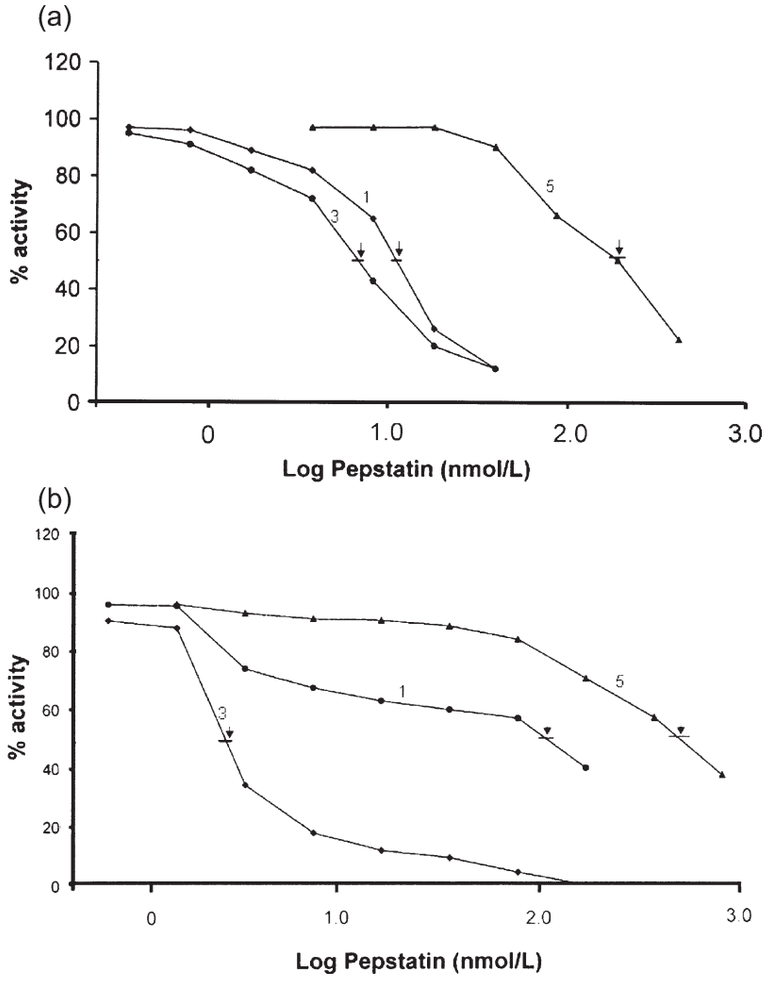

FIGURE 1 (a) Effect of increasing concentrations of pepstatin on the proteolytic activity of human pepsins: ◆ Human pepsin 1, ● human pepsin 3, ▲ human pepsin 5. (b) Effect of increasing concentrations of pepstatin on the proteolytic activity of human pepsinogens:- ● human pepsinogen 1, ◆ human pepsinogen 3, and ▲ human pepsinogen 5. ↓ indicates the point of 50% inhibition (1 nmol l^{-1} pepstatin is approximately 0.7 μg l^{-1}).

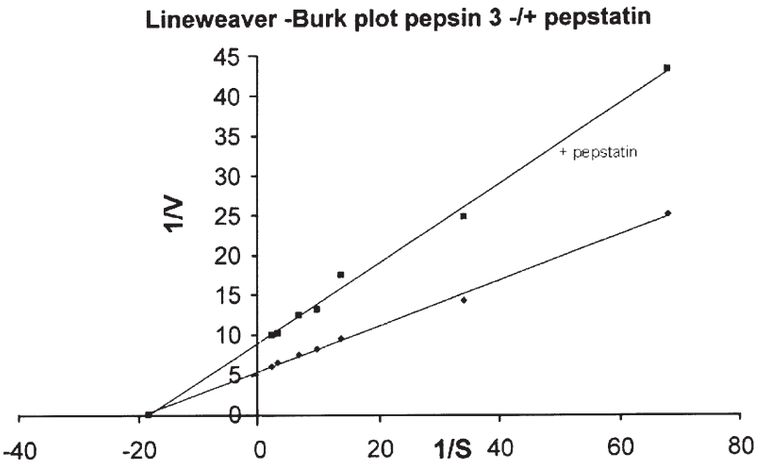

FIGURE 2 Effect of pepstatin upon the substrate-velocity curve for human pepsin 3 and bovine haemoglobin. The results are plotted according to Lineweaver and Burk.[30] Calculations of linear regression indicate that without pepstatin the abscissal, intercept is −20.0, and with pepstatin −19.5. ◆ control, ■ +pepstatin (1.76×10^{-9} M).

concentration of inhibitor than does pepsin 1 and pepsinogen 3 a 5-fold lower concentration than does pepsin 3. These differences probably relate to the amount of active against inactive enzyme or non-enzyme protein present in each preparation.

Calculation of the molar ratios of pepsin to pepstatin binding at 50% inhibition (Figure 1) were for pepsin 1, 1:0.69, pepsin 3, 1:0.75 and pig pepsin A, 1:0.24. For human pepsin 5 the enzyme to inhibitor ratio is 1:40 and the affinity much less.

Inhibition Constants

The Lineweaver and Burk plot for human pepsin 3 with or without pepstatin is shown(Figure 2). The extrapolation of the two lines gave an intercept within experimental error, on the x-axis inferring a non-competitive inhibition. For pig pepsin the Lineweaver and Burk plot for pepstatin inhibition was angulated, thus analysis of the intercept was not possible and no useful information about the type of inhibition was gained.

However, these studies indicate that pepstatin is an inhibitor with a high affinity for pepsin. The inhibitor constants were therefore, determined using the literature method.[15] The mean of the distances between the intersection points V/2, V/3 and V/4 were calculated as inhibitor concentrations. This in effect gives the inhibitor constant Ki, which was 3.15×10^{-9}M for pepsin 1 (Figure 3a). Human pepsins 3 and 5 gave Ki's of 2.44×10^{-9}M and 3.53×10^{-7}M respectively. Thus pepstatin has a very high affinity for the enzyme which is independent of competition from the substrate as shown in (Figure 3b) where the concentrations of pepstatin giving 50% inhibition are relatively constant for varying Hb concentrations for human pepsins 1, (3 and 5 data not shown).

Effect of pH on Inhibition with Pepstatin

The pH-activity curves for the individual human pepsins, and pig pepsin were compared with and without pepstatin (at approximately 50% inhibition of peptic activity measured at pH 2.0) and showed that pepstatin inhibits the pepsins to the same extent over the pH-activity range of each enzyme (Figures 4 and 5). Inhibition remained at approximately 50% of the control values throughout the pH range although at pH's above 3.8 inhibition was not as effective, decreasing at pH 4.3 to 20% or less.

Comparative Pepstatin-binding to Pepsins and Pepsinogens

Human pepsin 3 elutes from Sephadex G-50 after albumin and before myoglobin, and without loss of activity whereas very little of the activity is recovered when the enzyme has previously been incubated with pepstatin (Figure 6a). Pig pepsin behaved similarly, as was also shown by Kunimoto *et al.*[16] However, in the separation of pig pepsinogen after preincubation with pepstatin at pH 7.4, the pepsinogen eluted without loss of activity upon subsequent activation (Figure 6b). Pepstatin is, therefore, absent from the eluant fractions containing pepsinogen, and does not bind to this molecule at pH 7.4. The human pepsinogens 3 and 5 behaved similarly.

Effect of Carbenoxolone and Pepstatin Upon the Pepsins

Pre-incubation of the individual human pepsins with carbenoxolone showed the previously described loss of proteolytic activity when compared with the controls.[7] Addition of pepstatin to

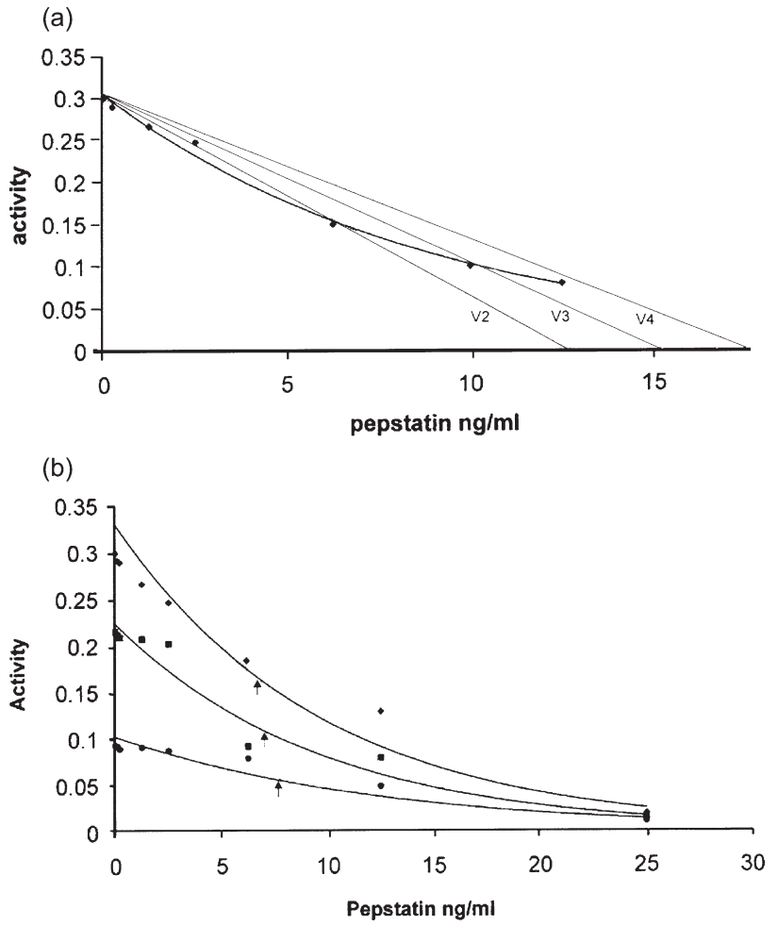

FIGURE 3 (a) Determination of the pepstatin inhibitor constant (Ki).[15] For a non-competitive inhibitor the mean of the distances between the intersection points(V/2, V/3 and V/4) on the baseline give the value of the Ki, namely 3.15×10^{-9} M for human pepsin1 and for pepsins 3 and 5 2.44×10^{-9} M and 3.53×10^{-7} M (respectively data not shown). (b) Effect of varying haemoglobin concentrations on the concentration of pepstatin to give 50% inhibition (shown by the arrow ↑) of human pepsin 1 (pepsins 3 and 5 data not shown). Hb concentrations were ◆ 0.33%, ■ 0.075% and ● 0.03% graphs.

the substrate solution after pre-incubation with carbenoxolone showed further inhibition of human pepsins 1,3 and 5 and of pig pepsin. The sensitivity of the enzymes to pepstatin remained and the approximate concentrations of pepstatin giving 50% inhibition of peptic activity were not altered significantly by pre-treatment with carbenoxolone Table I.

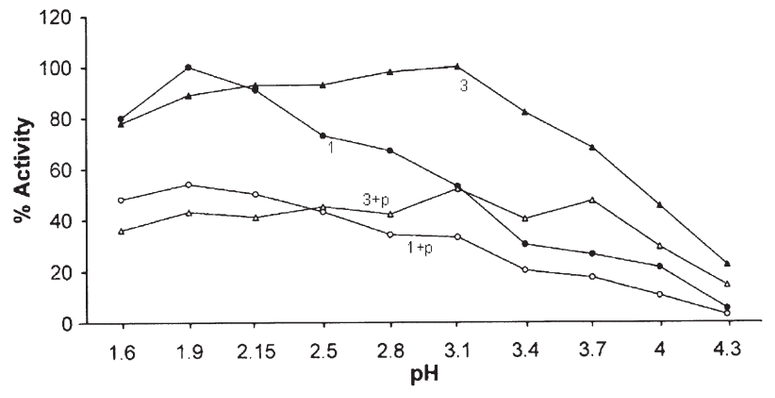

FIGURE 4 Effect of pH on the pepstatin inhibition of human pepsins 1 and 3. ● human pepsin 1 (0.85 ug.) ○ + pepstatin, ▲ human pepsin 3 (0.97 ug.) △ + pepstatin. Pepstatin concentration used was 8.8×10^{-9} M.

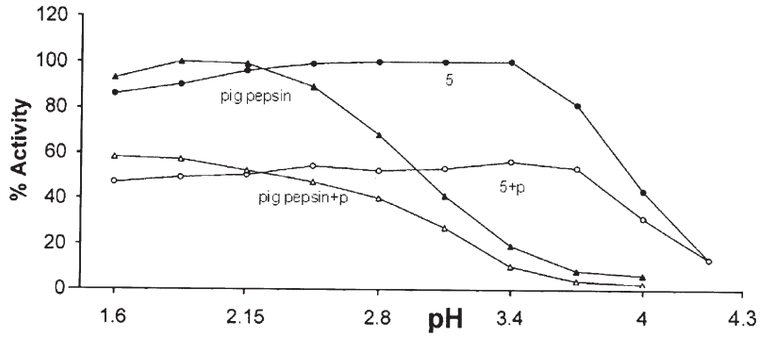

FIGURE 5 Effect of pH on the pepstatin inhibition of human pepsin 5 and pig pepsin. ● human pepsin 5, ○ +pepstatin (353×10^{-9} M), ▲ pig pepsin, △ +pepstatin (3.53×10^{-9} M); 0.81 ug and 1.15 ug were the amounts taken for human pepsin 5 and pig pepsin respectively.

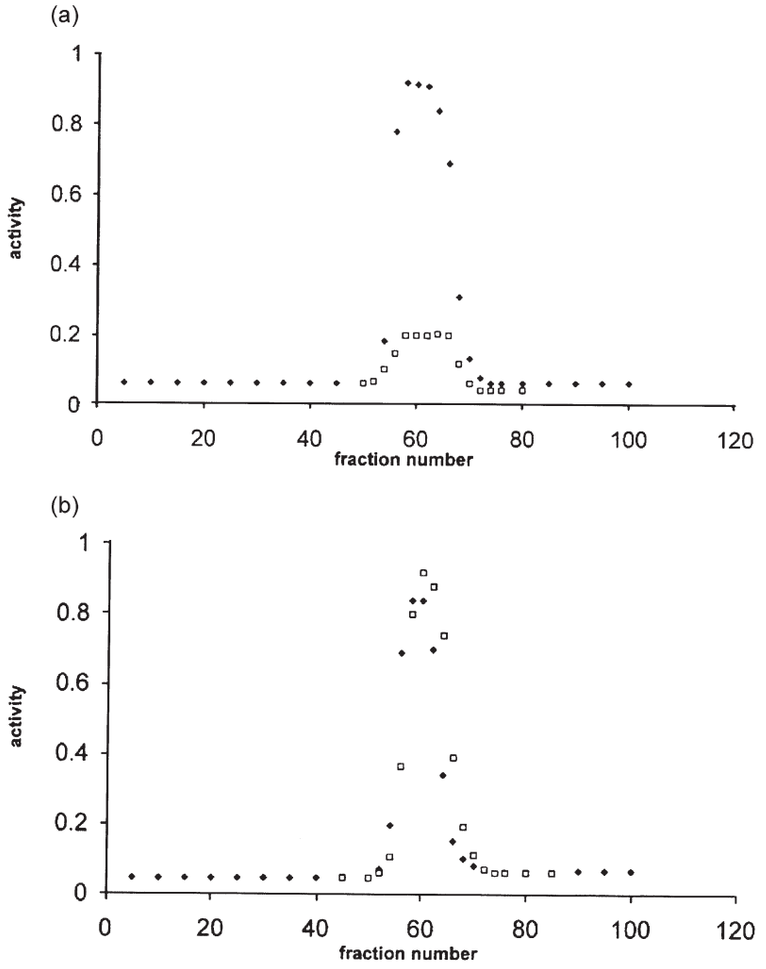

FIGURE 6 (a) Chromatography on Sephadex G-50 at pH 2.0 of a human pepsin 3 pepstatin mixture. Human pepsin 3 (1.0 mg) was pre-incubated with pepstatin sodium salt (0.3 mg) at pH 2.0 in a volume of 2.0 ml and passed down a Sephadex column at pH 2.0 as indicated in the methods. ◆ control pepsin 3 alone, □ pepsin 3 + pepstatin. (b) Chromatography on Sephadex G-50 at pH 7.4 of a pig pepsinogen/pepstatin mixture. Pig pepsinogen 0.3 mg was pre-incubated with pepstatin sodium salt (0.3 mg) at pH 7.4 in a volume of 2.0 ml and passed down a Sephadex column at pH 7.4 as indicated in the methods ◆ control i.e. pepsinogen alone, □ pepsinogen + pepstatin.

TABLE I Inhibition of human pepsins 1,3 and 5 and pig pepsin by pepstatins. Molar ratios of pepsin to pepstatin at 50% inhibition without pre-incubation

	Reaction mixture contents		
	Enzyme moles $\times 10^{-11}$	Pepstatin moles $\times 10^{-11}$	Enzyme/Pepstatin ratio (mol:mol)
Human pepsin 1	3.1	2.14	1:0.69
Human pepsin 1(a)	1.7	1.19	1:0.70
Human pepsin 1(b)	1.7	1.19	1:0.70
Human pepsin 3	2.04	1.44	1:0.71
Human pepsin 3(a)	3.9	2.12	1:0.54
Human pepsin 3(b)	3.9	2.12	1:0.54
Human pepsin 5	1.65	66.4	1:40.2
Human pepsin 5(a)	1.9	77.7	1:40.7
Human pepsin 5(b)	1.9	73.5	1:38.7
Pig pepsin	3.6	0.85	1:0.24
Pig pepsin (a)	3.6	0.92	1:0.26
Pig pepsin (b)	3.6	0.92	1:0.26

Molecular masses used were: pepstatin (sodium salt) 709; human pepsin 1, 43800; human pepsin 3, 37,000; human pepsin 5, 34,600[17] pig pepsin 34,644 Da.[29] (a) and (b) indicate the value obtained after pre-incubation of the enzyme with carbenoxolone at 0.81 mmoll^{-1} and 3.24 mmoll^{-1} respectively (0.5 and 2.0 mg ml^{-1}). The assays were carried out in triplicate with coefficient of variation $<10\%$.

DISCUSSION

These results confirm the earlier observations[2] that pepstatin inhibits human pepsin 3 (human "pepsin") and pepsin 5 ("gastricsin") and that human pepsin 1 is inhibited similarly to pepsin 3. Human pepsin 1 would appear, therefore, to be an enzyme more similar to human pepsin 3 than 5, so far as its inhibition by pepstatin is concerned.

Although the kinetic analysis may have been inappropriate using a large substrate with more than one possible cleavage site, the inhibitory constants obtained for pepsin 1,3 and pig pepsin were similar to the value of 3.0×10^{-9}M determined using *N*-acetyl-L-phenylalanyl-L-diiodotyrosine as substrate.[16] The slightly lower affinity for pepsin 3 compared with the homologous pig pepsin A may relate to the presence of some inactive enzyme in the pepsin 3 preparations. If pepsins 1, 3 and 5 are taken to have molecular masses, respectively, of 43,800, 37,150 and 34,600 Da[17] the pepsin to pepstatin ratios for 50% inhibition can be calculated as 1: 0.69, 1:0.70, 1:40 (Table I) respectively. Thus in the case of pepsins 1 and 3, the inhibition is achieved by a molecule to molecule interaction compatible with pepstatin binding at the active site of the enzymes. This behaviour contrasts sharply with the inhibitory action of carbenoxolone, for which the pepsin to inhibitor ratio is low, leading to the hypothesis[18] that carbenoxolone is binding to amino-acids away from the active site, but hindering access of substrate to the site. This is probably how ecabet Na (a compound similar to carbenoxolone) acts on and reduces pepsin activity.[19] The markedly lower affinity of pepstatin to pepsin 5(gastricsin) needs further explanation but unfortunately data on sequence and structural analysis is still relatively sparse for this enzyme. We therefore, used the Swiss—Model Program (www.expasy.ch) to predict how the inhibitor might be attached to gastricsin (Figure 7a). This was based on homology modelling of human pepsin 3a[20] in comparison with amino-acid differences for the active site region of gastricsin as shown from the sequence analysis of the progastricsin.[21] A close-up of the active site cleft is shown in Figure 7b with the residues that differ significantly from the pepsin archetype. The majority of the active site residues are conserved in both enzymes. However, various significant differences can be seen. For the S3 pocket the most notable mutations are Phe111Asn and Glu12Ala (pepsin numbering) which would be expected to enlarge this pocket significantly. At S2 the pocket becomes smaller and more polar in gastricsin due to the mutations Met289Thr and Gln287Glu. At S1 the gastricsin pocket may be slightly smaller than in pepsin due to the mutation Val30Leu. Thus such differences we suspect must be important as explanation(s) for the reduced affinity of pepstatin to the gastricsin active site.

The dissociation constants for pepstatin inhibition confirm the high affinity of pepstatin for the pepsins, and this affinity explains the failure to separate pepsins from pepsin/pepstatin mixtures on Sephadex columns. In contrast, the pepsinogens can be recovered from pepsinogen-pepstatin mixtures by column chromatography. It is likely therefore, that in the inactive precursor zymogens, as the active site is "protected" from pepstatin by the inhibitory peptide present at the *N* terminus of the molecule[21,22] this impedes access for pepstatin to bind. The reported inhibition of the pepsinogens by pepstatin is thus only explanable by inhibition of the pepsin molecules as they are formed by activation in an acid medium, rather than by a direct action upon the precursors themselves.[23] The pepsin pH activity curves with

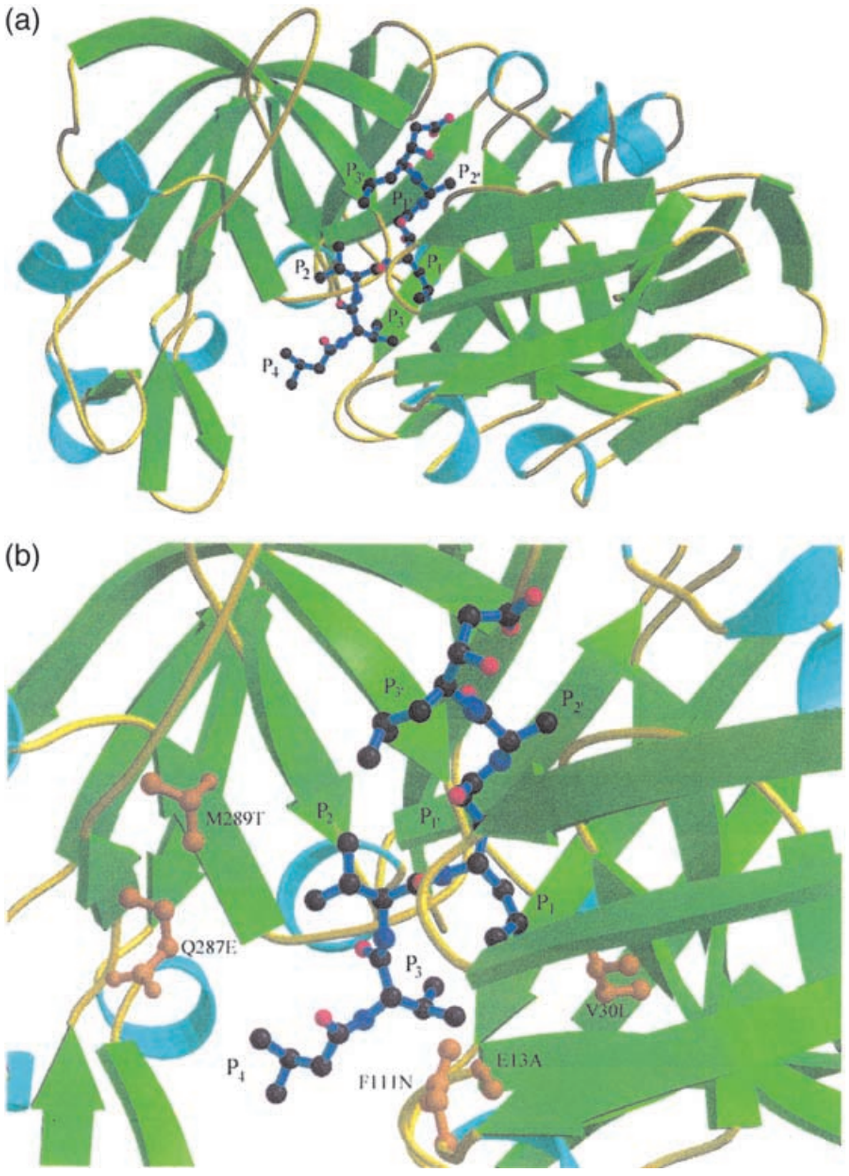

FIGURE 7 Swiss Model Program (www.expasy.ch) to predict pepstatin interaction with the enzymic active site of gastricsin as based on the pepsin 3 model. (a) The figure shows the tertiary structure of the gastricsin model by homology modelling with human pepsin 3. The inhibitor pepstatin is shown bound in the active site cleft. (b) The figure shows a close-up of the pepsin 5(gastricsin) active site cleft with the residues that differ significantly from the pepsin archetype.

and without pepstatin show the same general trend similar to those described previously.[17] The pepstatin binding has relatively constant avidity over the pH range of 1.6–3.8. Similar binding affinities of pepstatin to pepsin at pH 2.0 and at pH 5.5 have been previously observed.[16] However, the present results suggest that pepstatin inhibition tends to decrease at the higher pH's *i.e.* pH 3.8 and above. The apparent changes with increasing pH and the reduced affinity to pepsin 5(gastricsin), as already alluded to, infers more detailed structural analysis is required, as carried out for pepsin (equivalent to pepsin 3).[24]

If the human pepsins play an aetiological role in peptic ulceration, pepstatin, being a potent inhibitor of them all, may be effective therapeutically. Unfortunately pepstatin is relatively insoluble and more soluble forms need to be used which might affect their inhibitory properties. Nevertheless the preventative action of pepstatin (insoluble form) upon gastric ulceration in the pylorus-ligated rat[1] has been observed. Recent observations also confirmed protection from pepsin induced ulceration in a rat model.[25] Interestingly the rat only secretes one pepsin and that behaves electrophoretically as gastricsin.[26] A single study of pepstatin on

the healing rate of gastric ulceration in man showed no protective effect.[27] However, this negative result may have been related to the insolubility of pepstatin in gastric juice with little effect on the proteolytic activity. Clearly more detailed clinical trials of pepstatin in humans are still required maybe in combination with acid secretory inhibitors such as H2-receptor antagonists or proton pump inhibitors. This maybe relevant as pepsin secretion mediated by the vagus may still be functional[28] even though acid output has been reduced.

In conclusion, pepstatin inhibitor studies on the individual human pepsins and pepsinogens 1,3 and 5(gastricsin) show that the pepsin 1 enzyme behaves as pepsin 3 and pig pepsin A. Inhibition of the respective zymogens by pepstatin occurs after activation of the inactive precursor, since binding does not occur with the zymogen. Explanation for the reduced affinity of pepstatin to pepsin 5 requires further detailed structural analysis from enzyme/inhibitor co-crystallisation studies.

Acknowledgements

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