Pepsin 5 in gastric juice: determination and relationship to the alkali-stable peptic activity

V. WALKER AND W. H. TAYLOR

From the Department of Chemical Pathology, Royal Liverpool Hospital, Liverpool

SUMMARY Pure human pepsins 1 and 3 are inactivated by incubation at pH 7.1-7.3 for 30 minutes, losing 90% or more of activity. Pepsin 5 is alkali-stable, retaining 100% of activity. Mixtures of pure pepsins 1 and/or 3 with pepsin 5 were found to have greater alkali-stable activity than predicted. Two published methods for determining the alkali-stable fraction of the peptic activity of gastric juice gave, respectively, in our hands values of 45.4-80.0% and 27.5-43.9% of the total activity. These values seemed too high to be attributable only to pepsin 5 in gastric juice, as agar gel electrophoresis shows pepsin 3 to have the principal activity. Electrophoretograms of alkaline incubated gastric juice revealed that large amounts of pepsin 3 retained activity as well as pepsin 5, and a proteolytic zone '4' appeared between them. Alkali inactivation thus does not allow the estimation of pepsin 5 individually in gastric juice. Pepstatin, at a final concentration of 100 to 170 pmol/ml, may be used to estimate pepsin 5 in gastric juice and gave values of 18.0 to 27.6% of the total peptic activity. Pepsin 5, in gastric juice and in mixtures of pepsins, appears to protect pepsin 3 from alkaline-inactivation, and to a lesser extent from pepstatin inhibition.

Now that the multiplicity of the pepsins of man is established (see review by Taylor, 1962), the need to estimate the pepsins individually in gastric juice is apparent (Taylor, 1970; Walker, 1976; Walker and Taylor, 1976). Turner *et al.* (1967) observed that the 'pepsin 1' of Seijffers *et al.* (1964) was stable at pH 7.0 and showed only a slow loss of activity between pH 7.0 and pH 7.5, whereas pepsins IIA, IIB, and III showed a rapid loss of proteolytic activity at pH 7.0. Turner *et al.* (1967) therefore developed a technique for estimating 'pepsin 1' in gastric juice, based on its alkali-stability at pH 7.24-7.26. Seijffers and Tkatch (1970) reported an alternative method based on the same principle, but using weak alkali and an incubating pH of 7.1.

Because the study of the pepsins has been confused by the use of different systems of nomenclature, Etherington and Taylor (1967) proposed that the pepsins be numbered in order of decreasing mobility to the anode on agar gel electrophoresis. They found subsequently (Etherington and Taylor, 1969) that the 'pepsin 1' of Seijffers *et al.* (1964) was mainly their pepsin 5, the slowest migrating pepsin, as was the 'gastricsin' of Richmond *et al.* (1958). Roberts and Taylor (1973) and Roberts (1975) demonstrated the stability of an electrophoretically homogeneous preparation of pepsin 5 to alkali, and

Received for publication 16 May 1979

the alkali-lability of preparations of the more electronegative pepsins, pepsin 1 and 3.

The primary aim of the present investigation was to determine whether the technique of alkali-inactivation is a reliable way of estimating pepsin 5 individually in a gastric juice. As the shortcomings of the method became apparent, preliminary experiments were undertaken to determine whether use of the acid proteinase inhibitor, pepstatin (Umezawa *et al.*, 1970) might provide a better alternative. Aoyagi *et al.* (1971) had found that, whereas human 'pepsin' prepared from human gastric juice by the method of Richmond *et al.* (1958) is readily inhibited by pepstatin, 'gastricsin' is approximately 100 times more resistant to inhibition than is pepsin. Roberts (1975) found that pepstatin readily inhibited preparations of the electronegative pepsins, pepsins 1 and 3, and to a similar extent, whereas pepsin 5 was approximately 40 times less sensitive to inhibition.

Methods

GASTRIC JUICE

Gastric juice was collected over 15 minute intervals by aspiration *via* nasogastric tube, basally and after pentagastrin injection (6 µg/kg body weight) or intravenous insulin injection (0.15 units/kg body weight).

HUMAN PEPSINS

Electrophoretically homogeneous preparations of human pepsins 1, 3, and 5 and a mixture of pepsins 3 and 5 were kindly donated by Dr N. B. Roberts, who prepared them by chromatography of pooled human gastric juice on DEAE (diethylaminoethyl) cellulose using chloride gradient elution (Etherington and Taylor, 1969; Roberts and Taylor, 1978).

PEPSTATIN

Pepstatin (iso-valeryl-L-valyl-L-valyl-4 amino-3 hydroxy-6-methylheptanoyl-L-alanyl-4 amino-3 hydroxy-6 methyl heptanoic acid) was kindly donated as the sodium salt by Dr K. Goto and Dr H. Umezawa of the Banyu Pharmaceutical Co., Tokyo, Japan.

ESTIMATION OF PROTEOLYTIC ACTIVITY

The method was that of Anson and Mirsky (1932), as modified by Hanley *et al.* (1966) and further by Etherington and Taylor (1969). One 'pepsin unit' is arbitrarily defined as that amount of peptic activity which releases tyrosine and tyrosine-containing peptides from bovine haemoglobin, colorimetrically equivalent to 1 mg standard tyrosine under the conditions of assay.

ALKALI INACTIVATION

Method of Turner et al. (1967)

By preliminary assay, a dilution of pepsin solution or gastric juice was determined with activity in the linear part of the standard proteolytic activity curve for swine pepsin. Solutions of twice this strength were prepared in 0.1 M acetate buffer, pH 5.0. Half was mixed with an equal volume of 0.001 M acetate buffer pH 5.0 (control). The pH of the remainder was increased to 7.12–7.20 by mixing with an equal volume of 0.1 M phosphate buffer, pH 7.80 (test). In some instances 1 or 2 drops of 0.25 M sodium hydroxide were needed to adjust the pH to within this range and an equal volume of pH 5.0 buffer was then always added to the control tube. The samples were mixed well and left at room temperature (21°–23°C). At intervals from one to 48 minutes after addition of alkaline buffer, 0.1 ml of test and control (each in duplicate) was removed and assayed for peptic activity.

This procedure differed slightly from that originally described, in that: (a) preliminary dialysis of the gastric juice samples was omitted, as this was not a procedure used in measuring the total peptic activity of the samples, with which the results were ultimately to be related; (b) the temperature of alkaline incubation was room temperature (21° to 23°C) and not 25°C as described by Turner *et al.*.

Seijffers and Tkatch (1970)

Gastric juice was diluted 1 in 5 with 0.1 M acetate buffer, pH 5.3, and this mixture was further diluted (1 in 10) with 0.02 M phosphate/0.01 M citric acid buffer, pH 7.8, to give a final pH of 7.10 to 7.30. The tubes were covered and left at room temperature (21°–23°C). At exactly 90, 105, and 120 minutes after addition of alkali, 0.1 ml was removed and assayed for peptic activity in duplicate. The mean residual—that is, alkali-stable—activity was calculated. The 'control' activity of each gastric juice—that is, not inactivated—was estimated by diluting each gastric juice first 1 in 5 in 0.1 M acetate buffer pH 5.3, and further diluting this mixture (1 in 10) in 0.02 M phosphate/0.01 M citric acid buffer pH 7.0, with rapid mixing, giving a final dilution of 1 in 50, and a pH of 6.5 to 6.6. Immediately after mixing, 0.1 ml was assayed in duplicate for peptic activity.

ALKALI INACTIVATION FOLLOWED BY AGAR GEL ELECTROPHORESIS

Gastric juice and pepsins were incubated for 30 minutes at room temperature at pH 7.10–7.20 using the method of Turner *et al.*, described above, but with higher concentrations of enzyme solution. Zymograms of the alkaline-preincubated pepsin solutions and of the 'native' pepsin solutions, diluted to the same strength in 0.001 M hydrochloric acid, were obtained by agar gel electrophoresis at pH 5.0, using the technique of Etherington and Taylor (1969) with the following small changes: 15 g/l Ionagar no. 2 was used to prepare the gels; electrophoresis was for two hours 10 minutes, and a human globin substrate concentration of 3.3 g/l in pH 2.0 buffer was used to demonstrate zones of proteolytic activity.

INHIBITION OF HUMAN PEPSINS AND PEPTIC ACTIVITY OF GASTRIC JUICE BY PEPSTATIN

50 µl of each of a series of solutions of pepstatin containing 0.001–25 µg pepstatin/ml (0.0014–35.26 nmol/l) in 0.05 M acetate buffer pH 4.0 was added respectively to tubes containing 1.9 ml bovine haemoglobin substrate pH 2.0; 50 µl of buffer without pepstatin was added to a further, control tube. After mixing, the tubes were incubated at 37°C for five to 10 minutes in a water bath, 0.1 ml diluted pepsin solution or gastric juice was added, and proteolytic activity was determined by the modified Anson and Mirsky procedure.

Results

SENSITIVITY OF INDIVIDUAL PEPSINS TO ALKALI

Using the method of Turner *et al.*, pepsins 1 and 3 were both readily inactivated by preincubation at

pH 7.10–7.30, losing 90% or more of activity. Pepsin 5 was alkali-stable, retaining 100% of activity (Fig. 1). These results confirm the findings of Roberts and Taylor (1973). A mixture of pepsins 3 and 5, on the other hand, retained 63% of the initial activity, despite the preponderance of pepsin 3

solutions, a 'predicted value' for the alkali-stable activity of each pepsin mixture was calculated. In Table 1, the 'predicted activities' of the mixtures are compared with the alkali-stable activities actually observed. In the control assays, the pepsin mixtures had activities close to the predicted. On alkalisiation,

Fig. 1 Sensitivity of human pepsins to alkali. Solutions of pepsins 1, 3, 5 and a mixture of pepsins 3 and 5 were preincubated at pH 7.10–7.30 for 48 minutes at room temperature (21°–22°C). Samples were removed at intervals and assayed with bovine haemoglobin (pH 2.0) for 30 minutes at 37°C for residual peptic activity (A700 nm). *—* pepsin 1. ○—○ pepsin 3. ■—■ pepsin 3+5 mixture. ●—● pepsin 5. An agar gel electrophoretogram of the initial 3+5 mixture is shown.

in the mixture (see accompanying electrophoretogram).

SENSITIVITY OF MIXTURES OF PEPSINS 1, 3, AND 5 TO ALKALI

Solutions of the individual pepsins and mixtures of these solutions were (1) preincubated at pH 7.20 for 30 minutes at room temperature (23°C), using the method of Turner *et al.*, and residual peptic activity was estimated, and (2) preincubated for 30 minutes at pH 5.3 before assay (controls). Knowing the proportions by volume of the solutions of pepsins 1, 3, and 5, respectively, comprising each mixture and the alkali-stable activity of the component pepsin

the mixture of pepsins 1 and 3 behaved almost as predicted, with loss of 89% of peptic activity. Mixtures containing pepsin 5, however, all had more residual activity after alkaline preincubation than predicted. It is clear from Table 1 that the alkali-stable peptic activity is a poor guide to the proportion of pepsin 5 present in the mixture.

ALKALI-INACTIVATION OF PEPSINS IN GASTRIC JUICE

Fifteen minute gastric juice samples collected during the course of pentagastrin tests were each analysed for total and alkali-stable peptic activity by the modified method of Turner *et al.* (15 samples from

Table 1 Effects of alkaline preincubation* and of pepstatin on mixtures of pepsins 1, 3, and 5 (pepstatin concentration: 35.3 pmol/ml)

Pepsin or pepsin mixture (proportions by volume)	Control activity (units/ml)		Alkali-stable activity (units/ml)		Pepstatin-resistant activity (units/ml)	
	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	0.52		0.02		0.10	
3	0.64		0.06		0.12	
5	0.64		0.66		0.62	
1+3 (1:1)	0.54	0.58	0.06	0.04	0.08	0.11
1+5 (1:1)	0.64	0.58	0.50	0.34	0.44	0.36
3+5 (1:1)	0.65	0.64	0.50	0.37	0.42	0.37
1+3+5 (1:1:1)	0.64	0.60	0.46	0.25	0.38	0.28
1+3+5 (1:3:2)	0.69	0.62	0.48	0.26	0.41	0.29
1+3+5 (1:4:1)	0.65	0.62	0.31	0.15	0.31	0.21

*Method of Turner *et al.* (1967) with 30 minute preincubation.

two tests) or by that of Seijffers and Tkatch (six samples from a third test).

The alkali-stable activity ranged from 45.4% to 80.0% of the total activity by the modified method of Turner *et al.*, and from 27.5–43.9% of the total activity by the method of Seijffers and Tkatch.

Electrophoretograms of gastric juice samples incubated at pH 7.10–7.18 for 30 minutes at 22°C (Fig. 2) revealed that large amounts of pepsin 3 retained activity as well as pepsin 5, and that a proteolytic zone '4' appeared between them. Under these conditions this may simply be due to partially

and the resistance of pepsin 5 to pepstatin are shown in Table 1, thus confirming the observations of Roberts (1975).

INHIBITION OF MIXTURES OF PEPSINS 1, 3, AND 5, BY PEPSTATIN

The mixed pepsin solutions tested for alkali-sensitivity were at the same time investigated for their resistance to pepstatin, at a final concentration of 35.3 pmol/ml (Table 1). The mixture containing only pepsins 1 and 3 lost 85% of activity. Mixtures containing pepsin 5 had greater residual activity than

Fig. 2 Agar gel electrophoretograms of swine pepsin and gastric juice samples after preincubation at 22°C for 30 minutes at (a) pH 4.75–5.10; (b) pH 7.10–7.18. Sample 1—swine pepsin, final dilution 17 µg/ml; samples 2, 3, and 4, gastric juice samples applied at a final dilution of 1 in 14. Electrophoresis was for two hours 10 minutes at pH 5.0. 3.3 g/l human globin at pH 2.0 was used as substrate to demonstrate zones of proteolytic activity. NB: Pepsin 1 was not present in this patient. A proteolytic zone 4 is seen in electrophoretograms 3b and 4b.

denatured pepsin 3. Alternatively, it could represent a pepsin/inhibitor complex which migrates in a similar position on electrophoresis (Etherington and Taylor, 1969).

INHIBITION OF INDIVIDUAL PEPSINS BY PEPSTATIN

The sensitivities of preparations of pepsins 1 and 3

predicted, although the differences between the observed and predicted values were smaller than was found for the alkali-preincubated samples.

INHIBITION OF PEPTIC ACTIVITY OF GASTRIC JUICE BY PEPSTATIN

Pepsins 1 and 3 were not completely inhibited by pepstatin at a final concentration of 35.3 pmol/ml

Table 2 Effect of pepstatin on peptic activity of gastric juice collected during insulin/pentagastrin test

Gastric juice sample	Total peptic activity (no inhibitor) (units/ml)	Pepstatin-resistant activity	
		(units/ml)	(as per cent of total activity)
Resting juice	48.4	13.2	27.3
Basal	9.6	2.4	25.0
Basal	20.0	3.6	18.0
Post-insulin 3*	122.4	28.8	23.5
4*	112.0	28.0	25.0
5*	100.0	20.0	20.0
6*	78.4	21.6	27.6
7	59.2	15.2	25.7
8	54.4	12.8	23.5
Post-pentagastrin 1	36.8	8.8	23.9
2	40.8	9.6	23.5
3	30.8	8.0	26.0
4	34.0	8.0	23.5

Final pepstatin concentration in the assay was 176.3 pmol/ml.

*Samples diluted 1 in 200 for assay; all others diluted 1 in 100.

(see above). Using a higher final concentration (176.3 pmol/ml), pepsin 1 retained 6.0%, pepsin 3, 18%, and pepsin 5, 86% of initial activity. The peptic activities of 13 15-minute gastric juice samples, collected during a combined insulin-pentagastrin test, were estimated with and without pepstatin at 176.3 pmol/ml final concentration (Table 2). The pepstatin-resistant activity represented 18.0–27.6% of the combined peptic activity, and this proportion was remarkably constant for widely differing 'control' peptic activities of the samples.

Discussion

From the results presented, it appears unlikely that either of the two methods used for alkali inactivation measured the activity of pepsin 5 individually in gastric juice samples, for the following reasons: firstly, electrophoretograms of gastric juice samples which had been preincubated at pH 7.10–7.18 for 30 minutes showed that, in addition to pepsin 5, relatively large amounts of pepsin 3, and of a pepsin migrating in the position of zone 4 of Etherington and Taylor, retained activity. Secondly, mixtures of solutions of the individual pepsins, which included pepsin 5, had higher alkali-stable activities than was predicted from the alkali-stable activities of the constituent solutions. The explanation for this observation is not known, although the possibility that there may have been some protective interaction between the pepsin molecules cannot be excluded. Such an interaction might also be anticipated between the pepsins of gastric juice, which is a pepsin 5-containing mixture. Thirdly, the values obtained for the percentage of total peptic activity of gastric juice which is alkali-stable – 45.4% to 80.0%, using the modified method of Turner *et al.*, and 27.5% to 43.9% using the method of Seijffers and Tkatch – seem too high to be attributable only to pepsin 5 (Table 3). Tang *et al.* (1959) by subjecting gastric juice to chromatography on Amberlite, estimated the ratio of 'pepsin' to 'gastricsin' to be approximately 4:1. Etherington and Taylor (1969), however, have since shown that 'gastricsin' prepared in this

way, although containing mainly pepsin 5, contains some pepsin 3 also. On visual inspection of agar gel electrophoretograms, pepsin 5 usually, subjectively, accounts for 20–25% of the total proteolytic activity, although the proportion varies between samples. By semi-quantifying pepsin 5 from agar gels in terms of swine pepsin equivalent, and comparing this value with the total peptic activity of gastric juice, Walker (1976) found that pepsin 5 accounted for 4.0–17.0% of the total activity. This range is possibly a little low, as results from two different methods of assay were compared in the calculation.

Turner *et al.* did not quote values for the percentages of peptic activity of gastric juice which was alkali-stable, although in one figure shown in their paper, a gastric juice sample has approximately 33% of residual activity after alkaline preincubation. Seijffers and Tkatch found ratios for the alkali-labile to alkali-stable peptic activity in 20 gastric juice samples of 1.47 to 4.25, so that the percentage of alkali-stable activity was 19.1% to 40.5%. However, 14 of the samples had ratios of 2.03 or lower, giving alkali-stable activity between 33.3% and 40.5%, which would seem too high to be attributable only to pepsin 5. Although these workers found close agreement between the alkali-stable: alkali-labile peptic activity ratios and the ratios of the activities of their 'pepsin 1' to the summated activities of all the other pepsins eluted during chromatography of gastric juice on DEAE cellulose, 'pepsin 1' prepared in this way was found to be a mixture of pepsins 3 and 5 (nomenclature of Etherington and Taylor, 1967) when examined by agar gel electrophoresis, and it is moreover unlikely that the most electro-negative of the pepsins (pepsins 1 and 2) were eluted from the column (Etherington and Taylor, 1969).

From the experiments with pepstatin, it was concluded that the use of this inhibitor may provide a better means of measuring pepsin 5 selectively in gastric juice. No preliminary dialysis of the gastric juice samples is necessary and the total peptic activity and pepstatin-resistant activity may be estimated by the same procedure. Clearly the concentration of inhibitor used is of critical im-

Table 3 Determinations of pepsin 5 ('gastricsin' and 'Seijffers' pepsin 1) as percentage of total peptic activity of gastric juice

Method	Authors	Per cent of total peptic activity
Chromatography on Amberlite	Tang <i>et al.</i> (1959)	Approx. 20
Alkali-inactivation: method of Turner <i>et al.</i>	Turner <i>et al.</i> (1967)	33.0 (approx.; one sample only)
	Walker (1976)	45.4–80.0
Alkali-inactivation: method of Seijffers and Tkatch	Seijffers and Tkatch (1970)	19.1–40.5
	Walker (1976)	27.5–43.9
APDT hydrolysis*	Chiang <i>et al.</i> (1966)	Basal secretion: 24.8–41.9; histamine-stimulated secretion: 23.8–51.0
Agar gel electrophoresis	Walker (1976)	4.0–17.0 (approx.)
Resistance to pepstatin	Walker (1976)	18.0–27.6

*Acetyl phenylalanyl L-di-iodotyrosine.

Fig. 3 Inhibition of pepsins 1, 3, and 5 by pepstatin. Final concentrations of pepstatin are given. *—* pepsin 1. O—O pepsin 3. ●—● pepsin 5. (after Roberts, 1975).

portance, if pepsins 1 and 3 are to be inhibited maximally and pepsin 5 is to retain full activity. A final pepstatin concentration of 176.3 pmol/ml almost met the requirements; from the data of Fig. 3 it would seem that final concentrations from about 100 pmol/ml upwards would give a similar result; pepsin 1 is almost completely inhibited at these levels, pepsin 3 retains about 15% of its activity, and pepsin 5 loses about 15% so that the latter two effects tend to balance out each other.

The protective action of pepsin 5 on pepsin 3 during exposure to alkaline conditions is a barrier to our analytical techniques, but is, nevertheless, a remarkable biochemical phenomenon which is at present unexplained. Physiologically, the pepsins of gastric juice meet an alkaline environment as soon as they enter the duodenum. It is widely believed that their proteolytic action then ceases because they are progressively destroyed and because they are in any case inactive above pH 4.0. Our observations (for 30 minutes at 21–23°C) suggest that destruction may not be as complete as might be imagined but it is still difficult to conceive of any further physiological, or pathological, role for the pepsins, as the hydrogen ion concentration in the intestine is unfavourable.

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