

Ovalbumin Digestion by Human Pepsins 1, 3 and 5

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1. Of the three major human pepsins, pepsin 1 has greater proteolytic activity towards ovalbumin than has pepsin 3. Pepsin 5 has low activity towards this substrate. 2. Proteolytic pH-activity curves show only one pH maximum, about pH 1.4 for pepsin 1, pH 1.4–1.5 for pepsin 3 and pH 1.2–1.4 for pepsin 5. The curve for pepsin 3 has a shoulder between pH 2.4 and 3.4. 3. The rate of digestion of ovalbumin by pepsin 1 is approximately three times slower than are those of bovine haemoglobin or human globin. 4. The results suggest that there may be a physiological advantage in having more than one pepsin.

Man secretes at least five pepsins, which may be numbered 1, 2, 3a, 3 and 5 in order of decreasing electrophoretic mobility at pH 5.0 (Etherington & Taylor, 1969). Pepsins 1, 2, 3 and 5 have been isolated (Roberts & Taylor, 1972; Roberts & Taylor, 1978), and knowledge of the properties of the individual pepsins is accumulating (Roberts, 1975). Pepsin 1, the most electronegative pepsin, is associated with chronic peptic ulceration, being secreted more often and in increased quantity, after histamine stimulation, by patients with ulcer than by normal subjects, (Taylor, 1970). In previous work, Taylor (1956, 1959) found that, with egg albumin as a substrate, the pH-proteolytic activity curves of gastric juice from patients with peptic ulceration often had two pH maxima (at pH 1.5–1.6, and pH 2.6–3.1), whereas those of gastric juice from normal subjects had only a single pH maximum (at pH 1.5–1.8). The question arises therefore as to whether pepsin 1 might be responsible for the second pH maximum of patients with peptic ulceration. If this were so, digestion of egg albumin at this pH might be a useful means of determining pepsin 1 in gastric juice in the presence of the other pepsins.

Experimental

Gastric juice was obtained via nasogastric tube from patients with chronic peptic ulceration during tests of acid output after pentagastrin or insulin stimulation. 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-cellulose by using chloride gradient elution, as described by

Etherington & Taylor (1969) and Roberts (1975). Porcine pepsin was a crystalline product from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.

Determination of proteolytic activity

(1) *Bovine haemoglobin as substrate.* The method was that of Anson & Mirsky (1932) as modified by Hanley *et al.* (1966), and further by Etherington & Taylor (1969). Diluted gastric juice or pepsin solutions were incubated for 30 min at 37°C with bovine haemoglobin (Armour Pharmaceuticals Eastbourne, East Sussex, U.K.), 3.3 g/litre in 0.2 M-glycine/0.1 M-NaCl buffer adjusted to pH 2.0 with 0.2 M-HCl. A unit of pepsin is arbitrarily defined as that amount which releases tyrosine and tyrosine-containing peptides from the substrate colorimetrically equivalent to 1 mg of standard tyrosine under the conditions of assay in 30 min.

(2) *Ovalbumin as substrate.* Substrate solutions of the required pH (in the range 1.0–5.0) were prepared by the dropwise addition of ovalbumin (3.3 g/litre; electrophoretic purity 99%; BDH Chemicals, Poole, Dorset, U.K.) dissolved in 0.2 M-HCl, to ovalbumin (3.3 g/litre) in 0.2 M-glycine/0.1 M-NaCl buffer, the mixture being stirred continuously. Proteolytic activity was determined as in (1) above, but incubation was for either 4 or 16.5 h. The tubes were capped to minimize water loss by evaporation, and were shaken throughout the incubation period.

Agar-gel electrophoresis

Gastric juice and pepsin solutions, appropriately diluted in 1 mM-HCl, were examined by agar-gel electrophoresis, as described by Etherington & Taylor (1969) with the following modifications:

Ionagar no. 2 (15g/litre; Oxoid, London E.C.4, U.K.) was used to prepare the gels; electrophoresis was for 2h 10min, and the substrate used to demonstrate proteolytic activity after electrophoresis was usually human globin (3.3g/litre; prepared by the method of Ito *et al.*, 1964), in 0.2M-glycine/0.1M-NaCl/0.2M-HCl buffer, pH2.0. In one series of experiments, ovalbumin (3.3g/litre; in pH1.4 buffer) was the substrate. A total incubation period of 1.5h was used with globin as substrate, and of 13.5h with ovalbumin. After staining with Ponceau S, the gels were transferred to transparent film (Dupont Photo Products, St. Neots, Huntingdon, Cambs., U.K.), dried at 60–70°C, and negative photographs obtained by using a standard office copier (Dalcopier, W. A. Goddard, Manchester 12, U.K.).

Results

pH-proteolytic activity curves

In Figs. 1(a) and 1(b) are shown the pH-proteolytic activity curves of human pepsins 1, 3, and 5, and of one gastric-juice sample, acting on ovalbumin over the pH range 1.0–5.0, and with incubation at 37°C for 4h. An agar-gel electrophoretogram (globin substrate) corresponding to the gastric juice sample is shown; it can be seen that pepsin 1 was present in moderate amount in this sample, which was aspirated from a patient stimulated by insulin hypoglycaemia. The purified pepsin solutions were diluted so that they had similar peptic activities

when assayed for 30min with bovine haemoglobin by the modified Anson & Mirsky (1932) method; thus pepsin 1 had 0.43 pepsin unit of activity/ml, pepsin 3, 0.55, and pepsin 5, 0.49 unit/ml. The diluted gastric juice was more active, having 0.74 unit/ml.

The pepsins were each found to have only a single pH maximum, around pH1.4 for pepsin 1, pH 1.4–1.5 for pepsin 3, and pH1.2–1.4 for pepsin 5. The curve of pepsin 3 had a shoulder between pH2.4 and 3.4. No additional pH maxima were found for any of the pepsins when a longer incubation period (16.5h) was used. Pepsin 1 was approximately three times as active as pepsin 3 when compared with the activities of the enzymes towards haemoglobin, whereas pepsin 5 had relatively little activity towards ovalbumin. The pH-activity curve of the gastric juice showed peak activity between pH1.2 and 1.4, and also had a shoulder as the pH increased to 3.0. The appearance of the curve was that which might be expected from a mixture containing pepsins 1 and 3.

Peptic digestion of ovalbumin at pH 1.4

Figs. 2(a), 2(b) and 2(c) compare the proteolytic activities of pepsins 1, 3 and 5 respectively with the substrates bovine haemoglobin (pH2.0), human globin (pH2.0) and ovalbumin (pH1.4). The pH values approximate to the respective pH maxima with each substrate (Roberts & Taylor, 1972). The proteolytic activity of all three pepsins is greater with

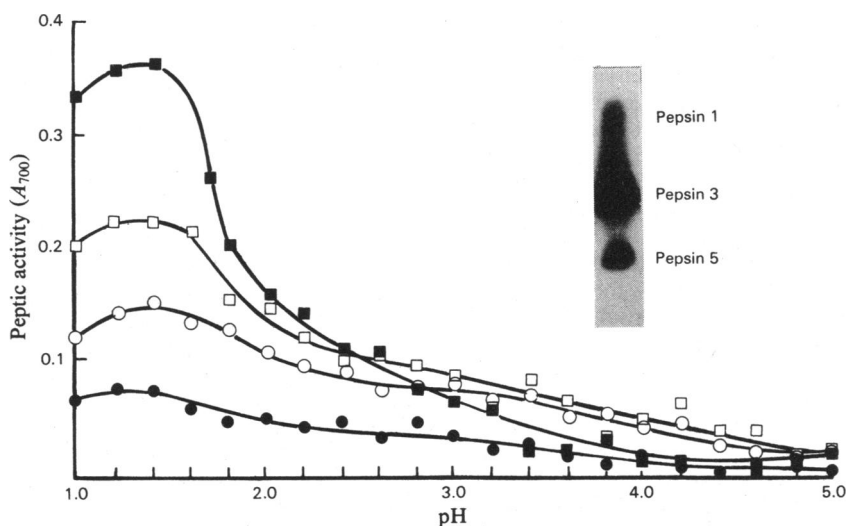


Fig. 1. *pH-activity curves of a sample of human gastric juice and human pepsins 1, 3 and 5 towards ovalbumin as substrate* Incubation was for 4h at 37°C. The pepsin-1 solution had 0.43 unit of peptic activity/ml in the standard proteolytic assay (haemoglobin substrate, 30min incubation), and the solutions of pepsin 3, pepsin 5 and the diluted gastric juice, activities of 0.55, 0.49 and 0.74 unit/ml respectively. The inset shows an agar-gel electrophoretogram of the gastric juice. Key to symbols: □, human gastric juice; ■, pepsin 1; ○, pepsin 3; ●, pepsin 5.

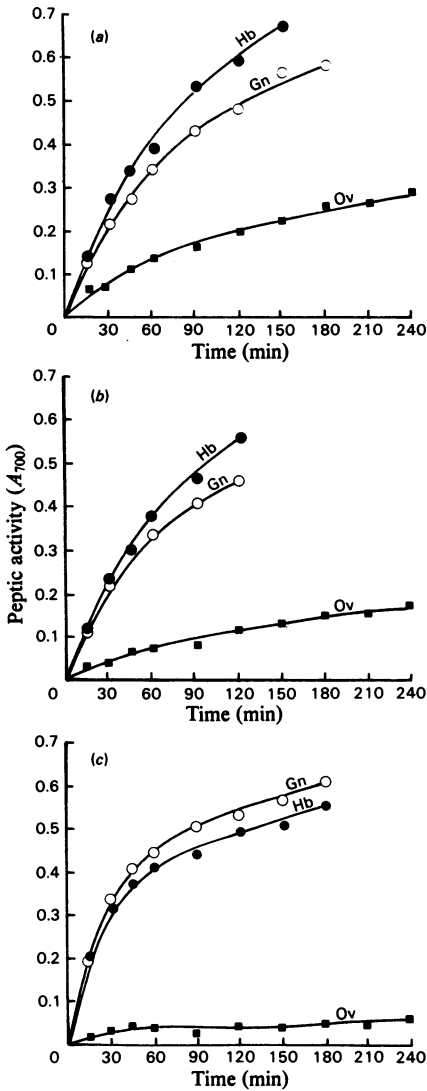


Fig. 2. Time course of the proteolytic activities of pepsins 1, 3 and 5 towards ovalbumin (Ov, ■), bovine haemoglobin (Hb, ●) and human globin (Gn, ○)

Final substrate concentrations were 3.3g/litre. The pepsin solutions used in the experiments had the following activities in the standard proteolytic assay (haemoglobin substrate, 30min incubation): pepsin 1 (a), 0.58 unit/ml; pepsin 3 (b) 0.53 unit/ml (human globin and bovine haemoglobin experiments); pepsin 5 (c), 0.58 unit/ml (ovalbumin experiment); pepsin 5 (c), 0.68 unit/ml. Bovine haemoglobin and human globin were digested at pH2.0, and ovalbumin at pH1.4.

5. Ovalbumin has a probable mol.wt. of 43000 (Castellino & Barker, 1968), which is lower than that of haemoglobin, so that this effect is seen despite the somewhat higher molar concentration of ovalbumin than of the other substrates in the digestion mixtures.

The time courses of proteolysis with ovalbumin as substrate show that pepsin 1 was about twice as active as pepsin 3, as compared with bovine haemoglobin, and was more than four times as active as pepsin 5.

Specific activities of human pepsins 1, 3 and 5 for ovalbumin

Roberts (1975) found the specific activities for bovine haemoglobin of the pepsins 1, 3 and 5 used in the present study to be respectively 0.23, 0.37 and 0.45 μmol of tyrosine released/h per μg of protein.

Specific activities for ovalbumin were determined in duplicate, by using the 60-min points from the data of Fig. 2 and from a second experiment. The values were: pepsin 1, 0.083 and 0.079, mean = 0.081; pepsin 3, 0.075 and 0.073, mean = 0.074; pepsin 5, 0.034 and 0.044, mean = 0.039 μmol of tyrosine liberated/h per μg of protein. Pepsin 1 thus digests this substrate more readily than do the other pepsins.

Peptic digestion of ovalbumin after electrophoresis

Solutions of human pepsins, porcine pepsin and human gastric-juice samples were subjected to

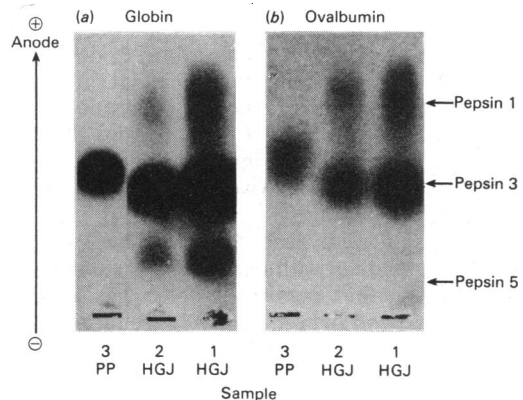


Fig. 3. Agar-gel electrophoretograms of human gastric juice (HGJ, samples 1 and 2) diluted 1 in 15, and porcine pepsin (PP, sample 3,) 17 $\mu\text{g}/\text{ml}$

The gels were incubated with (a) human globin (3.3 g/litre; pH2.0) for 1.5h at 37°, or (b) ovalbumin (3.3g/litre; pH1.4) for 13.5h at 37°C after electrophoresis to demonstrate zones of proteolytic activity. The amount of pepsin 1 in gastric juice is less than that of pepsin 3 so that, even with ovalbumin, pepsin 3 gives the denser spots. The pepsin 1 spots have increased in density, relative to the pepsin 3 spots, with ovalbumin as substrate.

bovine haemoglobin and human globin than with ovalbumin, and this difference is greatest for pepsin

electrophoresis at pH5.0. Two identical gels were prepared, and the same dilution of each pepsin solution was applied to each plate. After electrophoresis, one gel was incubated with human globin substrate, pH2.0, for 1.5h, and the second with ovalbumin substrate, pH1.4, for 13.5h. Representative electrophoretograms for porcine pepsin and human gastric juice are shown in Fig. 3. Although pepsin 3 appears as the predominant pepsin in the gastric-juice samples on both gels, the proteolytic zone on the 'ovalbumin' gel is smaller and less intense relative to the pepsin-1 zone than it is on the 'globin' gel. Porcine pepsin clearly digests ovalbumin less well than does pepsin 1, yet the opposite is the case with globin as substrate. Pepsin 5, having little action on ovalbumin, is not seen on the 'ovalbumin' gel.

Discussion

With enzyme concentrations that gave approximately equal activities towards haemoglobin, pepsin 1 was found to be two or three times more active than pepsin 3 towards ovalbumin, whereas pepsin 5 had little activity with this substrate. Tang *et al.* (1967) had previously observed that the rate of hydrolysis of egg albumin by 'pepsin' was twice that by 'gastricsin', but no conditions of assay were defined. The effect of pepsin 1 on egg albumin has not been studied previously.

The rate of proteolytic digestion of ovalbumin, even by pepsin 1, is considerably slower than the rate of hydrolysis of bovine haemoglobin or of human globin (Figs. 2a-2c). Nevertheless, the present observations provide, for the first time, evidence that there may be a physiological advantage in having more than one pepsin; the demonstration of a greater activity of pepsin 1 than pepsins 3 and 5 towards ovalbumin might indicate a role for pepsin 1 in initiating the breakdown of this and other similar proteins difficult to digest.

The pepsins were each found to have only a single pH maximum with ovalbumin as substrate. It would appear that pepsin 1, which has a single maximum around pH1.4, probably would not have accounted for the second pH maximum at pH2.6-3.1 observed by Taylor (1959) when gastric juice from patients with peptic ulceration digested egg albumin. Pepsin 3, which has a 'shoulder' in the pH-proteo-

lytic activity curve between pH2.4 and 3.4, might have been responsible. However, proteolytic activity in the present work was assayed as the amount of tyrosine and soluble tyrosine-containing peptides released from the substrate, whereas Taylor (1959) used the technique of direct formol titration, which determines the carboxy groups of all split peptide bonds. Moreover, the egg albumin substrate used by Taylor (1959) was not electrophoretically pure, showing two major and a number of minor components on paper electrophoresis with barbiturate buffer at pH7.9 (Taylor, 1956). Possibly the differences in analytical techniques may have accounted for the failure to demonstrate clearly a second peak of activity for any of the pepsins in the present study.

The finding that pepsin 1 is more active towards ovalbumin than are the other pepsins adds to the existing evidence (Roberts & Taylor, 1972, 1978) that this enzyme has a separate identity from the other pepsins. This conclusion is supported by the fact that it arises from a separate zymogen, pepsinogen 1 (Etherington & Taylor, 1970).

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