Minor Proteases in the Stomach of the Pig

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1. Pepsins C and D and probably pepsin B as well as the major enzyme pepsin have been detected on starch-gel electrophoretograms of concentrates of the gastric contents of four pigs. 2. The presence of pepsin B is further indicated by a high ratio of peptidase activity to haemoglobin-digesting activity after treatment at pH 6.9.

Previous reports have described the isolation of three minor gastric proteolytic zymogens, pepsinogen B (Ryle, 1965a), pepsinogen C (formerly called parapepsinogen II) (Ryle, 1960) and pepsinogen D (Lee & Ryle, 1963; Ryle, 1965a), from the gastric mucosa of the pig and the isolation of pepsins B and C (formerly parapepsins I and II) from crude commercial pepsin (Ryle & Porter, 1959). The third minor enzyme, pepsin D, has also been found in crude and crystalline commercial pepsin preparations (D. Lee & A. P. Ryle, unpublished work).

Since commercial pepsin preparations are apparently made by acid extraction of the gastric mucosa, the reports quoted above do not provide any evidence that the minor zymogens, or their corresponding enzymes, are secreted into the stomach. This paper presents evidence that the minor enzymes are, as expected, found in the stomach contents.

EXPERIMENTAL

Materials. Haemoglobin was bovine haemoglobin enzyme substrate powder (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex). Acetyl-L-phenylalanyl-L-di-iodotyrosine was prepared as described by Baker (1951) and had m.p. 231° (decomp.) and $[\alpha]_D^{20} + 30.5 \pm 0.8^\circ$ (c 2 in pyridine).

Hydrolysed starch was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

Dialysis tubing (Visking) was obtained from Hudes Merchandising Corp. Ltd., London, W. 1.

Polyethylene glycol (Carbowax 20M), mol.wt. 15000–20000, was obtained from Union Carbide Ltd., London, W. 1.

Preparation of samples. The stomachs were removed from castrated male bacon pigs about 5 months old as soon as possible after slaughter, i.e. 15–20 min. after electrical stunning, and the contents were emptied into jars kept on ice for transport to the laboratory. The pH of the contents was measured, the total contents were then filtered with the aid of Hyflo Super-Cel and the filter was washed with 0.1 M-acetate buffer, pH 5.6. The filtrate and washings

were combined and concentrated to about 20 ml. by dialysis in 24/32 in. Visking tubing against polyethylene glycol and the proteolytic activity against haemoglobin was then measured.

The activation of any remaining zymogens was now completed by acidification to pH 2 with dilute HCl for 30 min. at room temperature. The pH was then readjusted to 5 by addition of M-acetate buffer, pH 5.6, and the preparations were dialysed against 0.1 M-acetate buffer, pH 5.6, and finally concentrated by dialysis against polyethylene glycol in 9/32 in. Visking tubing to give about 1 ml. of dark-green solution.

Although polyethylene glycol is known to enter the dialysis sac (Ryle, 1965b) its presence was found not to interfere with the activity determinations and electrophoresis.

Electrophoresis in starch gel. Samples (0.01 ml.) of the concentrated preparations were applied to segments (9 mm. $\times$ 5 mm.) of Whatman 3MM filter paper, which were then inserted into a horizontal gel prepared in the usual way beside samples of pepsin and of pepsins B, C and D, all of which were 2 mg./ml. After the electrophoretic run the gel was sliced horizontally and the two cut surfaces were used for detection of the enzymic activities.

Detection of activity with haemoglobin. The method used is based on that of Uriel (1960). The gel was covered with a piece of Whatman no. 1 filter paper moistened with a mixture of 2.5% haemoglobin and 0.315 N-HCl (4:1, v/v). The paper was removed after 20 min. and after a further 20 min. at room temperature the gel was stained for protein with Naphthalene Black 10B (G. T. Gurr Ltd., London, S.W. 6) in the usual fashion. The enzymes are detected as a white area on a blue ground.

Detection of activity with gelatin. A warm solution of 5% gelatin in 0.05 N-HCl containing sufficient Congo Red to give a deep-purple-blue colour was poured on to the gel and allowed to set. After 15–20 hr. at room temperature the gel was gently washed under the cold tap, leaving white areas where the enzymes had liquefied the gelatin. On the original gel these areas were clearly distinguishable from the light areas caused by the uneven thickness of the gelatin layer.

Determination of activity with acetyl-L-phenylalanyl-L-di-iodotyrosine and haemoglobin. These assays were carried out as described by Ryle (1965a) and Ryle & Porter (1959)

respectively. The units used are μ moles of di-iodotyrosine liberated/min. and proteolytic units ([P.U.]^{ab}).

RESULTS

Preparation of samples. Four stomachs were examined; the total activity found in each was about 0.8 [P.U.]^{ab}, corresponding to 30–40 mg. of pepsin, so that the solutions applied to the starch gel were equivalent to 30–40 mg. of pepsin/ml. This is much more than is necessary for the detection of a single enzyme but a considerable overload was used to allow the detection of minor components. The pH of the stomach contents varied from 4.5 to 5.1, and since it was thus possible that they contained incompletely activated zymogens they were treated at pH 2 at room temperature for 30 min. to complete the activation before electrophoretic examination.

Electrophoresis in starch gel. In preliminary experiments I failed to find any conditions in which all four enzymes could be separated in a single run. Pepsin and pepsin D can be separated by electrophoresis at pH 3.2 but at this pH the separation of pepsins B and C from one another is marginal. These two enzymes can be separated from one another at pH 5.6, when they migrate towards the anode (Ryle & Porter, 1959), but it was found that the faster-migrating pepsin in the concentrates left a trail that interfered with the detection of pepsins B and C. It was decided to use pH 3.2 for separation of pepsin, pepsin D and pepsins B plus C and to rely on the differential specificity and stability of the enzymes for the identification of the last pair.

Figs. 1 and 2 show the results from a single run. In Fig. 1 it is apparent that the pepsin and pepsin

D standards are each contaminated with the other but the enzymes are detected at 1.8 and 0.7 cm. in the direction of the anode respectively. Pepsin B has little or no activity against haemoglobin, the weak activity being possibly due to contaminating pepsin C. All four samples of stomach contents clearly show pepsin C at 7 cm. in the direction of the cathode, and despite the heavy load of pepsin it was just possible in the original to detect pepsin D in all four samples as well.

In Fig. 2 the presence of pepsin and pepsin D is clear in all four samples. On the cathodic side of the origin there is a suggestion of two zones of activity of which the slower, more intense one would be due to pepsin B and the faster, weak one due to pepsin C, which has about one-fifth the gelatin-liquefying activity of pepsin B as measured by fall in viscosity of a solution (Ryle & Porter, 1959). However, the separation of pepsin B from pepsin C under these conditions is too slight to allow the certain identification of pepsin B in the presence of pepsin C.

Judged by the intensity of the spots of pepsin C and pepsin D these enzymes may each be about 1% of the total.

Activity after treatment at pH 6.9. Pepsin C has little or no activity with acetyl-L-phenylalanyl-L-di-iodotyrosine as substrate at pH 2 and is slowly inactivated at pH 6.9 (Ryle & Porter, 1959), whereas pepsin B attacks this substrate at pH 2 and is stable at pH 6.9. Both pepsin D (D. Lee & A. P. Ryle, unpublished work) and pepsin are rapidly inactivated at pH 6.9. Thus pepsin B can be identified by its activity (stable at pH 6.9) with the acetyl-peptide as substrate at pH 2.

Samples of the concentrated stomach contents were dialysed against 0.02 M-phosphate buffer, pH 6.9, in the presence of toluene to inhibit bac-

Fig. 1. Starch-gel electrophoresis of concentrates of gastric contents. The conditions used were: 0.05 M-citrate buffer, pH 3.2; 2.5 v/cm.; 22 hr. Activity was detected with haemoglobin. B, C, D and P: Markers of pepsins B, C and D and of pepsin; 1–4: four concentrates. A tracing of a photograph is shown.

Fig. 2. Starch-gel electrophoresis of concentrates of gastric contents. The conditions and symbols were as defined in Fig. 1, but the activity was detected with gelatin. A tracing of a photograph is shown.

terial growth for 24 hr. at 2° and then for 39 hr. at room temperature. A precipitate was removed from each by centrifugation and the activities of the supernatants against the acetyl-peptide and against haemoglobin were determined.

The ratios (μ moles of di-iodotyrosine liberated/min./[P.U.]¹⁰⁰) found were 41, 46, 53 and 90, whereas the value for pepsin or pepsin D is 6.8–7.0. The activity of the samples against haemoglobin was decreased to less than 0.1% of the initial value but it is not clear why even this small activity remained. It is possible that one or more of the enzymes is stabilized by the presence of some other material in the concentrated gastric contents.

If the activity against the acetyl-peptide after treatment at pH 6.9 is correctly ascribed to pepsin B the amount found indicated that this enzyme accounted for at least 0.15–0.5% of the total enzyme in the stomachs. The value would be higher if any activity was lost with the precipitate removed by centrifugation.

DISCUSSION

All the proteolytic components detected in concentrates of pig stomach contents by electrophoresis in starch gel or by their specificity and stability properties can be accounted for as one or

another of the known enzymes pepsins B, C or D or pepsin itself. All four have been detected shortly after death of the animals in the stomach of each of the four pigs examined although the amounts (relative to pepsin) of the minor components pepsins B, C and D appear to be rather less than those (4, 12 and 6% respectively; Ryle, 1965a) of the corresponding zymogens found in neutral extracts of the gastric mucosa. However, the estimate of the relative amounts of the enzymes made here is not at all accurate.

It appears likely that the three minor zymogens are secreted into the stomach during life although the possibility has not been excluded that they are only liberated during the trauma of death.

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REFERENCES

- Baker, L. E. (1951). *J. biol. Chem.* **193**, 809.
- Lee, D. & Ryle, A. P. (1963). *Biochem. J.* **87**, 44p.
- Ryle, A. P. (1960). *Biochem. J.* **75**, 145.
- Ryle, A. P. (1965a). *Biochem. J.* **96**, 6.
- Ryle, A. P. (1965b). *Nature, Lond.*, **206**, 1256.
- Ryle, A. P. & Porter, R. R. (1959). *Biochem. J.* **73**, 75.
- Uriel, J. (1960). *Nature, Lond.*, **188**, 853.