Demonstration of Chymosin (EC 3.4.23.4) in the Stomach of Newborn Pig

By BENT FOLTMANN, PETER LØNBLAD and NILS H. AXELSEN
*Institute of Biochemical Genetics and The Protein Laboratory, University of Copenhagen,
Copenhagen, Denmark*

(Received 24 October 1977)

The stomach of newborn pig contains a proteinase that is immunologically closely related to calf chymosin (rennin) (EC 3.4.23.4). None of the pepsins from the stomach of adult pig is present in the newborn pig. Pig chymosin has optimal general proteolytic activity around pH 3.5. The ratio of milk-clotting activity to general proteolytic activity is about 30-70 times higher than that of pyloric and fundic pepsins.

Information about foetal or neonatal proteinases in mammalian gastric juice other than that of ruminants is only sparse. From investigations on proteolytic and milk-clotting activities Holter & Andersen (1934) concluded that no chymosin-like (rennin-like) enzyme was present in the gastric juice of young dogs and children. Similarly, Malpress (1967) was unable to observe a chymosin-like enzyme in the gastric juice of children, whereas Hirsch-Marie *et al.* (1976), using immunological methods, observed a human foetal pepsinogen that did not cross-react with antibodies against adult human pepsinogens. Henschel (1973) and Kotts & Jenness (1976) observed that the proteolytic activity of the gastric juice of young rabbits and rats has a pH optimum around 4. From this they inferred the presence of a chymosin-like enzyme in the gastric juice of these young animals.

In the present paper we demonstrate the presence of a neonatal proteinase in the stomach of piglets. The apparent similarity to calf chymosin is so great that we will use the name pig chymosin. For comparison, extracts of mucosa from adult pig are also examined.

Materials and Methods

Stomachs from piglets were obtained from either fully developed but still-born piglets or piglets that died during their first 8 h of life. The stomachs were removed as soon as death was observed. After being flushed with water they were stored frozen.

Stomachs from 6-month-old pigs were removed immediately after slaughter, and turned inside out, washed with tap water and stored frozen.

Extraction of mucosa

The mucosa was macroscopically of the same type throughout the piglet stomach, and all mucosa was scraped off. From six stomachs 13 g of mucosa was

obtained. Extraction with 130 ml of distilled water was carried out in a Potter-Elvehjem homogenizer for 5 min in an ice bath. The tissue was removed by centrifugation (7700g for 15 min at 2°C) and 113 ml of turbid extract (pH 6.3) was obtained. Samples were taken for immunological tests, and mucous substances were removed by formation of an aluminium phosphate gel *in situ* (Foltmann, 1970). After centrifugation (12000g) the extract was concentrated by ultrafiltration to 60 ml. For comparison two stomachs of adult pig were analysed. From each, 2 g of fundic and 2 g of pyloric mucosa were extracted separately with 20 ml of distilled water in the same way as the piglet mucosa. After centrifugation these extracts were used without further treatment.

The zymogens were converted into active enzymes by addition of 0.05 M-HCl to pH 2; such samples were left for 20 min at room temperature (20°C) before tests were carried out.

Crystalline calf chymosin (Foltmann, 1970) was used as reference material. Specific antisera against calf chymosin and pig pepsins A, B and C were obtained from Chr. Hansens Laboratory, Copenhagen, Denmark.

Immunoelectrophoresis

The methods were those described in the manual of Axelsen *et al.* (1973). Anti-(calf chymosin) was of the same type as that used by Rothe *et al.* (1976).

The specificities of the pig antisera were tested by crossed tandem immunoelectrophoresis under the same conditions as described in the legend to Plate 1. In each experiment one of the wells contained a sample of the gastric-mucosal extract from which the antigen was prepared, and the other well contained a sample of the purified antigen. In the second dimension the gel contained antiserum against the purified antigen. One continuous fused precipitation zone was

observed in each of the experiments with anti-(pepsin A) and anti-(pepsin C). With anti-(pepsin B) one predominant fused precipitation zone was observed, but with the sample of gastric extract precipitation of small amounts of non-pepsin component was also observed, and the area below this second precipitation zone was about 10% of that below the fused arc. Finally the identities were confirmed in experiments that included pig pepsin A (Sigma Chemical Co., St. Louis, MO, U.S.A.) and pepsinogens B and C (Ryle, 1970) obtained from Dr. A. P. Ryle (Department of Biochemistry, University of Edinburgh Medical School, Edinburgh, Scotland, U.K.).

Proteolytic activities were determined with acid-denatured haemoglobin as substrate. After precipitation in 0.2M-trichloroacetic acid the A_{280} values of the digests were measured (Foltmann, 1964). Samples with absorbances between 0.3 and 1.0 at the optimum pH were used. Milk-clotting activities were determined and expressed in chymosin units as described previously (Foltmann, 1970).

Results and Discussion

The extracts were tested for contents of proteolytic enzymes by rocket and by crossed immunoelectrophoresis.

For rocket immunoelectrophoresis four plates with antisera against pig pepsins A, B and C and against calf chymosin were run. On each plate samples of fundic, pyloric and piglet stomach extract were applied both as zymogens and after treatment at pH 2. The amounts of the individual components were not quantified. The fundic extract precipitated anti-(pepsins A, B and C). Pyloric extract precipitated anti-(pepsins A and C). None of the adult pig extracts precipitated anti-(calf chymosin), whereas the extract of piglet stomach precipitated this antiserum only. The morphology of the precipitates of pepsinogen A and prochymosin were different from those of the active enzymes. The former had a blurred precipitate inside the rocket, whereas the latter had a clear inside. This phenomenon was not pronounced for pepsinogen B and pepsin B. It is especially noteworthy that our antiserum against pepsin C did not precipitate the zymogen.

Plate 1 illustrates the immunological analyses of pig chymosin in relation to calf chymosin. The two enzymes have the same electrophoretic mobility in agarose and are clearly related, with calf chymosin spurring over pig chymosin (Plate 1b). The faint fuzzy precipitates around the dense peaks are due to split products formed during the activation.

The proteolytic activities with acid-denatured haemoglobin as substrate are shown in Fig. 1. The results demonstrate a radical difference in the pH-dependence of activities of proteinases from newborn and adult pigs. The former has a pH optimum

Fig. 1. Degradation of acid-denatured haemoglobin by gastric proteinases

The activities are expressed as percentages of the absorbance of the digest at the optimum pH for proteolytic activity. Δ, Extract of piglet stomachs; ○, extract of adult pig fundic mucosa; ●, extract of adult pig pyloric mucosa.

of about 3.5, which is nearly the same as that of calf chymosin when tested under the same conditions (Foltmann, 1964).

The samples were also tested for milk-clotting activity. The results are presented in the following as the ratio between milk-clotting activity (chymosin units/ml) and the proteolytic activity measured as the absorbance of the digests produced by 1 ml of extract at the optimum pH. As reference a sample of calf chymosin was also tested. The ratios were: fundic extract, 0.13:1; pyloric extract, 0.30:1; piglet extract, 10.0:1; calf chymosin, 2.5:1.

The properties of the reference material from adult pig stomachs reflect differences in concentration and enzymic properties of the adult pepsins. Studies on other species (especially man; reviewed by Samloff, 1971) show that pepsin C predominantly occurs in the pyloric part of the stomach. Our results indicate a relatively lower milk-clotting activity of pepsins A and B.

Extract of piglet stomach has a ratio of milk-clotting activity to general proteolytic activity that is more than an order of magnitude higher than that of extracts of adult pig gastric mucosa. It is noteworthy that the relative milk-clotting activity of the extract from piglet stomach is greater than that found for calf chymosin.

Taken together, the observations provide sufficient evidence for the presence of a neonatal pig proteinase

EXPLANATION OF PLATE I

Tandem crossed immunoelectrophoresis of activated extract of piglet stomach and calf chymosin

In the wells (bottom left) 5 μ l of the following samples were applied: 1 and 6, blank (0.1 M-NaCl); 2 and 4, piglet stomach extract with pig chymosin (PC); 3 and 5, calf chymosin (CC). The bar in (a) indicates 1 cm. First-dimension electrophoreses (anode to the right) were run at 10 V/cm at 12°C for 35 min in agarose gel (type HSA, 1% w/v; Litex, Glastrup, Denmark) with Tris/veronal buffer, pH 8.6, *I*0.02. Second-dimension electrophoreses (anode at the top) were run at 2 V/cm at 12°C overnight, with the same agarose gel and buffer as for the first dimension, but with rabbit antibodies against calf chymosin.

that is closely related to calf chymosin in immunological as well as in enzymic properties; thus we consider it proper to use the name pig chymosin.

We thank Mrs. Inga Vaarst Andersen for perfect assistance in the immunological assays. We are grateful to Chr. Hansens Laboratory and to Dr. A. P. Ryle for providing antisera and reference enzymes respectively, and to Mr. Viggo Danielsen (The Pig Experiment Station, 'Sjælland III', near Roskilde, Denmark) for delivery of piglet stomachs.

References

- Axelsen, N. H., Krøll, J. & Weeke, B. (1973) *Scand. J. Immunol.* **2**, Suppl. 1, 1-164
- Foltmann, B. (1964) *C.R. Trav. Lab. Carlsberg* **34**, 319-325
- Foltmann, B. (1970) *Methods Enzymol.* **19**, 421-436
- Henschel, M. J. (1973) *Br. J. Nutr.* **30**, 285-296
- Hirsch-Marie, H., Loissillier, F., Touboul, J. P. & Burtin, P. (1976) *Lab. Invest.* **34**, 623-632
- Holter, H. & Andersen, B. (1934) *Biochem. Z.* **269**, 285-299
- Kotts, C. & Jenness, R. (1976) *J. Dairy Sci.* **59**, 1398-1400
- Malpress, F. H. (1967) *Nature (London)* **215**, 855-857
- Rothe, G. A. L., Axelsen, N. H., Jøhnk, P. & Foltmann, B. (1976) *J. Dairy Res.* **43**, 85-95
- Ryle, A. P. (1970) *Methods Enzymol.* **19**, 316-336
- Samloff, M. I. (1971) *Gastroenterology* **60**, 586-604