Pepsinogen D

A FOURTH PROTEOLYTIC ZYMOGEN FROM PIG GASTRIC MUCOSA

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Methods are described for the isolation and purification of pepsinogen D, a minor zymogen occurring to the extent of about 5% of the potential proteolytic activity in neutral extracts of the pig gastric mucosa. The physical and chemical properties of this zymogen indicate that it is very similar to, if not identical with, dephosphopepsinogen.

There are several recent reports which indicate the existence in the gastric mucosa of a multiplicity of zymogens with potential proteolytic activity. Ryle (1960, 1964) and Ryle & Hamilton (1966) reported the isolation of pepsinogens B and C (formerly called parapepsinogens I and II respectively) as well as the main zymogen pepsinogen from the gastric mucosa of the pig, and there is evidence to indicate that there are several zymogens in the human gastric mucosa (Seijffers, Segal & Miller, 1963*a,b*; Kushner, Rapp & Burtin, 1964; Hanley, Boyer & Naughton, 1966). Levchuk & Orekhovitch (1963) have also detected a multiplicity of zymogens in the chick stomach where there are three or possibly four zymogens which give rise to enzymes with proteolytic activity at pH 2.

Lee & Ryle (1963) have given a preliminary report of the isolation of a fourth zymogen, pepsinogen D, from the pig gastric mucosa and this paper gives a more detailed account of its isolation, purification and properties.

EXPERIMENTAL

Materials. Haemoglobin was bovine haemoglobin enzyme substrate powder obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

Acetyl-L-phenylalanyl-L-di-iodotyrosine was purchased from YEDA Research and Development Co. Ltd., Rehovoth, Israel, and was designated lot PHTY 6.

The milk powder used was Gayelord Hauser dried skim milk which was purchased locally.

DEAE-cellulose was prepared as described by Peterson & Sober (1956) and had about 0.6 m-equiv. of basic groups/g. Visking dialysis tubing 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.

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

1-Fluoro-2,4-dinitrobenzene was obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Phosphatase B (potato) was obtained as described by Ryle (1965).

'Isoamyl alcohol' used for chromatography was British Drug Houses laboratory reagent and was described as 3-methylbutan-1-ol with 15–30% of 2-methylbutan-1-ol.

Buffer solutions. Except where otherwise stated buffer solutions were made from sodium salts and their concentrations in molarities refer to the total concentration of the buffering component.

METHODS

Activity determination with haemoglobin. Assays of activity against haemoglobin were made in duplicate at pH 1.7 by the method described by Ryle (1960), the increase in extinction at 280 m μ of the deproteinized solutions being converted into proteolytic units ([P.U.]^{HB}) by reference to a standard curve prepared with a solution of crystalline pepsin standardized by the method of Anson (1938). There was no significant difference in the standard curve obtained with crystalline pepsin and that obtained with a sample of pepsin D when it became available, and it was only above 0.1 m[P.U.]^{HB} that the standard curve obtained with pepsin C differed significantly from that of the other two enzymes.

Activity determination with acetyl-L-phenylalanyl-L-di-iodotyrosine. Assays of activity against this substrate were done in duplicate at pH 2.0 in the manner described for acetyl-DL-phenylalanyl-L-di-iodotyrosine by Ryle (1960), except that the concentration of the acetyl-peptide was halved to 1 mM in 0.01 N-NaOH. Extinctions at 570 m μ were converted into proteolytic units ([P.U.]^{APD}) by reference to a standard curve prepared with a solution of crystalline pepsin and, in this case also, it was confirmed that there was no significant difference between the graph given by pepsin and that given by pepsin D. One proteolytic unit as determined by haemoglobin digestion was arbitrarily made

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equal to one proteolytic unit as determined with this substrate. By reference to the ninhydrin colour produced by known amounts of di-iodotyrosine, it was found that $1 \text{ m[P.U.]}^{\text{APD}}$ corresponded to the release of $7.0 \mu\text{moles}$ of di-iodotyrosine/min.

Determination of activity by the clotting of milk. Gayelord Hauser dried skim milk (20g.) was worked into a paste with water and, after the addition of 10ml. of 1.0M-sodium acetate-1.0M-acetic acid buffer and 5ml. of 0.2M-calcium chloride, the solution was made up to 100ml. A portion (5ml.) of this solution (pH 5.1) at 35.5° was added to 0.5ml. of the enzyme solution at the same temperature and, after shaking, the solution was inspected at regular intervals by tilting the tube about 30° to the vertical. The end point was taken as the time required for the first appearance of floccular material in the film of milk that clung to the wall of the tube. In this assay, one proteolytic unit ($[\text{P.U.}]^{\text{Ren}}$) was defined as the amount of enzyme that would clot 11 ml. of the assay mixture in 1 min. (Herriott, 1938).

Extraction of zymogens from the gastric mucosa. The method used was basically that of Ryle (1965) but it included some small modifications which enabled the procedure to be concluded in a shorter time. Pig stomachs were obtained from the abattoir immediately after death, packed in ice and transported to the laboratory where they were treated in the following manner at $2-5^\circ$. The stomachs were washed with water and the red or brown part was cut out. The mucosa of these so-called 'fundic' or body regions was removed from the muscularis, scraped free of mucus with a stout piece of polythene, cut into small pieces, minced, weighed and suspended in 4 vol. of 0.02M-phosphate buffer, pH 6.9. To this suspension was added benzyl penicillin (5 units/ml.), streptomycin sulphate (50 $\mu\text{g.}/\text{ml.}$), Hyflo Super-Cel (50g./l.) and Filter-Cel (100g./l.), the volume referring to the volume of buffer solution used. The mixture was stirred for 1 hr. before centrifugation at 500g and 0° for 5 min. and the residue was extracted for a further 1 hr. with a like volume of the same buffer before centrifugation as before. The pooled extracts were then filtered on a Buchner funnel through a piece of Whatman no. 54 filter paper. The paper was found to clog up after about 500ml. had passed and it was then necessary to use a fresh piece. The potential activity of the filtrate was determined by using haemoglobin as substrate but it was not possible to determine the potential activity against acetylphenylalanyldi-iodotyrosine because of the high blank values.

Chromatography of the mucosal extract. Chromatography of the mucosal extract and rechromatography of the zymogens from the main zymogen peak was carried out in the manner described by Ryle (1965), except that a column of wider diameter was used in the ion-exchange rechromatography.

Concentration of zymogen solutions. Solutions of the zymogens were concentrated either by dialysis in Visking $\frac{3}{16}$ in. or $1\frac{1}{2}$ in. dialysis tubing against Carbowax 20 M or by ultrafiltration, under 60 cm. Hg pressure, through Visking $\frac{3}{16}$ in. dialysis tubing which was supported by a sleeve of silk net. Concentration steps after the last chromatography on DEAE-cellulose were always performed by ultrafiltration to preclude any contamination by Carbowax.

Electrophoresis in starch gel and detection of enzymic or potential enzymic activity within the gel. Samples of the zymogen or enzyme solutions were applied to pieces (10mm. $\times$ 5mm.) of Whatman no. 3MM filter paper and

inserted into a horizontal gel prepared in the usual way (Smithies, 1955). After electrophoresis the gel was sliced horizontally and the enzymic activity or potential enzymic activity detected by the ability to digest haemoglobin. The cut surface of the gel was covered with a piece of Whatman no. 2 filter paper moistened with a 2% solution of haemoglobin in 78mN-HCl. The paper was removed after 15 min. and the gel was stained for protein with Naphthalene Black 10 B (G. T. Gurr Ltd., London, S.W.6). Areas of enzymic activity were detected as white areas on a blue background.

Determination of total nitrogen. Nitrogen was determined by the micro-Kjeldahl procedure on samples containing about 0.3 mg. of N.

Determination of N-terminal amino acid residues. N-Terminal amino acid residues were determined with 1-fluoro-2,4-dinitrobenzene (Porter, 1957). The ether-soluble DNP-amino acids were first identified by paper chromatography with the solvent systems of Blackburn & Lowther (1951) and of Levy (1954) and finally by using the thin-layer method (system no. 4) of Brenner, Niederwieser & Pataki (1961). Attempts were also made to identify any water-soluble DNP-amino acids by extraction into butan-1-ol-ethyl acetate (1:1, v/v) (Brenner, Niederwieser & Pataki, 1964), the extracts being taken to dryness and examined by chromatography on Whatman no. 3MM paper in the solvent system 'isoamyl alcohol'-pyridine-water (7:7:6, by vol.) (Ryle, 1965).

Determination of C-terminal amino acid residues. Pepsinogen D (20mg.) was dissolved in 0.5ml. of water and denatured by heating in a boiling-water bath for 3 min. After cooling there was added 0.63ml. of 0.1M-phosphate buffer, pH 7.6, and 0.13ml. of a suspension of carboxypeptidase (0.75 μmole) (Sigma lot C110B-055) that had been preincubated for 1 hr. at 37° with a 50-fold molar excess of di-isopropyl phosphorofluoridate in 0.1M-phosphate buffer, pH 7.6. The reaction mixture was incubated at 37° and samples were taken at the time of mixing and at 2, 4, 6, 8, 10 and 22 hr. thereafter. Protein was precipitated by addition of an equal volume of 0.6N-HCl in ethanol and the released amino acids were identified by chromatography on Whatman no. 4 paper by using the solvent systems 80% (w/v) phenol-0.3% ammonia and butanol-acetic acid-water (63:10:27, by vol.).

Determination of phosphate. Phosphate was determined by the method of Allen (1940) scaled down so that the final volume was 2.5 ml. After perchloric acid digestion, however, 0.5 ml. of water was added and the reaction mixture was heated for 30 min. to allow hydrolysis of any pyrophosphate that had been formed.

Treatment of zymogens with potato phosphatase B. The method of dephosphorylation was based on the findings of Perlmann (1958). Freeze-dried samples of each zymogen were dissolved to a concentration of 7mg./ml. in 0.2M-acetate buffer, pH 5.6, which was 0.02M with respect to MgCl_2 , and to these solutions was added an equal volume of a solution of phosphatase B (0.025 unit/ml.) dissolved in acetate buffer, 0.02 M, pH 4.5. These solutions, together with controls containing no phosphatase, were incubated at 37° for 24 hr.

'Fingerprint' analysis of the peptides produced by digestion of oxidized zymogens with α -chymotrypsin. The zymogens were denatured by incubation in 0.02N-NaOH for 10 min. at 35.5° and the disulphide bonds subsequently oxidized

with performic acid for 2 hr. at 0°. After the addition of 10 vol. of water, the solutions were dialysed against water and freeze-dried. About 2.5 mg. of the oxidized proteins was dissolved in 0.1 ml. of 0.2 M-ammonium carbonate (pH 8.5) containing 0.05 mg. of α -chymotrypsin (Sigma lot C49-66) and incubated for 3 hr. at 35.5°. The resulting peptides were separated on a sheet of Whatman no. 3MM chromatography paper by using high-voltage electrophoresis between water-cooled metal plates in pyridine-acetic acid-water (25:1:475, by vol.) buffer, pH 6.5, and chromatography in the 'isoamyl alcohol'-pyridine-water (7:7:6, by vol.) system (Baglioni, 1961). After drying the peptides were rendered visible by spraying with ninhydrin.

Stability of pepsinogen D in solutions of alkaline pH. Phosphate and borate buffer solutions were prepared according to Gomori (1955) but adjusted so that the concentration was constant and with sufficient NaCl added to bring the ionic strength to 0.2. One volume of each buffer solution at 35.5° was added to 1 vol. of a solution of pepsinogen D (0.25 mg./ml.) in 0.7 mM-phosphate buffer, pH 6.9, and each mixture was incubated at 35.5° for 10 min. The residual potential activity against haemoglobin and acetylphenylalanyldi-iodotyrosine was determined in the manner described above except for the fact that the concentration of HCl had to be adjusted to obtain the required pH in the assay mixtures. Also, in the synthetic-substrate assay, blank values were taken from reactions in which 0.01 N-NaOH replaced the substrate solution.

RESULTS

Chromatography of zymogens. Fig. 1 shows the chromatography of the zymogens from a single gastric mucosa. This elution pattern is very similar to that obtained by Ryle (1965) and shows the separation of a peak of pepsinogen C at 3.7 l. from the main zymogen peak at 2.4 l. As expected, the leading edge of the main zymogen peak displayed a greater potential activity against acetylphenylalanyldi-iodotyrosine than against haemoglobin and when the fractions from this region of the chromatogram were pooled, concentrated, dialysed and subjected to rechromatography, the elution pattern shown in Fig. 2 was obtained. This shows peaks of pepsinogen B, pepsinogen D and pepsinogen in order of elution. The resolution of these three zymogens tended to be variable and in some cases only a single zymogen peak was obtained. This variation was traced to the variable amounts of pepsinogen included in the cuts taken for rechromatography and good resolution was always obtained if cuts were restricted so that they contained only 10–15% of the total potential proteolytic activity recovered from the first chromatogram. The absolute amount of the zymogens subjected to rechromatography appeared to be of little consequence and good resolution was obtained with loads up to 30 [P.U.]^{Hb}.

The material used in the following studies of pepsinogen D was obtained by pooling the fractions contributing to the central peak in chromatograms

Fig. 1. Chromatography of the zymogens (14 [P.U.]^{Hb}) in the extract of the body region of one pig gastric mucosa on DEAE-cellulose in 0.02 M-phosphate buffer, pH 6.9. The diameter of the column was 4.8 cm. and the zymogens were adsorbed on to DEAE-cellulose, which gave a column of 5 cm. on top of a 5 cm. column of fresh DEAE-cellulose. The gradient was 0→0.45 M-NaCl, the mixing vessel being 2 l. The flow rate was approx. 70 ml./hr. and 35 ml. fractions were collected. O, Potential activity against acetyl-L-phenylalanyl-L-di-iodotyrosine; ●, potential activity against haemoglobin; —, E_{280} . The bar indicates that portion of the effluent which was used for the isolation of pepsinogen D. The calculated concentration of chloride in the effluent is shown above.

such as that shown in Fig. 2; to reduce contamination caused by overlapping of the three peaks, fractions lying close to the minima were not included. The zymogen solution was then dialysed against 0.5 mM-ammonia (as dialysis against water resulted in some activation) and freeze-dried.

Exclusion chromatography of pepsinogen D. Further purification of the fraction containing pepsinogen D (Fig. 2) was achieved by gel filtration on Sephadex G-100, which separated small amounts of material of greater and smaller molecular weights. The pepsinogen D prepared in this way was slightly contaminated with pepsinogen, which has a very similar elution volume on Sephadex G-100 to that of pepsinogen D. It thus seems likely that the molecular weight of pepsinogen D is close to that of pepsinogen, 41 000 (Arnon & Perlmann, 1963).

Electrophoresis in starch gel. In starch gels prepared with 0.02 M-phosphate buffer, pH 6.9, or with 0.05 M-citrate buffer, pH 5.6, the electrophoretic mobilities of pepsinogen and pepsinogen D

Fig. 2. Rechromatography of the zymogens ($2.5 \text{ [P.U.]}^{\text{APD}}$) obtained from the leading edge of the main zymogen peak shown in Fig. 1. The material was applied to a column ($11 \text{ cm.} \times 4.8 \text{ cm. diam.}$) of DEAE-cellulose in 0.02 M -phosphate buffer, $\text{pH } 6.9$, and eluted with a gradient of $0 \rightarrow 0.45 \text{ M}$ -NaCl, the mixing vessel being 2 l . The flow rate was approx. 150 ml./hr. and 38 ml. fractions were collected. ○, Potential activity against acetyl-L-phenylalanyl-L-diiodotyrosine; ●, potential activity against haemoglobin; —, E_{280} . The bar indicates the typical cut required to obtain pepsinogen D which was contaminated with only small amounts of pepsinogen. The calculated concentration of chloride in the effluent is shown above.

Fig. 3. Starch-gel electrophoresis of about 1 mg. of zymogens and enzymes in a gel prepared with 0.05 M -citrate buffer, $\text{pH } 3.2$. Enzymic activity against haemoglobin was detected after running for 34 hr. at 2.8 v/cm.

were very similar, but it can be seen from Fig. 3 that the mobility of 'pepsinogen D' was only half that of 'pepsinogen' when the gel was prepared with 0.05 M -citrate buffer, $\text{pH } 3.2$. Inverted commas are used here as it is probable that we were observing the migration of enzymes produced by the activation of these zymogens at $\text{pH } 3.2$. The mobility was not dependent on the time-interval between dissolving the zymogens and their insertion in the gel. Fig. 3

also shows that there was little difference in the mobility of the enzymes produced by activation at $\text{pH } 3.2$ and those isolated from commercial crystalline pepsin by chromatography on DEAE-cellulose (Lee & Ryle, 1967) and probably produced by activation at a pH close to 2. At $\text{pH } 3.2$ 'pepsinogen B' and 'pepsinogen C' both migrate toward the cathode (Ryle, 1966).

Specific potential activity of pepsinogen D. The specific potential activities of pepsinogen D were found to be $0.25 \text{ [P.U.]}^{\text{APD}}/\text{mg.}$ of nitrogen and $0.18 \text{ [P.U.]}^{\text{HB}}/\text{mg.}$ of nitrogen. The former value corresponds to the liberation of $1.75 \mu\text{moles}$ of di-iodotyrosine/min./mg. of nitrogen and is close to the value of 1.84 found by Ryle (1965) for pepsinogen B. However, at $0.18 \text{ [P.U.]}^{\text{HB}}/\text{mg.}$ of nitrogen the potential specific activity of pepsinogen D is lower than the $0.22 \text{ [P.U.]}^{\text{HB}}/\text{mg.}$ of nitrogen which was reported for pepsinogen (Herriott, 1938).

N-Terminal analysis. The only visible ether-soluble DNP-amino acid spot behaved on paper chromatography in the same manner as DNP-leucine and DNP-isoleucine and it was finally identified as the former when it separated from DNP-isoleucine but not from DNP-leucine on thin-layer chromatography. By using an internal control of added DNP-leucine to assess the destruction of DNP-leucine during hydrolysis of the dinitrophenylated pepsinogen D it was found that 0.9 mole of DNP-leucine was obtained/mole of pepsinogen D (assuming molecular weight 41000).

The fraction extracted with butanol-ethyl acetate contained only ϵ -DNP-lysine and a small amount of yellow material which migrated just ahead of the lysine derivative. On further hydrolysis the yellow colour became extractable with ether and now behaved on thin-layer chromatography as DNP-leucine or DNP-isoleucine. It is concluded that this material was a DNP-peptide and that pepsinogen D contains no *N*-terminal histidine or arginine.

C-Terminal analysis. During digestion with carboxypeptidase the first amino acid released from the denatured pepsinogen D was alanine (about 1 mole/mole) and this was followed by a slower release of two amino acids, which were probably valine and leucine.

Relationship between pepsinogen D and dephosphopepsinogen. Direct determination of the phosphorus content of one pepsinogen D preparation showed it to contain $0.65 \text{ g. atom/mole}$, but phosphate buffer had been used in its chromatographic isolation and it seemed probable that this simply represented contamination with phosphate which was not in covalent linkage with zymogen. This idea was confirmed when a value of $0.26 \mu\text{g. atom/41 mg.}$ was found with a sample which had been subjected to gel filtration on a column of Sephadex

G-25 prepared in tris-citrate (0.1 M-tris-0.05 M-citric acid-0.054 N-sodium hydroxide) buffer, pH 6.7. The latter value is clearly less than 1 g. atom/mole and this led us to compare the properties of pepsinogen D and dephosphopepsinogen.

Samples of pepsinogen and pepsinogen D were incubated with phosphatase B and then submitted to electrophoresis in a block of starch gel prepared with 0.05 M-citrate buffer, pH 3.2. This treatment was found to have no effect on the mobility of the activation product of pepsinogen D although it converted pepsinogen into a form in which its activation product migrated with a mobility similar to that of the activation product of pepsinogen D.

This similarity between pepsinogen D and dephosphopepsinogen was extended when it was found that chromatography of a mixture of dephosphopepsinogen and pepsinogen on a column of DEAE-cellulose in 0.02 M-phosphate buffer, pH 6.9, with a sodium chloride gradient, gave rise to two peaks, and that the material in the peak eluted first gave rise to an activation product which behaved in the same manner as the activation product of pepsinogen D on electrophoresis in starch gel at pH 3.2.

'Fingerprint' analysis of the peptides produced by digestion of oxidized pepsinogen D and of oxidized dephosphopepsinogen with α -chymotrypsin. As it was not possible to distinguish pepsinogen D and dephosphopepsinogen by their behaviour on electrophoresis in starch gel or by chromatography on DEAE-cellulose it was decided to submit the performic acid-oxidized proteins to 'fingerprint' analysis. Fig. 4 shows the great similarity in the 'fingerprint' patterns obtained from α -chymotryptic digests of the two oxidized proteins. Both gave at least 23 peptides which occupied similar positions on the peptide map and although the digest from oxidized dephosphopepsinogen gave an additional peptide (arrowed in Fig. 4) this spot was stained rather faintly and it is possible that its counterpart in the digest from oxidized pepsinogen D was just below the limit of detection of the staining method.

Stability of pepsinogen D in solutions of alkaline pH. The residual potential haemoglobin-digesting activity found after exposure of pepsinogen D to solutions of alkaline pH is shown in Fig. 5. The activity remaining after exposure to pH 6.5 was taken as representing 100% activity and it is seen that there was only a small decrease in activity at values up to pH 7.5. However, above the latter pH an increasing proportion of the potential activity was lost until at pH 10.6 less than 10% of the original activity remained.

The loss of potential activity was also studied with acetylphenylalanyldi-iodotyrosine as substrate. In these assays blanks in which 0.01 N-sodium hydroxide replaced the usual solution of acetyl-peptide were used as some increase in

Fig. 4. Tracing of the ninhydrin-stained peptides obtained after separation of the peptides produced by α -chymotryptic digestion of 2.5 mg. of (A) oxidized pepsinogen D, (B) oxidized dephosphopepsinogen. The significance of the arrowed peptide is discussed in the text. The electrophoresis was at pH 6.5, 63 v/cm. for 1 hr. and the chromatography in 'isoamyl' alcohol-pyridine-water (7:7:6, by vol.).

ninhydrin colour was found to be due to digestion of denatured protein by the enzyme; however, these blanks may not be strictly valid as the rate of digestion may not be the same in the absence as in the presence of the acetyl-peptide substrate. It appeared that the loss of potential activity at pH 9.2 was the same as the loss against haemoglobin and that the loss of potential activity was the same irrespective of whether the exposure was for 10 min. or for 30 min. Also, on returning the solution from pH 9.2 to pH 7.3 for 10 min. at 35.5° the potential activity rose from about 20% to about 50% of its original value. These preliminary results suggest that the alkaline inactivation of pepsinogen D, like that of pepsinogen (Herriott, 1938), is at least partly reversible.

Activation of pepsinogen D. Pepsinogen D possessed little or no ability to clot milk whereas pepsin D was active in this respect (D. Lee & A. P. Ryle, unpublished observation), and it was therefore possible to trace the activation of pepsinogen D

Fig. 5. Stability of pepsinogen D. Percentage of original activity against haemoglobin remaining after exposure for 10 min. at 35.5° to buffers of 1.0 and various pH values. ●, 0.025 M-phosphate buffers; ■, 0.05 M-borate buffers.

by the appearance of milk-clotting activity. Fig. 6 shows the time-course of the appearance of milk-clotting activity when pepsinogen D was incubated in 0.01 M-citrate buffer, pH 3.7, at 35.5°.

Before it had been exposed to the buffer of pH 3.7 the zymogen solution had an activity 0.028 [P.U.]^{Ren}/ml. at the pH of the milk solution (5.1), and substitution of this value and the equilibrium value of 4.18 [P.U.]^{Ren}/ml. in the equation for an autocatalytic process gave the theoretical curve shown in Fig. 6. The experimental points are seen to lie close to this curve.

The open circle in Fig. 6 is not on the time-scale and it indicates the milk-clotting activity obtained when the zymogen solution was acidified to pH 2 and left for 5 min. at room temperature before being brought back to pH 5. An identical activity was obtained when the pH 3.7 activation mixture was acidified in the same manner and it would appear that the product of activation and its activity depend on the pH used for activation.

The activation of pepsinogen D in 0.27 M-acetate buffer, pH 4.6, and at 35.5° proceeded more slowly than at pH 3.7 and in this case the appearance of activity appeared, at least in the initial stages, to be

Fig. 6. Activation of pepsinogen D (0.43 mg./ml.) in 0.01 M-citrate buffer, pH 3.7. The line drawn is that for an autocatalytic reaction with initial and final activities of 0.028 and 4.18 [P.U.]^{Ren}/ml. The open circle is not on the time-scale and its significance is discussed in the text.

directly proportional to the time of exposure at this pH.

DISCUSSION

Figs. 1 and 2 demonstrate the isolation of pepsinogens B, C and D from the gastric mucosa of a single pig and show that these zymogens are not aberrant forms of pepsinogen originating separately in the gastric mucosa of different pigs.

From the properties described above, pepsinogen D is much more similar to pepsinogen than are pepsinogens B and C and, indeed, pepsinogen D appears to be very similar to, if not identical with, dephosphopepsinogen. The *N*-terminal and *C*-terminal residues of leucine and alanine respectively are the same as those found in pepsinogen (Van Vunakis & Herriott, 1957; Orekhovitch, Lokshina, Mant'ev & Trietskaya, 1956) and the molecular weight must be close to that of the main zymogen. This information, together with the fact that the phosphorus content of pepsinogen D was only 0.26 g. atom/mole led us to investigate the possibility of the identity of pepsinogen D and dephosphopepsinogen. Pepsinogen D behaved in the same manner as dephosphopepsinogen on electrophoresis in starch gel at pH 3.2 and on chromatography on DEAE-cellulose at pH 6.9, and although the 'fingerprints' of oxidized pepsinogen D and oxidized dephosphopepsinogen were not identical,

the additional peptide from oxidized dephosphopepsinogen was only just detectable and it may be that its counterpart in the digest from oxidized pepsinogen D was just below the limit of detection. It appears therefore that pepsinogen D is very similar to, if not identical with, dephosphopepsinogen.

There has been no previous report of the isolation of dephosphopepsinogen from the pig gastric mucosa although Perlmann (1958) has reported dephosphorylation of pepsinogen and of pepsin *in vitro*. In accord with her results we find that the lack of covalently bound phosphate in pepsinogen D does not prevent its conversion into the active enzyme, although the potential specific activity of 0.18 [P.U.]^{HB}/mg. of nitrogen is slightly lower than the 0.22 [P.U.]^{HB}/mg. of nitrogen reported for pepsinogen (Herriott, 1938). The apparent potential specific activity against the synthetic substrate is 0.25 [P.U.]^{APD}/mg. of nitrogen and, although the activity against both substrates should theoretically be the same, this high value probably results from a contribution to the ninhydrin colour from the digestion of the peptides of activation as well as the added substrate.

The stability to alkali of the potential haemoglobin-digestion activity of pepsinogen D is similar to that of pepsinogen (Herriott, 1938), although it may be slightly less stable than the main zymogen; however, the conditions of our experiment were somewhat more severe than those used by Herriott so that there may be no real difference in the stability.

The close similarity between pepsinogen D and pepsinogen is also seen in the fact that the activation of pepsinogen D at pH 3.7 displays autocatalytic kinetics. Pepsinogen has been reported to be activated autocatalytically at all pH values (Herriott, 1939) and although the activation of pepsinogen B at pH 4.0 and of pepsinogen C at pH 4.7 also shows some autocatalytic character, these zymogens differ from pepsinogen in that first-order kinetics seem to be involved when the activation pH is reduced to 2.0 and 4.1 respectively (Ryle, 1960, 1965).

The phenomenon of incomplete activation of pepsinogen D at pH 3.7 has been paralleled in the activation of pepsinogen C at pH 4.1 and, although this phenomenon may be explained by the existence of an enzyme-inhibitor complex similar to that produced in the activation of pepsinogen, it is difficult to reconcile this idea with the fact that the peptide inhibitor from pepsinogen is digested with a pH optimum of 4 (Herriott, 1941).

Sanger & Hocquard (1962) have presented evidence which indicates that dephospho-ovalbumin

is an intermediate in the synthesis of ovalbumin and it could be that pepsinogen D is an intermediate in the synthesis of pepsinogen, but we have no evidence to indicate whether this is in fact the case or whether pepsinogen D is the product of the dephosphorylation of a completed pepsinogen molecule.

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