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Parapepsins: Two Proteolytic Enzymes Associated with Porcine Pepsin

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Several groups of workers have reported the presence of other proteolytic enzymes associated with pepsin, either in gastric juice or in extracts of the gastric mucosa. Freudenberg (1940), Pope & Stevens (1951), Buchs (1954) and Taylor (1959) have shown that activity may be found at pH values above 3.0 and this activity has generally been attributed to a cathepsin. These reports are discussed by Taylor (1959). Although Pope & Stevens (1951) were able to obtain a fraction showing a sharp pH optimum at pH 1.7 they were unable to purify a fraction with optimum in the range pH 3.0–4.0. Merten, Schramm, Grassmann & Hannig (1952), by precipitation and electrophoresis, and Richmond, Tang, Wolf, Trucco & Caputto (1958), by passage through a cation-exchange resin, obtained partial separations of the fractions with optimum activity below pH 2.0 and above pH 3.0.

Northrop (1932) obtained a fraction from pepsin which had a gelatin-liquefying activity much greater than that of pepsin.

We have fractionated crude pepsin by ion-exchange chromatography on diethylaminoethyl-cellulose and have isolated two minor components which differ from pepsin in specificity but are similar to it in other properties. One of these enzymes has also been found in a batch of crystalline pepsin.

This paper reports the isolation and characterization of these enzymes; a preliminary account of this work has already appeared (Ryle, 1958).

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EXPERIMENTAL

Abbreviations used in this paper are as follows: acetyl-DL-phenylalanyl-L-di-iodotyrosine, APD; haemoglobin, Hb; dinitrophenyl, DNP; amino acid residues, those used by Brand & Edsall (1947).

Where the activities of the enzymes are expressed as m[F.U.] (defined below) without indication of the substrate, it is to be understood that APD was the substrate for parapepsin I and Hb for parapepsin II; for pepsin the units are equal for the two substrates.

Materials

The crude pepsin used was pepsin B.P. (sold by Hopkin and Williams Ltd., Chadwell Heath, Essex, and manufactured solely from pig gastric mucosae). Several different batches have been used, but no significant difference has been observed between them.

Diethylaminoethyl-cellulose was prepared according to Peterson & Sober (1956) and had about 1 m-equiv. basic groups/g.

Haemoglobin was bovine haemoglobin enzyme-substrate powder (Armour and Co. Ltd., Hampden Park, Eastbourne, Sussex), and was dialysed against water before use.

Acetyl-DL-phenylalanyl-L-di-iodotyrosine was kindly given by Mrs R. V. Pitt-Rivers of this Institute. This stereoisomeric mixture has not been crystallized, but was precipitated five times by acidification of an alkaline solution. It gave no reaction with ninhydrin.

Cysteyltyrosine and tryosylcysteine were also given by Mrs Pitt-Rivers.

Bovine plasma albumin was crystallized material obtained from Armour and Co. Ltd.

The milk powder was Gayelord-Hauser skim-milk powder, obtained locally from Boots Ltd.

Pepsin inhibitor was material obtained from porcine pepsinogen and very kindly sent by Professor R. M. Herriott of Johns Hopkins University.

Gelatin was a sample (no. 141) obtained through the generosity of Mr A. G. Ward, of the British Gelatin and Glue Research Association, and had a viscosity at 6.67%, pH 7.0 and 40° of 6.67 centistokes, and isoelectric point at pH 5.0.

Methods

Instruments. Extinction values were determined with either a Hilger Uvispek or a Unicam spectrophotometer.

pH determinations were made with an E.I.L. (Richmond, Surrey) direct-reading pH meter or a Cambridge pH meter, standardized with Cambridge buffer tablets.

Buffer solutions. Except where otherwise stated, buffers were made from sodium salts. Their concentrations are stated with respect to the anionic component.

Activity determination by haemoglobin. Haemoglobin assays in duplicate were based on the method of Northrop, Kunitz & Herriott (1948), modified by scaling down the volume of each assay to 1.2 ml., to conserve material, and by reading the extinction of the filtrate obtained after adding 5 ml. of 4% trichloroacetic acid (w/v) without dilution, to reduce the labour involved in assaying a large number of chromatographic fractions. The increments in extinction found were converted into milliproteolytic units ($m[P.U.]^{Hb}$) by reference to a standard curve obtained with a preparation of crystalline pepsin which had been standardized by the original method of Anson (1938). Assays were restricted to the region of near-proportionality of amount of enzyme to extinction.

Determination of activity by acetyl-DL-phenylalanyl-L-di-iodotyrosine. Ninhydrin reagent was freshly prepared each day by mixing equal volumes of 4% (w/v) ninhydrin in 2-methoxyethanol (methyl Cellosolve) (distilled from $SnCl_2$) and a buffer mixture containing both the acetate buffer (2M) of Moore & Stein (1954) and the citrate buffer (0.05M) of the same authors (Moore & Stein, 1948) and 160 mg. of $SnCl_2 \cdot 2H_2O/100$ ml. The acetate buffer was used because of its high buffering power, but it was found that unless some citrate was added colour yields only about 60% of the theoretical (Troll & Cannan, 1953) were obtained. With the amount of citrate used the colour yield of leucine was 93% of the theoretical and the colour obtained with di-iodotyrosine 90% of the theoretical.

The procedure adopted for the enzymic assay was as follows: to 0.5 ml. of enzyme solution at 35.5° was added 0.25 ml. of HCl of concentration calculated to give finally 0.01N, followed 10–15 sec. later by 0.25 ml. of 0.002M-acetyl-DL-phenylalanyl-L-di-iodotyrosine in 0.01N-NaOH. The mixture was incubated for 20 min., 1.0 ml. of ninhydrin reagent was added and the colour was developed by heating at 100° for 15 min. After dilution with 5 ml. of 60% (v/v) ethanol the extinction at 570 $m\mu$ was determined. Assays were performed in duplicate, and duplicate blank determinations were made by adding the ninhydrin reagent to the enzyme before the HCl and substrate and omitting the 20 min. incubation. A standard curve was prepared relating $m[P.U.]^{Hb}$ of pepsin to increase in extinction. This curve was linear to 0.9 $m[P.U.]^{Hb}$ with a slope corresponding to a release of 4.1×10^{-3} μ moles of di-iodotyrosine/ $m[P.U.]^{Hb}/min$. One $m[P.U.]^{APD}$ has been defined as the activity of one $m[P.U.]^{Hb}$ of pepsin, and the activities of

enzyme solutions have been expressed as $m[P.U.]^{APD}$ by reference to this standard curve.

Milk-clotting activity. Determinations of milk-clotting activity were made by the method of Herriott (1938), except that following his present practice (personal communication) a slightly more alkaline buffer (equimolar acetic acid and sodium acetate) was used. The pH of the milk solution was 5.6. $[P.U.]^{Ren}$ is as defined by Herriott (1938) but is not strictly comparable because of the slight difference in pH.

Gelatin-liquefying activity. The change in viscosity of gelatin solution at pH 5.0 produced by the enzymes was determined in conditions as close as possible to those used by Northrop (1932). A sample (15 ml.) of 2.5% (w/v) gelatin solution was mixed with 0.3 ml. of 0.1N-HCl and 0.3 ml. of enzyme in 0.1M-acetate buffer, pH 5.6, at $35.2 \pm 0.1^\circ$ and the viscosity was measured over the following 30 min. in an Ostwald viscometer ($t_{H_2O} = 47.3$ sec.). The pH, determined after the digestion, was 4.95. Blank determinations with no enzyme were also performed and gave no change in viscosity. Gelatin-liquefying activity is given as $[P.U.]^{Gel V}$ (the percentage fall in the additional viscosity/min.) as calculated by Northrop (1932), but the units may not be comparable with his units because of probable differences in the gelatin used.

Chromatography of crude pepsin. Pepsin B.P. (500 g.) was dissolved in 1 l. of 0.5N- H_2SO_4 , the solution was filtered and the pepsin was precipitated by the addition of 1 l. of saturated $MgSO_4$ solution. The precipitate was washed with $\frac{2}{3}$ -saturated $MgSO_4$, suspended in water and brought back into solution by the cautious addition of 0.5N-NaOH (Northrop, 1930). This solution (about 250 ml.) was then dialysed in the cold against 0.1M-acetate buffer, pH 5.6, in Visking (Visking Corp., Chicago, Ill., U.S.A.) 24/32 tubing. A small precipitate was then centrifuged off and the supernatant, containing 6–8 g. of pepsin (determined by haemoglobin assay), was run into a column of 50 g. of diethylaminoethyl-cellulose (5 cm. $\times$ 35 cm.) previously equilibrated with 0.1M-acetate buffer, pH 4.0. Under these conditions the pepsin was adsorbed as a faintly visible band at the top of the column. The column was then washed with 0.1M-acetate buffer, pH 4.0, until the extinction at 280 $m\mu$ of the effluent was falling sharply. A gradient of rising NaCl concentration, obtained by running 0.1M-acetate, pH 4.0, containing 0.2M-NaCl into a 2600 ml. mixing vessel filled with 0.1M-acetate, pH 4.0, was then used to elute the adsorbed material from the column. Fractions (45 ml.) were collected from the point at which the gradient was applied and the protein concentration of all the fractions was measured by readings of extinction at 280 $m\mu$. The activities against haemoglobin and acetylphenylalanyl-di-iodotyrosine of alternate fractions in the regions of the extinction peaks were determined.

The materials in the active peaks were pooled separately and concentrated by ultrafiltration through 18/32 Visking dialysis tubing supported by a sleeve of silk net under 60 cm. Hg pressure. Towards the end of this concentration some protein sometimes precipitated but was redissolved on dialysis against 0.1M-acetate, pH 5.6. The pepsin from these chromatograms was dialysed against water and freeze-dried. All these operations were carried out in the cold room.

Re-chromatography of parapepsins. The concentrated solutions obtained as described above were, in the earlier part of the work, re-chromatographed directly, but in the

later stages parapepsin I was adjusted to pH 6.9 in diethyl barbiturate buffer (0.1M), allowed to stand for 20 hr. at room temp., returned to pH 5.6 by addition of HCl and dialysed against 0.1M-acetate, pH 5.6. This treatment at pH 6.9 destroys any contaminating parapepsin II.

The re-chromatography is a scaled-down version of the first (for details see the legend to Fig. 2) so that the gradient of NaCl concentration along the column is nearly the same for all the chromatograms. Suitable fractions were pooled, concentrated by ultrafiltration, dialysed against 0.1M-acetate, pH 5.6, centrifuged to remove dust, dialysed against ion-exchange water and freeze-dried.

Electrophoresis in starch gel. Electrophoresis of the enzymes in starch gel was performed by the method of Smithies (1955) in 0.05M-citrate buffer, pH 5.6.

The protein samples (0.08 ml. of solution containing 10 mg. of protein/ml.) were applied to 0.6 cm. $\times$ 1.3 cm. segments of Whatman no. 3MM filter paper and inserted, side by side, into a cut near the middle of a starch block 25 cm. $\times$ 4 cm. $\times$ 0.6 cm. After the electrophoresis the block was split into upper and lower halves. One half was stained with amidoschwarz 10 B (George T. Gurr, London) but only bands corresponding to the parapepsins could be detected. The other half of the block was stained by an adaptation of the method used by Reindel & Hoppe (1954) for detecting peptides on paper strips. The freshly cut gel was placed in a dish over the HCl-KMnO₄ mixture for 5 min. It was then allowed to stand for 5 min. in the air before being immersed in the KI-o-tolidine solution. Blue bands appeared, of which those due to parapepsins I and II were in the same position as those detected with amidoschwarz. If the gel was transferred directly from the chlorine atmosphere to the KI a strong blue background was obtained, and the formation of this background was prevented by allowing the gel to stand for 5 min. in the air. Such standing even for long periods was of no avail, however, if the gel was prepared with acetate in place of citrate buffer; apparently the citrate reduces the excess of chlorine and hypochlorite without reducing the labile chlorine bound in the peptide bonds.

Electrophoresis on ethanolyzed cellulose. Electrophoresis on ethanolyzed cellulose columns was performed by the method of Porath (1956) in 0.05M-citrate buffer, pH 5.6. The enzyme solutions were applied in 0.5 ml. of water, washed into the column with 0.5 ml. of the citrate buffer and subjected to a potential gradient of about 10 v/cm. The eluate from the column was collected in small (approx. 1ml.) fractions and analysed, after dilution, for extinction and enzymic activity.

Attempted removal of amino acids and small peptides from parapepsins I and II by ion-exchange resins. A solution of about 20 mg. of the enzyme in water was passed at 1° through a column (0.85 cm. $\times$ 9 cm.) of Zeo-Karb 225 and De-Acidite FF (The Permutit Co. Ltd.), both 20–40 mesh, mixed 1:2 (v/v). The effluent was frozen and dried from the frozen state. The recovery of activity from the column was 95% for parapepsin I and 80% for parapepsin II.

Attempted removal of amino acids and small peptides from parapepsins by precipitation with trichloroacetic acid. To 25–30 mg. of the enzyme dissolved in 3 ml. of 0.1M-acetate buffer, pH 5.6, was added 0.3 ml. of 40% (w/v) trichloroacetic acid. The cloudy solution was then heated at 100° for 5 min. to complete precipitation. The precipitate was washed three times with 4% (w/v) trichloroacetic acid,

once with ethanol-ether (1:1, v/v), three times with ether and was then air-dried.

Identification and estimation of N-terminal residues. The N-terminal residues of the two parapepsins and of some of the peptides obtained from digests of the B chain of oxidized insulin were determined by the fluorodinitrobenzene method. Dinitrophenyl (DNP)-proteins obtained were hydrolysed both with and without the addition of the DNP derivative of the amino acids, previously identified in qualitative experiments as occupying the N-terminal position, in order to obtain breakdown-correction factors for these DNP-amino acids. The DNP-amino acids were separated and identified by chromatography on paper in *tert.*-amyl alcohol (Blackburn & Lowther, 1951) and, where necessary, in phosphate buffer (Levy, 1954). The methods used throughout this phase of the work are described in detail by Porter (1957).

Digestion of fraction B of oxidized insulin. Insulin (1.3 g.) was oxidized for 20 min. at room temp. with 50 ml. of formic acid [47.5 ml. of 98% formic acid + 2.5 ml. of H₂O₂ (100 vol.), allowed to stand 1 hr.] and was then fractionated by precipitation (Sanger, 1949).

A portion (11 mg.) of the fraction B obtained was dissolved in 2 ml. of 0.01N-HCl, the required amount of enzyme was added in a small volume of buffer and the pH was adjusted to 2.0 by the addition of N-HCl. A trace of thiomersal was added to those mixtures allowed to digest for 20 hr.

Identification of peptides obtained from digests of B chain of oxidized insulin. The digests were evaporated to dryness on a rotary still and dissolved in a small amount of formic acid, the solutions were transferred to strips of filter paper (Whatman no. 3 or no. 3MM) and subjected to electrophoresis in pyridine-acetic acid buffer, pH 6.5 (Ryle, Sanger, Smith & Kitai, 1955). The separated bands were detected with ninhydrin on guide strips cut from the edge of the paper and eluted with N-acetic acid. Samples were hydrolysed (5.7N-HCl, 105°, 18–20 hr.) and chromatographed two-dimensionally in 80% (w/v) phenol-aq. 0.3% NH₃ soln. and butanol-acetic acid-water (1260:200:540, by vol.) on 36 cm. $\times$ 28.5 cm. sheets of Whatman no. 1 paper for identification of the amino acids. In some cases the peptide could be identified from consideration of its constituent amino acids, its electrophoretic behaviour and the known structure of the B chain of insulin (Sanger & Tuppy, 1951).

Some of the peptide bands were further fractionated by electrophoresis at pH 3.6 in pyridine-acetic acid buffer (Ryle *et al.* 1955); N-terminal residues were identified and estimated by the 1-fluoro-2,4-dinitrobenzene method and non-N-terminal amino acids were estimated as their DNP derivatives, separated by two-dimensional chromatography. In some cases C-terminal residues were identified by use of carboxypeptidase treated with diisopropyl phosphorofluoridate (Harris, 1955). The peptides from the peptic digests were not studied in detail. Their structures have been deduced from their amino acid content and from the results of Sanger & Tuppy (1951) and Brown, Sanger, Smith & Kitai (1955).

RESULTS

Behaviour of the ion-exchange chromatographic system. Fig. 1 shows the changes in pH and chloride concentration (determined by titration with silver

nitrate) occurring in the effluent from a column of smaller size but of the same proportions as those used for separating the enzymes.

Chromatographic separation of the enzymes. Fig. 2 (A) shows a typical chromatogram of crude pepsin. No activity against haemoglobin could be detected in any part of the effluent appearing before the gradient was applied, but it was not possible to assay this part of the chromatogram for activity against acetylphenylalanyldi-iodotyrosine because the blank values were too high. At least some of the material eluted before application of the gradient is therefore of relatively low molecular weight.

After the application of the gradient of sodium chloride concentration three active peaks are found. The first to emerge is active against acetylphenylalanyldi-iodotyrosine but has little activity against haemoglobin, and has been named parapepsin I. The second peak, parapepsin II, has little activity against the synthetic substrate but readily digests haemoglobin. The third peak has both activities and is pepsin itself. Although the two parapepsins are well separated from pepsin they are not usually well separated from one another and they have therefore been further purified by a second chromatography in the same system (Fig. 2B and C).

The parapepsin I, of which the re-chromatography is shown in Fig. 2 (B), had been allowed to stand overnight at room temperature at pH 6.9; this treatment, as is shown below, destroys parapepsin II. It nevertheless retains some proteolytic activity against haemoglobin, but the ratio of this activity to the activity against acetylphenylalanyldi-iodotyrosine is only 2.5% of that for pepsin.

Fig. 1. Changes in pH and chloride concentration in effluent from chromatographic column. Column: diethylaminoethyl-cellulose, 1.2 cm. $\times$ 27.5 cm. Buffer: 0.1M-acetate, pH 4.0. Gradient: 0.02M-NaCl in 0.1M-acetate, pH 4.0; mixing vessel, 125 ml.; pH of effluent (right ordinate), $\square$; chloride concentration, calc., $\circ$, found, Δ .

We have not found any such simple treatment by which to free parapepsin II of contaminating parapepsin I, but, by taking suitable cuts from the chromatograms, samples of parapepsin II can be obtained in which the ratio $m[P.U.]^{APD}:m[P.U.]^{HB}$ is less than 4% of the ratio for pepsin, so that the extent of contamination with parapepsin I or with pepsin cannot exceed 4%, and may be less if parapepsin II has some intrinsic activity against acetylphenylalanyldi-iodotyrosine.

The freeze-dried materials obtained from these and similar chromatograms, or solutions of the same material before freeze-drying, have been used for the studies reported here.

The recovery of the activity against haemoglobin of the solution of crude pepsin at the magnesium sulphate-precipitation step was 70%. In two experiments in which a sample of the filtrate was dialysed and chromatographed no active peak other than that of pepsin was found. It was not possible directly to determine the recovery of parapepsins I and II at the precipitation step, or on

Fig. 2. Gradient-elution chromatography of enzymes on diethylaminoethyl-cellulose. In each the buffer was 0.1M-acetate, pH 4.0, and the gradient was 0.02M-NaCl in the same buffer. (A) Chromatography of crude pepsin. Approx. 6 g. on 5 cm. $\times$ 35 cm. column. Mixing vessel 2.6 l. (B) Re-chromatography of parapepsin I. 4500 m[P.U.] on 2.4 cm. $\times$ 36 cm. column. Mixing vessel 560 ml. (C) Re-chromatography of parapepsin II. 5000 m[P.U.] on 2.4 cm. $\times$ 40 cm. column. Mixing vessel 560 ml. $\circ$, Activity versus acetylphenylalanyldi-iodotyrosine; $\bullet$, activity versus haemoglobin. Experimental points have been omitted from the extinction curve ($E_{280\text{ m}\mu}$) for the sake of clarity.

the first chromatogram, because the high proportion of pepsin present made independent assays of the parapepsins impossible. Assays by acetylphenylalanyldi-iodotyrosine were also not possible at these stages because of the high ninhydrin blank of the crude material.

The recovery of activity of parapepsins I and II on re-chromatography was usually 70–80%, recovery of activity on freeze-drying (relative to extinction) was 95% and the overall yield from 500 g. of crude pepsin has been about 30 mg. of parapepsin I and 90 mg. of parapepsin II. These amounts probably represent about 50% recovery of the parapepsins present in the starting material.

Chromatography of crystalline pepsin. Three batches of commercial crystalline pepsin have been chromatographed on 2.4 cm. × 30 cm. columns of diethylaminoethyl-cellulose. No parapepsin II has been found in any of them, but one batch contained parapepsin I accounting for 4% of the total activity against acetylphenylalanyldi-iodotyrosine.

Specific activities. The specific activities of the parapepsins, measured against their better substrates, are of the same order as that of freeze-dried pepsin obtained from the first chromatogram without re-chromatography (see Table 5). This pepsin itself has 120% of the activity on a weight basis of commercial crystalline pepsin, and taking a nitrogen content for dry, ash-free pepsin of 14.6% (Brand, 1948), and the determined moisture content of freeze-dried pepsin of 14%, the specific activity of $23 \text{ m[P.U.]}_{\text{mg.}}^{\text{Hb}}$ corresponds to $183 \text{ m[P.U.]}_{\text{mg. N}}^{\text{Hb}}$ which compares well with $184 \text{ m[P.U.]}_{\text{mg. N}}^{\text{Hb}}$ found by Anson & Mirsky (1932) for pepsin crystallized five times.

Product and extent of digestion of acetyl-DL-phenylalanyl-L-di-iodotyrosine by parapepsin I. The ninhydrin-positive product recovered from acetylphenylalanyldi-iodotyrosine extensively digested with parapepsin I does not separate from di-iodotyrosine on chromatography in phenol-aq. NH_3 soln., butanol-acetic acid, amyl alcohol-pyridine-water (7:7:6, by vol.) or collidine-water (1000:355, by vol.) in the presence of ammonia vapour. It does not separate either on electrophoresis on paper in N-formic acid, and the same material, eluted from butanol-acetic acid chromatograms and hydrolysed in 5.7N-HCl (105°, 20 hr.), gave no spot corresponding to phenylalanine on paper chromatograms. It is concluded that this material is di-iodotyrosine and that parapepsin I hydrolyses the phenylalanyldi-iodotyrosine peptide bond in the synthetic substrate.

When the progress of digestion was followed by ninhydrin estimations on the whole digest at suitable time intervals it was found that digestion had practically ceased when 46% of the di-iodotyrosine had been liberated. The enzyme ap-

parently hydrolyses only one of the diastereoisomers, presumably the L-L, but no attempt has been made to determine which is in fact hydrolysed.

Electrophoresis of parapepsins I and II. Since these enzymes are bound to diethylaminoethyl-cellulose at pH 4.0 they are apparently acidic proteins, too acid to allow them to be chromatographed on a cation-exchanger such as carboxymethyl-cellulose as a further step in their purification, and in fact it was found that parapepsin I passed unretarded through a column of carboxymethyl-cellulose in 5 mM-acetate buffer, pH 4.5.

To test their purity they were therefore subjected to electrophoresis on a column of ethanolysed cellulose (Porath, 1956) and in starch gel (Smithies, 1955).

On the cellulose column each enzyme appeared as a single symmetrical peak of extinction at $280 \text{ m}\mu$, coincident with the peak of enzymic activity. In the starch gel only a single band was found for each enzyme. After electrophoresis for 5 hr. at 2.5v/cm. the bands were about 0.3 cm. wide and had moved from the origin towards the anode as follows: parapepsin I 2.5 cm., parapepsin II 2.9 cm., pepsin 4.7 cm.

Ultracentrifuging of parapepsins I and II. We are indebted to Dr P. A. Charlwood of this Institute, who performed the ultracentrifugal investigations for us in the Spinco model E analytical ultracentrifuge.

The solvent used was acetate buffer, pH 5.6, μ 0.20. Sedimentation patterns showed both parapepsins to be monodisperse ($S_{20, w}$ for parapepsin II 3.32 at 0.3%) and molecular-weight determinations were made by the Archibald (1947) method as used by Charlwood (1957). In a single run parapepsin II gave a set of five values clustered round the mean (40 700), but the values for parapepsin I showed a steady fall with time of centrifuging from 55 000 to 39 000, indicating the presence of some more rapidly sedimented impurity. A 1.1% solution of parapepsin I was therefore centrifuged in the bucket cell at 100 000 g (average) for 16 hr. at 2.9–4.7°. The solution in the cell was then carefully removed with a syringe so that three roughly equal fractions, from the top, the middle and the bottom of the cell, were obtained. The middle and lower fractions were re-dialysed against buffer and examined in the analytical cell. The lower fraction gave molecular-weight determinations falling from 63 000 to 43 000 and, on high-velocity sedimentation, a small, rapidly-spreading peak which moved ahead of the main peak in the earlier pictures. This impurity was not more than 2–3% of the total material in the cell.

The middle layer was monodisperse during sedimentation ($S_{20, w}$ 3.26 at 0.42%) and gave a molecular weight (average 38 600) independent of

Table 1. *N-Terminal residues of parapepsins*

In addition to residues shown here traces of the DNP-derivatives of Asp, Glu, Thr, Val and Phe were found. No DNP-glycine was found in samples of the DNP-proteins hydrolysed for 4 hr. No DNP-arginine was found in the aqueous fraction of the 24 hr. hydrolysates.

Treatment	Hydrolysis time (hr.)	Moles found/mole of protein, not corrected for moisture content			
		Parapepsin I		Parapepsin II	
		Ala	Leu/Ileu*	Ser	Leu/Ileu*
None	19	0.68	0.19	0.77	0.36
Ion-exchange†	20	0.74	0.12	1.08	0.83
Pptn. with trichloroacetic acid†	24	0.98	0.31	0.78	1.13

* The DNP-derivatives of these amino acids cannot be separated by paper chromatography and we have not determined which isomer is present.

† See Experimental.

Table 2. *Stability of parapepsins*

Each enzyme was diluted in the appropriate solvent to the indicated initial activity. After standing at room temperature (about 20°) for the indicated time the residual activity was determined. Parapepsin I: initial activity 1.3 m[P.U.]/ml. Time, 49 hr. pH 2.0, 0.01 *N*-HCl; pH 4.0 and 5.6, 0.1 *M*-acetate; pH 6.9, 0.04 *M*-diethylbarbiturate. All contained NaCl to give μ 0.10. Parapepsin II: initial activity 0.75 m[P.U.]/ml. Time, 98 hr. pH 2.0, 0.01 *N*-HCl; pH 4.0, 0.1 *M*-acetate; pH 5.6, 0.05 *M*-citrate; pH 6.9, 0.04 *M*-diethylbarbiturate-0.06 *M*-NaCl.

pH	Percentage of activity remaining	
	Parapepsin I	Parapepsin II
2.0	87	80
4.0	95	85
5.6	95	94
6.9	100	0

Fig. 3. Inactivation of parapepsin II at pH 6.9, 25°. Initially 7.3 m[P.U.] per ml. 0.04 *M*-diethylbarbiturate-0.06 *M*-NaCl.

time of centrifuging, and this fraction had therefore been satisfactorily freed of the heavier components.

N-terminal residues of parapepsins I and II. Preliminary semi-quantitative experiments showed

that the *N*-terminal residues of parapepsins I and II were respectively alanine and serine but later experiments showed the presence of *N*-terminal leucine or isoleucine in parapepsin II as well. Table 1 shows the molar proportions of the main DNP-amino acids found, corrected for breakdown on hydrolysis, in three experiments.

Phosphorus content of parapepsins I and II. Phosphorus analyses were made on duplicate 3–4 mg. samples of the two parapepsins by the method of Allen (1940), scaled down to a final volume of 3 ml. Not more than 0.03 g. atom of phosphorus/mole of protein was found.

Stability of parapepsins I and II. Strong solutions of the two parapepsins were diluted in buffers of various pH values and allowed to stand at room temperature. Table 2 shows the percentage of the activity remaining at the times shown. The inactivation of parapepsin II at pH 6.9 is shown in greater detail in Fig. 3; the enzyme has a half-life of 70 min. The difference in stability of parapepsins I and II has been utilized in the purification of parapepsin I (see Methods section: re-chromatography of parapepsins).

A parapepsin II solution with 75% of its activity destroyed by treatment at pH 6.9 does not recover any activity on standing at pH 5.6 or pH 2.

Like pepsin the two parapepsins appear to be rather sensitive to surface denaturation; it has been noticed when pooling chromatographic fractions that a skin of insoluble protein tends to collect on the surface as successive fractions are added and some part of the loss of activity during chromatography is probably due to the same cause.

pH optima of parapepsins. The relative initial rates of digestion of acetylphenylalanyldi-iodotyrosine by parapepsin I are plotted against pH in Fig. 4.

Fig. 5 shows the progress of digestion of haemoglobin at three pH values by parapepsin II and by pepsin. The peptic digestion proceeds smoothly to an extinction well beyond 0.6, but with parapepsin

II the rate of reaction falls at E 0.5 and then follows a linear course for some time. The relative slopes of these linear phases of the reaction at these and some other pH values are plotted against pH in Fig. 4 as 'later reaction'. By using a lower concentration of enzyme the initial rate of digestion was measured at different pH values. These relative initial rates are also plotted in Fig. 4.

It can be seen that the pH optima for parapepsin I and for the later part of the digestion of haemoglobin by parapepsin II lie close to pH 1.8, whereas the optimum for the initial digestion of haemoglobin is at pH 3.0. Experiments in which the pH has been adjusted only by the addition of sodium hydroxide to acid-denatured haemoglobin show

Fig. 4. pH optima of parapepsins at 35.5°. (A) Parapepsin I on acetylphenylalanyldi-iodotyrosine, 1.3 m[P.U.]/ml. At pH 3.80 and 5.62 the mixture contained 0.05 M-acetate, at pH 2.75 0.05 M-citrate; lower pH values were obtained with HCl only. All solutions contained NaCl (to μ 0.10) and 0.5 mm-acetyl-DL-phenylalanyl-L-di-iodotyrosine. (B) Parapepsin II on haemoglobin. $\circ$, Initial reaction 0.083 m[P.U.]/1.2 ml.; $\bullet$, later reaction 0.33 m[P.U.]/1.2 ml. All digestion mixtures were made from 15 ml. of 2% haemoglobin (acid-denatured) + 3.0 ml. of (enzyme + buffer + HCl or NaOH). At pH 3.0 and 3.5 the mixture contained 0.02 M-citrate, at pH 3.9 and 4.5 0.01 M-acetate. At lower pH values no buffer was present.

Fig. 5. Digestion of haemoglobin by parapepsin II and pepsin at 35.5°. (A) Parapepsin II 0.33 m[P.U.]/1.2 ml. (B) Pepsin 0.46 m[P.U.]/1.2 ml. For details of solutions see parapepsin II, Fig. 4(b). $\square$, pH 1.1; $\triangle$, pH 1.7; $\circ$, pH 3.1.

that in the absence of buffer salts the initial digestion of haemoglobin by parapepsin II is still 1.3 times as fast at pH 3.0 as it is at pH 3.5.

Digestion of bovine plasma albumin. The digestion of bovine plasma albumin produced in 10 min. with varying amounts of pepsin and the two parapepsins up to 0.4 m[P.U.]/1.2 ml. was studied in mixtures made up as in the haemoglobin assay except that haemoglobin was replaced by plasma albumin.

Parapepsin I produced almost no digestion. Parapepsin II showed a linear relationship between increase in extinction at 280 $m\mu$ and amount of enzyme (ΔE_{280} 0.19 m[P.U.]). Pepsin was more active; the increase of extinction with 0.2 m[P.U.] was 0.135 but increased with increasing amount of enzyme slightly more rapidly than in a linear relationship.

Digestion of cysteilytyrosine and tyrosylcysteine. Harington & Pitt-Rivers (1944) showed that pepsin would digest cysteilytyrosine and tyrosylcysteine, though only slowly. An attempt was made to determine whether either parapepsin would digest these substrates: digests made with the two enzymes were chromatographed on paper in butanol-acetic acid in the presence of *N*-ethylmaleimide (Hanes, Hird & Isherwood, 1950). However, even after digestion for 24 hr. at 35.5° at pH 2.0 or 4.0 with an enzyme: substrate ratio of 1:10 (w/w), no free tyrosine or cysteine could be detected in digests made with either enzyme.

Effect of thiol and thiol-binding reagents. Pre-incubation of parapepsin II with 20 mm-thioglycolate and mm-ethylenediaminetetra-acetic acid in 0.1 M-acetate, pH 5.6, at 35.5° for 30 min. did not alter the activity of the enzyme (due allowance being made for the increase in the blank occurring on incubation of the haemoglobin substrate alone under the same conditions).

Similarly, pre-incubation of parapepsin I or II with mm-*N*-ethylmaleimide or with 0.1 mm-*p*-chloromercuribenzoate in 0.1 M-acetate, pH 5.6, at 35.5° for up to 2 hr. produced no change in the activity of either enzyme against acetylphenylalanyldi-iodotyrosine and haemoglobin respectively.

Milk-clotting activity and inhibition by pepsin inhibitor. Both parapepsins were found to clot milk, but less readily than does pepsin. The relationship between milk-clotting activity and activity against the substrates used for the regular assay is shown in Table 5. Preincubation of parapepsin II with the pepsin inhibitor (10 μ g./66 μ g. of parapepsin II) at 35.5° for 20 min. produced 35% inhibition of the milk-clotting activity of the enzyme. No such inhibitory effect on parapepsin I could be detected with inhibitor:enzyme ratios up to 1:5.

Gelatin-liquefying activity of parapepsins at pH 5. The gelatin-liquefying activities of parapepsins I and II relative to their activities against acetyl-phenylalanyldi-iodotyrosine and haemoglobin respectively and the same data for chromatographed pepsin are shown in Table 5. Assuming parapepsins I and II to have the same water content (14%) and nitrogen content (14.6%, Brand, 1948) as pepsin these figures can be converted into 3900, 520 and 17 m[P.V.]_{mg, N}^{gel V} for parapepsin I, parapepsin II and pepsin respectively.

Sites of attack on the B chain of oxidized insulin. Fig. 6 shows the peptide bands, detected by ninhydrin, found on paper-electrophoresis patterns of digests of the B chain of oxidized insulin made with

pepsin and the two parapepsins under conditions of extensive and of mild digestion. The peptides found (Table 3) in corresponding electrophoretic bands from digests made by the three enzymes are, as far as has been determined, the same. The digests differ in the relative amounts of the different peptide bands found.

Comparison of the data of Fig. 6 and Tables 3 and 4 for the three enzymes allows several conclusions to be drawn. The lack of precise specificity of pepsin is found also with the two parapepsins, which attack the same bonds in the insulin B chain as pepsin does, though with a change in the relative rates so that the parapepsins are somewhat more selective than pepsin. From examination of the

Fig. 6. Peptide bands found on electrophoresis of digests of the B chain of oxidized insulin. All digests 0.17 m[P.V.] of enzyme + 5 mg. of B chain/ml. Upper group: 20 hr., 36°. Lower group: 3 hr., 20°. Electrophoresis was in pyridine-acetic acid, pH 6.5; 33 v/cm.; 2.5 hr. The strength of the ninhydrin colour of the band is represented by the blackened area on the diagram.

Table 3. *Peptides found in digests of the B chain of oxidized insulin*

Peptides from the digests made with parapepsin I and parapepsin II are indicated by the prefixes I and II. Where the presence or absence of terminal residues has not been conclusively demonstrated they are placed in square brackets.

Band	Structure of peptide
A	Tyr. Thr. Pro. Lys. Ala. IIA not certainly identified
B	Phe. Tyr. Thr. Pro. Lys. Ala. IB and IIB have only one Phe per molecule
C	Phe. Val. Asp(NH ₂). Glu(NH ₂). His. Leu. CySO ₃ H. Gly. Ser. His. Leu. IC and IIC: Phe. Val. Asp(NH ₂). Glu(NH ₂). His. Leu. CySO ₃ H. Gly. Ser. His. [Leu. Val.]
D	Leu. Val. CySO ₃ H. Gly. Glu. Arg. Gly. Phe. Phe. Tyr. Thr. Pro. Lys. Ala. ID: N-terminal Leu. Probably one Leu per molecule. IID: Leu N-terminal. Trypsin digestion gives: Leu. Val. CySO ₃ H. Gly. Glu. Arg. Gly. Phe. Phe. Tyr. Thr. Pro. Lys and Ala
E	Phe. Val. Asp(NH ₂). Glu(NH ₂). His. Leu. CySO ₃ H. Gly. Ser. His. Leu. Val. Glu. Ala and Phe. Val. Asp(NH ₂). Glu(NH ₂). His. Leu. CySO ₃ H. Gly. Ser. His. Leu. Val. Glu. Ala. Leu. IE: Mostly the longer peptide. Much Leu liberated by carboxypeptidase. IIE: Mostly the shorter peptide. Find 2 Leu per molecule but some Leu liberated by carboxypeptidase
F	Leu. Val. CySO ₃ H. Gly. Glu. Arg. Gly. Phe. Tyr. Leu. Val. CySO ₃ H. Gly. Glu. Arg. Gly. Phe and related peptides (cf. Brown, Sanger, Smith & Kitai, 1955)
G	Val. Glu. Ala. Leu. IIG: Leu. Val. Glu. Ala?
H	Val. Glu. Ala

peptides produced under mild hydrolysis it can be seen that parapepsin I splits the phenylalanyl-phenylalanine (B24-B25) bond most readily whereas parapepsin II directs its major attack to the alanyl-leucine (B14-B15) and tyrosyl-leucine (B16-B17) bonds (numbers in parentheses indicate the position of the peptide bond in the B chain).

Examination of the peptides produced by the more prolonged digestion shows some peptides liberated by pepsin which are not produced, or are produced much more slowly, by the parapepsins. Thus both parapepsins form Phe.Tyr.Thr.Pro.Lys.Ala (band B) as the main fragment from the C-terminal end of the chain whereas pepsin gives rise mainly to Tyr.Thr.Pro.Lys.Ala (band A). The parapepsins do not readily attack this phenylalanyl-tyrosine bond (B25-B26). Although parapepsin I produces band E it does not give rise to the peptide in band C in any quantity, nor does it form bands G and H. These differences indicate that this enzyme does not readily hydrolyse the leucyl-valine (B11-B12) bond, which is hydrolysed by pepsin and probably by parapepsin II.

DISCUSSION

The curves shown in Fig. 1 illustrate a danger inherent in gradient-elution methods; even when changes of salt-concentration are made gradually in the eluting buffer the nature of the ion-exchanger is such that sudden changes of ion concentration and of pH may occur in the effluent. The changes shown in Fig. 1 must arise from the displacement of acetate bound to the DEAE-cellulose by chloride from the eluting buffer, and they could lead to spurious effects in the elution diagram. In the chromatograms presented here the rise in pH would merely lead to a stronger adsorption of the anionic proteins on to the diethylaminoethyl-cellulose, so that the proteins would move more slowly until the wave of raised pH had passed. However, the proteins with which we are concerned do not emerge (Fig. 2) until the volume of effluent exceeds 1 hold-up volume plus 0.68 times the volume of the mixing vessel, whereas the pH change and rapid change of chloride concentration are over before 1 hold-up volume plus 0.58 times the volume of the mixing vessel has passed. The proteins therefore emerge in a normal part of the gradient.

Three kinds of evidence for the purity of parapepsins I and II has been obtained. Electrophoresis, either on a column of ethanolysed cellulose or in starch gel, did not reveal any secondary components; the results with the starch gel are particularly satisfactory as the method of staining, with chlorine, might be expected to detect small peptides, or peptides which did not contain aromatic amino acid residues which might escape

detection by amidoschwarz staining or measurements of extinction.

The ultracentrifugal analyses show that parapepsin II is homogeneous whereas parapepsin I has a small amount of material which sediments more rapidly and which is itself heterogeneous. However, this material did not represent more than 2-3% of the total protein even in the lower layer of the ultracentrifuge cell into which it had been concentrated.

The results of the N-terminal analyses showed that the enzymes were contaminated with material which could be partly removed by ion-exchange resins or by precipitation of the protein with trichloroacetic acid. Since the enzymes are proteolytic it is not unlikely that they should bind small peptides or amino acids at their active centres, and a high molar ratio of such contaminating peptides would, of course, represent only a very small contamination on a weight basis. That pepsin can bind peptides in this way is indicated by the fact that whereas pure pepsin migrates to the anode in 0.1N-hydrochloric acid, in the presence of digestion products it migrates to the cathode at pH 1.5 (Herriott, Desreux & Northrop, 1940).

These results, taken in conjunction with the absence of phosphorus from the parapepsins and the difference in their relative rates of hydrolysis of haemoglobin and acetylphenylalanyldi-iodotyrosine, indicate that the proteins have been obtained free from pepsin and one another and nearly free of other protein contaminants.

The parapepsins are strikingly similar to pepsin in some properties but nevertheless show important differences in others. These relationships are summarized in the data shown in Table 5. Pepsin is a very acidic protein (Herriott *et al.* 1940) and the two parapepsins behave as anions at pH 5.6 on electrophoresis and at pH 4.0 on chromatography. Their electrophoretic and chromatographic behaviour suggests that they are less acid than pepsin, parapepsin I being the least acid of the three, and this difference accords well with the fact that the parapepsins contain no phosphorus whereas pepsin contains 1 residue of phosphate/mol.

In their molecular weights and sedimentation constants the two parapepsins are similar to pepsin, but whereas pepsin (Van Vunakis & Herriott, 1957; Heirwegh & Edman, 1957) and parapepsin I have only one N-terminal residue parapepsin II has two, which suggests that this enzyme must consist of two polypeptide chains or one branched one.

In their stability at pH 6.9, the three enzymes differ markedly. Pepsin is very rapidly inactivated at this pH (Steinhardt, 1937) but parapepsin I is completely stable. Parapepsin II lies somewhere between, being inactivated according to a uni-

molecular reaction (Fig. 3) but more slowly than pepsin. It is perhaps only coincidental that the order of instability of these enzymes at pH 6.9 is the same as that of their mobility at pH 5.6.

Parapepsin I digests acetylphenylalanyldi-iodotyrosine with a pH optimum close to that found for pepsin (Baker, 1951), but parapepsin II on haemoglobin shows two optima: at pH 3.0 for the initial part of the digestion and at pH about 1.8 for the later part. Such a change does not necessarily indicate any heterogeneity of either the enzyme or the protein substrate. It is possible that the peptide bonds most readily hydrolysed are such that the pH optimum is different from that for the digestion of a second group of peptide bonds which are more slowly hydrolysed. In other words, although the protein used as a substrate may be homogeneous it contains a heterogeneous mixture of susceptible peptide bonds which are the true substrates of the enzyme.

Although the parapepsins are clearly differentiated from one another and from pepsin by the differences in their rates of hydrolysis of haemoglobin and of acetylphenylalanyldi-iodotyrosine, consideration of the peptides found in their respective digests of the *B* chain of oxidized insulin indicates that the three enzymes have related specificities, parapepsin I and II each showing part of the activity of pepsin.

In connexion with the observed differences in specificity of the enzymes two points should be noted. First, it must be emphasized that though parapepsin I showed little activity against haemoglobin or bovine plasma albumin this does not mean that no hydrolysis of these proteins occurs. It may be that a few peptide bonds are extensively hydrolysed but that the fragments produced are precipitated by trichloroacetic acid or do not contain tyrosine or tryptophan residues. In either case their formation would not be detected by the method used here, so that it cannot yet be concluded that parapepsin I has little or no activity against protein substrates.

Secondly, it is rather surprising that, although parapepsin I hydrolyses the phenylalanyl-di-iodotyrosine bond in the synthetic substrate and the phenylalanyl-phenylalanine (*B*24-*B*25) bond in the *B* chain of oxidized insulin it does not cause as much hydrolysis of the phenylalanyl-tyrosine (*B*25-*B*26) bond as pepsin does. This effect may be due not to a fundamentally low rate of hydrolysis of this bond so much as to a relatively greater rate of hydrolysis of the neighbouring phenylalanyl-phenylalanine bond, so that the phenylalanyl-tyrosine bond now lies next to a free amino group which may be inhibitory. It is possible that a phenylalanyl-tyrosine bond found in a different location would be readily hydrolysed.

Both the parapepsins digest casein and gelatin, but at rates widely different from that found with pepsin; however, the most remarkable similarity of specificity is that the milk-clotting activity of parapepsin II is inhibited by pepsin inhibitor, whereas Herriott (1941) found that chicken pepsin was not inhibited by porcine or chicken inhibitor, although the latter would inhibit both bovine and porcine pepsins. The porcine inhibitor was without effect on any of the other proteolytic enzymes tested. Thus parapepsin II is inhibited by the inhibitor hitherto found specific for pepsins of some species only.

The two parapepsins are similar to pepsin in many ways and we must consider whether either of them might be chemically related to pepsin in having a largely common structure. The possibility that either of these enzymes is a fragment of pepsin is eliminated by the results of the molecular-weight determinations, but it is still possible that they may be derived from pepsinogen, which has a molecular weight of 42000 (Herriott, 1938). In this case the peptides removed would have to be smaller than those removed in the activation to pepsin and would have to include the phosphate residue which remains with the protein when pepsin is formed. Amino acid analyses of the parapepsins, when available, will help to clarify this question of their possible origin in pepsinogen.

Parapepsin I is similar to the gelatinase of Northrop (1932), in that it liquefies gelatin much more readily than pepsin does, and parapepsin II is similar to the cathepsin of various workers discussed in the introduction, in that its pH optimum for the initial attack on haemoglobin lies above pH 2.0 (although we have not found it to be as high as pH 3.5, the value usually reported for the cathepsin). It is not really possible to relate the gelatinase or the cathepsin to either of the parapepsins, since the first two enzymes have not yet been sufficiently characterized.

SUMMARY

1. Two proteolytic enzymes have been isolated and shown to be minor components of crude pepsin.
2. The enzymes are similar to pepsin in their physicochemical properties, their low pH optimum and their sites of attack on the *B* chain of oxidized insulin. One is inhibited by pepsin inhibitor.
3. The enzymes differ from pepsin in their *N*-terminal residues, their phosphorus content, their stability at pH 6.9 and their activity against various substrates.
4. The possible relationships of these enzymes to pepsin and to other enzymes from gastric sources are discussed.

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The Denaturation of Ovalbumin

CHANGES IN OPTICAL ROTATION, EXTINCTION AND VISCOSITY DURING SERIAL DENATURATION IN SOLUTIONS OF UREA

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Anfinsen & Redfield (1956) suggested an A-shaped structure for the native ovalbumin molecule, one leg of the A terminating in the carbohydrate moiety and the other bearing the C-terminal group. This structure was supported by a study of the increasing chemical reactivity of the ϵ -amino groups of lysine in ovalbumin during serial ethanol denaturation (Steven & Tristram, 1958). The authors postulated an internal link, which ruptured when the concentration of ethanol reached 50-55% (v/v). In earlier work, Maurer & Heidelberger

(1951) observed a critical degree of deamination of ovalbumin above which the molecule appeared to uncoil, stressing the importance of amino groups in the maintenance of the native structure.

The present work is a study of the serial denaturation of ovalbumin in urea solutions complementary to the studies of Simpson & Kauzmann (1953), who observed that the rate of denaturation of a solution of ovalbumin at pH 7.6 increased rapidly in 7M-urea. It seemed probable that the extent of denaturation of ovalbumin as measured