The Purification and Properties of a Single Chicken Pepsinogen Fraction and the Pepsin Derived from it

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1. Evidence is given for the presence of at least five pepsinogens in a crude extract of mixed chicken stomachs. One of these was purified and could be activated to yield a single pepsin. 2. The molecular weights of the pepsinogen and pepsin were 36000 and 34000 respectively. The pepsin associated at low pH values and low ionic strength. 3. The amino acid analyses of both proteins are given. The pepsin was devoid of phosphate but contained carbohydrate. 4. The *N*-terminal amino acids of pepsinogen and pepsin were serine and threonine respectively. Five amino acids were released by carboxypeptidase A and it was deduced that serine may be the *C*-terminal one. 5. Each protein contained one thiol group per molecule as determined by titration with *p*-chloromercuribenzoate. The rate of the reaction was very rapid with pepsin, but much slower with pepsinogen, although the same group appeared to react in both instances. The enzymic activity of pepsin was unaffected by the modification. 6. The isoionic point of the pepsin was close to pH 4.0 and the enzyme was stable for long periods at pH values up to 7.0. 7. The enzyme hydrolysed bis-phenyl sulphite almost as rapidly as did pig pepsin A.

The stomachs of all vertebrates investigated secrete a number of enzymes with proteinase activity at acid pH values (Taylor, 1968). The two stomach proteinases that have been studied in greatest detail are pig pepsin A (EC 3.4.4.1) and calf rennin (EC 3.4.4.3). Pig pepsin A is easily crystallized, the general properties and mechanism of action have been studied in considerable depth and its sequence has been partially determined (Fruton, 1971). Despite this, the tertiary structure of the molecule has only recently begun to be investigated. This is presumably due to the difficulty in placing the heavy atoms needed for X-ray-crystallographic analysis into defined locations in the crystal. This problem appears to have been solved by Andreeva *et al.* (1971), who have published the three-dimensional structure of the molecule at about 0.5 nm resolution. A similar difficulty has been experienced with calf rennin; although large crystals can be prepared in two forms and X-ray-crystallographic studies of these have been made (Bunn *et al.*, 1971), attempts to place heavy atoms into the crystals at defined locations have not yet been successful (Bunn *et al.*, 1970).

Chicken pepsin is the only one of the stomach acid proteinases so far investigated that appears easily accessible to X-ray-crystallographic analysis. This is because a single thiol group is present in both the chicken pepsin and pepsinogen described by Bohak (1969) and in at least one of the three chicken pepsinogens, and the derived pepsin, isolated by Donta & Van Vunakis (1970). Bohak (1969) observed that the thiol group in pepsin reacted with *p*-chloromercuri-

benzoate, thus showing that a heavy atom could be fixed at a specific location in the molecule.

The present paper describes the isolation of a pure chicken pepsinogen fraction and its conversion into a single pepsin. Some of the properties of both the pepsin and its zymogen are described. The presence of a single thiol group, which could be made to react with *p*-chloromercuribenzoate, has been established in both. A preliminary report of this work has been published (Green & Llewellyn, 1972).

Experimental

Materials

Chicken stomachs (proventriculae) were obtained from Ross Poultry Ltd., Aldershot, Hants., U.K., within 1 h of slaughter of the birds. The fat was dissected from them and they were washed and stored at -20°C until required. DEAE-cellulose (DE-52) was obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K., and Sephadex G-100 from Pharmacia, Uppsala, Sweden. Agarose, cytochrome *c* (horse heart) and crystalline chymotrypsinogen A (bovine) were products of Miles-Seravac Ltd., Maidenhead, Berks., U.K. Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Pig pepsin (twice crystallized), crystalline ovalbumin (grade V) and carboxypeptidase A (bovine pancreas), haemoglobin (bovine powder type II), *p*-chloromercuribenzoate and 5,5'-dithiobis-(2-nitrobenzoic acid) were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

Polyamide layer sheets, chloride-free toluene-*p*-sulphonic acid and 3-(2-aminoethyl)indole hydrochloride were from British Drug Houses Ltd., Poole, Dorset, U.K. 3-(2-Aminoethyl)indole was obtained from its hydrochloride as described by Liu & Chang (1971). Bis-phenyl sulphite was prepared as described by Stein *et al.* (1971). Iodoacetic acid was recrystallized from light petroleum (b.p. 60–80°C) and stored in the dark.

Buffers. For all buffers used the concentration given is that for all forms of the buffering ion.

Methods

Enzyme assays. Proteolytic activity was assayed by the hydrolysis of haemoglobin at pH 1.8 and 30°C (Herriott, 1955), unless otherwise indicated: 1 unit of enzymic activity was defined as that required to increase the E_{660} by 1.0 unit in 10 min. Potential proteolytic activity of pepsinogen was assayed after activation to the enzyme by incubation at pH 1.8–2.0 for 30 min at 0°C. Assay of activity in hydrolysing bis-phenyl sulphite was carried out at 30°C as described by Stein *et al.* (1971). Milk-clotting activity was assayed at 30°C as described by Berridge (1952) with milk powder reconstituted in 0.01 M-CaCl₂ as substrate.

Protein determinations. Elution of protein from columns was monitored by measurement of the E_{280} . All other protein determinations were carried out by the biuret micro-method of Itzhaki & Gill (1964).

Agarose-gel electrophoresis. Unless otherwise indicated electrophoresis and detection of potential proteolytic activity were carried out as described by Green (1972) except that 0.05 M-Tris-HCl buffer, pH 8.3, was used in the preparation of the gels and in the electrode compartments. When the purity of pepsinogen and pepsin was being assessed modifications of this method were used. Electrophoresis was carried out on a pair of 16 cm × 5 cm agarose-coated plates, with either 0.05 M-Tris-HCl, pH 8.3 (for pepsinogen), or 0.05 M-sodium acetate, pH 5.0 (for pepsin), as gel and electrode buffer. One of the pair of plates was stained for protein and the other for enzymic or potential enzymic activity. The plate to be stained for protein was treated as described by Chrambach *et al.* (1967). The plate to be treated to reveal the location of enzymic or potential enzymic activity was carefully covered with a layer of 0.2% (w/v) casein in 0.2 M-glycine-HCl buffer, pH 1.9, which was held there by surface tension, and incubated for 30–45 min at room temperature. It was then treated as described by Chrambach *et al.* (1967). The enzymic or potential enzymic activity was located as a light or colourless area, surrounding a dark protein spot, in a dark background of stained protein.

Activation of pepsinogen to pepsin. Two methods were used. (1) Pepsinogen was dialysed against 0.1 M-

sodium acetate buffer, pH 4.0, for 24 h at 4°C. The precipitate formed was redissolved during a further dialysis against 0.1 M-sodium phosphate buffer, pH 6.0. (2) Pepsinogen was adjusted to pH 2.0 by the addition of HCl and incubated for 10 min at 23°C. Unless otherwise indicated the activation peptide was removed by dialysis against 0.1 M-sodium phosphate buffer, pH 6.0.

Molecular-weight determinations. (1) These were done by using a column (90 cm × 2.5 cm) of Sephadex G-100 equilibrated with 0.1 M-sodium phosphate buffer, pH 6.9, and calibrated with cytochrome *c* (mol.wt. 13 400), chymotrypsinogen A (mol.wt. 23 600), ovalbumin (mol.wt. 43 500) and bovine serum albumin (mol.wt. 67 000) (Sober, 1968) as standards. The molecular weights of pepsin and pepsinogen were calculated as described by Andrews (1970). (2) Molecular weights were determined as described by Yphantis (1964) in a Beckman model E analytical ultracentrifuge. Several protein concentrations in 1 mM-sodium phosphate buffer (pH 7.0)–99 mM-NaCl were centrifuged at 35 600 rev./min and 20°C for 21 h.

Sedimentation coefficients. Sedimentation velocity analyses were made in a Beckman model E analytical ultracentrifuge. Schlieren optics were used and analyses were carried out at 20°C at 52 640 rev./min. Sedimentation coefficients were determined from the slope of a plot of the natural logarithm of the ordinate of the maximum against time.

Amino acid compositions of proteins. Proteins were reduced and carboxymethylated by a modification of the method of Anfinsen & Haber (1961). Guanidine hydrochloride was used as the denaturant and the reduction was allowed to proceed at 37°C for 4 h. After carboxymethylation the proteins were dialysed against 1 mM-NaHCO₃, pH 8.0. Hydrolyses with toluene-*p*-sulphonic acid were carried out as described by Liu & Chang (1971) and the amino acid analyses were performed on a Jeolco model 5AH amino acid analyser.

Determination of carbohydrate content of pepsin. Amino sugars were determined, together with amino acids, on the protein hydrolysates prepared and analysed as described above. Neutral sugars were determined by the anthrone method (Spiro, 1966) by using mannose as standard.

Determination of phosphate content of pepsin. Phosphate was determined by the method of Nakamura (1952) except that the protein was digested by heating at 95°C for 1 h with the 70% (w/v) HClO₄ and cooled before the other reagents were added.

N-Terminal analyses. Preparation of the dansyl derivatives of pepsin and pepsinogen and their hydrolyses were carried out as described by Gray (1967). T.l.c. on polyamide sheets with three solvents, as described by Hartley (1970), was used to identify the Dns-amino acids.

C-Terminal analyses. Hydrolysis by carboxypepti-

dase A (EC 3.4.2.1) was carried out as described by Ambler (1967) under the following conditions: enzyme/substrate ratio, 1:170 (mol/mol), pH 8 for pepsin, pH 9 for pepsinogen, at 30°C. The reaction was terminated by addition to Amberlite 1R-120 (H⁺ form) resin which lowered the pH value to about 2.0. The released amino acids were identified and determined by g.l.c. of their trifluoroacetylated methyl ester derivatives by a modification of the method of Darbre & Islam (1968).

Determination of the thiol groups in pepsin and pepsinogen. (1) Titration with *p*-chloromercuribenzoate. The method of Benesch & Benesch (1962) was used, the concentration of *p*-chloromercuribenzoate being determined from the E_{232} (Boyer, 1954). For the titration of pepsin two pairs of cells were used, the same volumes of *p*-chloromercuribenzoate being added to the reference cell containing buffer alone and to the sample cell containing buffer+protein. For pepsinogen, a series of solutions of the same protein and buffer concentration but containing increasing amounts of *p*-chloromercuribenzoate were incubated for 24 h at 4°C. The E_{255} of each was then determined. (2) Use of 5,5'-dithiobis-(2-nitrobenzoic acid). The thiol determination was carried out as described by Ellman (1959).

Results

Number of chicken pepsinogens detected and the purification of one of them

Loss of one pepsinogen in the preliminary fractionation. The crude extract of chicken stomachs prepared as described by Bohak (1969) (steps 1 and 2, and step 3 up to, but not including, application to the DEAE-cellulose column) was compared with the supernatant fraction of a chicken stomach homogenate in 0.05M-sodium phosphate buffer, pH 7.5. The preliminary fractionation procedure of Bohak (1969) was found to have resulted in the loss of about 28% of the protein and 34% of the potential enzymic activity. Agarose-gel electrophoresis of the two extracts (Plate 1) showed that one pepsinogen had been completely removed by the preliminary fractionation, although a complex mixture of proteinases remained. The pepsinogen removed was probably the pepsinogen C described by Donta & Van Vunakis (1970), since that fraction was eluted last from a DEAE-cellulose column (Donta & Van Vunakis, 1970) and therefore probably represented the most highly negatively charged material. Since Donta & Van Vunakis (1970) found no free thiol groups in pepsin C, it seemed best, for the purposes of the present work,

Fig. 1. Salt-gradient elution of the chicken pepsinogen mixture from a DEAE-cellulose column in step 3 of the purification procedure

The column was eluted with a linear gradient prepared by siphoning 2 litres of 0.02M-sodium phosphate buffer (pH 6.9)–0.1 mM- NaN_3 –0.3M- NaCl into 2 litres of phosphate buffer– NaN_3 –0.1M- NaCl . Other experimental details are described in the text. The numbered bars indicate the pooled fractions. —, E_{280} ; ○, potential enzymic activity.

Table 1. Recoveries of protein and potential enzymic activity from step 3 of the purification procedure

The starting material was that recovered from step 2 and the fractions were those indicated in Fig. 1. Methods are described in the Experimental section.

Fraction	Total protein		Total potential enzymic activity		Potential specific activity (units/mg of protein)
	(g)	(% recovered)	(units)	(% recovered)	
Starting material	2.08	—	189000	—	90.5
1	0.122	6	8350	4	68.3
2	0.234	12	26600	14	113
3	0.527	25	55500	29	105
4	0.553	27	68500	36	124

to use the preliminary fractionation procedure of Bohak (1969) as a first purification step.

Purification of a pepsinogen fraction. Step 1. A crude extract was obtained from 400g of chicken stomachs by the preliminary fractionation procedure of Bohak (1969) (see above).

Step 2. The crude extract in 0.05M-sodium borate buffer, pH7.6, was applied to a column (20cm × 5.6cm) of DEAE-cellulose equilibrated with the same buffer. The column was washed with 0.05M-borate buffer (pH7.6)–0.1M-NaCl until the E_{280} of the effluent was below 0.2, and the effluent was discarded. The pepsinogen was then eluted from the column with 0.05M-borate buffer (pH7.6)–0.3M-NaCl.

The rest of the purification procedure was modified from that of Donta & Van Vunakis (1970).

Step 3. The pepsinogen fraction from step 2 was dialysed against three changes of 5 litres of 0.02M-sodium phosphate buffer (pH6.9)–0.1mM-Na₃ and applied to a column (20cm × 4.2cm) of DEAE-cellulose equilibrated with the same buffer. The column was washed with 0.02M-sodium phosphate buffer (pH6.9)–0.1mM-Na₃–0.1M-NaCl until the E_{280} of the effluent had fallen below 0.2, and the effluent was discarded. The protein was then eluted with a linear gradient of 0.1–0.3M-NaCl in the phosphate buffer–Na₃ and 30–50ml fractions were collected (Fig. 1). Four potential enzymically active fractions were obtained from fractions pooled as shown in Fig. 1. The recoveries of protein and potential enzymic activity in each fraction are shown in Table 1. Each represented a different protein, since the mobility of the potential enzymes on agarose-gel electrophoresis increased in the order of elution from the column.

Step 4. Fraction 4 from step 3 was dialysed against three changes of 4 litres of 0.02M-sodium phosphate buffer (pH6.9)–0.1mM-Na₃ and applied to a column (20cm × 3.5cm) of DEAE-cellulose equilibrated with the same buffer. The column was washed with phosphate buffer–Na₃–0.15M-NaCl until the

E_{280} of the effluent was below 0.2, and the effluent was discarded. A linear gradient prepared by siphoning 1.5 litres of phosphate buffer–Na₃–0.3M-NaCl into 1.5 litres of phosphate buffer–Na₃–0.15M-NaCl was then applied to the column and fractions of 20–35ml were collected. Two incompletely separated peaks of potential enzymic activity, coinciding with protein peaks, were obtained. The first peak, normally the smaller, may have represented contaminating fraction 3 pepsinogen from step 3. The fractions from the latter part of the second peak, judged to be free of fraction 3 pepsinogen, were combined and concentrated to 8–10ml by vacuum dialysis against 0.1M-sodium phosphate buffer (pH6.9)–0.1mM-Na₃.

Step 5. After further dialysis against 0.1M-sodium phosphate buffer (pH6.9)–0.1mM-Na₃, the concentrated pepsinogen solution was applied to a Sephadex G-100 column and eluted from it (Fig. 2). Very minor peaks sometimes preceded the symmetrical major protein peak, which coincided with the potential enzymic activity. The fractions comprising the peak were combined as indicated in Fig. 2. The solution was dialysed against three changes of 2 litres of 0.05M-sodium borate buffer, pH7.6, and stored at –20°C.

The recoveries and potential specific activities obtained at the various stages of a typical purification procedure are given in Table 2.

Purification of chicken pepsin

In a few instances the above purification procedure did not give a pure product, as judged by the criteria described below. Then the pepsinogen, prepared as described above, was activated to pepsin and a further purification step was carried out. The pepsin solution was dialysed against 2 litres of 0.02M-sodium phosphate buffer, pH6.9, then against 2 litres of 0.02M-sodium phosphate buffer (pH6.9)–0.1mM-Na₃. It was applied to a column (20cm × 1.4cm) of DEAE-cellulose equilibrated with the

EXPLANATION OF PLATE I

Agarose-gel electrophoretogram of extracts resulting from the preliminary fractionation procedure described by Bohak (1969) (1) and the supernatant fraction of a crude chicken stomach homogenate (2)

Electrophoresis was carried out at 5°C for 1.5 h at 15.6 V/cm with 0.05 M-sodium acetate buffer, pH 5.0, as both gel and electrode buffer. The gel was stained for potential enzymic activity. Further details are given in the Experimental section.

EXPLANATION OF PLATE 2

Agarose-gel electrophoretograms of pepsinogen and pepsin stained for protein and potential enzymic or enzymic activity

Electrophoresis was carried out at 5°C for 1.5 h at 15.6 V/cm with the buffer indicated as both gel and electrode buffer. Further details are in the Experimental section. (a) and (b) Pepsinogen, 0.05 M-Tris-HCl buffer, pH 8.3; (c) and (d) pepsin, 0.05 M-sodium acetate buffer, pH 5.0. (a) and (c) are stained for protein; (b) and (d) are stained for potential enzymic or enzymic activity.

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Fig. 2. Elution of fraction 4 pepsinogen from a Sephadex G-100 column

The protein solution was applied to a column (92 cm $\times$ 2.5 cm) of Sephadex G-100 equilibrated with 0.1 M-sodium phosphate buffer (pH 6.9)–0.1 mM- NaN_3 and eluted with the same buffer solution, 10 ml fractions being collected. The bar indicates the fractions combined. —, E_{280} ; ○, potential enzymic activity.

same buffer solution. The column was washed with phosphate buffer– NaN_3 –0.2 M- NaCl until the E_{280} of the effluent was below 0.2. Then the pepsin was

eluted by application of a linear gradient prepared by siphoning 300 ml of phosphate buffer– NaN_3 –0.4 M- NaCl into 300 ml of phosphate buffer– NaN_3 –0.2 M- NaCl , fractions of 10–20 ml being collected. A single protein peak was obtained, which coincided with the enzymic activity. The enzymically active material was combined, concentrated to about 25 ml by ultra-filtration, dialysed against two changes of 1 litre of 0.02 M-sodium phosphate buffer, pH 6.0, and stored at -20°C .

Evidence of purity of the pepsinogen and pepsin fractions

The pepsinogen and pepsin fractions obtained both gave single peaks on chromatography on DEAE-cellulose columns and on gel filtration on Sephadex G-100 columns. In each instance, agarose-gel electrophoresis by the duplicate-plate method gave a single protein spot and a single enzyme or potential enzyme spot coincident with the protein spot (Plate 2).

Comparison of the pepsins produced by the two methods of activation

Pepsinogen was activated at pH 4.0 and 2.0 as described in the Experimental section. In each instance the product was applied to gel-filtration columns equilibrated at each of pH 3.0 and 6.9. From each column a single protein peak, coincident with the enzymic activity, was obtained. The elution volumes are given in Table 3. The protein-containing tubes from each column were combined and concentrated by vacuum dialysis against the solution used for elution. The observed sedimentation coefficients, determined by analytical ultracentrifugation, of the products eluted from the two columns at pH 6.9 are given in Table 3, but the products were insufficiently soluble at pH 3.0 for ultracentrifugal analysis. Agarose-gel electrophoresis by the duplicate-plate method was carried out on each product and mixtures of two in all possible combinations. All samples gave single enzyme and protein spots and therefore appeared identical.

Properties of pepsinogen and pepsin

Molecular weights. The molecular weights of pepsinogen and pepsin were determined by the elution volume from a Sephadex G-100 column calibrated by four standards, and by analytical ultracentrifugation by the meniscus-depletion method (Yphantis, 1964). The results are shown in Table 4. The molecular weights determined by the average of the results for the Yphantis (1964) method were 36000 for pepsinogen and 34000 for pepsin.

Sedimentation coefficients for pepsin at pH values between 3.0 and 7.0. The sedimentation coefficients

Table 2. Recoveries and potential enzymic activities obtained in a typical purification procedure of pepsinogen 4

Methods are described in the Experimental section.

Purification Step	Protein			Potential enzymic activity			Specific activity (units/mg of protein)
	(mg/ml)	Total (mg)	Recovery (%)	(units/ml)	Total (units)	Recovery (%)	
1	14.8	10210	100	489	337000	100	33.0
2	2.75	5200	51	166	314000	93	60.3
3	2.4	1080	10.6	189	85100	25	78.8
(fraction 4)							
4	1.4	249	2.4	121	21500	6.4	86.4
5	3.5	189	1.9	313	16900	5.0	89.5

Table 3. Comparison of the pepsins produced by activation of pepsinogen at pH 2.0 and 4.0

For the gel filtration, columns (90cm×2.5cm) of Sephadex G-100 were used, equilibrated and eluted with either 1M-HCl or 0.1M-sodium phosphate buffer, pH6.9, 10ml fractions being collected. The samples applied were the products formed from 15 mg of purified pepsinogen by activation at either pH4.0 or pH2.0 as described in the Experimental section. When the activation was carried out at pH2.0, the samples were applied to the columns immediately; when it was carried out at pH4.0, the samples were applied to the columns after dialysis against 0.1M-sodium phosphate buffer, pH6.0.

	Sample	
	Activated at pH2.0	Activated at pH4.0
Elution vol. from column, pH3.0 (ml)	234	231
Elution vol. from column, pH6.9 (ml)	227	225
<i>s</i> (observed) pH6.9 (S)	3.2	3.4

were determined for pepsin over the pH range 3.0–7.0 at ionic strengths of 1.0 and 0.1. The results (Table 5) indicate that pepsin aggregates as the pH decreases. The association appeared more favoured at the lower ionic strength than the higher one. At *I* 0.1, pepsin existed in the associated form at pH6.0 and lower pH values; at *I* 1.0, the associated form existed at pH4.6 and 3.0, with an intermediate form at pH6.0. This intermediate form may have been an equilibrium mixture, in which case the equilibrium must have been very rapidly established, since only a single peak was obtained. This was confirmed by agarose-gel electrophoresis of pepsin in 0.05M buffers of between pH1.0 and 7.0. In each instance only a single spot was obtained.

Table 4. Molecular weights of pepsinogen and pepsin determined by gel-filtration and Yphantis (1964) methods

The elution volumes of pepsinogen and pepsin from the Sephadex G-100 column were determined from measurements of both E_{280} and potential enzymic or enzymic activity. In each instance the peaks coincided. The elution volumes of the standard proteins were determined in a separate experiment with the same column. Plots of elution volume against log molecular weight were calculated by the method of least squares. Other experimental details are described in the text.

Protein concentration (mg/ml)	Molecular weights	
	Pepsinogen	Pepsin
Gel-filtration method		
—	41900	40800
Yphantis (1964) method		
0.175	35300	—
0.2	—	34500
0.25	—	31800
0.35	37300	—
0.4	—	33700
0.5	35700	36600

Amino acid and amino sugar compositions of pepsinogen and pepsin. Acid hydrolyses were carried out for 22, 48 and 72h and each hydrolysate was analysed for amino acids and amino sugars. The results (Table 6) are expressed in terms of mol of amino acid or amino sugar/mol of protein, by using the molecular weights determined by the Yphantis (1964) method. The nearest integer was calculated by averaging the results for the three hydrolysates except for tryptophan and serine, which are known to be destroyed during the hydrolysis (Liu & Chang, 1971), and galactosamine, which appears to decrease during the hydrolysis. For these three compounds the

nearest integer was calculated by extrapolation to zero time.

Neutral carbohydrate and phosphate contents of

Table 5. *Sedimentation coefficients of pepsin at pH values between 3.0 and 7.0 and I between 1.0 and 0.1*

For *I* 1.0, the buffers used were at 10mM and contained 990mM-NaCl. For *I* 0.1, the buffers were at 1mM and contained 99mM-NaCl. The buffers used were: pH 3.0, glycine-HCl; pH 4.6, sodium acetate; pH 6.0 and 7.0, sodium phosphate. The protein concentrations were about 4.5mg/ml for *I* 1.0 and 2.7mg/ml for *I* 0.1.

pH	$s_{20,w}$ (S)	
	<i>I</i> 1.0	<i>I</i> 0.1
3.0	5.7	5.8
4.6	5.5	6.1
6.0	4.9	6.0
7.0	4.1	4.0

pepsin. Analysis of pepsin for neutral carbohydrate gave 5–6 'mannose equivalents'/34000g and analysis for phosphate revealed less than 0.1 mol/34000g.

N-Termini of pepsin and pepsinogen. T.l.c. of an acid hydrolysate of Dns-pepsin gave a spot that ran coincident with Dns-threonine, therefore showing that threonine was the *N*-terminal amino acid of the pepsin. For pepsinogen it was necessary to reduce and carboxymethylate the protein before treating it with dansyl chloride. T.l.c. of an acid hydrolysate then gave a spot coincident with Dns-serine, showing that serine was *N*-terminal in the pepsinogen.

C-Termini of pepsin and pepsinogen. The release of amino acids from pepsin and pepsinogen by carboxypeptidase A is shown in Table 7. The comparative rates of release of the five amino acids require comment. First, there are possible inaccuracies in the results; (1) the blanks contained small amounts of serine, phenylalanine and tyrosine, although these were not more than 10% of the 1 min value in any instance; (2) the analytical method used gave erratic results with tyrosine, leading to uncertainty in the yields of this amino acid. Secondly, all the amino

Table 6. *Amino acid and amino sugar analyses of pepsin and pepsinogen*

Each protein was reduced and carboxymethylated, and a sample (4.0mg) of each was hydrolysed for each of the times indicated. The hydrolysates were made up to 5ml and 1ml of each was analysed. Other experimental details are described in the text. CmCys, carboxymethylcysteine.

Amino acid or amino sugar released (mol/34000g)

Amino acid or amino sugar	Hydrolysis time ...	Pepsinogen				Pepsin			
		22h	48h	72h	Nearest integer	22h	48h	72h	Nearest integer
Trp		3.2	2.7	2.8	3	3.4	1.9	—	5
Lys		12.8	13.7	13.9	13	9.0	8.8	7.3	8
His		5.7	6.1	6.3	6	3.8	3.9	3.2	4
Arg		5.1	5.4	5.8	5	4.3	4.5	3.8	4
Asp		33.2	33.1	33.4	33	36.2	32.6	27.2	32
Thr		20.9	21.7	22.0	22	23.7	23.0	18.8	22
Ser		32.4	31.5	31.0	33	35.7	31.9	25.6	38
Glu		20.7	20.9	21.1	21	22.1	20.5	16.9	20
Pro		14.7	14.7	15.4	15	14.2	12.8	10.3	12
Gly		26.2	26.2	26.4	26	29.1	26.7	22.6	26
Ala		14.0	14.0	16.6	15	15.4	14.1	11.7	14
Val		14.1	18.2	19.6	17	15.6	18.4	15.9	17
Met		7.7	7.4	7.8	8	8.2	7.9	6.0	7
Ile		11.6	15.2	16.9	15	13.5	15.8	14.2	14
Tyr		20.1	20.7	21.8	21	22.4	21.1	17.1	20
Phe		15.0	15.9	16.7	16	16.7	16.7	13.9	16
Leu		20.7	22.1	23.1	22	18.9	18.7	15.9	18
CmCys		4.9	4.8	5.0	5	5.8	5.6	4.7	5
NH ₃		36.3	38.4	41.0	39	37.6	50.1	61.6	50
Galactosamine		6.7	5.8	5.4	7	4.9	4.1	3.0	6

Table 7. Release of amino acids from pepsin and pepsinogen by carboxypeptidase A

The experimental details are described in the text. The results were corrected by subtraction of blanks containing all components of the reaction mixture except substrate.

Reaction time (min)	Amino acids released (mol/mol)									
	Pepsin					Pepsinogen				
	Leu	Ser	Phe	Val	Tyr	Leu	Ser	Phe	Val	Tyr
1	0.42	0.38	0.27	0.00	0.41	0.34	0.31	0.33	0.11	0.28
5	0.72	0.48	0.42	0.03	0.31	0.49	0.36	0.57	0.18	0.43
30	0.98	0.55	0.43	0.10	0.31	0.58	0.50	0.58	0.16	0.48
100	—	—	—	—	—	1.00	0.65	0.82	0.52	0.72
240	1.51	1.01	0.56	0.42	0.50	1.27	1.05	0.86	1.00	0.79

acids released, except serine, are in the group rapidly released by carboxypeptidase A (Ambler, 1967). Further, it is known that the penultimate amino acid affects the rate of release of the C-terminal one (Ambler, 1967), which probably explains the rapid release of serine.

Because five amino acids were rapidly released from each protein by the enzyme, it is not possible to deduce a unique sequence in either instance. Further, the results suggest that, in both instances, more than one residue of leucine was released. Since leucine, assuming the release of two residues, serine and phenylalanine were all released at approximately the same rate from both proteins, and the release of serine would be expected to be rate-determining, the results suggest that serine may be the C-terminal amino acid. Valine and tyrosine were released from both proteins at a perceptibly slower rate, suggesting that these two amino acids are farther from the C-terminus than the other three. Therefore, although the results do not permit the deduction of an unambiguous C-terminal residue or sequence, the following sequence appears in greatest accord with the results: -(Val,Tyr)-(Phe,Leu,Leu)-Ser-OH. The results do suggest, however, that both the pepsin and pepsinogen have the same C-terminal sequence.

Determination of thiol groups in pepsin and pepsinogen. The thiol group of pepsin appeared to be extremely susceptible to oxidation by O₂. This was avoided by carrying out thiol analyses on pepsin prepared by activation of pepsinogen at pH 2.0 and then immediately dialysed against buffer that had been flushed with N₂, and performing the analysis rapidly. When this was done and the results were analysed by the least-squares method, titration of pepsin with *p*-chloromercuribenzoate revealed 1.02 ± 0.01 (s.d.) thiol equivalents/34000 g in 0.1 M-sodium phosphate buffer, pH 7.0, and 1.00 ± 0.16 thiol equivalents/34000 g in 0.1 M-sodium acetate buffer, pH 4.6. A difference spectrum performed after

removal of the excess of *p*-chloromercuribenzoate by dialysis against 0.05 M-sodium phosphate buffer, pH 6.0, showed that the mercuribenzoate moiety had been retained.

An attempt to apply the same method to pepsinogen was unsuccessful, since the reaction did not reach completion in 1 h at room temperature. However, experiment showed that the reaction was complete after 24 h at 5°C. Least-squares analysis of extinctions of mixtures of pepsinogen incubated for 24 h at 4°C with increasing amounts of *p*-chloromercuribenzoate in 0.1 M-sodium phosphate buffer, pH 7.0, indicated the presence of 0.99 ± 0.12 thiol equivalents/36000 g. The fully reacted pepsinogen was adjusted to pH 2.0 to activate it to pepsin and then dialysed against 0.02 M-sodium phosphate buffer, pH 6.0, to remove the activation peptide and excess of *p*-chloromercuribenzoate. A difference spectrum of the resulting solution showed that the mercuribenzoate moiety had been retained. The enzymic activity was unchanged in both the modified pepsin and the pepsin produced from the modified pepsinogen.

The reaction of pepsin with 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.1 M-sodium phosphate buffer, pH 7.0, at room temperature was extremely slow. The reaction appeared to reach completion after 2.5 h, after which 0.54 thiol equivalents had reacted/34000 g of pepsin. The presence of 6 M-urea had little effect on the reaction rate and the product appeared to be unstable.

Isoionic point of pepsin. The isoionic point of pepsin, determined as described by Green (1971) by using 7.8 mg of pepsin, was pH 4.2 ± 0.3 (s.e.m.). This is in accordance with the point of minimum solubility of the protein, which was close to pH 4.0.

Stability of pepsin and pepsinogen. Pepsinogen in 0.05 M-sodium borate buffer, pH 7.6, and pepsin in 0.02 M-sodium phosphate buffer, pH 6.0, were stable for several months at -20°C. Pepsin showed no measurable loss of activity on storage at 5°C in 0.02 M-

sodium phosphate buffer, pH 6.0, and 6.9, for 10 days, but a 30–40% loss of activity was observed for pepsin in 0.02M-sodium acetate buffer, pH 4.6. Further, pepsin showed no loss of activity in 0.1M-glycine-HCl buffer, pH 2.0 and 3.0, 0.1M-sodium acetate buffer, pH 5.0, and 0.1M-sodium phosphate buffer, pH 6.0 and 7.0, in 4 days at 23°C.

Assay of pepsin by the clotting of milk and the hydrolysis of bis-phenyl sulphite. Chicken pepsin both clotted milk and hydrolysed bis-phenyl sulphite. The specific activities in the assays used, expressed as a percentage of the specific activity of crystalline pig pepsin, were 58% for milk clotting at pH 6.2, 92% for the hydrolysis of bis-phenyl sulphite at pH 3.6 and 160% for the proteolysis of haemoglobin at pH 1.8.

Discussion

Number, identity and purity of chicken pepsinogens and pepsins

It appears from the present work that there are at least five chicken pepsinogens; one was removed during step 1 of the purification procedure and the other four were separated by DEAE-cellulose column chromatography. Each was characterized by its mobility on agarose-gel electrophoresis at pH 8.3. The proventriculæ used in this work were probably obtained from a mixture of breeds; however, the proportion of the different pepsinogens was remarkably constant in different batches. Other workers have obtained evidence for different numbers of chicken pepsinogens. Donta & Van Vunakis (1970) separated three pepsinogens by column chromatography on DEAE-cellulose at pH 6.9, which they judged to be pure by gel filtration and polyacrylamide-gel electrophoresis. In Fig. 2 (b) of their paper a shoulder of potential enzymic activity can be seen at the beginning of the first pepsinogen peak. In the present work, the salt gradient used was considerably expanded from that used by Donta & Van Vunakis (1970), resulting in improved resolution of the peaks. It seems probable that the first shoulder observed by Donta & Van Vunakis (1970) represented a mixture of the minor pepsinogens 1 and 2 observed in the chromatogram obtained in step 3 of our procedure. The pepsinogens 3 and 4, observed in step 3 of our procedure, probably correspond to their pepsinogens D and A respectively. Pepsinogen C (Donta & Van Vunakis, 1970) was eluted from DEAE-cellulose only at higher ionic strengths than the other pepsinogens, indicating that it had the highest negative charge. This probably corresponds to the zymogen of highest electrophoretic mobility, which was removed in step 1 of our procedure. Levchuk & Orekhovich (1963) also separated three pepsinogen fractions by DEAE-cellulose column chromatography. Although the conditions

used by these workers differed from those of the present work, it is probable that their fractions 1, 2 and 3 represent our fractions 3 and 4 and pepsinogen C of Donta & Van Vunakis (1970) respectively. Bohak (1969) reported only one chicken pepsinogen, judged to be pure by DEAE-cellulose column chromatography, gel filtration and polyacrylamide-gel electrophoresis. It was shown in the present work that pepsinogen C would have been removed early in the purification procedure of Bohak (1969), but it does not appear possible to relate his remaining pure fraction to any of those obtained by other methods.

In the present work pepsinogen 4 appeared to be pure as judged by its emergence as a single peak from DEAE-cellulose and gel-filtration columns and by agarose-gel electrophoresis. Further, it could be activated by two different procedures to a single pepsin judged to be pure by the same criteria. This accords with the work of Levchuk & Orekhovich (1963), in which the corresponding pepsinogen was activated to a single pepsin, with only slight contamination. Pepsinogen A isolated by Donta & Van Vunakis (1970) could not be activated by them to a single pepsin; multiple bands were observed on polyacrylamide-gel electrophoresis.

Properties of pepsinogen 4 and the derived pepsin

The molecular weights of both the pepsinogen and pepsin isolated in the present work appeared higher by the gel-filtration method than by the ultracentrifugal meniscus-depletion method (Yphantis, 1964). This suggests that the proteins are non-spherical. The true molecular weights were taken as those given by the latter method, i.e. 36000 and 34000 for pepsinogen and pepsin respectively. These compare with a molecular weight of about 42000 reported for both pepsinogen A and the pepsin complex derived from it by Donta & Van Vunakis (1970) and molecular weights of about 43000 and 35000 for the pepsinogen and pepsin prepared by Bohak (1969).

Sedimentation analyses in the ultracentrifuge indicated that the purified pepsin prepared in the present work associated at low pH values. The equilibrium between the associated and non-associated forms seemed to be rapidly established, and association was favoured by low ionic strength, indicating that electrostatic interactions were involved. No conclusions can be drawn as to whether both forms were enzymically active, since pepsin was probably mainly or partially associated in all the enzymic assays used.

From the difference between the amino acid compositions of pepsinogen and pepsin it can be deduced that the activation peptide contained the following charged residues: five lysine, two histidine, one arginine, one aspartic acid and one glutamic acid. Therefore the activation peptide must be positively

charged, with the corollary that pepsin has a higher negative charge than pepsinogen. This is in accord with experiment; pepsin showed a higher electrophoretic mobility than pepsinogen and was eluted from DEAE-cellulose columns at pH 6.9 at higher salt concentrations than pepsinogen.

Because of the scatter in the results for the different times of hydrolysis, the amino acid compositions of the two proteins cannot be deduced with certainty. This particularly applies to the results for tryptophan, aspartic acid, threonine, serine, glutamic acid, valine, methionine and isoleucine. Taking account of the range of error in the results and adjusting for differences in assumed molecular weights, the amino acid compositions of the two proteins determined in the present work were close to those reported by Donta & Van Vunakis (1970) for pepsinogen and pepsin A and by Bohak (1969) for the purified pepsinogen and pepsin that he had isolated. The evidence indicated that phosphate was absent from the pepsin, as found by Levchuk & Orekhovich (1963) for all their fractions, but that carbohydrate was present. The number of residues and nature of the carbohydrate were similar to those found by Bohak (1969) in pepsinogen and pepsin, except that Bohak (1969) detected two residues of glucosamine and no galactosamine, whereas in the present work about six residues of galactosamine and no glucosamine were detected.

Bohak (1969) found serine to be the *N*-terminal residue of pepsin, whereas in the present work threonine and serine were found to be *N*-terminal in pepsin and pepsinogen respectively. The dansylation reaction used for the *N*-terminal analysis only occurred with pepsinogen after its reduction and alkylation, which, since it involved treatment with guanidine hydrochloride, may have denatured the protein. This agrees with the observation of Bohak (1969) that no *N*-terminal amino acid could be detected after direct dansylation of his purified pepsinogen. The *C*-terminal amino acid of neither pepsinogen nor pepsin could be identified with certainty, but the evidence suggested that it might be serine in both proteins, with leucine and other amino acids also being released by carboxypeptidase A. This accords with the results of Bohak (1969) who detected both serine and leucine in carboxypeptidase A digests of both proteins and suggested that serine was probably the *C*-terminal amino acid in each. The similarity in the *C*-terminal residues between zymogen and enzyme and the dissimilarity between the *N*-terminal ones indicates that activation of the zymogen occurs by release of a peptide from the *N*-terminal end. This is in accord with the activation mechanism of pepsinogens of other species (Taylor, 1968). Further conclusions can be drawn about the activation mechanism from the difference in the rates of reaction of the pepsinogen and pepsin with *p*-chloromercuribenzoate. The evidence suggests that

the same thiol group reacted in each instance and that it was sufficiently distant from the active site to have no effect on the enzymic activity. Therefore, the difference in reaction rate suggests that the proteins are conformationally different. Wang & Edelman (1971) arrived at a similar conclusion for pig pepsinogen and pepsin by means of fluorescence studies. Further, the *N*-terminal residue of chicken pepsinogen was not accessible to dansyl chloride suggesting that the *N*-terminal part of the polypeptide chain is buried in the interior of the molecule. Since the peptide released on activation of chicken pepsinogen had a molecular weight of only about 2000, suggesting that the bond hydrolysed during activation may be sufficiently near the *N*-terminus to be similarly buried, it seems possible that any conformational change taking place during activation precedes the release of the activation peptide. Funatsu *et al.* (1972) suggested that such a mechanism occurs in the activation of pig pepsinogen, on the basis that the presence of a pepsin substrate decreases the rate of release of the activation peptide, indicating that a substrate-binding site is present before the activation peptide is released.

Levchuk & Orekhovich (1963), Bohak (1969) and Donta & Van Vunakis (1970) found that chicken pepsin did not hydrolyse certain small peptide substrates that are hydrolysed by pig pepsin. In the present work, the only small substrate tested was a sulphite diester, bis-phenyl sulphite. This was hydrolysed by chicken pepsin nearly as rapidly as by pig pepsin. The evidence available from inhibition studies and the pK_a values of the groups involved in the enzymic reaction suggests that such esters are hydrolysed by pig pepsin by the same mechanism as are peptides (Reid & Fahrney, 1967). As observed by Levchuk & Orekhovich (1963) and Donta & Van Vunakis (1970) for a probably identical enzyme, the pepsin isolated in the present work was more active in hydrolysing haemoglobin and less active in clotting milk than is pig pepsin.

Comparison of pepsinogens and pepsins from chicken with those from other species

All species on which detailed investigations have been made secrete more than one gastric proteinase. This applies to the human, pig (Taylor, 1968), dogfish (Merrett *et al.*, 1969) and ox (Lang & Kassell, 1971; Antonini & Ribadeau-Dumas, 1971), and the chicken is no exception.

As far as can be assessed from the results reported, the specificity of gastric proteinases increases with their isoelectric point. Thus pig pepsin C has both a lower electrophoretic mobility (Ryle & Hamilton, 1966) and a greater specificity than pig pepsin A (Ryle *et al.*, 1968). Calf rennin, of isoelectric point pH 4.6 (Foltmann, 1966), is even more specific (Hill, 1969; Raymond *et al.*, 1972). Chicken pepsin is more

specific than pig pepsin A (Levchuk & Orekhovich, 1963; Bohak, 1969; Donta & Van Vunakis, 1970), but there is insufficient information to compare it precisely with other gastric proteinases.

The stability of gastric proteinases at high pH values also seems to be related to the isoelectric point, e.g. calf rennin is stable at higher pH values than is pig pepsin A (Foltmann, 1966). However, chicken pepsin seems exceptional in this regard, in that it was stable for long periods at pH 7.

Chicken pepsin is unique among the gastric proteinases so far studied in having a single thiol group. In the present work, one thiol group per molecule was found for both the pepsinogen and the pepsin, and the thiol group appeared equally reactive in the associated and non-associated forms of pepsin. Since the thiol group in both the zymogen and the enzyme reacted with *p*-chloromercuribenzoate, it is possible that both will prove suitable for X-ray-crystallographic studies and thus might act as models for gastric proteinases and zymogens in general. Investigation of this possibility requires crystallization of both the pepsin and the pepsinogen and further study of the properties of the enzyme, in particular those of the active site.

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