Pepsinogen C and Pepsin C

FURTHER PURIFICATION AND AMINO ACID COMPOSITION

By A. P. RYLE AND M. PAULINE HAMILTON*

*Department of Biochemistry, University of Edinburgh Medical School,
Teviot Place, Edinburgh*

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Pepsinogen C and pepsin C from the pig have been further purified by chromatography on DEAE-cellulose and by exclusion chromatography and the specific activities (with haemoglobin substrate) found are higher than those previously reported. The final preparations are homogeneous on electrophoresis in starch gel at three pH values except for contamination with less than 4% of pepsinogen and pepsin respectively. Pepsinogen C, like pepsin C, contains no phosphate. The amino acid compositions show some marked differences from those of pepsinogen and pepsin especially in the content of basic amino acids, glutamic acid, aspartic acid, leucine and isoleucine. The molecular weights of the enzyme and zymogen, obtained from the amino acid compositions, are 41400 and 36000 respectively, similar to those of pepsinogen and pepsin.

The isolation of two minor pig gastric proteases, which were formerly called parapepsins I and II and are now (Ryle, 1965) called pepsin B (EC 3.4.4.2) and pepsin C (no number given by the Enzyme Commission) respectively, has been reported (Ryle & Porter, 1959).^{*} Pepsin B was then thought to be a single enzyme but has since been shown (Ryle, 1965) to contain two active components that can be separated by electrophoresis in starch gel. Pepsin B, pepsin C and a third minor enzyme, pepsin D, have been detected in the gastric contents of the pig (Ryle, 1966).

Pepsin C is derived from a zymogen, pepsinogen C (formerly parapepsinogen II), whose isolation by ion-exchange chromatography from gastric mucosa has also been described (Ryle, 1960). The present paper describes the further purification of both enzyme and zymogen by exclusion chromatography and the results of some analyses of the two proteins.

EXPERIMENTAL

Buffer solutions and materials

Buffer solutions were made from sodium salts except where otherwise indicated. The concentration refers to the total molarity of the buffering component.

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

Acetyl-L-phenylalanyl-L-di-iodotyrosine was prepared by the method described by Baker (1951) and had m.p. 231° (decomp.) and $[\alpha]_D^{20} + 30.5 \pm 0.8^\circ$ (c 2 in pyridine).

5,5'-Dithiobis-(2-nitrobenzoic acid) was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.

Sephadex was obtained from Pharmacia Ltd., Uppsala, Sweden.

DEAE-cellulose was prepared as described by Peterson & Sober (1956) and had about 1 m-equiv. of basic groups/g.

Hydrolysed starch for use in electrophoresis experiments was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

Methods

Activity determination with haemoglobin. Assays of activity against haemoglobin were made in duplicate at 37° and pH 1.7 by the method used in the earlier work (Ryle, 1965, based on Anson, 1948). The increases in extinction of the deproteinized solutions at 280 m μ were converted into proteolytic units (haemoglobin substrate), designated [P.U.]^{hb}, by reference to a standard curve prepared for purified pepsin C. The acid used for acidification of the haemoglobin for assay of enzyme or zymogen dissolved in the different buffers was as follows: for 0.1 M-acetate, pH 5.6: 0.39 N-HCl; for 0.02 M-phosphate, pH 6.9: 0.315 N-HCl; for tris-citrate buffer: 0.454 N-HCl.

Activity determinations with acetyl-L-phenylalanyl-L-di-iodotyrosine. These were carried out as described by Ryle (1965) except that, to conform with the recommendations of the International Commission on Enzymes, the temperature was 37°. When the enzyme was dissolved in 0.1 M-acetate buffer, pH 5.6, the acid used for acidification was 0.23 N-HCl; for assay of zymogen dissolved in 0.02 M-phosphate buffer, pH 6.9, the acid was 0.088 N-HCl. Activity is expressed as μ moles of di-iodotyrosine liberated/min.

Purification of pepsin C and pepsinogen C. The operations described below were performed in the cold room and the

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buffers at pH 6.9 were kept saturated with toluene to prevent growth of bacteria.

Pepsin C, prepared by ion-exchange chromatography and rechromatography (Ryle & Porter, 1959), was further fractionated by exclusion chromatography on Sephadex G-100 with 0.1 M-acetate buffer, pH 5.6. Suitable fractions were pooled, dialysed against water and freeze-dried.

Pepsinogen C was previously (Ryle, 1960) prepared by ion-exchange chromatography of a fraction of mucosal extract obtained by precipitation with $(\text{NH}_4)_2\text{SO}_4$. Pepsinogen C can also be obtained by chromatography of the whole mucosal extract as in the preparation of pepsinogen B (Ryle, 1965), but it is then contaminated with much inactive material absorbing at 280 m μ . Batches of material obtained in this second way were subjected to rechromatography on DEAE-cellulose columns (about 25 cm. $\times$ 2.4 cm.) in 0.02 M-phosphate buffer, pH 6.9, with a gradient of NaCl obtained by passing buffer containing NaCl (0.45 M) into a closed mixing vessel containing 2 l. of buffer without NaCl. Suitably pooled material from such chromatograms was concentrated, chromatographed on Sephadex G-75, rechromatographed on DEAE-cellulose as before, concentrated again and finally chromatographed on Sephadex G-100. The buffer used was 0.02 M-phosphate, pH 6.9, throughout, except for the final gel filtration of the preparation used for the analyses described here when the buffer used was tris-citrate, pH 6.9 (0.1 M-tris-0.05 M-citric acid-0.054 N-NaOH). Pooled fractions from the ion-exchange chromatographic steps were concentrated by ultrafiltration under 60 cm. Hg pressure in Visking 24/32 in. tubing supported by a sleeve of silk net. The final pooled material was dialysed against water and freeze-dried.

Electrophoresis in starch gel. This was performed in horizontal gels in 0.02 M-phosphate buffer, pH 6.9, or in 0.05 M-citrate buffer, pH 5.6 or pH 3.2, by the method of Smithies (1955). The insertion of the samples and detection of protein by the chlorine method and of activity against haemoglobin were done as described before (Ryle, 1965).

Determination of total nitrogen. Nitrogen was determined by the micro-Kjeldahl procedure on samples containing about 0.2 mg. of nitrogen.

Determination of total phosphate. Total phosphate was estimated by a modification (T. S. Work, personal communication) of the method of Allen (1940) in which the protein is digested with 2 ml. of 2 N- H_2SO_4 and 2 drops of 60% (w/v) HClO_4 and after hydrolysis of pyrophosphate is made up to 5 ml. with 1 ml. of the amidol-bisulphite reagent [1% (w/v) amidol in 20% (w/v) sodium metabisulphite], 1 ml. of 3.3% (w/v) ammonium molybdate and water.

Oxidation of protein with performic acid. The oxidation procedure was that of Hirs (1956). About 10 mg. of the protein was dissolved in 0.25 ml. of 98% (w/v) formic acid and 0.05 ml. of methanol was added. To the solution cooled to -10° was added 0.5 ml. of performic acid solution at -10° . The performic acid solution was prepared by allowing 1 vol. of 30% (w/v) H_2O_2 to stand for 1 hr. at room temperature with 19 vol. of 98% (w/v) formic acid. Oxidation was allowed to proceed for 2.5 hr. at -10° and was then stopped by the addition of 20 ml. of ice-cold water. The solutions (that from pepsin C was cloudy) were subjected to freeze-drying but even after adding a further 20 ml. of water the mixtures did not remain well frozen but tended to melt at the surface.

Hydrolysis of proteins. The native proteins (about 10 mg.) were dissolved in 1.5 ml. of water and the oxidized proteins (about 10 mg.) were dissolved in 1.5 ml. of approx. 0.01 M- Na_2CO_3 . Then 1.5 ml. of conc. HCl (A.R.) was added to each and a finely divided precipitate of protein appeared. The suspensions were rapidly frozen, evacuated to 50 μ Hg pressure, sealed up and heated in a constant-temperature oven at 105° for 24 or 48 hr. The protein had all redissolved after 2 hr. The HCl was removed *in vacuo* over NaOH.

Amino acid analysis. Automatic amino acid analysis was carried out by the accelerated method of Spackman (1963) with a Beckman-Spinco model MS amino acid analyser.

Spectrophotometric determination of tryptophan and tyrosine. Determinations of the tyrosine and tryptophan content of the proteins were made by determination of the extinction at 280 and 294.4 m μ of solutions in 0.1 N-NaOH with correction for non-specific absorption made by extrapolation from the extinction values at 340 and 370 m μ as described by Goodwin & Morton (1946). The extinction measurements were made with a Unicam SP.500 spectrophotometer.

Determination of thiol content. The method used employed the reagent of Ellman (1959). Protein (6–7 mg.) in 1 ml. of water was mixed with 2 ml. of 6 M-urea solution in phosphate buffer, pH 8 and 10.15, and with 0.5 ml. of 0.01 M-5,5'-dithiobis-(2-nitrobenzoic acid). The extinction of the solution at 419 m μ was read against a blank containing water in place of the protein solution immediately and at intervals over 16 hr.

RESULTS

Purification of pepsin C by exclusion chromatography. The chromatography of pepsin C is shown in Fig. 1. The activity against haemoglobin emerges as a single peak with an almost constant ratio of activity to extinction at 280 m μ at about 1.5 times the excluded volume of the column and is followed by a broader peak of inactive material. The material in the active peak shows a ratio of activities (μ moles of di-iodotyrosine/min./[P.U.]^{ab}) 0.2. The ratio for pepsin is about 6.9 so that contamination of pepsin C with pepsin cannot exceed 3%. Fractions of high activity were pooled for further study. The material in the pooled fractions represented 86% of the activity against haemoglobin applied to the column and, after dialysis and freeze-drying, 61% of the applied weight. The recovery of extinction at 280 m μ over the whole chromatogram was 93%.

Purification of pepsinogen C by ion-exchange and exclusion chromatography. Rechromatography on DEAE-cellulose of combined batches of pepsinogen C taken from chromatograms of crude mucosal extracts showed a single peak of potential activity against haemoglobin incompletely separated from inactive ultraviolet-absorbing material. The ratio of potential activity against acetylphenylalanyldi-iodotyrosine to potential activity against haemoglobin was higher in the leading than in the trailing

Fig. 1. Exclusion chromatography of pepsin C. Pepsin C (418mg. in 10ml.) was applied to a column (43.5 cm. $\times$ 2.2 cm.) of Sephadex G-100. The buffer was 0.1 M-acetate, pH 5.6. Fractions (approx. 8 ml.) were collected at a flow rate of about 20 ml./hr. ●, Activity against haemoglobin (curve not drawn in); ○, activity against acetyl-L-phenylalanyl-L-di-iodotyrosine; Δ, E_{280} ; —, E_{260} (experimental points omitted). The bar shows the fractions pooled for freeze-drying.

Fig. 2. Exclusion chromatography of pepsinogen C. Pepsinogen C (25.1 potential [P.U.]^{Hb}) in 8.3 ml. of 0.02 M-phosphate buffer, pH 6.9, was applied to a column (97 cm. $\times$ 2.6 cm.) of Sephadex G-75 packed in the same buffer. Elution was with the same buffer at approx. 6 ml./hr. and 10 ml. fractions were collected. ●, Potential activity against haemoglobin (curve not drawn in); —, E_{280} (experimental points omitted). The bar shows the fractions pooled for further treatment.

edges of the zymogen peaks, indicating incomplete separation from another zymogen, probably pepsinogen. The recovery of potential activity from such chromatograms was 70–80%.

Material obtained in this way was fractionated by chromatography on Sephadex G-75, and one such run is shown in Fig. 2. The pooled fractions here contained 80% of the applied potential activity. In two other runs the early inactive peak was not so well separated from the zymogen but appeared as a shoulder on the leading edge.

The material from these three runs was combined and subjected to a final rechromatography on DEAE-cellulose, shown in Fig. 3. (In this particular chromatogram the buffer was inadvertently omitted from the reservoir and 0.45 M-sodium chloride alone was used. This has made no noticeable difference to the chromatographic behaviour.) Again the potential activity against acetylphenylalanyldi-iodotyrosine shows that pepsinogen C is incompletely separated from pepsinogen. Summation of the activities against the two substrates over the pooled fractions shows that the contamination with pepsinogen does not exceed 4%. The recovery of potential activity against haemoglobin was 87%.

The final step was chromatography on Sephadex G-100 (Fig. 4) on which it was hoped to obtain a better separation of the zymogen from the early inactive material than was obtained on Sephadex G-75. The column was packed with tris-citrate buffer because it was intended to use the preparation for estimation of phosphate in the protein and advantage was taken of the gel filtration to separate inorganic phosphate from the zymogen. No separation of inactive material was observed but all the fractions had nearly the same ratio of potential activity to extinction so that the earlier separation on Sephadex G-75 appears to have been effective. As expected there was a very good separation of protein from inorganic phosphate. The recovery of potential activity against haemoglobin was 101%.

Electrophoresis of pepsin C and pepsinogen C in starch gel. Both enzyme and zymogen were submitted to electrophoresis at pH 5.6 in 0.05 M-citrate buffer beside samples of pepsin and pepsinogen. Each protein (0.3 mg.) was applied across 1 cm. of gel. The positions of the bands detected both by chlorine and by activity against haemoglobin are shown in Table 1. The pepsin C and pepsinogen C both showed only a single band by the chlorine

method and no contamination of the pepsin C by pepsin was detected by activity against haemoglobin. (The substrate used was here only 0.06N with respect to hydrochloric acid and the activity of the zymogens was only weakly detected, presumably because of incomplete activation, so that no valid conclusion could be drawn about possible contamination of pepsinogen C with pepsinogen.)

The pepsinogen C was also examined by electrophoresis in 0.02M-phosphate buffer, pH 6.9, beside a sample of pepsinogen (0.5 mg. of each/cm. of gel). Only a single band was detected by the chlorine

method (Table 1), but the test with haemoglobin showed very weak activity at about the lower limit of detection in the pepsinogen C sample at the position of pepsinogen.

The enzyme and zymogen (0.5 mg.) were also examined by electrophoresis in starch gel in 0.05M-citrate buffer, pH 3.2, and each showed only a single band by the chlorine method (Table 1), but both showed some activity against haemoglobin that

Fig. 3. Rechromatography of pepsinogen C. Pepsinogen C (1770mg.) was applied to a column (25cm. x 2.7cm.) of DEAE-cellulose in 0.02M-phosphate buffer, pH 6.9. Elution was with a gradient obtained by passing 0.45M-NaCl into a closed mixing vessel containing 2l. of 0.02M-phosphate buffer, pH 6.9. Fractions (24ml.) were collected at approx. 50ml./hr. ●, Potential activity against haemoglobin (curve not drawn in); ○, potential activity against acetyl-L-phenylalanyl-L-di-iodotyrosine; —, E_{280} (experimental points omitted). The bar shows the fractions pooled for further treatment.

Fig. 4. Final exclusion chromatography of pepsinogen C. Pepsinogen C (43 potential [P.U.]^{Hb}) in 16 ml. of phosphate buffer, pH 6.9, was applied to a column (43cm. x 2.2cm.) of Sephadex G-100 packed in tris-citrate buffer, pH 6.9, and was washed through with the same buffer at approx. 20ml./hr. Fractions (10ml.) were collected. ●, Potential activity against haemoglobin (curve not drawn in); —, E_{280} ; ■, concn. of inorganic phosphate. The bar shows the fractions pooled for freeze-drying.

Table 1. Electrophoresis of enzymes and zymogens in starch gel

The positions of the bands were detected both by the chlorine method and by activity or potential activity against haemoglobin. Mobilities are expressed as distances (cm.) in the direction of the anode (positive values) or cathode (negative values).

Conditions	Mobility (cm.)			
	Pepsin C	Pepsin	Pepsinogen C	Pepsinogen
pH 5.6, 1.4 v/cm., 14 hr.	+6.8	+8.5	+5.0	+6.8
pH 6.9, 4.0 v/cm., 7.5 hr.	—	—	+9.5	+11.7, +12.1*
pH 3.2, 3.0 v/cm., 20 hr.	−6.8	+3.6	−7.2	+3.6

* These two components were detected by the chlorine method but were not resolved by the stain for potential activity against haemoglobin. The presence of two components has been noted before (Ryle, 1965).

had migrated towards the anode rather more slowly than pepsin. Under these conditions it is probable that zymogens are converted into enzymes and migrate as such.

Specific activity of pepsin C and pepsinogen C. The freeze-dried preparation of pepsin C had a specific activity of 0.41 [P.U.]^{Bb}/mg. of N and a m-[P.U.]^{Bb}/ml./ E_{280} ratio 35.1, the extinction being measured in 0.1M-acetate buffer, pH 5.6. The freeze-dried preparation of pepsinogen C had 0.37 [P.U.]^{Bb}/mg. of N and a m-[P.U.]^{Bb}/ml./ E_{280} ratio 40.2, the extinction being measured in 0.02M-phosphate buffer, pH 6.9. These values for the specific activities of pepsin C and pepsinogen C are higher than those (0.31–0.34 and 0.28–0.32 [P.U.]^{Bb}/mg. of N respectively) previously reported for materials that had not been purified by chromatography on Sephadex (Ryle, 1960).

Phosphate content of pepsinogen C. Digestion and

analysis of a 23.5mg. sample of the zymogen freed from inorganic phosphate by gel filtration in tris-citrate buffer showed less than 0.02mole of phosphate/41000g.

Amino acid composition of pepsinogen C and pepsin C. Analyses were performed on a 24hr. hydrolysate of the unoxidized proteins and on 24 and 48hr. hydrolysates of the oxidized proteins. In the determination of the molar proportions of the amino acid residues a first calculation was made assuming the zymogen and enzyme to contain respectively 2 and 1moles of histidine/mole. The results obtained for five other amino acid residues were sufficiently consistent and nearly integral to allow the indicated integral values for the group to be used as a basis for recalculation of the values for all the amino acid residues. In this way errors that might have been introduced had a single amino acid residue been used as the basis of the calcu-

Table 2. *Amino acid composition of pepsinogen C*

The Table shows, for each analysis, the number of moles of each amino acid found/mole of protein, calculated as described in the text. The Table also shows, for comparison, some data for pepsinogen obtained by (a) Arnon & Perlmann (1963) and (b) van Vunakis & Herriott (1957). The best integers include corrections for serine, threonine, tyrosine and ammonia (see the text). CySO_3H , cysteic acid; MetO_2 , methionine sulphone; Tyr-XX, halogenated tyrosine.

Hydrolysis...	Amino acid composition (mole/mole of protein)					
	Not oxidized	Oxidized		Best integer	Pepsinogen	
		24hr.	48hr.		(a)	(b)
Lys	12.9	11.6	11.6	12	11	12
His	2.0	2.0	2.0	2	3	4
Arg	7.1	6.9	6.5	7	4	3
CySO_3H	—	5.5	5.3	—	—	—
Asp	30.7	30.3	29.9	30	46	46
MetO_2	—	5.3	5.4	—	—	—
Thr	24.9	24.4	23.9	25	28	28
Ser	32.5	33.6	32.0	35	47	53
Glu	44.5	46.1	46.3	46	30	32
Pro	19.6	20.1	20.4	20	19	20
Gly	35.0	35.0	35.0	35	36	36
Ala	23.3	23.3	23.5	23	21	27
CyS	3.1*	—	—	6	6	(6)
Val	22.3	21.1	22.0	22	25	27
Met	5.4	—	—	5	~4	(5)
Ile	15.9	15.4	16.5	16	25	64
Leu	39.4	40.0	40.0	40	33	
Tyr	19.9	19.1	18.6	22†	17	20
Phe	23.9	24.5	24.5	24	15	20
Tyr-XX	—	2.5	2.4	—	—	—
Trp	6.7	—	—	6	6	6
NH_3	(36.1)	(36.3)	(38.1)	(34)		

From spectrophotometric data: Tyr (related through total N) 22.7; Trp (moles/22moles of Tyr) 5.8, 6.0; (related through total N) 5.3.

Sum: 376 residues, 15.2% N, 449g.atoms of N/mole, mol.wt. 41400.

* Not considered in choosing best integer.

† Account taken of Tyr-XX content.

lation were avoided. The amino acids and integers used as the basis of calculation were for pepsinogen C: Lys₁₂, His₂, Arg₇, Met₅, Pro₂₀, Leu₄₀; and for pepsin C: Lys₄, His₁, Arg₄, Met₄, Pro₁₈, Leu₃₄. The data thus calculated are shown in Tables 2 and 3, which include the results of two spectrophotometric determinations of tryptophan (relative to tyrosine) and one in which both the tyrosine and tryptophan were determined relative to total nitrogen and related to the chromatographic analyses through the total nitrogen applied to the chromatographic columns for the oxidized samples.

In taking values for the best integers shown in Tables 2 and 3 those for serine and threonine were obtained by linear extrapolation of the analytical results for the oxidized proteins to zero time of hydrolysis and by assuming 10% loss of serine and 5% loss of threonine for the single 24 hr. hydrolysis of the unoxidized proteins. The values for tyrosine were obtained by a similar extrapolation of the

data for the oxidized samples with correction for the tyrosine-XX (halogenated tyrosine) found. Values for half-cystine in unoxidized proteins are notoriously unreliable and they were not considered in choosing the best integer for half-cystine. Analysis for thiols with dithiobis-(2-nitrobenzoic acid) in alkaline urea solution showed less than 0.1 mole of thiol group/mole of protein for both enzyme and zymogen, leaving little doubt that the integral value for half-cystine in each is six, not five. The best integers for ammonia include a correction for ammonia arising from the breakdown of serine and threonine.

The analyses leaving the most room for doubt are those for tyrosine, especially for pepsin C, for which the spectrophotometric method indicated a distinctly higher value than did the chromatographic method. The data for the ammonia content can provide only an approximate value for the number of amide groups in the molecule.

Table 3. *Amino acid composition of pepsin C*

The Table shows for each analysis the number of moles of each amino acid found/mole of protein calculated as described in the text. The Table also shows the difference in composition from pepsinogen C, and, for comparison, some data for pepsin obtained by Blumenfeld & Perlmann (1959). The best integers include corrections for serine, threonine and ammonia (see the text). CySO₃H, cysteic acid; MetO₂, methionine sulphone; Tyr-XX, halogenated tyrosine.

Hydrolysis...	Amino acid composition (moles/mole of protein)					
	Not oxidized 24 hr.	Oxidized		Best integer	Pepsinogen C — Pepsin C	Pepsin
		24 hr.	48 hr.			
Lys	4.1	3.9	4.0	4	8	1
His	1.1	1.0	1.0	1	1	1
Arg	4.0	3.9	3.9	4	3	2
CySO ₃ H	—	5.7	5.7	—	—	—
Asp	27.8	28.0	27.3	28	2	44
MetO ₂	—	4.3	4.2	—	—	—
Thr	23.5	23.3	22.3	25	0	28
Ser	30.2	32.9	29.6	35	0	44
Glu	40.6	41.5	41.1	41	5	27
Pro	17.9	17.8	17.6	18	2	15
Gly	32.1	32.7	32.2	32	3	38
Ala	20.5	21.2	20.9	21	2	18
CyS	3.1*	—	—	6	0	~4
Val	19.6	19.3	19.8	20	2	21
Met	4.1	—	—	4	1	~5
Ile	13.8	13.4	14.3	14	2	27
Leu	33.8	34.0	34.2	34	6	28
Tyr	17.3	17.0	16.4	18	4	18
Phe	20.2	21.3	20.8	21	3	14
Tyr-XX	—	—	—	—	—	—
Trp	0.6	—	—	6	0	6
NH ₃	(32.2)	(33.4)	(35.0)	(32)	(2)	(36)

From spectrophotometric data: Tyr (related through total N) 20.1; Trp (moles/18 moles of Tyr) 5.8, 5.3; (related through total N) 5.6.

Sum: 332 residues, 15.0% of N, 387 g. atoms of N/mole, mol. wt. 36000.

* Not considered in choosing best integer.

The molecular weight of pepsinogen C calculated from the amino acid composition is 41 400, close to that (40 500) previously obtained from sedimentation studies by the Archibald procedure (Ryle, 1960), but the value 36 000 for pepsin C is lower than that (40 700) obtained earlier (Ryle & Porter, 1959) by the Archibald method.

DISCUSSION

Homogeneity of the preparations. Detection of only one band by the chlorine method in the preparations of pepsin C and of pepsinogen C under three different conditions of electrophoresis in starch gel indicates that both are free of any major protein contaminant. However, each preparation showed some activity or potential activity with the substrate acetylphenylalanyldi-iodotyrosine and this activity could be accounted for by the presence of 3% of pepsin in the preparation of pepsin C or 4% of pepsinogen in the preparation of pepsinogen C, on the assumption that pepsin C has no intrinsic activity against this substrate. No preparation of either pepsin C or pepsinogen C has been obtained devoid of activity against the acetylpeptide and if some of the activity found in the present preparations is in fact due to pepsin C or pepsinogen C themselves then the extent of contamination must be less than the estimates given above. The test for activity against haemoglobin after electrophoresis in starch gel can detect 5 μ g. of pepsin and did in fact detect a small amount, estimated at 2–3%, of activity probably due to pepsin or pepsinogen in the preparations of pepsin C and pepsinogen C respectively. It is concluded that the preparations are better than 95% homogeneous, and this conclusion is further supported by the near approach of the ratios of amino acids found in the hydrolysates to whole numbers, and by the low phosphate content reported here for pepsinogen C and earlier (Ryle & Porter, 1959) for pepsin C.

Analysis of proteins. The amino acid analysis shows that pepsinogen C is less acidic than pepsin C and that the two are less acidic than pepsinogen and pepsin respectively. This is in accord with the electrophoretic and chromatographic behaviour of the proteins, except that pepsinogen C is eluted from DEAE-cellulose at pH 6.9 at a higher ionic strength than is pepsinogen. This behaviour suggests that factors other than ion-exchange may play a part in the chromatographic separation. The absence of phosphate from pepsin C and from pepsinogen C is not an important cause of the differences in electrical properties from those of the major proteins since the differences in amino acid composition could account for greater differences in charge.

The molecular weights of the enzyme and zymogen obtained from the amino acid analysis are very similar to the values of 36 000 for pepsin (Blumenfeld & Perlmann, 1959) and 41 000 for pepsinogen (Arnon & Perlmann, 1963), and show that the loss in weight of pepsinogen C on activation is greater than was formerly supposed and is very similar to that for pepsinogen. If 1 mole of pepsinogen C on activation gives rise to 1 mole of pepsin C the (potential) molar activities of enzyme and zymogen should be identical. The data given above lead to [P.U.]^{EB}/m-mole values of 11.8 and 11.2 for pepsin C and pepsinogen C respectively.

It is not clear why the ratio of activity to E_{280} is lower for the enzyme than for the zymogen (both for the freeze-dried proteins and before freeze-drying; cf. Figs. 1 and 4), when the zymogen must contain at least as many chromophores. Differences in the solvent used when the extinction measurements were made or differences in the tertiary structures of the proteins may be involved.

Origin of pepsinogen C. Several authors (see review by Taylor, 1962; Seijffers, Segal & Miller, 1963; Hanley, Boyer & Naughton, 1966) have reported differences in the zymogen content of mucosa from different parts of the stomach of various species and in one pig we found a much higher ratio of pepsinogen C to pepsinogen in the pyloric mucosa than in the body mucosa (Ryle, 1964). It seems likely that pepsin C is the pyloric pepsin described by other authors. The most striking differences in the amino acid compositions of pepsinogen and pepsin on the one hand and pepsinogen C and pepsin C on the other are the inversion of the ratio of aspartic acid to glutamic acid and the large changes in the ratios of leucine and isoleucine. Since the corresponding proteins are of very similar polypeptide chain length such differences are difficult to reconcile with the suggestion of Hanley, Boyer & Naughton (1966) that all the zymogens are the product of a single gene and that the different zymogens are produced by subsequent modification of this single gene product.

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