Pepsin D

A MINOR COMPONENT OF COMMERCIAL PEPSIN PREPARATIONS

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Methods are described for the isolation and purification of pepsin D, an enzyme which accounts for about 10% of the enzymic activity in commercial preparations of pepsin. Pepsin D is similar to pepsin in having a molecular weight of about 35000, the same *C*-terminal amino acid sequence, and an *N*-terminal isoleucine residue. It differs in having no phosphate residue. Pepsin D is similar to pepsin in its ability to digest haemoglobin, acetyl-L-phenylalanyl-L-di-iodotyrosine and gelatin but it is twice as active as pepsin in the clotting of milk. It has the same specificity as pepsin in its action on the B-chain of oxidized insulin. It is probable that the pepsin D in commercial preparations of pepsin arises from the activation of gastric pepsinogen D.

In addition to pepsinogen, the pig stomach is known to contain small amounts of other zymogens, namely pepsinogens B, C and D (Ryle, 1960, 1965; Ryle & Hamilton, 1966; Lee & Ryle, 1967). The enzymes produced by activation of pepsinogens B and C have been isolated from commercial preparations of pepsin by ion-exchange chromatography at pH4 and their properties have been described by Ryle & Porter (1959). However, these authors did not detect any component of pepsin preparations which might have been the enzyme derived from pepsinogen D.

When it was found that pepsinogen and pepsinogen D on electrophoresis in starch gel at pH3.2 gave zones of enzymic activity which migrated at different rates towards the anode (Lee & Ryle, 1967), it seemed possible that preparations of pepsin could contain pepsin D which failed to separate from pepsin on chromatography at pH4 but which might do so at pH3.2. This paper gives an account of the isolation and properties of such an enzyme from crystalline preparations of pepsin. The enzyme is probably produced by activation of pepsinogen D.

EXPERIMENTAL

Materials. Gelatin was a sample (lot no. 141) generously given by Dr A. G. Ward.

Insulin was a six-times recrystallized sample (lot CBI 4245LL) kindly given by Boots Pure Drug Co. Ltd. The

sources of other materials were as given in the preceding paper (Lee & Ryle, 1967).

Buffers. Except where otherwise stated buffer solutions were made with sodium salts and their concentrations are expressed in terms of the total molarity of the buffering component.

Activity determinations. Assays of enzymic activity with haemoglobin, acetylphenylalanyldi-iodotyrosine and milk were performed as described earlier (Lee & Ryle, 1967).

Determination of activity by the liquefaction of gelatin. A solution (0.3 ml.) of pepsin D in 0.1 M-acetate buffer, pH5.6, at 35.5° was mixed in a boiling tube with 15 ml. of a 2.5% (w/v) solution of gelatin at the same temperature and filtered through a Pyrex SF1A1 sintered-glass disk into an Ostwald viscometer (t_{H_2O} 27.07 sec.). The viscosity of the solution was determined at intervals of 5–10 min. over the next hour and the gelatin-liquefying activity was calculated according to Northrop (1931–32).

Ion-exchange chromatography. The enzymes were chromatographed on columns of DEAE-cellulose packed in 0.1 M-citrate buffer, pH3.2, by using a gradient of NaCl obtained by passing buffer containing 0.1 M-NaCl into a 'constant-volume' mixing vessel, which initially contained 2 l. of NaCl-free buffer. The columns were packed with DEAE-cellulose described as the chloride form obtained by equilibration with 0.1 M-citrate buffer, pH3.2, which contained 0.1 M-NaCl, and subsequent washing with chloride-free buffer until chloride could not be detected in the effluent.

Electrophoresis in starch gel. Electrophoresis and detection of enzymic activity by the ability to digest haemoglobin were carried out in the manner described previously (Lee & Ryle, 1967).

Determination of phosphate. A modification of the method of Allen (1940) was used (Lee & Ryle, 1967).

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

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Determination of the pH optimum for the action of pepsin D on haemoglobin and on acetylphenylalanyldi-iodotyrosine. These determinations were carried out in a manner based on the routine assay procedures except that the pH of the reaction mixtures was varied by altering the concentration of HCl used. To obtain the initial velocities of the reactions the haemoglobin digestions were stopped after 1, 2 or 3 min. and the synthetic-substrate reactions after 1 or 2 min. The pH of the reaction mixtures was determined on control solutions prepared with a sample of enzyme that had been denatured by heating in a boiling-water bath for 15 min.

Digestion of the B-chain of oxidized insulin. A sample of the B-chain of oxidized insulin was prepared by oxidation of 500 mg. of six-times recrystallized insulin with 10 ml. of formic acid (9 ml. of formic acid + 1 ml. of 30%, w/v, H_2O_2) at room temperature for 1 hr. The chains were then separated on a column of DEAE-Sephadex A-50 according to the method of Fittkau (1963) and precipitated from the effluent fractions by addition of ethanol-ether (1:1, v/v). The precipitates were washed by centrifugation in ether and dried in air. The use of fluorodinitrobenzene showed that phenylalanine was the only N-terminal amino acid present in the sample of the B-chain.

Digestion of the B-chain was carried out by incubating at 35.5° for 3 hr. or for 20 hr. with conditions similar to those of Ryle & Porter (1959). At the end of the incubation period the reaction mixtures were frozen and dried from the frozen state under vacuum. The dried residues were dissolved in formic acid, subjected to paper electrophoresis, and the resulting peptide bands identified as described by Ryle & Porter (1959).

'Fingerprinting' of pepsin D and the enzyme produced by activation of pepsinogen D. The disulphide bonds of the enzymes were oxidized in the manner described for pepsinogen D (Lee & Ryle, 1967) but in this case there was no need for alkaline inactivation and oxidation was carried out simply by dissolving 10 mg. of the enzyme in 2.5 ml. of chilled performic acid and allowing the oxidation to proceed at 0° for 2 hr. Performic acid was destroyed by the addition of 22.5 ml. of water and the solution was freeze-dried.

About 10 mg. of the oxidized proteins were dissolved in 0.5 ml. of 0.2 M-ammonium carbonate (pH 8.5) containing 0.25 mg. of α -chymotrypsin (Sigma lot C49-66) and digested for 3 hr. at 35.5°. The resulting peptides were separated by high-voltage electrophoresis and paper chromatography in the manner described by Lee & Ryle, (1967).

RESULTS

Isolation of pepsin D from preparations of crystalline pepsin. Chromatography of crystalline pepsin at pH 3.2 resulted in the separation from pepsin of a minor component having activity against both haemoglobin and acetylphenylalanyldi-iodotyrosine but only if the DEAE-cellulose was previously loaded with chloride as described in the Experimental section. A typical chromatogram is shown in Fig. 1; the minor component, pepsin D, appears as the peak at an effluent volume of 11. and accounts for about 10% of the total enzymic activity.

The use of a pH as low as 3.2 for chromatography

Fig. 1. Chromatography of 1.5 g. of Mann alcohol-crystallized pepsin (lot J2211) on a column of DEAE-cellulose (16.0 cm. $\times$ 4.8 cm. diam.) in the chloride form. The column was packed and equilibrated with 0.1 M-citrate buffer, pH 3.2, and the gradient was 0 $\rightarrow$ 0.1 M-NaCl in the same buffer. Flow rate, 120 ml./hr.; fractions 37.5 ml. O, Activity against acetylphenylalanyldi-iodotyrosine; ●, activity against haemoglobin; ●, points for which activities against the two substrates coincide. The calculated salt gradient is shown above and the bar indicates that portion of the effluent which yielded a sample of pepsin D almost entirely free of pepsin.

increased the probability that one of the two enzyme peaks shown in Fig. 1 was an artifact produced by autodigestion of the other and to clarify the situation the fractions of the chromatogram containing pepsin D and those containing the main component were pooled, dialysed against 0.01 M-citrate buffer, pH 3.2, and subjected to chromatography under conditions similar to those used in their isolation. In both cases only one enzymic component was obtained and each was eluted in the part of the gradient in which it was expected.

Exclusion chromatography of pepsin D on Sephadex G-75. The exclusion chromatography of pepsin D on a column of Sephadex G-75 packed in 0.1 M-acetate buffer, pH 4.0, gave rise to only one peak of enzymic activity which pertained to a molecule which penetrated the gel bed to a small extent and thus probably had a molecular weight in the region of 35 000. There was also purification from a small amount of enzymically inactive material which penetrated the gel bed completely.

Comparative exclusion chromatography of pepsin

D and pepsin. To obtain a better assessment of the molecular weight of pepsin D a sample (16 [P.U.]^{Hb}) was run through a column (33 cm. $\times$ 2.3 cm. diam.) of Sephadex G-100 in 0.1 M-acetate buffer, pH 5.6. The volume of the enzyme solution applied was 31 ml. and the maximum of the enzyme peak emerged at 105 ml. On the same column pepsin (6.8 [P.U.]^{Hb} in 10 ml. of buffer) emerged at 100 ml. These elution volumes are not considered significantly different and it is concluded that the molecular weight of pepsin D is close to that of pepsin, 35 000.

Electrophoresis of pepsin D in starch gel. Fig. 2 shows the result of electrophoresis at pH 3.2 and pepsin D is seen to migrate with the same mobility as pepsinogen D, which is activated in the gel at this pH. The mobility is slightly more than half that shown by pepsin.

N-Terminal amino acid residue of pepsin D. Samples of pepsin D were dinitrophenylated and hydrolysed and the DNP-amino acids examined in the manner described by Lee & Ryle (1967). The main DNP-amino acid in our preparations was found to be DNP-isoleucine, although there was noticeable contamination by others, mainly those derived from aspartic acid, glutamic acid and valine. A quantitative determination of the *N*-terminal residue by using added DNP-isoleucine to assess hydrolytic breakdown gave a corrected value of 0.97 mole of DNP-isoleucine/35 000 g. of pepsin D and less than 0.1 mole of any other DNP-amino acid (uncorrected).

The butanol-ethyl acetate extract contained ϵ -DNP-lysine, no DNP-arginine or bis-DNP-histidine but a small amount of material which migrated slightly faster than bis-DNP-histidine and was probably a DNP-peptide.

Isoleucine has been identified as the *N*-terminal residue of pepsin (Van Vunakis & Herriott, 1957; Heirwegh & Edman, 1957; Stepanov, Vaganova & Kuznetsov, 1964).

C-Terminal amino acid sequence of pepsin D. Pepsin D was subjected to the action of carboxypeptidase and the released amino acids were identified as described by Lee & Ryle (1967). With digestion times of 10, 25 and 40 min. the first amino acid to be released was alanine and this was followed by one of the leucines. In another experiment with a digestion time of 2 hr. about 1 mole each of alanine, valine and leucine or isoleucine or both was released/mole of pepsin D (assuming molecular weight 35 000). Digestion for a further 24 hr. gave no further increase in the amounts of these amino acids and only a slight additional release of an amino acid which could have been either tryptophan or methionine. Thus the *C*-terminal sequence in pepsin D appears to beVal-^{Leu}Ile-AlaOH. The

same sequence was found in pepsinogen and pepsin by H. Van Vunakis & R. M. Herriott (unpublished work, cited by Herriott, 1962), although Vaganova, Levin & Stepanov (1964) have reported the sequencePro-Val-AlaOH for pepsin denatured in phenol.

Phosphorus content of pepsin D. Assuming a molecular weight of 35 000, pepsin D was found to contain 0.3 g. atom of phosphorus/mole. Thus, as with pepsinogen D, we have a fractional amount of phosphorus but, in view of the electrophoretic homogeneity of the enzyme, it appears that this simply represents inorganic phosphate which is avidly bound. To clarify the situation, however, samples of pepsin D and pepsin were subjected to the action of phosphatase B in the manner described by Lee & Ryle (1967). The result of electrophoresis of the reaction mixtures is shown in Fig. 3, where it is seen that the electrophoretic mobility of pepsin D remained unaltered whereas that of pepsin was reduced almost by half to correspond with the mobility of pepsin D. This suggests that pepsin D does not contain any covalently linked phosphate groups.

Fig. 2. Electrophoresis in starch gel. 'Haemoglobin staining' of enzymically active proteins after electrophoresis of about 1 mg. of each preparation for 34 hr. at 2.8 v/cm. in a starch gel prepared with 0.05 M-citrate buffer, pH 3.2. Electrode vessels contained 1.0 M-citrate buffer, pH 3.2.

Fig. 3. Action of phosphatase B on pepsin and on pepsin D. 'Haemoglobin staining' of enzymically active proteins after electrophoresis of the reaction mixtures for 12 hr. at 3.5 v/cm. in a starch gel prepared with 0.05 M-citrate buffer, pH 3.2. Electrode vessels contained 1.0 M-citrate buffer, pH 3.2.

Fig. 4. Effect of pH on the digestion of haemoglobin by pepsin D.

Fig. 5. Effect of pH on the digestion of *N*-acetyl-L-phenylalanyl-L-di-iodotyrosine by pepsin D.

pH optimum for the action of pepsin D on haemoglobin and on acetylphenylalanyldi-iodotyrosine. The pH-activity curves for the action of pepsin D on both of these substrates are shown in Figs. 4 and 5. The optimum lies close to 2 in each case. The curve shown in Fig. 4 is similar to that obtained by Northrop (1922-23) for the action of pepsin on haemoglobin although the fall-off in activity at higher pH values was greater with pepsin than with pepsin D. Baker (1951) found that the pH optimum for the action of pepsin on acetylphenylalanyldi-iodotyrosine lay close to pH 2 but in this case also the fall-off in activity towards higher pH values was greater with pepsin than with pepsin D.

Specific activity of pepsin D. The specific activities of pepsin D were found to be 0.20 [P.U.]^{Hb}/mg. of nitrogen and 0.23 [P.U.]^{APD}/mg. of nitrogen, the latter value corresponding to the release of

Fig. 6. Stability of pepsin D at pH values approaching neutrality. O, Activity against acetylphenylalanyldi-iodotyrosine; ●, activity against haemoglobin.

1.61 μmoles of di-iodotyrosine/min./mg. of nitrogen. The former value is similar to the 0.18 [P.U.]^{Hb}/mg. of nitrogen obtained for pepsin by Anson & Mirsky (1932).

Activity of pepsin D as determined by various assay procedures. The activities against other substrates were related to the activity against haemoglobin and the following values were found: [P.U.]^{Ren}/m[P.U.]^{Hb}, 1.35; [P.U.]^{Gel V-}/m[P.U.]^{Hb}, 0.01. With a sample of pepsin a ratio [P.U.]^{Ren}/m[P.U.]^{Hb} of 0.7 was found and although this is much lower than the value 1.2 reported by Ryle & Porter (1959) it should be pointed out that the conditions used here were slightly different from those used previously and that the recognition of the clotting-point is a subjective matter over which different individuals may not agree.

Ryle & Porter (1959), using conditions similar to those used in this instance, found [P.U.]^{Gel V-}/m[P.U.]^{Hb} ratios of 12.1, 2.5 and 0.15 for pepsin B, pepsin C and pepsin respectively and, in this respect, pepsin D is more similar to pepsin than to pepsins B and C.

Stability of pepsin D at pH values approaching neutrality. The procedure used in this experiment was that described for the study of the alkali-stability of pepsinogen D (Lee & Ryle, 1967). Pepsin D in 0.1 M-phosphate buffers of varying pH (*I* 0.1) was incubated for 10 min. at 35.5°. Thereafter, either haemoglobin or hydrochloric acid and the synthetic substrate was added and the assays were conducted in the normal fashion. The activity found after exposure to these pH values is shown in Fig. 6, where it is seen that there was a slight

Fig. 7. Diagram of the ninhydrin-stained peptides obtained from the digestion of the B-chain of oxidized insulin by pepsin and by pepsin D, after their separation by electrophoresis in pyridine-acetic acid-water (100:4:1900, by vol.) buffer, pH 6.5, for 2.5 hr. at 33 v/cm. The strength of the ninhydrin band is represented by the height of the blackened area in the diagram.

decrease in activity after exposure to pH 6.3 and that activity was almost completely destroyed by exposure to pH 6.5. This behaviour is similar to that reported for pepsin (Goulding, Borsook & Wasteneys, 1926-27; Northrop, 1929-30).

Digestion of the B-chain of oxidized insulin by pepsin and by pepsin D. The ninhydrin-staining bands obtained after electrophoresis of each digest are shown in Fig. 7 and the structure of the peptide(s) present in each band are shown in Table 1.

There appears to be no significant difference in the peptides obtained by digestion with pepsin D and those obtained with pepsin and the only major difference in the peptide patterns obtained with an extended digestion time was the appearance of band D.

Almost all of the bonds found to be split were in agreement with the findings of Sanger & Tuppy (1951) for the action of pepsin on the B-chain. However, we found the 14-15 Ala-Leu bond to be split rapidly whereas they found it to be split rather slowly, and we found a slight cleavage of the 3-4

Table 1. *B-chain of oxidized insulin and the probable structures of the peptides resulting from its digestion by pepsin or by pepsin D*

Phe-Val-Asn-Gln-His-Leu-CySO₃H-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-
Tyr-Leu-Val-CySO₃H-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

Band

Peptide structure

Peptides resulting from a 3 hr. digestion by pepsin or by pepsin D

A	Tyr-Thr-Pro-Lys-Ala
B	Tyr-Thr-Pro-Lys-Ala
C ₁	Phe-Val-Asn-Gln-His-Leu-CySO ₃ H-Gly-Ser-His-Leu
C ₂	Phe-Val-Asn-Gln-His-Leu-CySO ₃ H-Gly-Ser-His-Leu
E	Phe-Val-Asn-Gln-His-Leu-CySO ₃ H-Gly-Ser-His-Leu-Val-Glu-Ala
F	Leu-Val-CySO ₃ H-Gly-Glu-Arg-Gly-Phe and about 10-20% Tyr-Leu-Val-CySO ₃ H-Gly-Glu-Arg-Gly-Phe
G	Val-Glu-Ala-Leu
H	Val-Glu-Ala

Peptides resulting from a 20 hr. digestion by pepsin or by pepsin D

A	Tyr-Thr-Pro-Lys-Ala
B	Tyr-Thr-Pro-Lys-Ala
C ₁	Val-Asn-Gln-His-Leu-CySO ₃ H-Gly-Ser-His-Leu
C ₂	Phe-Val-Asn-Gln-His-Leu-CySO ₃ H-Gly-Ser-His-Leu
D	His-Leu-CySO ₃ H-Gly-Ser-His-Leu and about 30% Gln-His-Leu-CySO ₃ H-Gly-Ser-His-Leu
E	Phe-Val-Asn-Gln-His-Leu-CySO ₃ H-Gly-Ser-His-Leu-Val-Glu-Ala
F	Leu-Val-CySO ₃ H-Gly-Glu-Arg-Gly-Phe and about 30% Tyr-Leu-Val-CySO ₃ H-Gly-Glu-Arg-Gly-Phe
G	Val-Glu-Ala-Leu and about 10-20% Tyr-Leu-Val-CySO ₃ H-Gly-Glu-Arg-Gly-Phe
H	Val-Glu-Ala-Leu and about 10-20% Tyr-Leu-Val-CySO ₃ H-Gly-Glu

Abbreviation: CySO₃H, cysteic acid.

Asp(NH₂)-Glu(NH₂) and the 21-22 Glu-Arg bonds whereas they did not find these bonds to be split at all.

'Fingerprinting' of pepsin D and the enzyme produced by activation of pepsinogen D. The above results indicated that pepsin D was probably produced by activation of pepsinogen D but, to clarify the matter, it was decided to submit the enzymes to the 'fingerprinting' technique. The pepsin D used in this experiment was isolated and purified by chromatography and gel filtration in the manner described above, and the enzyme from activation of pepsinogen D was obtained by exclusion chromatography on a column of Sephadex G-50 of the products of activation resulting from the exposure of a solution of pepsinogen D to a pH of 1.3 for 5 min. at room temperature.

The resulting peptide patterns are shown in Fig. 8

Fig. 8. (A) Tracing of the ninhydrin-stained peptides seen after separation of the peptides produced by digestion of about 8 mg. of the enzyme produced by activation of pepsinogen D, with α -chymotrypsin. (B) Sketch of the ninhydrin-stained peptides seen after separation of the peptides produced by digestion of about 8 mg. of pepsin D (isolated from crystalline pepsin) with α -chymotrypsin. The dashed outlines represent fainter peptides; x, origin.

and, although the two are not completely identical, the differences between the two are small and are confined to the ill-defined group of acidic peptides which migrate rather slowly on chromatography.

DISCUSSION

Ion-exchange chromatography, exclusion chromatography and starch-gel electrophoresis have shown that our preparations of pepsin D are homogeneous and free from contamination by other proteases. Pepsin D accounts for about 10% of the enzymic activity recovered from crystalline pepsin preparations after chromatography on DEAE-cellulose and so far it has been detected in all commercial preparations of pepsin that have been tested.

The fact that pepsin D is eluted from DEAE-cellulose at a lower salt concentration than pepsin probably indicates that it is less acidic and this idea is strengthened by the fact that it has a lower anodic mobility in starch gel at pH 3.2. However, the difference in acidity is not due to a large difference in molecular size as exclusion chromatography showed pepsin D to be approximately the same size as pepsin.

Pepsin D is similar to pepsin in many of its chemical and enzymic properties. It has the same *N*-terminal and *C*-terminal amino acid residues and may have the same *C*-terminal sequence. Its specific activity against haemoglobin is close to that of pepsin and its pH optima with this substrate and with acetylphenylalanyldi-iodotyrosine are the same as those of pepsin. At pH 4 pepsin D has about 60% of its optimum activity against the acetyl-peptide but it has little activity against haemoglobin at this pH. Pepsin is likewise much more active against peptide substrates than against haemoglobin at pH 4 (Baker, 1951; Northrop, 1922-23). At pH values close to neutrality pepsin D, like pepsin, is rapidly inactivated. No difference was found between the specificities of attack of the two enzymes on the B-chain of oxidized insulin but pepsin D has a higher milk-clotting activity and a lower gelatin-liquefying activity than pepsin, and Steven (1963) found a significant difference in the abilities of the two enzymes to liquefy acid-soluble collagen.

The main difference between the two enzymes is in the phosphorus content: pepsin contains 1 g. atom/mole whereas the value found for pepsin D and for the zymogen pepsinogen D (Lee & Ryle, 1967) is about 0.3 g. atom/mole. It is likely that this analytically unsatisfactory figure is high because of bound inorganic phosphate since treatment with a phosphatase had no effect on the anodic mobility of pepsin D at pH 3.2, although it reduced that of pepsin.

Pepsin D behaves on electrophoresis in starch gel and on chromatography on DEAE-cellulose in the same way as enzymically dephosphorylated pepsin and the properties reported here for pepsin D are consistent with those reported by Perlmann (1958) for dephosphorylated pepsin. The patterns of peptides produced by chymotryptic digestion of pepsin D and of the enzyme formed on activation of pepsinogen D (Lee & Ryle, 1967) are very similar, and this and the other properties reported for the enzyme together with the zymogen lead to the conclusion that pepsin D isolated from commercial pepsin preparations is in fact the enzyme formed by activation of pepsinogen D of the gastric mucosa and that it is probably identical with dephosphorylated pepsin.

After this paper had been written a publication appeared (Rajagopalan, Moore & Stein, 1966) in which it is claimed that alanine is the only amino acid liberated by carboxypeptidase A from a sample of pepsin prepared by controlled activation of pepsinogen; nevertheless, this does not detract from the fact that the amino acids released from pepsin D are the same as those released from pepsin under similar conditions of preparation and investigation.

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