The Preparation and Purification of Individual Human Pepsins by using Diethylaminoethyl-Cellulose

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A procedure was devised for isolating human pepsins 1, 2, 3 and 5 from gastric juice by repetitive column chromatography on DEAE-cellulose. The combined yields in four different experiments varied from 14% to 90% of the total peptic activity of the starting material. The isolated individual pepsins were shown to behave as single homogeneous proteins on agar-gel electrophoresis at pH 5.0 and on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. There is preliminary evidence, requiring further study, of two other pepsins, one migrating between pepsins 1 and 2 and the other a pepsin-3 component associating closely with pepsin 5 on chromatography.

The isolation of two human pepsins from gastric juice was reported independently by Taylor (1956, 1959), using an electrophoretic technique, and by Richmond et al. (1958), using a cationic Amberlite ion-exchanger. Subsequently, Etherington & Taylor (1967) demonstrated that human gastric juice can contain up to at least five pepsins. The problems in the isolation of individual human pepsins from such complex mixtures have subsequently been well demonstrated by Etherington & Taylor (1969), and by Turner et al. (1970) for the pepsinogens of neutral mucosal extracts. Both groups, using a variety of preparative chromatographic procedures, showed that apparently symmetrical chromatographic enzyme-elution peaks contained a mixture of the human pepsins, after analysis on agar-gel electrophoresis.

The purpose of the present investigation was therefore to develop a chromatographic procedure that would yield single electrophoretically homogeneous human pepsins, particularly the minor pepsins, 1 and 2, in quantities greater than those obtained previously by preparative agar-gel electrophoresis (Etherington & Taylor, 1971; Roberts & Taylor, 1972). The system finally adopted uses DEAE-cellulose in sodium acetate buffers of various pH values. Lee & Ryle (1967) used a similar system for separating the minor components of commercial pig pepsin preparations.

Experimental

Materials

Human gastric juice was obtained by pernasal intra-gastric tube from patients undergoing augmented histamine tests (Kay, 1953) or pentagastrin stimulation tests, by using 6μg/kg body wt. intramuscularly (Jepson et al., 1968). The patients had given their informed consent. After the routine analysis of pH and total acidity, the acidic pre- and post-stimulation gastric-juice samples were pooled and stored at 4°C. Juices of pH higher than 5.0 were discarded. Storage at -20 to -15°C was avoided, as it is known that rapid freezing and thawing denatures pepsins (Berstad, 1972; Walker, 1976). The use of specimens contaminated with bile or blood was also avoided. The pooled samples of gastric juice were then concentrated up to 20-fold against Carbowax (50g/litre) at 0-4°C and equilibrated to the pH of chromatography by dialysis against the elution buffer for 17h at 4°C.

Pig pepsin was obtained as a crystalline product from Armour Pharmaceuticals, Eastbourne, Sussex, U.K., or from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. Bovine haemoglobin was obtained from Armour and was a standardized preparation for proteolytic assay. DEAE-cellulose was obtained as the preswollen grade DE-52 from Whatman Biochemicals, Maidstone, Kent, U.K., agar, as Ionagar no. 2, was from Oxoid, London SE1 9HF, U.K., and Carbowax (15000-20000 grade) was from Searle Scientific Services, High Wycombe, Bucks., U.K. All the other reagents used were of analytical grade, from British Drug Houses, Poole, Dorset, U.K.

Chromatographic procedure

The equilibrated samples were applied to a column (25cm×2.9cm) of DEAE-cellulose washed through with the starting buffer (0.1m-sodium acetate/acetic acid) at the pH indicated and then eluted with a series of linear gradients of NaCl as described in the Results section. The flow rate through the column
was maintained at 30–40 ml/h with a peristaltic pump. Fractions (11.0 ml) were collected on a Mini-escargot fraction collector (Anachem, Luton, Beds., U.K.). The protein absorption of each fraction was recorded at 280 nm by a recording spectrophotometer (Anachem). The pepsin composition of representative fractions was then ascertained by agar-gel electrophoresis at pH 5.0 (Etherington & Taylor, 1969). This is as sensitive a way of detecting peptic activity as is the measurement of total peptic activity, which was not carried out in order to conserve material. Appropriately pooled fractions were then concentrated at least 10-fold by dialysis at 4°C against a solution of Carbowax, and their pepsin composition was confirmed by agar-gel electrophoresis. The individual pepsins were then separated from each other as described in the Results section.

**Final treatment of the isolated individual pepsins**

Each solution of isolated pepsin was concentrated against Carbowax and its composition again confirmed by agar-gel electrophoresis. Extraneous Carbowax was removed by repeated dialysis against 0.001 m HCl (Ryle, 1965). The individual pepsins were then further concentrated by precipitation with (NH₄)₂SO₄. Precipitation of pepsins from 0.001 m HCl required 22% saturation of (NH₄)₂SO₄. Alternatively, precipitation of pepsin protein from the buffer-containing chromatographic solutions was found to require from 22% to 80% saturation of (NH₄)₂SO₄. This latter procedure could be used instead of concentration with Carbowax.

The suspensions of pepsin thus formed at 4°C were allowed to aggregate over at least 17 h, and for up to 5 days for solutions of low pepsin concentration. The suspensions were then centrifuged at 2000 g for 15 min and the supernatants discarded. The final precipitate was stored at 4°C in as small a volume as possible, at concentrations of up to 20 mg of protein/ml. This procedure was finally adopted in preference to concentration by freeze-drying because of the loss of proteolytic activity with the latter procedure.

**Preparative agar-gel electrophoresis**

The system used for the preparation of individual pepsins by electrophoresis was that originally devised by Smithies (1959) for starch and then modified for use with agar by Etherington & Taylor (1971) and Roberts (1975).

**SDS/polyacrylamide-gel electrophoresis**

The method used was a combination of those reported by Dunker & Rueckert (1969) and Weber & Osborn (1969), based on the method originally devised by Shapiro et al. (1967).

Standard proteins (albumin, ovalbumin, pig pepsin, chymotrypsinogen A, each 0.5 mg) were carefully dissolved in 1.0 ml of 0.01 m sodium phosphate buffer, pH 7.4, containing 2.0 g of SDS/l and 0.2–0.4% (v/v) mercaptoethanol.

Samples of the purified human pepsins were prepared in the same way as the standard proteins, but usually dissolved in a smaller sample volume (not exceeding 500 µl) of the SDS-containing buffer. Before application to the gels the samples were preincubated at 100°C for 3–5 min to abolish any autocatalytic activity. The samples were layered (after adding solid sucrose to give a concentration of 10%, w/v) on to cylindrical gels containing 10% acrylamide and the electrode buffer.

Electrophoresis was then carried out in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% SDS. Migration of the dye marker Bromophenol Blue was used to estimate the length of the run required, and 5.0 cm dye migration was ideal. The gels were then fixed and stained in Coomassie Brilliant Blue and destained in 7% (v/v) acetic acid/5% (v/v) methanol in water until the background was clear.

**Determination of proteolytic activity**

The proteolytic activity of the individual pepsins was measured by the method of Anson & Mirsky (1933) as modified by Etherington & Taylor (1969). Calculation of peptic activity in pig pepsin equivalents was carried out by comparison with the equivalent activity of known concentrations of pig pepsin A.

**Determination of total protein concentration**

The total protein of each individual pepsin solution was measured by a modification (Oyama & Eagle, 1956) of the method of Lowry et al. (1951), with dried bovine serum albumin as the standard.

**Identification of the individual pepsins**

Each pepsin is identified qualitatively by its relationship to a pig pepsin marker and to other pepsins on the electrophoretogram. Not every pepsin is present in every individual (Etherington & Taylor, 1967, 1969; Samloff & Townes, 1970). Pepsin 1 is, by definition, the pepsin in the gastric juice of patients with peptic ulcer that moves fastest to the anode. A further aid to identification was the relative mobilities of the individual human pepsins relative to pig pepsin, taken from the reference electrophoretogram of Etherington & Taylor (1967), namely: pepsin 1, 1.33; pepsin 2, 1.10; pepsin 3a, 0.99; pepsin 3, 0.90; pepsin 5, 0.54.

Pepsins were identified by these criteria initially in gastric juice and monitored by them during the separate procedures. The relative mobilities of
pure preparations obtained during this work confirmed the identification, as follows [mean (range) \( n = \) no. of observations]): pepsin 1, 1.33 (1.27–1.38) \( n = 8 \); pepsin 2, 1.12 (1.09–1.13) \( n = 6 \); pepsin 3a, 0.99 \( n = 1 \); pepsin 3, 0.89 (0.84–0.94) \( n = 8 \); pepsin 5, 0.56 (0.50–0.66) \( n = 9 \).

When pure preparations of each pepsin were pooled they ran as homogeneous bands on the electrophoretograms, thus confirming their identification.

During the separative procedures it became clear that a sixth pepsin was being isolated with a mobility between pepsins 1 and 2 (mean 1.22, range 1.18–1.25, \( n = 4 \)). The pooled preparations ran as a single homogeneous band. As explained below, this pepsin is tentatively referred to as 'slow' pepsin 1.

Occasionally, because of endosmotic or ionic effects, or possibly from interference by non-pepsin proteins, mobilities are distorted. An example is seen in fractions 31–95 (Fig. 1b); here the mobilities of pepsins 3 and 5 relative to each other are the same.

Results

Isolation of human pepsin 3

Pepsin 3 is the most abundant pepsin in gastric juice and can be isolated readily by successive chromatography on DEAE-cellulose at pH 5.3 and then at pH 4.1. Figs. 1(a) and 1(b) show that at pH 5.3 the pepsins are readily separable (Cl\(^{-}\) concentration above 0.12M) from non-pepsin protein (Cl\(^{-}\) concen-
Fractionation below 0.12M). Fractions over the range 0.21–0.25M-Cl\(^{-}\) contained pepsin 3 alone. Fractions above a concentration of 0.25M-Cl\(^{-}\) contained only pepsins 1, 2 and 3, and no pepsin 5. When these latter fractions are rechromatographed at pH 4.1 (Figs. 2a and 2b) a small quantity of non-pepsin protein is eluted initially (Cl\(^{-}\) concentrations up to 0.13M) and then a sharp peak follows (0.22–0.26M-Cl\(^{-}\), which consists only of pepsin 3), except for a small amount of pepsin 3a in the 'tail' of the peak. The fractions containing pepsin 3 alone are pooled and stored.

**Isolation of human pepsin 5**

Pepsin 5 is the second most abundant pepsin and is prepared by removing pepsin 3 from those chromatographic fractions at pH 5.3 containing only pepsins 5 and 3; this was achieved only after repeated chromatographic separations at pH 4.1. The fractions, from DEAE-cellulose chromatography at pH 5.3, eluted at up to 0.22M-Cl\(^{-}\) (Fig. 1a) were applied to a similar column at pH 4.1. Non-pepsin protein was eluted first, then two major but overlapping pepsin protein peaks. The first of these, up to fractions eluted with 0.13M-Cl\(^{-}\), was mainly human pepsin 5, with pepsin 3 as a contaminant, whereas fractions eluted over 0.13–0.18M-Cl\(^{-}\) consisted of pepsin 3, with pepsin 5 as a minor contaminant. Fractions eluted over 0.11–0.13M-Cl\(^{-}\) were therefore pooled, concentrated and refractionated at pH 4.1 on DEAE-cellulose. A small non-pepsin protein peak was eluted, and a larger symmetrical pepsin protein peak (Fig. 3a). Agar-gel electrophoresis at pH 5.0 showed that the fractions 35–51 of the latter peak, eluted at 0.11–0.13M-Cl\(^{-}\), contained only pepsin 5, and fractions 52–70, eluted

![Diagram](image-url)
Purification of human pepsins

(a) DEAE-cellulose chromatography at pH 4.1 of a human pepsin 5 and 3 mixture, after previous separation at pH 5.3 and 4.1. ——— Protein ($A_{280}$). (b) Analysis of the pooled concentrated fractions from (a) by agar-gel electrophoresis at pH 5.0. Pig pepsin (1 µg) is used as a marker. Electrophoresis was for 3 h at 2 mA/cm width and 11 V/cm length.

Fig. 3. Purification of human pepsin 5

Pepsin 1 is the pepsin associated with peptic ulceration and is best purified from gastric juice from such patients. After chromatography at pH 5.3 and then at pH 4.1, the fractions containing pepsins 1 and 2 and sometimes 3 and eluted over the range 0.30–0.80 M-Cl– are further fractionated on DEAE-cellulose at pH 3.6 (Fig. 4a). A low protein-elution profile was observed. Analysis by agar-gel electrophoresis (Fig. 4b) showed that fractions 171–250 (eluted between approx. 0.35 M- and 0.60 M-Cl–) contained only pepsin 1. The pepsin eluted over fractions 101–170 (approx. 0.22–0.35 M-Cl–) migrated at a slightly lower rate than the purified pepsin 1 and lower than the pepsin 1 as seen in gastric juice, and contained the 'slow' pepsin 1 referred to above. The fractions 1–100 consisted of pepsin 2 contaminated with pepsin 3.

Pepsin 1 was also purified on occasions by chromatography at pH 4.1 during the separation of pepsin 3 where, as shown above (Figs. 2a and 2b), fractions
Isolation of human pepsin 2

Pepsin 2 usually accounts for less than 5% of total peptic activity of gastric juice and is often absent. It is isolated by elution from DEAE-cellulose at pH4.1 at 0.25-0.35M-Cl⁻. Depending on the precise nature of the starting material, pepsin 2 may be the only component over this range (fractions 171-195, Fig. 2b) or may be contaminated with pepsin 3 (fractions 1-100, Fig. 4b). We found that pepsin 2, when contaminated in this way, cannot easily be freed from pepsin 3 by further chromatography, as the quantity present is often so small. Preparative agar-gel electrophoresis was therefore used to isolate pepsin 2 successfully from these mixtures, in order not to waste material.

Assessment of homogeneity

The most adequate method of assessing enzymic homogeneity was by agar-gel electrophoresis at pH5.0. The results presented show clearly that the individual pepsins could be readily obtained as single components. Further analysis of the purified pepsins by SDS/polyacrylamide-gel electrophoresis (Fig. 5) showed...
confirmed that the enzymes behaved as single homogeneous proteins, although as pepsins 3 and 5 migrate to similar distances with this technique, the technique does not offer proof that they are free from each other.

Yields of the individual human pepsins

The recoveries of pepsins 1, 3 and 5 together varied in four different experiments from 14% to 90% of the total pepsin (100–800 mg) in the starting samples of gastric juice. The individual amounts of pepsins 1, 3 and 5 recovered were 1.0–10% (8–20 mg), 10–74% (74–277 mg) and 2.5–16% (16–19 mg) respectively, of the activity of the starting materials. The chromatographic technique thus enables the recovery of mg quantities of the individual pepsins, whereas preparative agar-gel electrophoresis on a laboratory scale yielded only μg quantities of each enzyme (Roberts & Taylor, 1972). Recoveries of pepsin 2, involving the use of preparative agar-gel electrophoresis, were 50 μg and 200 μg in two experiments.

Specific activities of the individual human pepsins

The specific-activity values, defined as μmol of tyrosine liberated from bovine haemoglobin substrate/h per μg of pepsin protein, are given for the individual pepsins in Table 1. Human pepsin 5 showed a higher activity than did the pepsins 1 and 3. The commercially available pig pepsins had up to four times the specific activity of the individual human pepsins. Tang et al. (1967) similarly found that gastricsin (pepsin 5) had a higher specific activity than human pepsin (pepsin 3).

Discussion

The procedures described above make possible the isolation of the individual pepsins 1, 2, 3 and 5 as defined by the criteria of Etherington & Taylor (1967). Allowing for some variation depending on the pH of elution, pepsin 5 was eluted approximately at chloride concentrations of 0.10–0.15 M, pepsin 3 at 0.15–0.25 M, pepsin 2 at 0.25–0.35 M and pepsin 1 at concentrations usually above 0.30 M. The fractions containing pepsins 5 and 3 were usually easily detected from the protein-elution pattern, whereas pepsins 1 and 2, in the quantities recovered, were not easily detected by their A280.

The pepsins have now been successfully isolated by two separate procedures, (i) ion-exchange chromatography and (ii) preparative agar-gel electrophoresis (Etherington & Taylor, 1971; Roberts & Taylor, 1972). The former system enables the preparation of relatively large quantities of each pepsin and is the only one suitable for dealing with large starting volumes of gastric juice.

Individual pepsins

Human pepsin 3, the major proteinase secreted in human gastric juice, was successfully isolated free from pepsins 5 and 1 as a single electrophoretic component. Repeated chromatography was essential to remove all non-protein contaminants and the other pepsins. Various workers have used chromatographic materials other than DEAE-cellulose in the isolation of pepsins. Tang’s group (Richmond et al., 1958; Mills & Tang, 1967) reported the isolation of individual human pepsin and gastricsin (pepsins 3 and 5 respectively) by using the Amberlite cationic-ex-
change system, but were unaware of the existence of other pepsins, and it is possible that the latter may have been contaminants of their apparently symmetrical chromatographic peaks. Meitner & Kassell (1971) used hydroxyapatite columns and phosphate-gradient elution in the separation of individual ox pepsins. Seijffers et al. (1963a) used DEAE-cellulose, but were unable to separate the human pepsin 3, as were Etherington & Taylor (1969), also using DEAE-cellulose. The purification procedure presented therefore is the first successful use of DEAE-cellulose in the separation of human pepsin 3 free from other pepsin contaminants.

The isolation on DEAE-cellulose of human pepsin 5, the ‘gastricsin’ of Richmond et al. (1958), proved to be a much more complicated problem. Etherington & Taylor (1969) found that pepsin 5 was contaminated with a pepsin 3 after both cationic and anionic chromatography. The present study shows that pepsin 5 can be isolated free from its contaminating pepsin 3 only by repeated chromatography on DEAE-cellulose. The persistent contamination of pepsin 5 with a pepsin 3 would indicate that the two enzymes have some similar characteristics, and the possibility cannot be excluded that the pepsin 3 that does separate easily as a single enzyme is somehow different. The pepsin 3a found by Etherington & Taylor (1969) was shown on several electrophoretograms, and an attempt has not yet been made to isolate it; it clearly moves more rapidly to the anode than the easily separable pepsin 3 and the pepsin 3 that is associated with pepsin 5.

The more electrophoretically mobile pepsins first discovered by Etherington & Taylor (1967) and labelled 1 and 2 have now been successfully isolated for the first time by both DEAE-cellulose chromatography and preparative agar-gel electrophoresis. The rapid mobility of pepsins 1 and 2 towards the anode was considered to be an indication of their high net negative charge. This property was then used to devise the above procedure for their isolation on the positively charged DEAE-cellulose, by chromatography in sodium acetate-buffered solutions of fairly low pH, i.e. pH 4.1 and/or pH 3.6. The strength of the net negative charge was also evidenced by the high concentration of NaCl, greater than 0.30 M, required to elute the enzymes from the DEAE-cellulose, a concentration that is apparently relatively independent of the pH of chromatography. Whitecross et al. (1974) reported the isolation of pepsins from human gastric juice by using DEAE-cellulose at pH 4.2 in a citrate/phosphate buffer (0.014 M). Unfortunately, these workers used fairly low concentrations of NaCl (up to 0.23 M) to elute the pepsins, and under these conditions not all the pepsins present will be eluted, particularly pepsins 1 and 2. Figs. 2(b) and 4(b) indicate the possibility that there is a new enzyme, a ‘slow’ pepsin 1.

Pepsins 3 and 5 are quite different enzymes with differing physical and biochemical properties, as results from our laboratory and that of Tang et al. (1967) have repeatedly confirmed. However, the individuality and significance of the more electrophoretically mobile pepsins 1 and 2 have been regarded by some with suspicion. For example, Agunod & Glass (1972) and Whitecross et al. (1974) suggested that the latter enzymes were evidence of breakdown products of a larger enzyme, formed in the gastric juice. The evidence from the preparative studies now reported clearly suggests that the enzymes labelled 1 and 2 do have a definite identity and are therefore separate enzymes. The comparative biochemical studies previously reported (Roberts & Taylor, 1972, 1973) of human pepsins 1, 2, 3 and 5 confirm this finding. So far as pepsin 1 is concerned the matter has been put beyond all possible doubt by the demonstration that the enzyme, as prepared above, has a much greater ability to digest ovalbumin than have pepsins 3 or 5 (Walker, 1976). Etherington & Taylor (1969) further showed that gastric-mucosal extracts contain an individual pepsinogen precursor of pepsin 1. These enzymes should therefore be regarded as pepsins of a gastric cellular origin rather than as products formed in the gastric juice by autodigestion of some other pepsin.

In conclusion, a satisfactory procedure has been devised for isolating pepsins 1, 2, 3 and 5. The isolation of pepsin 3a has yet to be achieved. The chromatographic procedure has thrown up two questions requiring further study. (1) Is there a further enzyme migrating between pepsins 1 and 2, and not shown individually on routine agar-gel electrophoresis of gastric juice? (2) Is the pepsin 3 that behaves chromatographically rather like pepsin 5 different from the readily separable pepsin 3? It is possible that the answer to the latter question will explain some of the earlier observations (Tang & Tang, 1963; Seijffers et al., 1963a,b; Kushner et al., 1964), which have claimed that a pepsin can dissociate into two other pepsins.

References
PURIFICATION OF HUMAN PEPSINS