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IMPROVED SEPARATION OF HUMAN PEPSINS FROM GASTRIC JUICE BY HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A simple and precise separative procedure can now be used to isolate individual pepsins from gastric juice, and by measurement of the protein absorbance at 280 nm enable their direct quantitation. This will facilitate the study of pepsin secretion, particularly in patients with peptic ulcer disease.

INTRODUCTION

Newton *et al.*[1] recently reported the development of a high-performance ion-exchange chromatography (HPIEC) method for the separation of human pepsins 3 and 5 from gastric juice. However, the pepsin 1 component was not identified. In view of the marked mucolytic activity of human pepsin 1 (ref. 2) and the evidence that this enzyme may be involved in the pathogenesis of peptic ulcer disease[3] it is important to develop an analytical procedure that will identify and quantitate this enzyme in addition to the other pepsins.

Hutton *et al.*[4] quantified pepsin 1 after agar gel electrophoresis at pH 5.0 by eluting the enzyme from the gel and then measuring the resultant proteolytic activity. Thus, they confirmed the conclusion of Walker and Taylor[5], who used a semi-quantitative electrophoretic procedure, that pepsin 1 is increased in the gastric juice of patients with peptic ulcer disease. Such electrophoretic procedures are laborious, time consuming and relatively imprecise and restrict the processing of large numbers of samples required in investigative gastroenterology.

We now report a modified HPIEC procedure, in which a ternary pump with low pressure mixing is used to resolve the human pepsins, including pepsin 1. The procedure is rapid and relatively easy, thus enabling the direct quantitation of all the human pepsins present in gastric juice.

EXPERIMENTAL

Chemicals

All chemicals used were of AnalaR grade (British Drug Houses, Poole, U.K.). Naphthalene black, bovine haemoglobin, bovine globin, and swine pepsin were obtained from Sigma (Poole, U.K.). Agar was purchased from Oxoid (London, U.K.),

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and the polyacrylamide gradient gels (PAA 4/30) and low-molecular-weight markers from Pharmacia (Milton Keynes, U.K.).

Apparatus

The ion-exchange column used was a 7.5 × 0.75 cm, TSK DEAE 5PW, 10 μm (Toyo Soda Manufacturing, Tokyo, Japan) column, connected with a guard column packed with TSK guard gel DEAE .5PW. The high-performance liquid chromatography (HPLC) system included a CM4000 low-pressure mixing ternary pump (Milton Roy, Stone, U.K.). The eluent was monitored at 280 nm with a SM4000 variable-wavelength detector (Milton Roy). Samples were injected automatically, using a Gilson 231 autosampler (Anachem, Bedford, U.K.), through a 500-μl loop.

Sample preparation

Human gastric juice was obtained from patients undergoing routine gastric function tests with pentagastrin as the stimulant. For the analysis of individual samples of gastric juice, 1–3 ml aliquots were dialysed against 1 l of 50 m*M* sodium acetate (pH 4.1) for 16 h, and filtered through a 0.45-μm Gelman Acro LC 13 filter before injection in to the HPIEC system.

For the preparation of large quantities of pepsins 3 and 1, a modification of the column technique reported by Roberts and Taylor[6] was used. Pooled gastric juices (up to 4 l) were filtered through a Whatman 113V filter paper to remove large particulate matter and then concentrated to 800 ml by ultrafiltration, using a Sartorius tangential flow ultrafiltration apparatus (V. A. Howe, London, U.K.). The concentrated juice (up to 1 l) was dialysed against 10 l of 50 m*M* sodium acetate (pH 4.1) for 16 h, and then mixed with 100 g of DEAE cellulose previously equilibrated with 50 m*M* sodium acetate (pH 4.1). The slurry was stirred for 1 h, allowed to settle, and the supernatant was discarded. Pepsins 3 and 5 were eluted with 750 ml of 50 m*M* sodium acetate buffer (pH 4.1), containing 0.25 *M* NaCl. The pepsin 3 and 1 fraction was eluted with 750 ml of 50 m*M* sodium acetate buffer (pH 4.1) containing 1 *M* NaCl. The pepsin-rich supernatants were concentrated in 200-ml aliquots to 10 ml by ultrafiltration, using stirred cells (Amicon, Stonehouse, U.K.), then dialysed for 16 h against 1 l of 50 m*M* sodium acetate (pH 4.1) containing 50 m*M* NaCl, and filtered through a 0.45-μm filter before injection in to the HPIEC system.

The procedure for obtaining pure pepsin 5 was modified to improve the poor recovery obtained in the above method. Pooled or individual samples of gastric juice were filtered through a Whatman 113V filter paper, dialysed for 16 h against 1 l of 50 m*M* sodium acetate (pH 4.1), and concentrated by ultrafiltration in stirred cells. All preparative and dialysis procedures were performed at +4°C.

The concentrates (10 ml) were filtered through a 0.45-μm filter and pumped directly into the TSK DEAE 5PW column. Individual pepsin fractions were collected into 10-ml plastic tubes on ice, and dialysed against 50 m*M* sodium acetate (pH 4.1) before use in the chromatographic and analytical studies outlined.

High-performance ion-exchange chromatography

The elution of human pepsins was performed with a linear binary gradient profile (Table I). The flow-rate was 1 ml/min (pressure < 10 bar). All solvents were filtered through a 0.45-μm filter under vacuum and purged with helium for 10 min

TABLE I

ELUTION PROFILE FOR SEPARATION OF HUMAN PEPSINS

Solvent A = 50 m*M* sodium acetate (pH 4.1). Solvent B = A + 1 *M* NaCl.

Time (min)	*Solvent composition (%)*	
	A	*B*
0	100	0
5	100	0
30	70	30
30.1	0	100
40	0	100
42	100	0

before use. At the end of each day, the system was flushed with methanol for 10 min and then with deionised water for 10 min.

Chromatographic fractions were collected into 5-ml plastic tubes on ice, and analysed for protein[7], and for proteolytic activity using bovine haemoglobin as a substrate at pH 2.0 (ref. 8) (calibrated with a swine pepsin standard). Specific activity was expressed as the proteolytic activity in swine pepsin equivalents per mg of protein.

Agar gel electrophoresis

Agar gel electrophoresis was performed at pH 5.0, using a Panagel electrophoresis unit (Millipore, London, U.K.) for 1.5 h at 150 V and 40 mA on 1.5% (w/v) agar gels at +4°C, containing 25 m*M* sodium acetate pH 5.0, as described by Newton *et al.*[1]. The pepsins were visualised after incubation with bovine globin at pH 2.0[8].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed using the Pharmacia gel electrophoresis apparatus GE-2/4. Individual pepsins (100 μl) were prepared by the addition of an equal volume of 20 m*M* Tris buffer (pH 8.0), containing 2 m*M* EDTA, 5% sodium dodecyl sulphate (SDS) and 10% mercaptoethanol and heated to 100°C for 2–3 min. When cooled, a small amount 5% glycerol, containing 0.5% bromophenol blue, was added to the mixture. Gradient gels were pre-equilibrated in 40 m*M* Tris–20 m*M* sodium acetate (pH 7.4), containing 2 m*M* EDTA and 0.2% (w/v) SDS, by electrophoresis for 1 h at 70 V. Samples were then applied to the gel and electrophoresed at 125 V for 1 h after the tracking dye had migrated out of the gel.

RESULTS

Fig. 1 shows the resolution of pepsins 5, 3 and 1 by means of low-pressure mixing and the modified linear chloride gradient in Table I. Pepsins 5 and 3 were eluted between 0.15 and 0.3 *M* sodium chloride, and pepsin 1 after a rapid, stepwise increase to 1.0 *M* NaCl. Pepsin 1 was still separated from pepsin 3 when 15 mg of protein, from a pepsin 3 and 1 concentrate, was loaded onto the TSK column. Fig. 1 also shows the variable secretion of pepsins in separate gastric juices. In particular, pepsin 1 varied

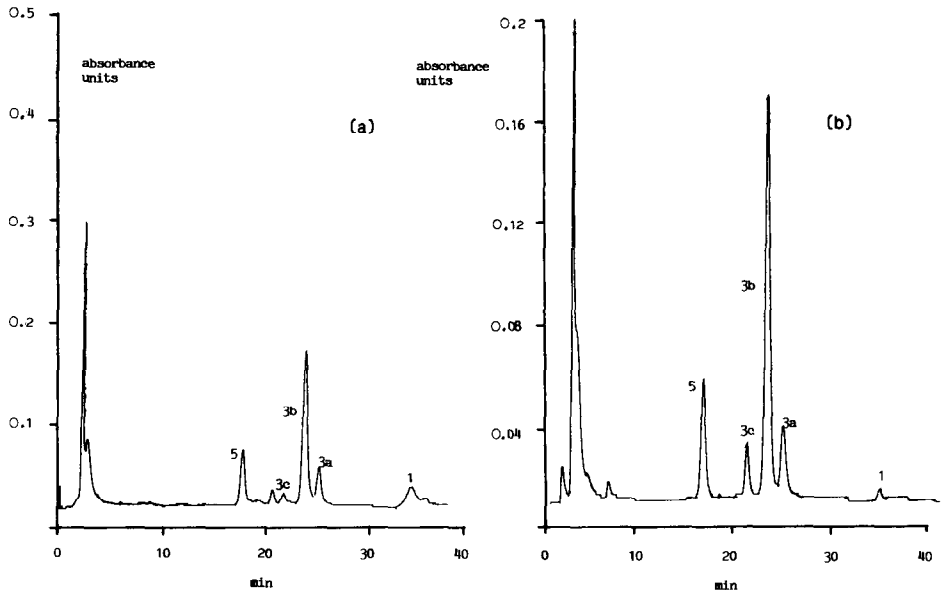

Fig. 1. The separation of human pepsins from gastric juice. The composition is expressed as the percentage of each pepsin peak area compared to the total pepsin peak area. Injection volume 500 μl. ——, UV 280 nm. (a) Patient 1: % composition for pepsin 5, 3c, 3b, 3a, and 1 was 16.5, 4.1, 56.8, 9.2 and 9.4, respectively. (b) Patient 2: % composition for pepsin 5, 3c, 3b, 3a, and 1 was 16.7, 6.9, 66.3, 9.5 and 0.6, respectively.

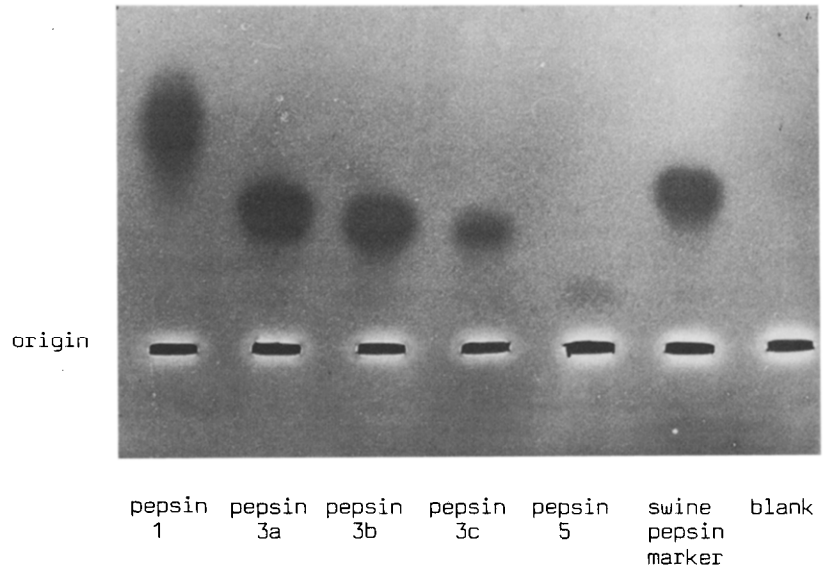

Fig. 2. Agar gel electrophoresis of individual human pepsins obtained by HPIEC. Enzymic activity is shown as zones of unstained areas (dark in the photograph) as a result of depletion of the substrate (bovine globin) contained in the gel.

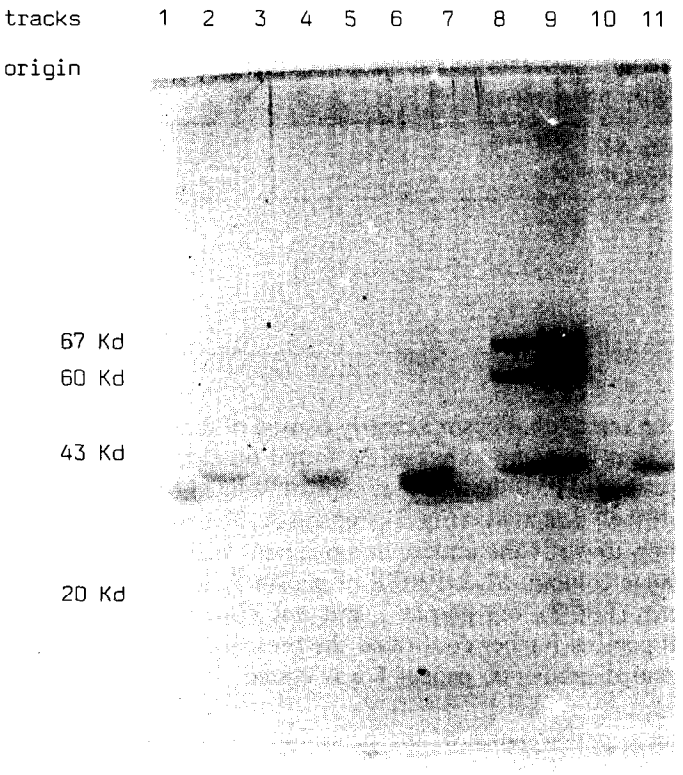

Fig. 3. SDS-PAGE of the individual human pepsins obtained by HPIEC. Tracks: 1 = pepsin 5; 2 = pepsin 3c; 3 = pepsin 3b; 4 = pepsin 3a; 5 = pepsin 1; 6 = gastric juice; 7 = swine pepsin; 8 = molecular weight markers; 9 = molecular weight markers; 10 = pepsin 5; 11 = pepsin 3c. Kd = Kilodaltons.

TABLE II

RETENTION TIMES, ANALYTICAL PRECISION AND RECOVERY OF PROTEOLYTIC ACTIVITY FOR HUMAN PEPSINS

Data from replicate injections (n = 6) of individual pepsins prepared by HPIEC. C.V. = coefficient of variation.

Pepsin	*Retention time (min)*		*Peak areas (arbitrary units)*		*Proteolytic recovery (%)*	
	Mean	*C.V.*	*Mean*	*C.V.*	*Mean*	*C.V.*
5	17.5	1.3	52.8	1.6	99.0	8.0
3c	22.6	0.7	30.0	3.6	82.0	8.0
3b	25.2	0.7	109.0	2.5	101.0	10.0
3a	27.4	0.7	44.5	1.5	69	3.4
1	35.7	0.2	11.2	3.2	101	3.6

TABLE III

SPECIFIC ACTIVITIES OF PURIFIED HUMAN PEPSINS

Values are the mean ± 1 S.D. for six measurements.

Pepsin	*Mean specific activity ± S.D. (μg swine pepsin equiv./μg protein)*
5	0.87 ± 0.05
3c	0.42 ± 0.01
3b	0.86 ± 0.09
3a	0.42 ± 0.01
1	0.20 ± 0.01

from 9.4 to 0.6% of the total secretion. Pepsin 3 clearly consist of three peaks, 3a, 3b and 3c, labelled such that 3a is the same as pepsin 3a found on electrophoresis[9].

Agar gel electrophoresis at pH 5.0 showed that pepsins isolated by HPIEC were present as single zones of proteolytic activity (Fig. 2). Pepsins 3c, 3b and 3a had slightly increasing rates of migration towards the anode, in agreement with their increased retention on the ion-exchange column. SDS–PAGE of pepsin 5, 3c, 3b and 3a also showed single protein bands (Fig. 3), but pepsin 1, was not visualized. Rechromatography of the individual pepsins further confirmed the presence of single components. However, with extended gradients, pepsin 1 was eluted as a broad band of unresolved peaks.

To assess the analytical performance of the procedure purified pepsins were reinjected into the HPIEC system. Table II shows good precision of retention time, mean peak area, and recovery of proteolytic activity. The recovery of proteolytic

TABLE IV

THE RELATIVE AMOUNTS OF HUMAN PEPSINS IN GASTRIC JUICE COLLECTED DURING A PENTAGASTRIN STIMULATION TEST

The relative percentage was calculated from peak areas.

Gastric juice	*pH*	*Vol. (ml)*	*Pepsin (relative %)*				
			5	*3c*	*3b*	*3a*	*1*
Basal							
0–15[a]	1.7	12.0	23.0	7.5	63.5	4.2	1.7
15–30	1.3	34.0	14.3	4.3	74.0	5.9	1.4
30–45	2.0	19.0	15.1	4.2	73.0	5.6	2.0
After Pentagastrin							
0	0.9	45.0	12.4	5.0	77.1	5.1	0.4
0–15	1.0	16.0	13.2	4.2	76.6	4.9	1.1
15–30	0.9	82.0	13.7	4.1	77.0	4.8	0.4
30–45	0.9	92.0	13.8	4.2	77.3	4.6	0.2
45–60	0.9	41.0	13.5	4.2	76.7	5.3	0.2
60–75	0.9	31.0	13.3	4.3	77.0	5.2	0.1

[a] Minutes.

activity of 3a and 3c was less than the *ca.* 100% obtained for pepsins 5, 3b and 1. However, there was no loss of protein for any of the pepsins after repeated chromatography.

The specific activities of purified pepsins are shown in Table III. Pepsin 1 has approximately one quarter the activity of pepsin 3b and pepsin 5. The lower specific activities of pepsins 3a and 3c compared with 3b may indicate some loss of activity during purification or different activities towards bovine haemoglobin.

The relative percentages of the individual pepsins secreted in a series of gastric juices, obtained from a patient undergoing a pentagastrin test, are given in Table IV. The percentage of pepsin 1 was maximal during the prestimulation period, with variable secretion of pepsin 5 and 3. However, after pentagastrin stimulation, the relative percentage of the different pepsins was remarkably consistent, the only change being the volume of juice secreted.

DISCUSSION

The chromatographic separation which we have developed allows the resolution of all the major human pepsins present in gastric juice, as confirmed by agar gel electrophoresis. We have previously shown the separation of pepsin 5 from pepsin 3b and 3c, but pepsins 3a and 1 were not identified. The successful resolution of pepsin 1 was achieved by a rapid stepwise change in gradient to 1.0 *M* NaCl, which resulted in the elution of this enzyme as a single peak. The improved separation of pepsin 5 and the three components of pepsin 3 was achieved with a modified chloride gradient formed by low-pressure mixing.

The specific activities reported are in agreement with those reported by Roberts[10]. The low specific activity observed for pepsin 1 would suggest the presence of non-pepsin protein *e.g.*, a proteoglycan[11], inactive enzyme, or activity towards haemoglobin different from that of swine pepsin.

The analysis of a pentagastrin test showed that there was little change in the percentage composition of pepsins following pentagastrin stimulation. However, during the prestimulation period, the composition was more variable. In particular, pepsin 1 was increased. Chromatograms of the basal secretion showed this to be a true increase in the amount of pepsin 1 compared with post stimulatory juices. In addition, pepsin 5 was present in all samples of gastric juice, unlike the earlier observation of Newton *et al.*[1] when this enzyme decreased after pentagastrin stimulation.

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