Journal of Chromatography, 476 (1989) 491–498
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1593

HETEROGENEITY OF HUMAN PEPSIN 1, AS SHOWN BY HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

We have shown that pepsin 1 can be prepared in milligram quantities from human gastric juice by semi-preparative high-performance ion-exchange chromatography. Further investigation into the elution of this enzyme using linear chloride gradients have shown it to be a heterogeneous mixture, the components of which all have peptic activity, but differing specific activities. These components are changed in number and retention time by incubation with hyaluronidase and aryl sulphatase, but not by neuraminidase or acid phosphatase, implying the presence of a sulphated proteoglycan.

INTRODUCTION

Pepsin 1 is present in human gastric juice in relatively low amounts, but is increased in patients with peptic ulcer disease[1]. It has different enzymic properties from the major human pepsin 3, particularly in its action on gastric mucoprotein[2] and bovine collagen[3]. We have shown that pepsin 1 contains up to 40–50% carbohydrate by weight[4], in contrast to human pepsins 3 and 5. "Fast" and "slow" moving components of pepsin 1 have been described[5], and subsequently Ryle and Foltmann[6] suggested that pepsin 1 is a mixture of pepsin–carbohydrate complexes produced as a result of differing amounts of carbohydrate attached to a common protein.

We now describe an investigation by high-performance ion-exchange chromatography (HPIEC) into the heterogeneity of pepsin 1 and the effect after pre-incubation with enzymes which might hydrolyse its carbohydrate component and any attached phosphate or sulphate groups.

EXPERIMENTAL

Chemicals

The chemicals used were of AnalaR grade (British Drug Houses, Poole, U.K.). Agarose was obtained from Oxoid (London, U.K.). DEAE-cellulose, naphthalene black, bovine haemoglobin, bovine globin, swine pepsin, acid phosphatase, neuraminidase, hyaluronidase, and aryl sulphatase were obtained from Sigma (Poole, U.K.).

0021-9673/89/$03.50

Apparatus

The ion-exchange column used was 7.5 × 0.75 cm, TSK DEAE 5PW 10 μm (Toyo Soda Manufacturing, Tokyo, Japan), connected with a guard column containing TSK guard gel DEAE 5PW. The high-performance liquid chromatographic (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 with a Gilson 231 autosampler (Anachem, Bedford, U.K.) through a 500-μl loop.

Sample preparation

Human gastric juice, obtained from patients undergoing routine Pentagastrin tests, was pooled and concentrated five times, using a Sartorious tangential flow ultrafiltration apparatus (V. A. Howe, London, U.K.). Approximately 1 l of concentrated gastric juice was then dialysed against 10 l of 50 m*M* sodium acetate (pH 4.1) for 16 h and mixed with 100 gm of DEAE cellulose, which had been equilibrated with 50 m*M* sodium acetate (pH 4.1). The slurry was stirred for 1 h at +4°C, allowed to settle, and the supernatant was discarded. The DEAE-cellulose was washed with 750 ml of 50 m*M* sodium acetate (pH 4.1), containing 0.25 *M* NaCl and the supernatant was discarded. The pepsin 3- and 1-rich fraction was then eluted with 750 ml of 50 m*M* sodium acetate (pH 4.1), containing 1 *M* NaCl, and concentrated in 200 ml aliquots to 10 ml by ultrafiltration, using stirred cells (Amicon, Stonehouse, U.K.). The concentrates were dialysed for 16 h against 1 l of 50 m*M* sodium acetate (pH 4.1), filtered through a 0.45-μm filter, and the pepsins isolated by HPIEC with gradient 1 (Table I). The purified pepsin 1 fractions were pooled, dialysed against 1 l of 50 m*M* sodium acetate (pH 4.1) for 16 h and then used for HPIEC studies. All the preparative and dialysis procedures were carried out at +4°C.

High-performance ion-exchange chromatography

The heterogeneity of pepsin 1 was investigated, using gradients 2 and 3 (Table I). The flow-rate was 1 ml/min and the back-pressure less than 10 bar. All solvents were filtered through a 0.45-μm filter under vacuum and purged with helium for 10 min before use. After use, the HPIEC system was flushed with methanol, followed by deionised water for 10 min each. For the proteolytic activity profiles, 0.5-ml fractions

TABLE I

ELUTION PROFILES FOR THE SEPARATION OF HUMAN PEPSIN 1

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

Gradient 1			*Gradient 2*			*Gradient 3*		
Time (min)	*A (%)*	*B (%)*	*Time (min)*	*A (%)*	*B (%)*	*Time (min)*	*A (%)*	*B (%)*
0	100	0	0	100	0	0	100	0
5	100	0	5	100	0	5	100	0
30	70	30	25	0	100	95	0	100
30.1	0	100	40	0	100	97	100	0
40	0	100	42	100	0			
42	100	0						

were collected into 5-ml plastic tubes on ice and assayed for protein[7] and for proteolytic activity, using bovine haemoglobin 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.*[9], and the pepsins were visualised after incubation with bovine globin at pH 2.0 (ref. 8).

Treatment of pepsin 1 with hydrolytic enzymes

Acid phosphatase (wheat germ, E.C. 3.1.3.6), neuraminidase (*Clostridium perfringens*, E.C. 3.2.1.18), hyaluronidase (bovine testes, E.C. 3.2.1.35), and aryl sulphatase (limpet, E.C. 3.1.6.1) were freshly prepared (1 mg/ml) in 50 m*M* sodium acetate (pH 4.1). Each enzyme was incubated with pepsin 1 (1:1, w/w; total volume 2 ml) for 1 h at 37°C. Immediately after incubation, the mixture was chromatographed in the HPIEC system; enzyme and pepsin 1 alone were used as controls.

RESULTS

Fig. 1 shows the purification of pepsin 1 (*ca.* 14% of the total pepsin content) from a pepsin 3- and 1-rich fraction by HPIEC. Pepsin 1 is well resolved from pepsin 3,

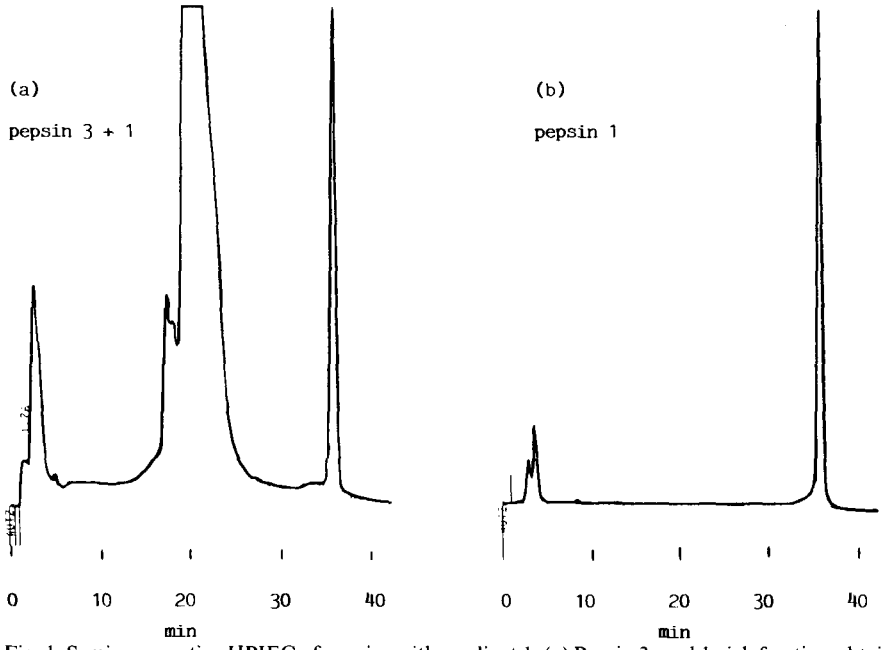

Fig. 1. Semi-preparative HPIEC of pepsins with gradient 1. (a) Pepsin 3- and 1-rich fraction obtained from concentrated gastric juice by the DEAE-cellulose preparative method, is shown to contain only pepsins 3 and 1. A 500-μl sample was injected, containing 15 mg of protein. (b) The pepsin 1 fraction obtained from Fig. 1a was rechromatographed to confirm its purity. A 500-μl sample was injected containing 1 mg of protein. UV 280 nm, 2.0 a.u.f.s.

and upon reinjection it was eluted as a single peak. This peak, when chromatographed with gradient 2 (Fig. 2), was eluted as a broad peak, consisting of several unresolved peaks. All fractions taken through this peak showed proteolytic activity against bovine haemoglobin (Fig. 2), but with differing specific activities (mean 0.2 ± 0.13 S.D.).

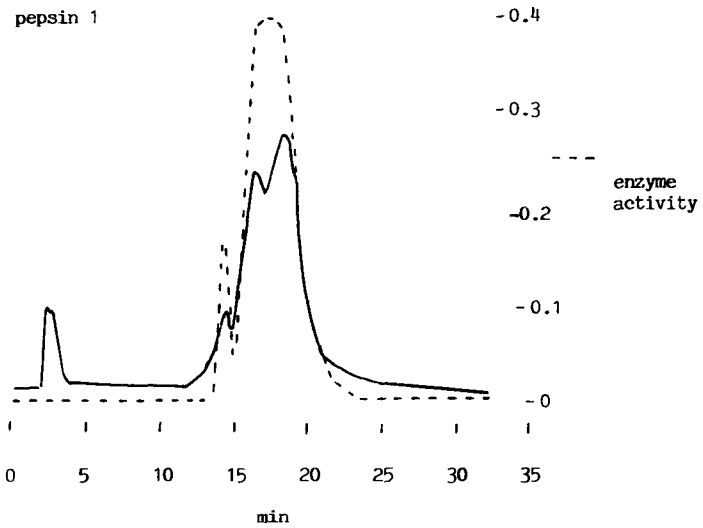

Fig. 2. HPIEC of purified pepsin 1 with gradient 2. (———) UV 280 nm, 0.2 a.u.f.s.; (– – –) proteolytic activity. A 500-μl sample was injected containing 500 μg of protein.

When the chloride gradient was extended to 95 min with gradient 3 (Fig. 3a), the elution of pepsin 1 extended over 33 min in at least six unresolved peaks.

Chromatography of pepsin 1 after treatment with various acid hydrolases is shown in Fig. 3. Neuraminidase and acid phosphatase had no effect on the elution profile of pepsin 1 (Fig. 3b and c). However, hyaluronidase changed the retention time and the elution profile, which now consisted of four large but still unresolved peaks eluted at a lower chloride concentration (Fig. 3d). When the hyaluronidase–pepsin 1 mixture was electrophoresed on agar gel, a slower migration to the anode was observed than for with pepsin 1 alone (Fig. 4). Aryl sulphatase (Fig. 3e) did not affect the onset of elution of pepsin 1, but significantly reduced the time for complete elution from 33 min to *ca.* 20 min. When the hydrolytic enzymes were chromatographed alone, no peaks were eluted in the pepsin 1 region.

DISCUSSION

Pepsin 1 can be readily obtained in milligram quantities by a combination of DEAE-cellulose batch adsorption and HPIEC. Pepsin 1 is observed as a single proteolytic zone on agar gel electrophoresis, but extended gradients in HPIEC show it to be heterogeneous, being eluted as a broad band, in which proteolytic activity can be detected in all fractions.

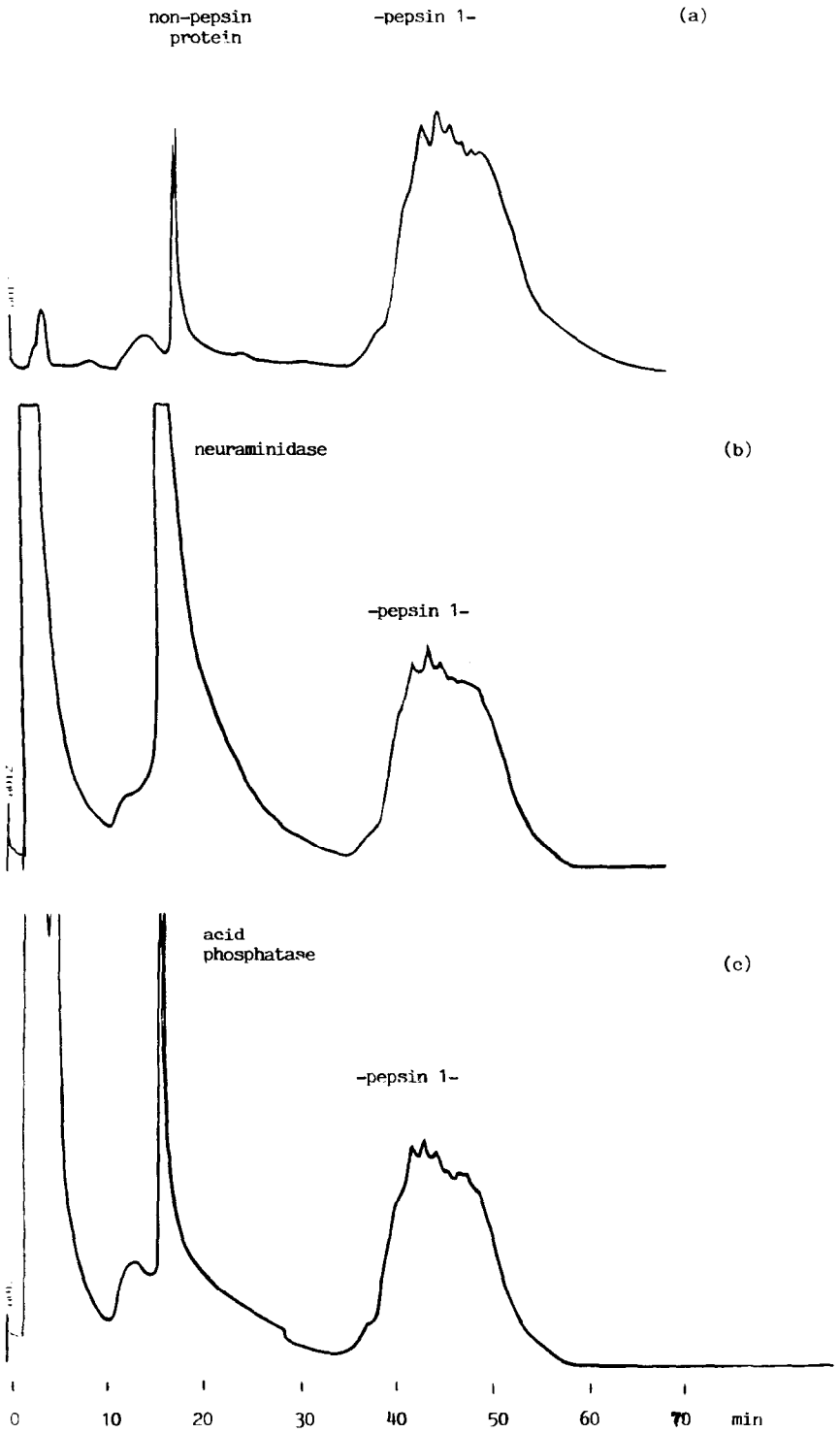

Fig. 3.

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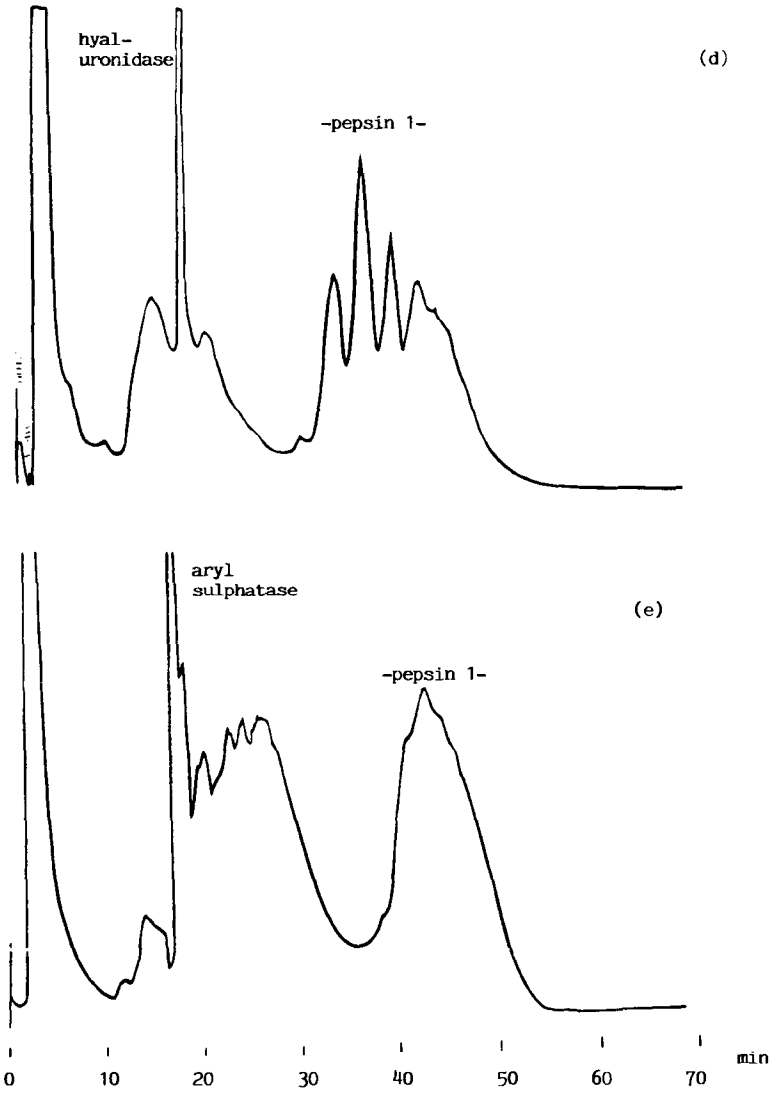

Fig. 3. Chromatogram of pepsin 1 with gradient 3 after pre-incubation with: (a) pepsin 1 control, (b) neuraminidase, (c) acid phosphatase, (d) hyaluronidase and (e) aryl sulphatase. UV 280 nm, 0.02 a.u.f.s. A 500-μl sample was injected containing 250 μg of pepsin 1.

Pepsin 1 is known to contain up to 50% carbohydrate[4], and Ryle and Foltmann[6] suggest that the variable substitution of carbohydrate onto a common protein may account for the heterogeneity observed on agar gels. We have investigated the action of various acid hydrolases on pepsin 1; aryl sulphatase and hyaluronidase had striking effects. In particular, hyaluronidase changed the elution profile and retention time of pepsin 1. Ryle and Foltmann[6] were the first to observe the effect of hyaluronidase on the electrophoretic mobility of pepsin 1, and showed that fragments were removed in association with a reduction in molecular weight.

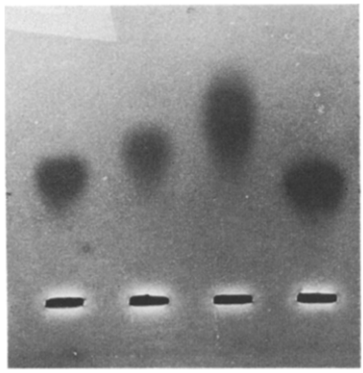

Fig. 4. Agar gel electrophoresis at pH 5.0 of pepsin 1 after treatment with hyaluronidase. Enzymic activity is shown as zones of unstained areas (dark in the photograph) as a result of depletion of the substrate (bovine globin) contained in the gel.

Initial studies by Pearson *et al.*[4] on the sugar analysis of the pepsin 1 complex showed the presence of uronic acid, N-acetylglucosamine, N-acetylgalactosamine and neutral sugars. In addition, they separated a proteoglycan from pepsin 1 by equilibrium centrifugation in a caesium chloride gradient containing 4 *M* guanidium chloride. They concluded that pepsin 1 was a complex of protein and proteoglycan, interacting ionically.

Hyaluronidase will degrade both hyaluronic acid and chondriotin sulphate, and the evidence we present indicates that pepsin 1 may contain one or both of these glycosaminoglycans. In addition, the action of aryl sulphatase would imply the presence of a sulphated proteoglycan.

The high electrophoretic mobility and strong adsorption on anion-exchange resins may thus result not only from the presence of phosphate[10], but from the presence of a proteoglycan moiety in which acidic sugars and sulphate groups produce an increased negative charge. The formation of this highly charged species may also affect the activity of pepsin 1 by interacting ionically with the substrate and allow more efficient proteolysis[2,3].

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