Journal of Chromatography, 599 (1992) 179–184
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2515

Peptide maps of five human pepsin isoenzymes and other aspartic proteinases

A. T. Jones* and N. B. Roberts

Department of Clinical Chemistry, Royal Liverpool University Hospital, Duncan Building, Prescot Street, Liverpool L7 8XW (UK)

ABSTRACT

Peptide maps of five individual human pepsins were developed using reversed-phase high-performance liquid chromatography after protein digestion with either *Staphylococcus aureus* proteinase (V8) or α-chymotrypsin. Human pepsins 3a, 3b and 3c produced almost identical peptide maps suggestive of proteins with very similar amino acid sequences. The map for human pepsin 1 was similar to pepsin 3b (the most predominant human pepsin) but less than half the expected amount of each equivalent peptide fragment was generated, indicating that the actual mass of digested protein used was less than the dry weight measurement would suggest, probably as a result of carbohydrate attached to pepsin 1. Comparison of human pepsin 3b maps with other aspartic proteinases confirmed a significant homology with swine pepsine A but not with endothiapepsin. The α-chymotrypsin digests compared with V8 gave more complex peptide maps as a result of its broader bond cleavage specificities.

INTRODUCTION

The separation and purification of five proteolytically active human pepsins (1, 3a, 3b, 3c and 5, where 5 is equivalent to gastricsin, using the pepsin classification based on mobility on agar gel electrophoresis [1]) has been reported previously [2]. Keen *et al.* [3] subsequently sequenced up to 37 N-terminal amino acids for five human pepsins and indicated no differences between pepsin 1, 3a, 3b and 3c. Pepsin 5 was clearly different and was identified as gastricsin. Athauda *et al.* [4] confirmed these findings by analysis of the N-terminal sequences of human pepsinogens. However, little information is available on the complete primary sequence of these individual human pepsins from protein analysis, and particularly pepsin 1, the ulcer-associated pepsin [5].

Human pepsin gene studies have been able to identify three pepsin (3) genes which were found to differ in up to only four amino acids, and one gastricsin gene [6–7], but there is no information from human pepsin gene studies on the amino acid sequence of pepsin 1.

Enzymic cleavage and reversed-phase high-performance liquid chromatography (HPLC) peptide mapping are well established techniques for preliminary studies of protein structures. The predicted amino acid sequence of three human pepsins from gene analysis [6] shows only three arginine residues located predominantly at the C-terminus and no lysine residues. The use of trypsin for pepsin digestion was therefore unsuitable. Glutamic acid residues, however, are distributed throughout the pepsin sequence (fourteen in pepsin 3) and cleavage at these residues would theoretically produce up to fifteen fragments. The proteinase V8 from *Staphylococcus aureus* can be specifically used for cleavage at glutamic acid residues [8]. We have therefore investigated the development of peptide map profiles using reversed-phase HPLC after V8 cleavage of the individual pepsins to highlight possible differences in their protein structures. Of particular interest was the degree of peptide map analogy between human pepsin 1 and the major isoenzyme 3b. Other aspartic proteinases with established amino acid sequences were investigated to identify any similar peptide fragments and thus sequence similarities with the human pepsins. Peptide maps developed after digestion with α-chymotrypsin (having a

0021-9673/92/$05.00

broader bond cleavage specificity) were also studied as a further comparison.

EXPERIMENTAL

Chemicals

Urea (molecular biology reagent), dithiothreitol (DTT), iodoacetamide (IAA), swine pepsin A, bovine pancreas α-chymotrypsin and *Staphylococcus aureus* V8 proteinase (V8) were obtained from Sigma (Poole, UK). Ammonium hydrogencarbonate and ammonium acetate (AnalaR grade) were obtained from British Drug Houses (Poole, UK), trifluoroacetic acid from Pierce and Warriner (Chester, UK) and acetonitrile (HPLC grade S) from Rathburn Chemicals (Walkerburn, UK). Human pepsins were prepared according to the method of Peek and Roberts [2], then dialysed against 1 mmol/l hydrochloric acid and dried by freeze-drying. Endothiapepsin from the fungus *Endothia parasiticia* was obtained from Dr. J. Cooper, Department of Crystallography, Birkbeck College, University of London.

Staphylococcus aureus proteinase (V8) digests

Aspartic proteinases were digested with V8 using modifications of the established methods of Drapeau [9] and Stone *et al.* [10]. An aliquot, 500 μg, of dry enzyme was dissolved in 500 μl of 0.1 mol/l ammonium acetate containing 8 mol/l urea, 50 μl of 45 mmol/l DTT were added and the mixture was incubated at 50°C for 15 min. After cooling to room temperature, 50 μl of 100 mmol/l IAA were added and the solution was incubated at 20–25°C for 10 min. Water (1.4 ml) was added, followed by 16.5 μl of 1 mg/ml V8 (1:33 enzyme: substrate ratio). The digest (final pH 6.21) was incubated at 37°C for 8 h. All reactions were stopped by either freezing to −20°C or immediate chromatography. The following enzymes were digested with V8: swine pepsin A, endothiapepsin, human pepsins 1, 3a, 3b, 3c and human gastricsin.

α-Chymotrypsin digests

Chymotrypsin digests of the same proteins were performed according to the method of Stone *et al.* [10]. The method was similar to that described for V8 digests except that ammonium acetate was replaced with 0.4 mol/l ammonium hydrogencarbonate and the digests (final pH 8.34) were terminated after 24 h.

Reversed-phase HPLC

Peptides, after V8 and chymotrypsin digestion, were separated on an Exsil 300Å C_{18} (5 μm) reversed-phase column (15 × 0.46 cm I.D.) (Jones Chromatography, Hengoed, UK). Peptides were detected at 220 nm (SM 3000 UV detector; LDC, Stone, UK). Binary linear gradients were developed on a low-pressure tertiary mixing pump (Model CM 4000; LDC). Chromatograms were recorded on a CI 4000 computing integrator (LDC).

RESULTS

V8 digests

The V8 maps for the human pepsins 1, 3c, 3b, 3a and gastricsin are shown in Fig. 1a–g and the elution times of the major peptide fragments are summarized in Table I. The peptide profiles for pepsins

TABLE I

COMPARISON OF PEPTIDE RETENTION TIMES IN REVERSED-PHASE HPLC AFTER V8 DIGESTS OF FIVE INDIVIDUAL HUMAN PEPSINS AND SWINE PEPSIN A

Human pepsins 3c, 3b, 3a, 1, gastricsin (GTN) and swine pepsin A (SPA) were the individual enzymes used. Dashes indicate the absence of a peak.

Retention time (min)					
3c	3b	3a	1	GTN	SPA
–	–	–	–	–	22.1
–	–	–	–	24.4	–
–	–	–	–	24.8	–
–	–	26-7	–	–	–
28.3	28.6	–	28.9	–	–
–	–	30.7	–	–	–
33.0	33.2	33.5	33.2	–	–
34.0	34.4	34.3	34.2	34.8	–
35.0	35.4	35.3	35.5	36.1	–
36.7	37.1	37.0	37.0	36.9	37.0
39.3	40.0	40.0	39.6	39.8	39.6
–	41.2	–	41.8	–	41.8
–	–	–	–	–	43.6
57.3	58.3	58.5	57.0	57.1	58.6
59.4	59.1	59.6	58.1	59.1	60.3
62.1	61.8	62.2	62.0	–	62.3
–	–	–	–	74.0	–

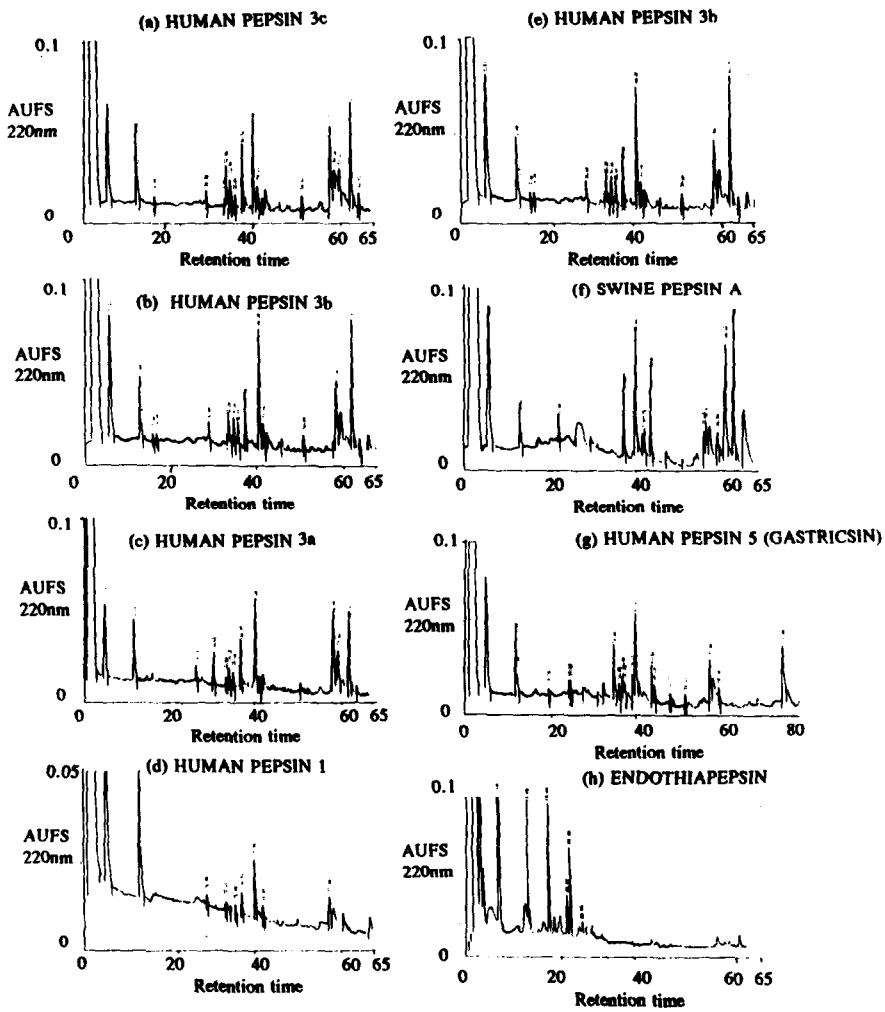

Fig. 1. Peptide maps after V8 digestion of five human pepsins, swine pepsin A and endothiapepsin, separated by reversed-phase HPLC. Injection volume, 350-μl (corresponding to 87 μg of digested protein); flow-rate, 1.5 ml/min; operating back-pressure, 1400–2000 psi (9653–13 870 kPa). Solvent A = trifluoroacetic acid–water (0.1:99.9, v/v); solvent B = solvent A–acetonitrile (40:60, v/v). Linear gradient: 100% A for 5 min, 0–30% B over 20 min and 30–80% B generated over 50 min. AUFS = Absorbance units full-scale; retention times in minutes.

3a, 3b and 3c were very similar although 3a produced specific peptides eluting at 26.7 and 30.7 min and none at 28 min. The pepsin 1 map (Fig. 1d) was also very similar to that of human pepsin 3b although the relative amount of the peptide eluting at 62 min was markedly reduced, as was the overall amount of eluting material (the AUFS scale was set at 0.05 for pepsin 1 compared with 0.1 for the other pepsins).

The human gastricsin and swine pepsin A maps showed a number of peptide similarities compared with the other human pepsin maps. The peptide map for endothiapepsin (Fig. 1h), however, was different, with all the major peptide eluting before 22 min.

Chymotrypsin digests

Chymotryptic maps (Fig. 2a–d) confirmed the marked similarities between human pepsins 1, 3c, 3b and 3a. For pepsin 1, however, the fragment at

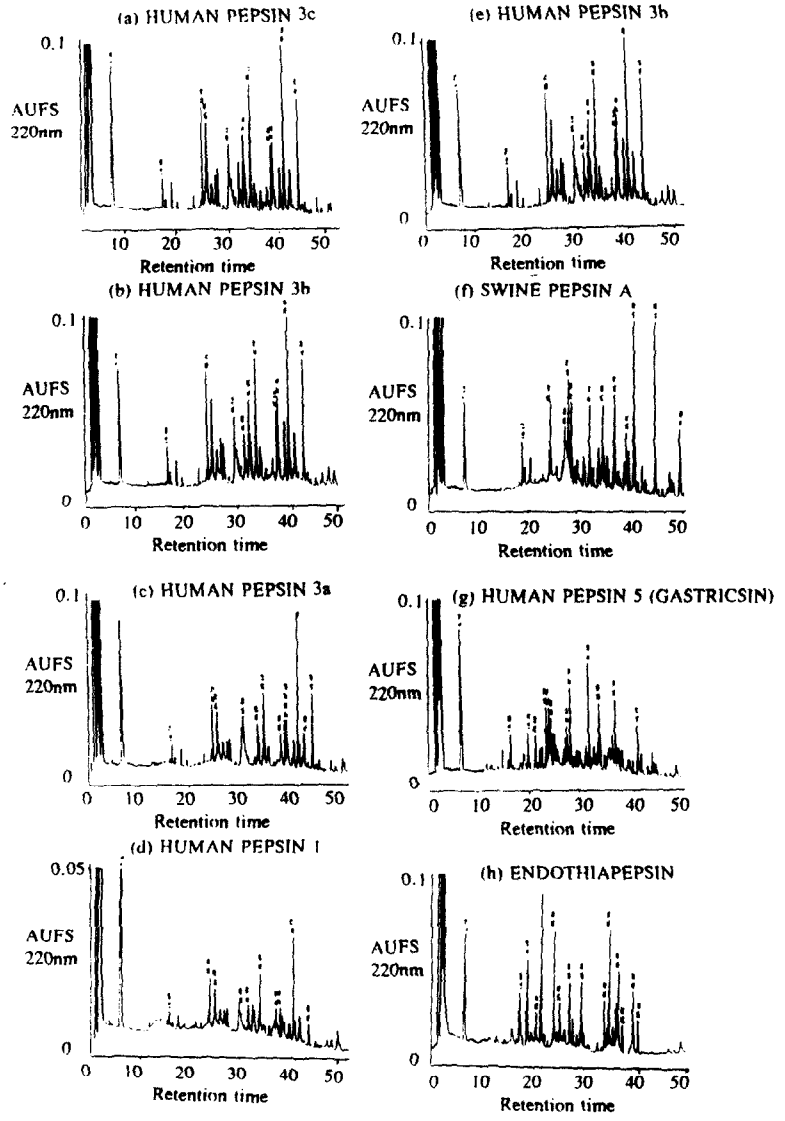

Fig. 2. Peptide maps after chymotrypsin digestion of five human pepsins, swine pepsin A and endothiapepsin, separated by reversed-phase HPLC. Injection volume, 250 μl (corresponding to 63 μg of digested protein); flow-rate, 1.5 ml/min; operating back-pressure, 1400–2000 psi (9653–13 780 kPa). Solvents A and B as in Fig. 1. Linear gradient: 100% A for 5 min, 0–75% B generated over 45 min. AUFS = Absorbance units full-scale; retention times in minutes.

42.6 min was significantly reduced compared with 3b, as was the overall amount of peptides. The human gastricsin map (Fig. 2g) gave a different profile in comparison with the other human pepsins.

Swine pepsin A (Fig. 2f) showed a similar overall for endothiapepsin had a significant proportion of early-eluting peptides, although some peptides eluted at similar times to the other pepsins, *viz.*, 23–25, 33 and 40 min.

The early-eluted peaks (Figs. 1 and 2) were asso-

a V8-related self-cleavage fragment eluting between 11 and 14 min (Fig. 1), but no other peaks were produced.

DISCUSSION

We have developed enzyme digest procedures using V8 and chymotrypsin, and reversed-phase HPLC peptide maps to investigate the similarities in amino acid structures between five human pepsins and other aspartic proteinases, namely swine pepsin A and endothiapepsin.

A relatively low proteolytic activity of V8 was observed when digests were performed in 0.1 *M* ammonium hydrogencarbonate (pH 8.1), a commonly used buffer [11], but was much improved when replaced with 0.1 *M* ammonium acetate (pH 6.2). A buffer-related susceptibility to bond cleavage has been described previously for this enzyme [12]. The HPLC gradient was extended for the V8 maps to improve the separation for subsequent peptide collection, human pepsin amino acid sequence analysis and confirmation of the site(s) of bond cleavage by V8. The maps after chymotrypsin digestion were more complex, as expected, as this enzyme is able to hydrolyse several types of peptide bonds, thus generating many more peptides *i.e.*, about 30 fragments. Hitherto very few if any detailed studies have been made on human pepsins using these procedures, but differences in the N-terminal sequences of two human pepsinogens PGA-3 and PGA-5 were identified using peptide mapping after cleavage with endoproteinase Lys-C [13].

The marked similarities in the V8 and chymotrypsin maps of human pepsins 1, 3a, 3b and 3c in this study suggest a high degree of amino acid homology in these proteins. The human pepsin 3c and 3b maps are in fact almost indistinguishable, which suggests that they correspond to PGA-3 and PGA-5, respectively, which from gene structural analysis [6] were found to differ in only one substitution (valine–leucine) at residue 77 on pepsinogen or residue 29 on pepsin. Pepsin 3a, which showed some differences from the 3b map, could then correspond to PGA-4, which Evers *et al.* [6] found differed by three amino acid residues.

The V8 map for pepsin 1 was very similar to that for pepsins 3b and 3c in terms of the number and retention times of peptides separated in reversed-phase HPLC (Table I), which suggests that pepsin 1 has a similar primary structure to pepsins 3b and 3c. However, less than half the amount of each equivalent peptide (in terms of comparative peak areas) was formed, implying that the actual weight of pepsin 1 used was less than the dry weight would suggest, probably as a result of carbohydrate attached to pepsin 1 [14]. Peptide sequence studies should clarify whether human pepsins 3b and 1 have the same primary structure and therefore that some post-translational modification (*i.e.*, incorporation of carbohydrate) has occurred in pepsin 1, thus modifying its enzymic properties [15].

The peptide maps also confirm the high degree of amino homology (89%) [16] between swine pepsin A and human pepsins, and lower homology (52%) between human gastricsin and human pepsins [7]. The endothiapepsin maps do not share any peptide similarities to the pepsin maps, which confirms little sequence homology [17].

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support of the Science and Engineering Research Council UK to A.T.J., Professor T. L. Blundell of Birkbeck College, University of London, for making that possible and Professor A. Shenkin, in whose department the work was carried out.

REFERENCES

1 D. J. Etherington and W. H. Taylor, *Nature (London)*, 216 (1967) 279.
2 K. Peek and N. B. Roberts, *J. Chromatogr.*, 476 (1989) 291.
3 J. N. Keen, J. B. C. Findlay, K. Peek, N. B. Roberts and W. H. Taylor, *Clin. Sci.*, 77, Suppl. 22 (1989) 94.
4 S. B. P. Athauda, M. Tanji, T. Kageyama and K. Takahashi, *J. Biochem.*, 106 (1989) 920.
5 W. H. Taylor, *Nature (London)*, 227 (1970) 76.
6 M. P. J. Evers, B. Zelle, J. P. Bebelman, *Genomics*, 4 (1989) 232.
7 G. Pals, T. Azuma, T. K. Mohandas, G. I. Bell, J. Bacon, I. M. Samloff, D. A. Walz, P. J. Barr and T. Taggarrt, *Genomics*, 4 (1989) 137.
8 J. Houmard and G. R. Drapeau, *Proc. Natl. Acad. Sci. U.S.A.*, 69 (1972) 3506.
9 G. R. Drapeau, *Methods Enzymol.*, 47 (1977) 189.
10 K. L. Stone, M. B. LoPresti, J. M. Crawford, R. DeAngelis and K. Williams, in P. T. Matsudaira (Editor) *A Practical Guide to Protein and Peptide Purification for Microsequencing*, Academic Press, New York, 1987, pp. 31–47.

11 A. Aitken, M. J. Geisow, J. B. C. Findlay, C. Holmes and A. Yarwood, in J. B. C. Findlay and M. J. Geisow (Editors), *Protein Sequencing, a Practical Approach*, IRL Press, Oxford, 1989, pp. 43–68.
12 G. R. Drapeau, Y. Boily and J. Houmard, *J. Biol. Chem.*, 247 (1972) 6720.
13 R. A. Bank, B. C. Crusius, T. Zwiers, S. G. M. Meuwissen, F. Arwert and J. C. Pronk, *FEBS Lett.*, 238 (1988) 105.
14 J. P. Pearson, A. Allen, N. B. Roberts and W. H. Taylor, *Clin. Sci.*, 72, Suppl. 16 (1987) 33.
15 J. P. Pearson, R. Ward, A. Allen, N. B. Roberts and W. H. Taylor, *Gut*, 27 (1986) 243.
16 B. Foltmann, *Essays Biochem.*, 53 (1981) 52.
17 T. L. Blundell, A. Jenkins, B. T. Sewell, L. H. Pearl, J. B. Cooper, I. J. Tickle, B. Veerapandian and S. P. Wood, *J. Mol. Biol.*, 211 (1990) 919.