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Microbore liquid chromatography coupled to a flow fast atom bombardment probe for the on-line detection of the Tyr-Pro cleavage of a nonapeptide by recombinant HIV-1 protease

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ABSTRACT

The nonapeptide Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln has been reported as a model substrate for an aspartyl protease produced by the human immunodeficiency virus (HIV-1). Cleavage of this peptide at the Tyr-Pro linkage to produce tetra- and pentapeptide fragments is the basis of high-performance liquid chromatographic assays to detect HIV-1 protease activity. Confirmation of the cleavage site has been proved by using microbore liquid chromatography coupled to a dynamic fast atom bombardment interface. Comparison with fortified control incubates indicates that an approximate stoichiometric amount of the tetrapeptide was formed from the nonapeptide, confirming that the cleavage of the substrate by HIV-1 protease is both specific and quantitative.

INTRODUCTION

The nonapeptide Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln has been reported as a model substrate for an aspartyl protease produced by the human immunodeficiency virus (HIV-1) [1]. Cleavage of this peptide at the Tyr-Pro linkage to produce tetra- and pentapeptide fragments is the basis of high-performance liquid chromatographic (HPLC) assays to detect HIV-1 protease activity [1]. The nonapeptide I (Fig. 1) was incubated with recombinant HIV-1 protease and the resultant products were examined by HPLC using ultraviolet (UV) detection at 220 nm. Although HPLC peaks were observed that co-chromatographed with synthetic standards of the cleavage products II and III (Fig. 1), mass spectral evidence was required to confirm that the cleavage occurred specifically at the Tyr-Pro linkage. The use of the relatively established technique of thermospray liquid chromatography–mass spectrometry (LC–MS) was attempted, but it was not possible to detect the peptide fragments at sufficiently low concentration. The objective of this study was to determine whether LC coupled to dynamic fast atom bombardment (FAB) could be utilised to solve this problem.

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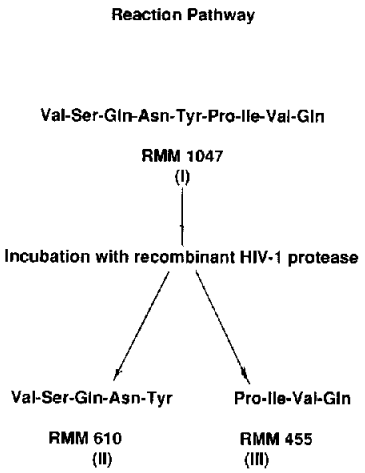

Fig. 1. HIV-1 aspartyl protease-catalysed cleavage mechanism.

EXPERIMENTAL

The incubation conditions for HIV-1 aspartyl protease are reported elsewhere [2]. Mass spectral analyses were conducted on a VG Trio-3 quadrupole mass spectrometer (VG, Manchester, U.K.), operating in the Q1 mode, scanning from 200 to 1500 a.m.u. over 3 s. Dynamic FAB spectra were recorded using Xenon at 8 kV and 1 mA. The dynamic FAB matrix was water–methanol (Rathburn, Walkerburn, U.K.)–glycerol (BDH, Poole, U.K.)–tetrafluoroacetic acid (TFA, Aldrich, Gillingham, U.K.) (50:50:5:0.1). The on-line FAB matrix was water–acetonitrile–glycerol–TFA (81:10:9:0.1). The LC column was a Techsphere 5 C8 5-μm, 25 cm × 2 mm I.D. (HPLC Technology, Cheshire, U.K.). The flow-rate was 100 μl/min provided by a Milton Roy Constametric 3000 pump. A Rheodyne loop injector (20 μl) and a Waters 490E UV detector were used. A home-made splitter, fitted between the UV detector and the mass spectrometer, was constructed from commercial stainless-steel HPLC fittings and fused-silica tubing. This allowed about 5 μl/min from the column flow into the dynamic FAB probe of the mass spectrometer.

Off line HPLC–MS sample preparation

The digest was dissolved in 70 μl of 6 *M* guanidine to denature the protease. Preparative HPLC of the digest was undertaken, collecting the fraction ($\sim$1 ml) eluting at the retention time of the tetrapeptide. This fraction was evaporated to dryness and reconstituted in the FAB matrix solvent (70 μl).

RESULTS AND DISCUSSION

The FAB spectra of synthetic standards of the three peptides, obtained from

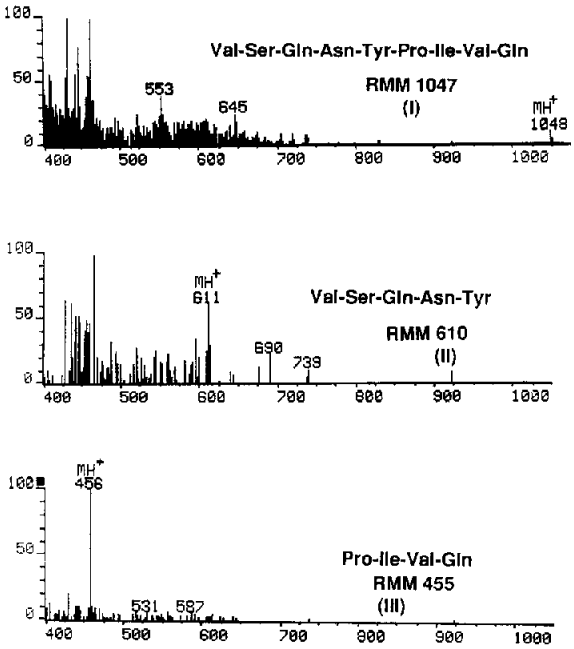

Fig. 2. Dynamic FAB spectra of the nonapeptide (I) and its two protease-catalysed cleavage products, pentapeptide (II) and tetrapeptide (III).

the same amount of sample on the probe in each case, (Fig. 2), differ in the intensity of the protonated molecular ion. The ion intensity in FAB may be considered as a function of the ease of transfer of the peptide from the solution to the surface of the matrix (the hydrophobicity of the peptide). Bull and Breese [3] have published a scale of hydrophobicities for amino acid residues; by summing the values for each residue in a peptide and dividing by the number of residues, one calculates an index related to its hydrophobicity and hence its availability for ionisation [4]. As can be seen from Table I, the tetrapeptide is more negative (more hydrophobic) than both the nonapeptide and the pentapeptide and so will more readily migrate to the surface of the matrix and be more available for

TABLE I

THEORETICAL HYDROPHOBICITIES OF THE PEPTIDES OBTAINED USING THE METHOD OF BULL AND BREESE [3]

Peptide sequence	B+B index
Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln	−954
Val-Ser-Gln-Asn-Tyr	−790
Pro-Ile-Val-Gln	−1160

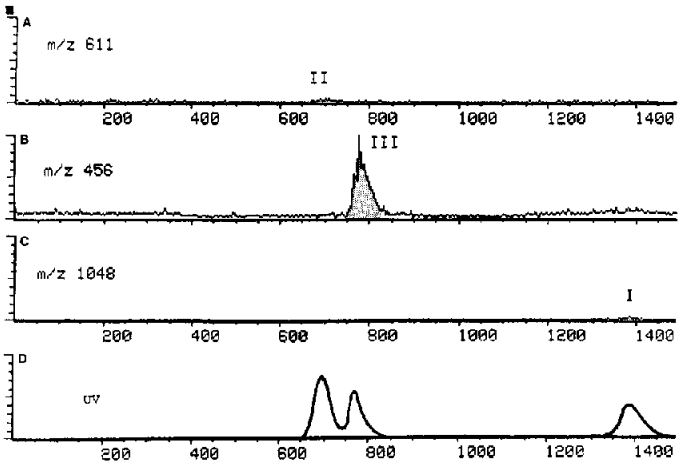

Fig. 3. On-line LC–MS of a mixture of the three peptides (3 μg each) showing the relative abundance of the protonated molecular ions. Extracted ion current profiles: (A) peptide II, *m/z* 611; (B) peptide III, *m/z* 456; (C) peptide I, *m/z* 1048. (D) shows the UV trace at 220 nm.

ionisation by FAB. This view is supported by the data in Fig. 3. The ion profiles for the MH^+ ions of each peptide have been normalised and the tetrapeptide is significantly more intense than the others. In view of the above it was decided to monitor for only the tetrapeptide (III) in the incubate.

Our initial approach to detecting the products of the protease digest was to examine the crude preparative HPLC fractions of the protease incubation (10 μg of the nonapeptide) using off-line dynamic FAB (see Experimental section). The sample was injected, via a 100-nl Valco loop injector on the dynamic FAB interface, into the mass spectrometer. The data obtained from the crude extract were not of good quality; background ions from the incubation were observed in the region of the MH^+ of the tetrapeptide which prevented us from detecting it at low concentrations. The relatively small proportion of the sample that could be injected via the small loop injector also contributed to the poor level of detection. It became clear that to obtain good quality data for this problem, "on-line" microbore LC–MS using dynamic FAB was necessary.

To investigate the feasability of on-line LC–MS three incubations were carried out. As before, incubation of the nonapeptide (10 μg) with aspartyl protease was carried out. A control incubation, without any of the nonapeptide, was also prepared. A second control incubation was also prepared and then fortified with 5 μg of the tetrapeptide. The digests were denatured by the addition of 6 *M* guanidine (70 μl), and 20 μl were injected onto the microbore LC column coupled the flow FAB interface by a 20:1 split.

Comparison of the control incubation with an incubation fortified with 10 μg of the nonapeptide (Fig. 4) by both UV (220 nm) and extracted ion current profile

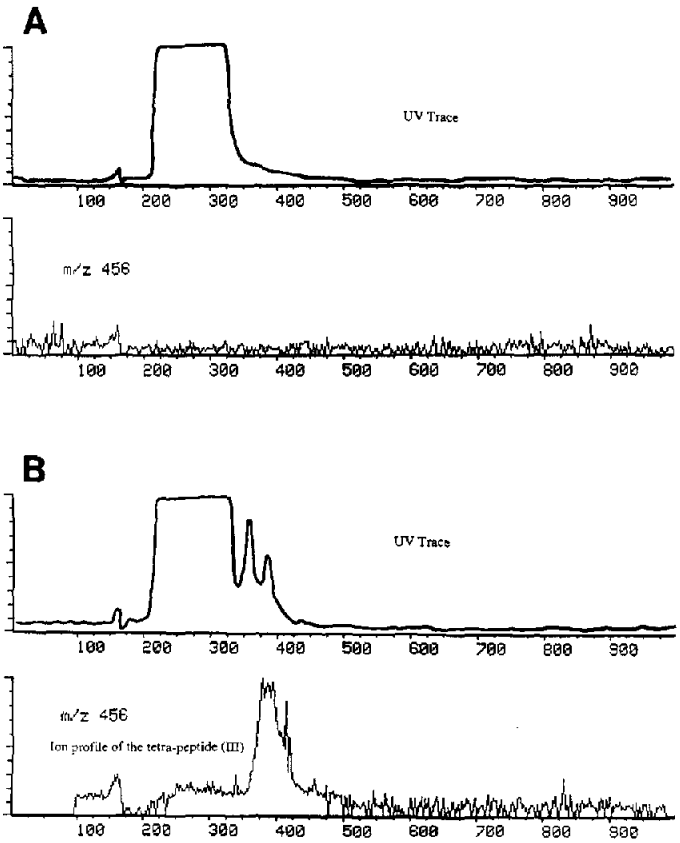

Fig. 4. $UV_{220\ nm}$ traces and extracted ion current profiles (m/z 456) for (A) a control incubation and (B) an incubation of the nonapeptide (10 μg) with HIV-1 aspartyl protease.

(m/z 456, MH^+ tetrapeptide) indicated that the tetrapeptide was formed from the nonapeptide. Intensity at m/z 456 was plotted against scan number, and the peak areas were recorded for all three samples. We were interested in obtaining a semi-quantitative estimate of the amount of the tetrapeptide being formed from the nonapeptide during the incubation. Using the peak area data from the sample fortified with the tetrapeptide, it was possible to obtain an estimate of amount of tetrapeptide formed in the incubation of the nonapeptide. The estimated yield of tetrapeptide was 4.1 μg, whereas the theoretical stoichiometric yield should be 4.3 μg. These data suggest that the cleavage of the nonapeptide by HIV-1 protease is both specific and quantitative.

CONCLUSIONS

"On-line" microbore LC–dynamic FAB has enabled us to detect the tetrapeptide (III) at low level (1.4 μg on-column) after incubation of the nonapeptide

(I) with HIV-1 protease. A 20:1 splitter was used after the LC column so the amount of the peptide entering the mass spectometer was approximately 70 ng. This compares favourably with thermospray LC–MS where 50 μg of the tetrapeptide were needed to give a similar response.

Interfering ions observed in the "off line" experiments have been shown to be artifacts of fraction collection and work-up; they were not due to co-eluting compounds. By installing a UV detector with a low-volume flow cell in line between the column and the dynamic FAB interface, we were able to monitor the column eluate and collect the bulk ($\sim$95%) of the sample from the splitter for further work. Comparison of an incubation containing the nonapeptide (I) with a control incubation fortified with the tetrapeptide (III) showed that an approximately stoichiometric amount of the tetrapeptide was formed from the nonapeptide. The cleavage of the nonapeptide (I) substrate by HIV-1 protease is both specific and quantitative.

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