Peptide Mapping of the Active Site of *Bacillus cereus* β-lactamase I by the Use of High Pressure Liquid Chromatography Coupled to Electrospray Ionisation Mass Spectrometry

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Electrospray ionisation mass spectrometry directly coupled to microbore HPLC is a powerful technique for the rapid identification of covalently modified peptides.

Electrospray ionisation mass spectrometry (ESIMS) has emerged as a powerful technique for the analysis of biopolymers.[1] When coupled to HPLC, it can be used for the detection of small molecules[2] and is of great potential in the peptide mapping of proteins.[3] ESIMS has also proved useful in the rapid characterisation of covalent enzyme : inhibitor[4,5] and enzyme : substrate complexes.[6] We now report the extension of the technique to the location of the modified active site using microbore HPLC directly coupled to ESIMS.

Radiolabelled 6β-bromopenicillanic acid has been used[7] to show that the inhibition of β-lactamase I from *Bacillus cereus* occurs through acylation of residue Ser 70 and probably results in the formation of a dihydrothiazine derivative (Fig. 1).[8] The formation of the dihydrothiazine derivative has recently been substantiated by an ESIMS study.[4] Herein, we report the use of HPLC–ESIMS to identify modified active site peptides of β-lactamase I inhibited with 6β-iodopenicillanic acid after proteolysis with either *Staphylococcus aureus* V8 (Glu-C) or *Pseudomonas aeruginosa* (Lys-C) proteases. Incubation of β-lactamase I [100 μg in 100 μl of Tris/HCl buffer (20 mmol dm^{-3}, pH 8.0)] with a 200-fold molar excess of 6β-iodopenicillanic acid **1** for 5 min was followed by digestion with either protease (at 37 °C with a protease : substrate ratio of 1 : 20). Aliquots (10 μl) were withdrawn at 1 h intervals, the digestion stopped by freezing at −80 °C and subsequently analysed by HPLC–ESIMS. The peptide mixture was separated by microbore HPLC and the column eluate was split so that 80% passed through a UV detector and the remaining 20% passed directly into the ESIMS interface. A comparison of the UV chromatogram and the total ion current chromatogram from the mass spectrometer showed a qualitative correlation (Fig. 1).

Mass spectra were scanned from m/z = 350 to 1400 every 2.1 s throughout the chromatographic separation. Analysis of the spectra associated with each peak in the total ion current chromatogram allowed the identification of peptides present by comparison of the observed masses with those calculated for predicted digest peptides from β-lactamase I. The results (Table 1) show the expected digest pattern for *S. aureus* V8 protease, with cleavage after every Glu residue. Lysis after Asp residues was less efficient and only cleavage after residues 114, 179 and 246 were identified.

Comparison of *S. aureus* V8 peptide digest samples from the inhibited and unreacted β-lactamase I showed one significant

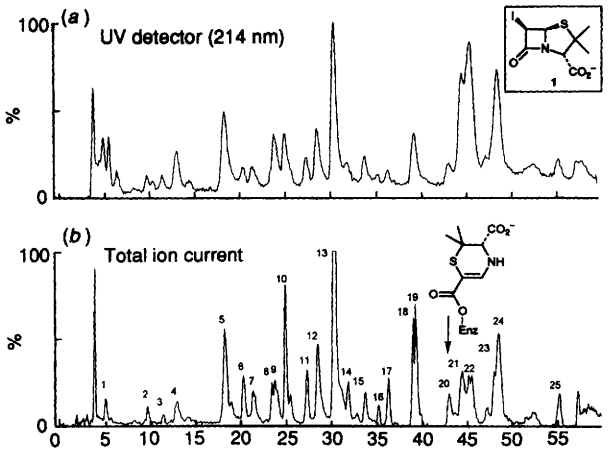

Fig. 1 Comparison of the UV chromatogram (*a*) and the total electrospray ion current chromatogram (*b*) of a Glu-C digest of β-lactamase I inhibited with 6β-iodopenicillanic acid **1** after 3 h digestion. The derivatised peptide is indicated with downward arrow. The numbered peaks correspond to the entries in Table 1.

Table 1 Assignment of peptides from *B. cereus* β-lactamase I digested with Glu-C protease for 3 h

Entry[a]	Retention time/min	Observed mass	n‡	Expected mass	Assignment[c]
1	5.07	1218.6	1	1220.4	E1
2	9.69	715.6			
3	11.43	514.8			
		737.9			
4	13.12	886.0	2	887.1	E14
		621.4	1	622.7	E2
		852.2	1	853.0	E5
5	18.27	1212.4	1	1214.4	E7
6	20.37	1096.7	2	1098.4	E18
7	21.56	595.2			
		893.5			
8	23.49	2076.5	2	2078.4	E10[b]
9	23.80	1136.2	1	1137.2	E6
10	25.01	2060.9	2	2062.4	E10
11	27.39	935.6			
		747.8			
		623.3			
12	28.52	1971.6	1	1972.2	E5–6
13	30.31	2578.6	2	2579.9	E9
		2954.9	2	2958.3	E3
14	31.85	3608.4	2	3610.1	115–149
15	33.74	3795.6	2	3795.2	210–246
16	35.10	2288.6	2	2289.6	180–202
		987.6			
17	36.26	3157.3	2	3158.7	180–209
18	39.02	3125.7	1	3127.6	E13
19	39.31	3995.1	3	3997.7	E13–14
20	43.01	3126.3	1	3126.5	Acylated E4
		2658.8	2	2660.2	221–244
21	44.38	6436.4	4	6437.4	E15–16
22	45.21	6178.3	3	6180.1	E15
23	48.12	10412.6	5	10416.0	E13–16
24	48.54	11546.6	4	11547.2	E13–17
		10156.5	5	10158.7	E13–15
25	55.27	12625.1	4	12627.6	E13–18
		12979.5	5	12984.0	E12–18
26	39.21	2928.0	1	2928.3	E4

[a] Entries 1–25 refer to a sample inhibited with 6β-iodopenicillanic acid and correspond to the peaks identified in Fig. 1. A control sample of uninhibited β-lactamase I differed significantly only in that it did not contain the acylated E4 peptide (entry 20) but did contain the unacylated active site peptide (entry 26). [b] E10 was tentatively assigned as the sulfoxide of one of the methionine residues of peptide E10. The values given for the unidentified species (entries 2, 3, 7, 11 and 16) are mass/charge ratios, as the charge state is unknown. The observed masses are measured from n‡ charge states. [c] Assignments (*e.g.* E12–13) correspond to the peptides indicated in Fig. 2, or refer to specific residues where an Asp-C cleavage has occurred (*e.g.* 180–209).

difference. An almost complete peptide map of the unmodified protein could be identified but in the case of the inhibited sample (Fig. 1 and Table 1) the peptide E4, containing the active site serine (Table 1, entry 26) was no longer present and a new peak (Table 1, entry 20) with a slightly greater retention time (t_R 43.01 min) was observed. The 'new' peptide had a mass 198.3 greater than the unacylated E4 peptide, consistent with the formation of the expected dihydrothiazine derivative.[4,7,8] An analogous result was obtained in the case of the *P. aeruginosa* Lys-C digest, where once again a single significant difference between the inhibited and control digests was observed, consistent with the formation of the dihydrothiazine derivative of peptide K6 (Table 2, entries 14 and 19). An 8 h time course of the digestion indicated that the modified active site peptides produced by both proteases underwent slow hydrolysis to the unmodified form. Digestion for a limited period of 2 or 3 h gave the most 'clear cut' results, with a significant digestion of the β-lactamase I but without significant hydrolysis of the modified active site peptides. The identity of the modified active site peptide from each digest was confirmed by automated Edman degradation of the purified acyl peptides (E4 and K6). The single serine residue common to both these peptides (Ser 70) was thus confirmed as the active site nucleophile (Fig. 2).

Partial digests also generated larger 'overlap' peptides in which some potential cleavage sites had not been fully digested (*e.g.* Table 1, entries 23 to 25). Few tri- and no di-peptides were directly observed, either because they were outside the mass range of the scan or because they co-eluted with the buffer salts in the solvent front (t_R 2.7 min). However, under the partial digestion conditions used, some of these short sequences could be identified as part of larger partially digested peptides. For example, the dipeptide E16 was not identified, but peptide E15-16 was observed (Table 1, entry 21). Other short peptides (4–6 residues, Table 2, entries 1, 2 and 3) were eluted very close to the solvent front. They gave principally the singly charged MH^+ ion and relatively weak signals.

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Table 2 Assignment of peptides from *B. cereus* β-lactamase I digested with Lys-C protease for 2 h

Entry[a]	Retention time/min	Observed mass	n‡	Expected mass	Assignment[b]
1	3.14	359.3	1	358.4	K24
		661.3	1	661.7	K18
		697.4	1	697.7	K3
		495.7	1	495.5	K12
2	3.91	470.6	1	470.6	K11
3	5.00	457.7	1	457.6	K23
4	10.03	787.1	1	786.9	K9
5	15.12	729.9	1	729.9	K14
6	17.19	880.2	1	879.4	K4
7	20.83	1501.8	1	1501.7	K15–16
8	22.65	1395.0	2	1394.5	K8
		1217.3	2	1217.4	K15
9	25.19	920.3	1	920.1	K17
10	27.76	1578.2	1	1578.7	K22
11	28.77	1640.4	1	1639.9	K19
12	34.51	2390.3	2	2390.6	K10
13	34.88	3848.5	3	3847.3	K13
14	37.01	3972.9	2	3972.2	Acylated K6
15	39.17	2562.3	2	2561.9	K7
16	42.20	3596.9	2	3597.1	K20–21
17	44.31	3224.2	2	3224.7	K20
18	56.64	7221.9	3	7219.3	K19–23
19	34.33	3773.0	2	3774.2	K6

[a] Entries 1 to 18 refer to a sample inhibited with 6β-iodopenicillanic acid. A control sample of uninhibited β-lactamase I differed significantly only in that it did not contain the acyl K6 peptide (entry 14) but did contain the unacylated active site peptide (entry 19). The observed masses are measured from n‡ charge states. [b] Assignments (*e.g.* K15) correspond to the peptides indicated in Fig. 2.

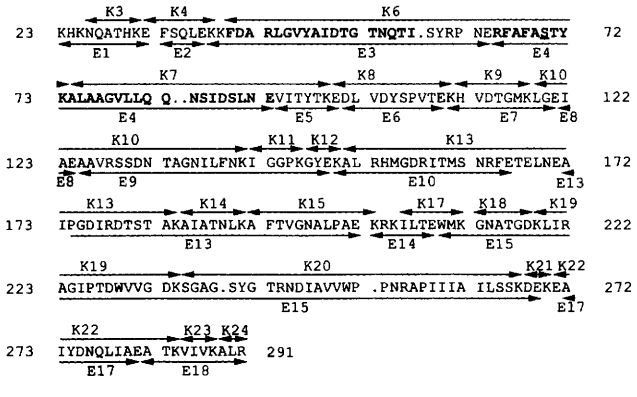

Fig. 2 The amino acid sequence[9] of β-lactamase I indicating the peptides produced by Glu-C digestion (indicated below the sequence, E1 to E18) and those produced by Lyc-C digestion (indicated above the sequence, K3 to K25). The sequences that have been confirmed by automated Edman degradation are shown in bold. The active site residue (Ser 70) is underlined.

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