An investigation of the binding of protein proteinase inhibitors to trypsin by electrospray ionization mass spectrometry

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Abstract The binding of BPTI and SBTI with trypsin has been investigated by ESI MS, using the mutant K15V-BPTI and the chemically modified RcamBPTI as controls. Although high cone voltages (+80 V) produce sharp spectra of BPTI, RcamBPTI, SBTI and trypsin alone, the complexes of BPTI, RcamBPTI and SBTI with trypsin undergo partial dissociation due to collisional activation. At lower cone voltages (+40 V) these non-covalent complexes are stable. The charge distribution on the trypsin and the inhibitors produced by gas phase dissociation of the complexes are markedly different from those of the components alone, indicating that ESI MS provides a novel probe for exploring the ionic interactions at the contact surface of proteins. Moreover, by determining the cone voltage at which the gas phase dissociation of complexes occurs it may be possible to use ESI MS to compare the binding energies of closely related complexes.

Key words: Trypsin; Bovine pancreatic trypsin inhibitor; Soybean trypsin inhibitor; Electrospray mass spectrometry; Non-covalent binding

1. Introduction

ESI MS is rapidly becoming a routine technique for the observation of non-covalent protein-small molecule and protein-protein complexes. Conditions have now been developed such that spectra can be obtained from picomole quantities of protein in their native conformations. The solvents are essentially at physiological pH and the source temperature is 40°C or below. This not only avoids the denaturing conditions that have been used previously, but also allows the study of proteins under conditions in which they can be detected as complexes with substrates, products or inhibitors. Initially, ESI MS was restricted to the study of complexes in which the ligand (substrate or product) was covalently bound [1], but increasingly non-covalently bound enzyme complexes are being studied [2–5]. However, these studies have principally been concerned with the binding of relatively small molecules to enzymes [6], or with protein-protein interactions in natural oligomers, e.g. haemoglobin [7].

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Abbreviations: ESI MS, electrospray ionization mass spectrometry; BPTI, bovine pancreatic trypsin inhibitor; SBTI, soybean trypsin inhibitor type I-S; K15V BPTI, the site-directed mutant of BPTI in which Lys-15 is replaced by Val; RcamBPTI, a chemically modified BPTI in which the Cys-14–Cys-38 disulfide bond is reduced and the resultant free thiols are carboxyamidomethylated; CV, cone voltage

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BPTI is a highly stable, basic protein consisting of a single polypeptide chain of 58 amino acids. The crystal structures of the BPTI complexes with β-trypsin and porcine kallikrein A have been solved [8,9]. These have shown that the region of interaction, as for many proteinase inhibitors, is between the active site cleft of the proteinase and an external loop region of the BPTI. The conformational complementarity of the loop region and the enzyme active site and the number of interactions made between the loop and the active site of trypsin are responsible for the high binding energy and very low inhibition constant, $K_i = 6 \times 10^{-14}$ M [10]. This is one of the lowest inhibition constants observed for a non-covalent protein inhibitor and for this reason it was selected as the initial target for analysis by ESI MS.

BPTI can also be relatively easily modified by both chemical and site-directed mutagenesis and in the work presented here, three different forms of BPTI were studied along with another inhibitor of trypsin, soybean trypsin inhibitor (SBTI), which is from a different family of proteinase inhibitors [11]. BPTI may be chemically modified by the reduction of a disulfide link (C14–C38) in the exposed binding loop region [12]. Reduction with sodium borohydride and reaction of the free thiols with iodoacetamide results in a modified form of BPTI (RcamBPTI) that has different physical and inhibitory properties e.g. $K_i = 1.3 \times 10^{-9}$ M [10]. The primary determinant of specificity for BPTI, and proteinase inhibitors in general, is the interaction between the P_1 residue of the inhibitor and the S_1 subsite of the proteinase [13]. Site-directed mutagenesis of the P_1 lysine to valine leads to a modified BPTI molecule which has totally different inhibitory properties, changing the specificity of inhibition from trypsin, which it no longer inhibits, to elastases (human leukocyte elastase-H.L.E. and porcine pancreatic elastase-P.P.E.) [14]. NMR data have confirmed that the conformation of the loop region does not change significantly in this mutant and that it is the nature of the P_1 residue that is critical to the strength of the interaction between the BPTI and trypsin. The K_i values of the inhibitors (against trypsin) discussed in this paper are given in Table 1. The data presented here show that ESI MS can not only be used to detect complex formation between proteins, but that by analysis of the gas-phase dissociated molecular ions, both the strength of the interaction can be probed and the nature of the contact region between the two proteins investigated.

2. Materials and methods

BPTI (aprotinin) was purchased from Boehringer Mannheim. Trypsin was purchased from Sigma (T 8642; TPCK-treated, from bovine pancreas). SBTI type I-S was purchased from Sigma (T 6522). K15V BPTI was expressed in *E. coli*; the production, isolation and characterisation are described elsewhere [15]. RcamBPTI was prepared ac-

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cording to the method of Kress and Laskowski [12]. ESI MS were measured on either a Micromass Quattro II or BioQ II mass spectrometer. The following standard conditions were used: samples were infused via a Harvard Model 22 syringe pump into the electrospray source (at 3 μl/min). Analyses were performed using cone voltages of +40, +60 or +80 V with a source temperature of 40°C and the mass spectrometer was scanned from 1000 to 4000 Da. Trypsin and inhibitors were made up in aqueous 2.5 mM ammonium acetate, pH 6.5–7.0, and preincubated for at least 20 min at a trypsin concentration of 10 pmol/μl. Spectra were recorded at molar inhibitor-trypsin ratios of 1:5, 1:2 and 1:1.

3. Results and discussion

The ESI mass spectral analysis of trypsin under denaturing conditions (Fig. 1c) showed the expected three components A* β-trypsin, B* α-trypsin and C* Ψ-trypsin with the typical broad charge state distribution from 23^+ to 10^+ expected for a denatured protein with all its residues exposed to the solvent. The differences in M_r are due to partial hydrolysis (at K125–S126 and K170–D171) of this disulfide-linked protein [16]. In marked contrast, analysis of trypsin from ammonium acetate solution showed a simple 'native state' spectrum Fig. 1a) dominated by the 10^+ and 9^+ charge states for the β-trypsin-Ca^{2+} (Note: hereafter 'trypsin' will refer to the β-trypsin-Ca^{2+} complex). Analysis at increased cone voltage resulted in a cleaner spectrum and some charge stripping to form the 8^+, 7^+ and 6^+ charge states (Fig. 1b).

ESI mass spectral analysis of the inhibitors from ammonium acetate solutions gave simple low charge state spectra Fig. 2a–c) as anticipated for the 'native state' inhibitors. The differences in the charge state distributions between BPTI (Fig. 2a) and RcamBPTI (Fig. 2b) reflect the chemical modification of the C14–C38 disulfide bond, the concomitant change in the molecule's structure and, in particular, the rigidity of the primary (binding) and secondary loop regions. This allows other residues more buried in the BPTI structure to become protonated.

The broad peaks observed for 'native state' SBTI (Fig. 2c) and the inhibitor-trypsin complexes (Fig. 3a, Figs. 4a and 5a) at low cone voltage (+40 V) result from incomplete desolvation of these ions prior to mass analysis. Desolvation by source collisional activation via increased cone voltage (+80 V) produces sharper well defined peaks for trypsin (Fig. 1b) and SBTI (observed $M_r = 19978.5 \pm 0.7$ and a minor component with $M_r = 20091.5 \pm 1.0$, spectrum not shown), but leads to dissociation of the inhibitor-trypsin complexes (Figs. 3d and 5c).

ESI mass spectral analyses of the products formed between trypsin and BPTI, RcamBPTI and SBTI were performed at a series of increasing cone voltages to evaluate the gas phase stability of the complexes to collisional activation.

At cone voltages of +40 V and at a 1:1 molar ratio of BPTI to trypsin, a second series of ions – series B (in addition to that corresponding to the native inhibitor – series C) are detected (Fig. 3a). This corresponds to a mass/charge ratio predicted for a 1:1 complex between the trypsin and the inhibitor. This shows that the complex between trypsin and this slow-tight binding inhibitor is sufficiently stable for analysis by ESI MS, as expected from its K_i value (Table 1).

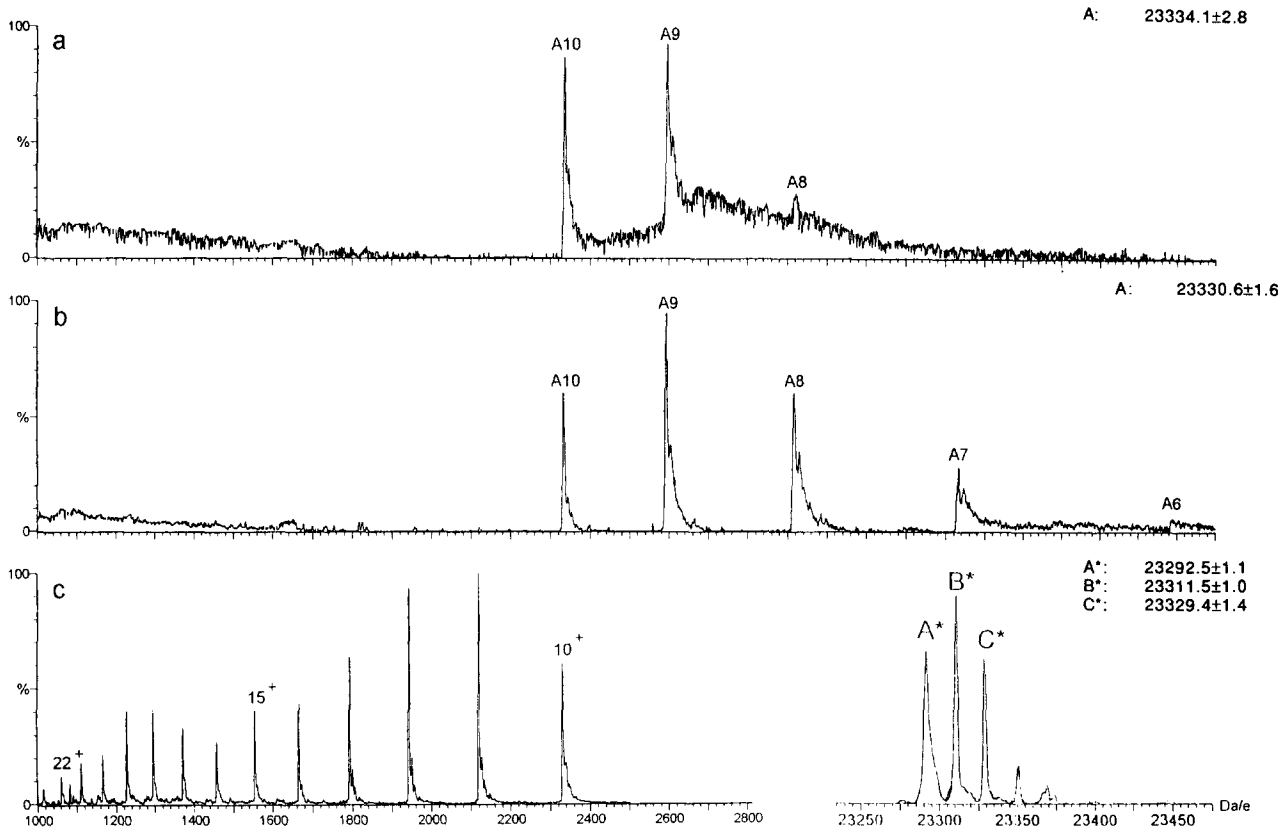

Fig. 1. ESI MS of trypsin: (a) native state, 10 pmol/μl, 2.5 mM NH_4OAc, CV = +40 V; (b) native state, 10 pmol/μl, 2.5 mM NH_4OAc, CV = 80 V (β-trypsin+Ca^{2+} = 23333.4 Da); (c) denatured state, 5 pmol/μl, H_2O/MeCN, 1:1, 0.2% HCO_2H. A = trypsin-Ca^{2+} complex; A* = β-trypsin (M_r=23293.3 Da), B* = α-trypsin (M_r = 23311.3 Da), C* = ψ-trypsin (M_r = 23329.4 Da).

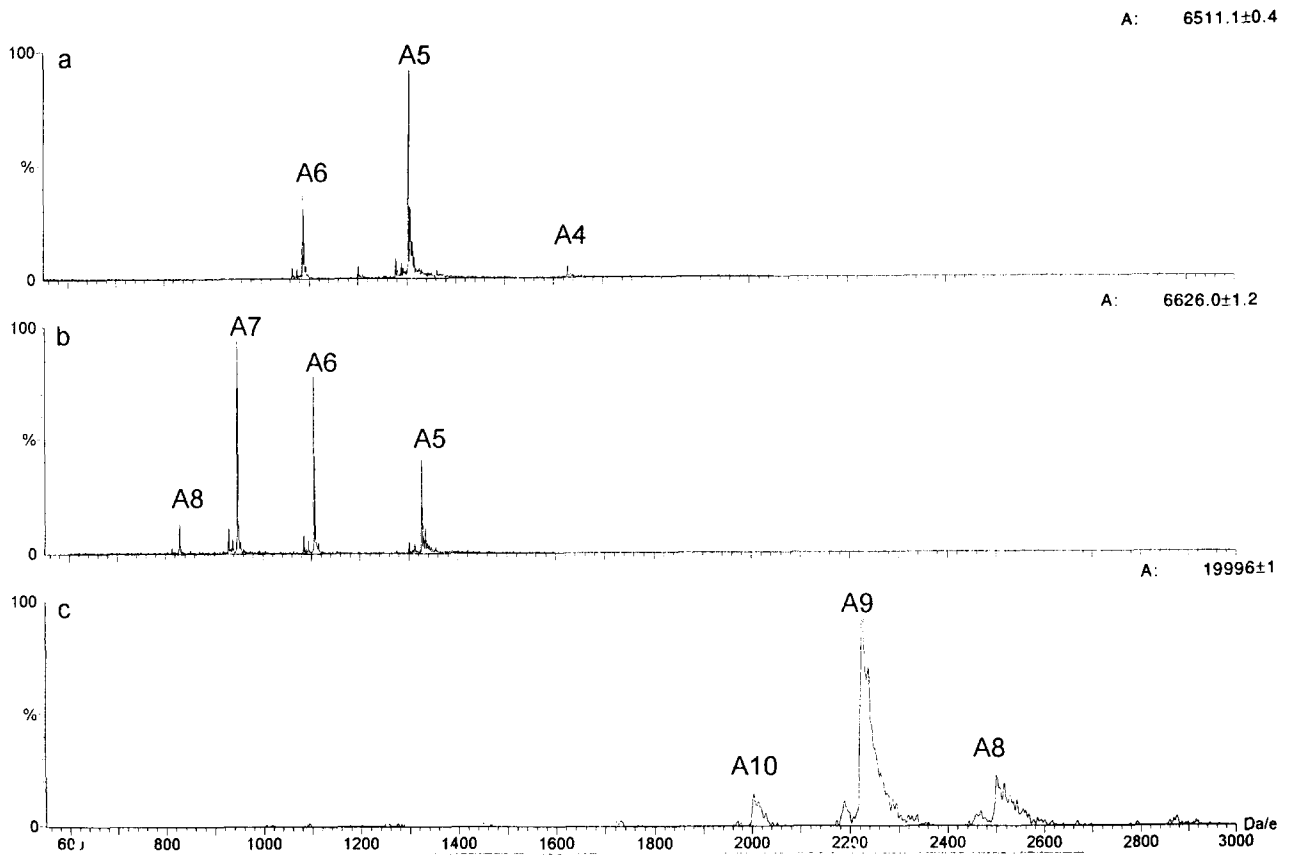

Fig. 2. ESI MS of trypsin inhibitors, 2.5 mM NH_4OAc, 10 pmol/μl: (a) BPTI (6511.5 Da), CV = +40 V; (b) RcamBPTI (6625.6 Da), CV = +40 V; (c) soybean trypsin inhibitor type I-S (19977.5 and 20090.7 Da), CV = +70 V.

As a control, the mutant K15V BPTI was preincubated with trypsin at a 1:5, 1:2 and 1:1 molar ratio. The resultant spectra (Fig. 3b; only the 1:1 molar ratio shown) show only two series of ions, one corresponding to the K15V BPTI (series C) and the other trypsin (series A). These series of ions are identical to those of the individual components run separately, showing that non-specific binding does not occur.

As the cone voltage is increased from +40 to +80 V, the spectra for the trypsin-SBTI become more clearly defined, with the peaks corresponding to the complex becoming sharper, especially for the charges states 11^+ and 12^+ (Fig. 4). It is noteworthy that, even at the highest cone voltage (+80 V), there is very little dissociation of this complex to the individual components.

For the trypsin complexes with the BPTI and RcamBPTI, the spectra are more complex. At a cone voltage of +80 V three series of ions are detected (Figs. 3d and 5c). Series B corresponds to the complex as detected at the lower cone voltage (+40 V; Figs. 3a and 5a, respectively) and series A and C correspond to the dissociated trypsin and BPTI/RcamBPTI, respectively. However, the series of ions for trypsin (series A) is significantly different from that observed for native trypsin (Fig. 1b). The series of ions has shifted to a lower charge state in which ions corresponding to 6^+, 7^+, 8^+ and 9^+ are identified with the dominant charge state being 7^+. This is clearly different from that observed for isolated trypsin in which ions corresponding to 6^+, 7^+, 8^+, 9^+ and 10^+ are identified with the dominant charge state being 9^+ and is in accord with the pH dependence of the association constant of BPTI for trypsin [17]. The shift in charge state distribution of the trypsin produced by the gas phase dissociation of the BPTI and RcamBPTI complex with trypsin suggests that at least two protonatable sites in native trypsin are masked from protonation in the trypsin-BPTI and trypsin-RcamBPTI complexes.

The significance of these results are two-fold. First, the detection of the protein-proteinase inhibitor complexes by ESI MS extends the utility of the technique to the detection of protein-protein non-covalent complexes. Where a protein exists in a variety of isoforms, it may be possible to confirm to which isoform(s) an inhibitor binds. In the case of trypsin, the results presented here suggest that all three isoforms bind to the inhibitor.

Secondly, the shift in charge state is also very significant as

Table 1
K_i values of trypsin-inhibitor complexes

Inhibitor	BPTI [10]	RcamBPTI [9]	SBTI [17,18]	K15V BPTI [14]
K_i (M)	6.0×10^{-14}	1.3×10^{-9}	1×10^{-11}	does not inhibit

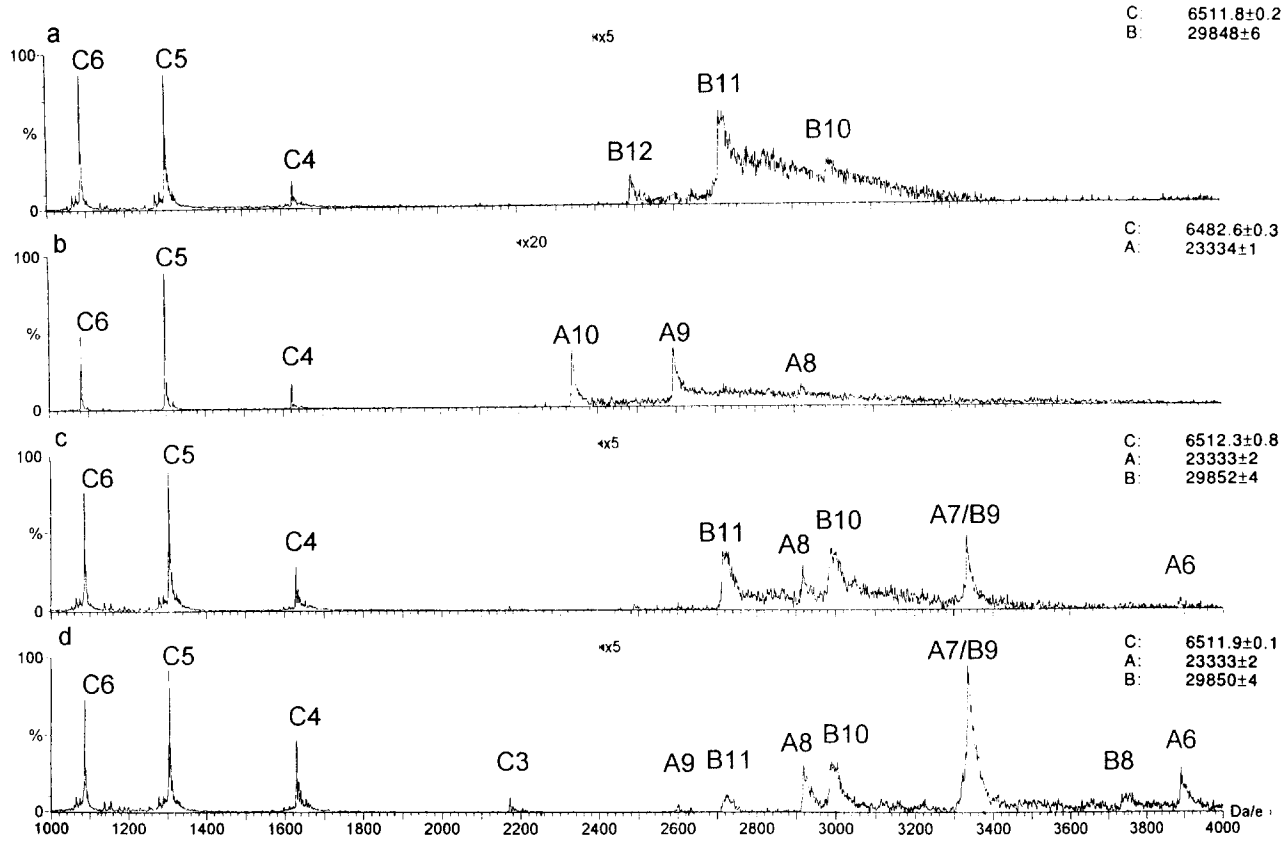

Fig. 3. ESI MS of trypsin plus BPTI and K15V BPTI (6482.5 Da), 2.5 mM NH_4OAc, 1:1, 10 pmol/μl: (a) trypsin+BPTI, CV = +40 V; (b) trypsin+K15V BPTI, CV = +40 V; (c) trypsin+BPTI, CV = +60 V; (d) trypsin+BPTI, CV = +80 V. A = trypsin-Ca^{2+}; B = trypsin-Ca^{2+}-BPTI complex; C = free inhibitor.

t gives an insight into the nature of the interaction between the contact surfaces of the two proteins in the complex. The nature of this interaction is reflected in the charge state distribution of the gas phase dissociated proteins, i.e. the BPTI leaves a 'footprint' on the trypsin. Data from the crystal structure of the trypsin-BPTI complex and other proteinase-pro-

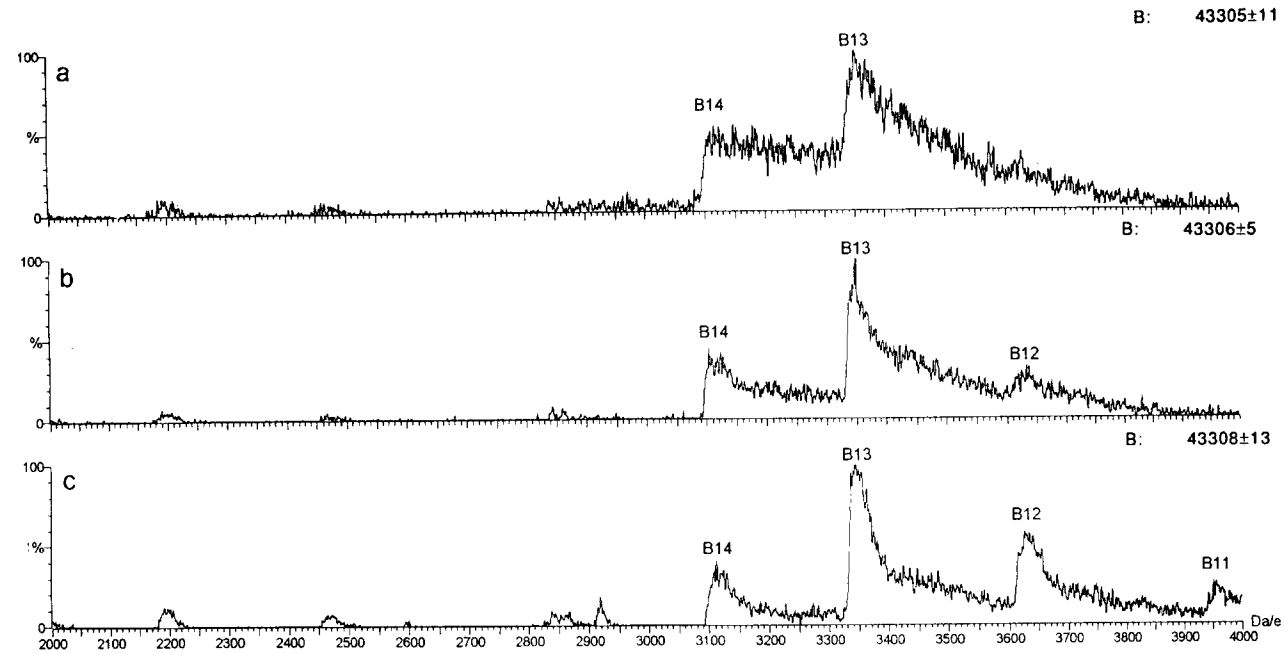

Fig. 4. ESI MS of trypsin plus soybean trypsin inhibitor type I-S, 2.5 mM NH_4OAc, 1:1, 10 pmol/μl: (a) CV = +40 V; (b) CV = +60 V; (c) CV = +80 V. B = trypsin-Ca^{2+}-soybean inhibitor complex.

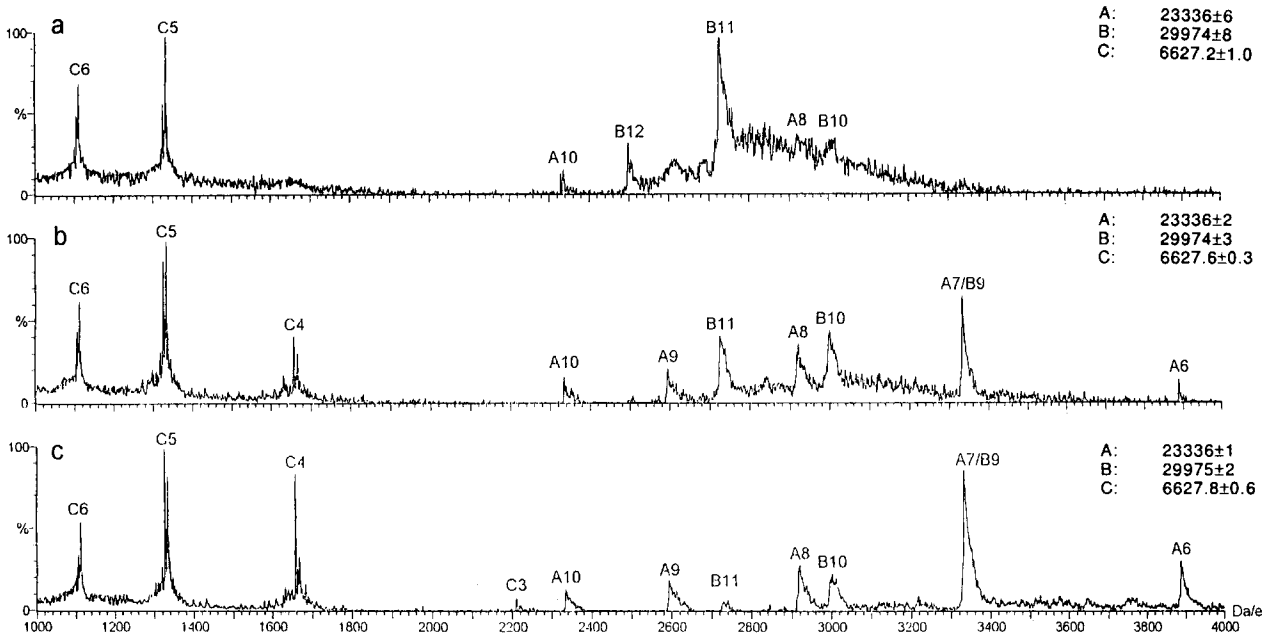

Fig. 5. ESI MS of trypsin plus RcamBPTI, 2.5 mM NH_4OAc, 1:1, 10 pmol/μl: (a) CV = +40 V; (b) CV = +60 V; (c) CV = +80 V. A = trypsin-Ca^{2+}; B = trypsin-Ca^{2+}-RcamBPTI complex; C = RcamBPTI.

tein proteinase inhibitor complexes suggest that 10–15 residues of the inhibitor form close contacts with 17–29 residues of the proteinase. This results in 600–950 Å^2 of the surface of each protein being buried [19]. For the trypsin-BPTI complex, 11 residues in BPTI and 19 residues in trypsin are buried in the complex. Changes in the relative charge state abundance are observed for BPTI produced by gas phase dissociation (Fig. 3d) compared to that of native BPTI (Fig. 2a). RcamBPTI shows a major shift to $5^+/4^+$ (Fig. 5c) from $7^+/6^+$ (Fig. 2b) when formed by gas phase dissociation of its trypsin complex.

The change in the charge state distribution observed for trypsin when produced by gas phase dissociation of the inhibitor complexes (Figs. 3d and 5c) may reflect a masking of some or all of the basic residues K176, K206 and K208 which are in close contact with BPTI and RcamBPTI in the complexes but are available for protonation in trypsin. Changes in the charge state distribution for BPTI and RcamBPTI when formed by gas phase dissociation suggest that some of the three residues (K15, R17 and R19), which are in close contact with trypsin in the complexes, are involved.

Comparision of the cone voltages at which BPTI and RcamBPTI dissociate from their complexes with trypsin (Figs. 3 and 5) suggests that ESI MS might be a useful way to order the strength of the binding interaction between closely related complexes, but most significantly ESI MS is a powerful probe for studying the ionic interactions that occur at the contact surface between proteins.

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