Studies on the Inhibition of Porcine Pancreatic Elastase using Electrospray Mass Spectrometry

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Electrospray mass spectrometry has been used to investigate the mode of inhibition of porcine pancreatic elastase by chloromethyl ketones and other inhibitors.

Elastases are serine proteases which have been implicated in various degenerative diseases such as rheumatoid arthritis,[1] pancreatitis,[2] emphysema[3] and other inflammatory diseases. As a result of their potentially destructive role in human systems extensive research has been carried out in a search for potent and specific inhibitors.

Previously we have demonstrated the potential of electrospray mass spectrometry (ESMS) for the analysis of intermediates in enzyme catalysed reactions.[4,5] ESMS has also been used to observe mass shifts consistent with the formation of *O*-acyl intermediates for chymotrypsin and subtilisin.[6] Herein, we report the results of our investigations using ESMS into the inhibition of porcine pancreatic elastase (PPE) with several known inhibitors. Three irreversible inhibitors, representing different structural types (chloromethyl ketones, isocoumarins and sulfonyl fluorides) were chosen for study. Incubation of these inhibitors with PPE allowed the observation of both enzyme-inhibitor intermediates and products, providing support and clarification for the proposed modes of action.

The positive ion ESMS mass transformed spectrum [Fig. 1(*a*)] of the PPE used in these studies indicated the presence of two components with molecular masses corresponding to native PPE, component B (observed M_r = 25 898.1 ± 1.5; calculated M_r = 25 898.1) and approximately 30% of a species, component A (observed M_r = 25 784.8 ± 2; calculated M_r = 25 784.1), corresponding to PPE without the *C*-terminal asparagine residue.† In the inhibition studies described subsequently, analogous mass shifts were observed corresponding to binding to both of these species.‡

Chloromethyl ketones are effective inhibitors of serine proteases and proposed modes of inactivation may be represented as in Scheme 1. ESMS studies of PPE incubated

† Electrospray mass spectra were measured on a VG BIO Q triple quadrupole atmospheric pressure mass spectrometer equipped with an electrospray interface. PPEI was purchased from Serva Feinbiochemica, Heidelberg/New York. Samples (10 μl) were injected into the electrospray source *via* a loop injector as a solution typically 20 pmol μl^{-1} in water : MeCN (1 : 1) at a flow rate of 2 μl min^{-1}. For experiments involving incubation of PPE with an inhibitor, sample solutions were prepared immediately prior to analysis by mixing in Milli-Q water (pH 7). The ratio of enzyme : inhibitor that resulted in the optimum spectra was found to vary with the inhibitor used. Typical values for this ratio were between 1 : 1 and 1 : 3. Aliquots were removed from the stock solution, mixed with an equal volume of MeCN and immediately analysed by positive ion ESMS using cone voltages of 50–80 V. The mass spectrometer was scanned over a mass range 1100 ± 450. The instrument was calibrated with horse heart myoglobin (20 pmol μl^{-1}, mass = 16 951.5).

‡ In general, the observed mass shifts on the major PPE species (component B, Fig. 1) were closer to the calculated values. The partial loss of a *C*-terminal Asn was confirmed by *C*-terminal sequencing.

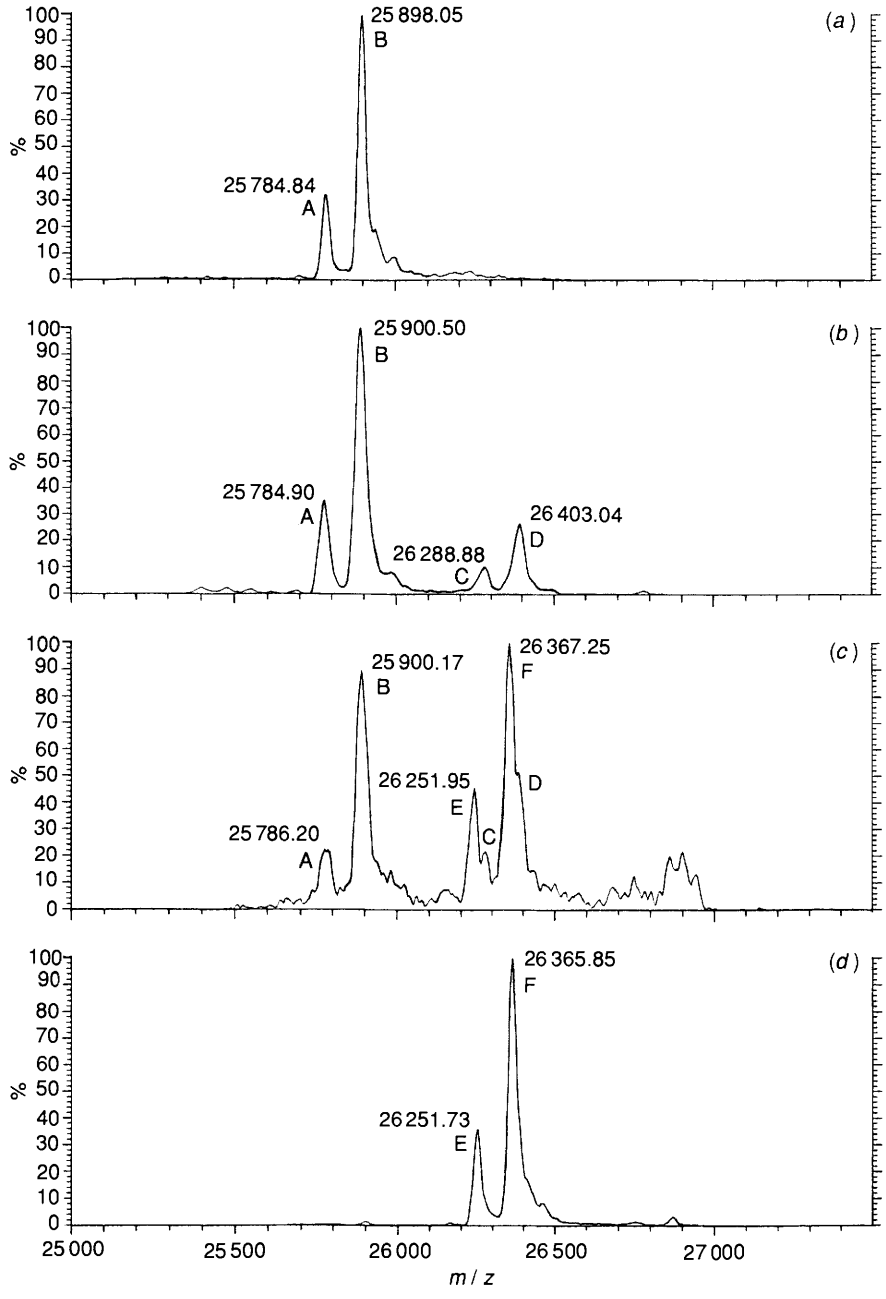
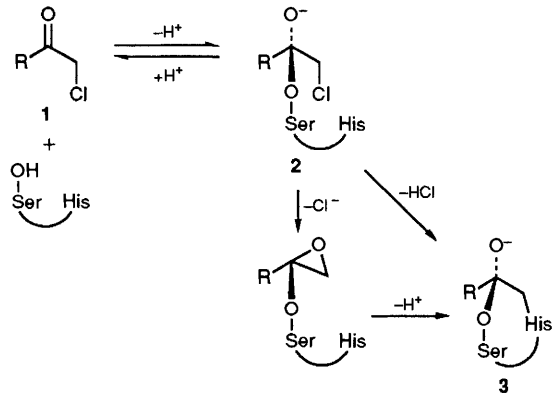


Fig. 1 Mass transformed ESMS data for the inhibition of PPE by chloromethyl ketone **1** (PPE : **1**, 1 : 3);† (*a*) mass transformed spectrum of uninhibited PPE; (*b*) incubation time $t < 1$ min; (*c*) $t = 1.5$ min; (*d*) $t = 20$ min. M_r values recorded on figure were accurate to $\leqslant \pm 2$. Mass shifts for (*b*): C − A = 504.0 ± 2, D − B = 502.5 ± 2; for (*c*): C − A = 504.0 ± 2, E − A = 465.8 ± 2, D − B = 498.7 ± 2, F − B = 467.1 ± 2. Calculated mass shifts for formation of hemiketal **2** = 502.5; for alkylated enzyme **3** = 466.0.

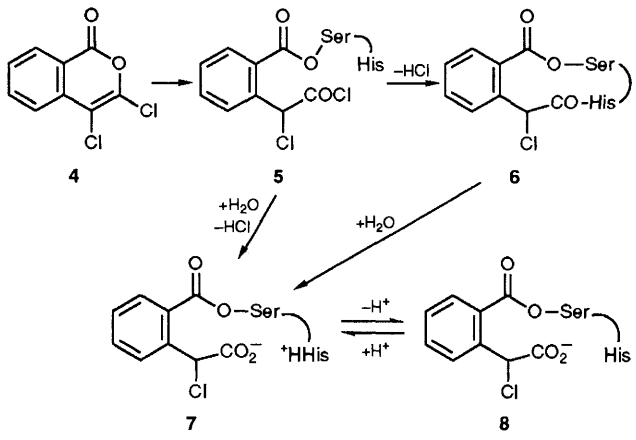
with the chloromethyl ketone inhibitor, MeOSuc-L-Ala-L-Ala-L-Pro-L-ValCH_2Cl **1**,[7,8] gave mass shifts consistent with the formation of both the hemiketal **2** [C and D, Figs. 1(*b*) and (*c*)] and the alkylated species **3** [E and F, Figs. 1(*c*) and (*d*)]. Furthermore the intensity of the peaks (E and F) corresponding to **3** increased with the incubation time.

The proposed mechanisms of inhibition of PPE by 3,4-dichloroisocoumarin **4**, the second inhibitor studied, are summarised in Scheme 2.[9] Previous studies were unable to distinguish between **6**, **7** and **8** as the structure(s) of the inhibited enzyme. ESMS studies on the inhibition of PPE by **4** were in accord with the formation of **7** and/or **8**. Typically, a mass shift of 196.5 ± 1 was observed on the major PPE species [component B, Fig. 1(*a*)] following incubation of PPE with **4** (calculated mass shift for formation of **7** = 196.6; of **8** = 195.6). Despite varying both the concentration of the enzyme and inhibitor and the incubation time, we were unable to detect the species **6**. When a solution of PPE and **4** was left to stand for 24 h an increase in the proportion of native PPE was observed, consistent with previous kinetic results.[9]

ESMS studies, at a cone voltage of 50 V, on the inhibition of PPE using phenylmethylsulfonyl fluoride (PMSF)[10] revealed mass shifts corresponding to sulfonation of the enzyme [observed mass shift = 154.8 ± 2 on the major PPE species {component B, Fig. 1(*a*)}; calculated mass shift = 154.2] and a further species consistent with formation of a phenylmethylsulfonic acid salt of the sulfonated enzyme [obs. mass shift = 329.3 ± 3 on the major PPE species {component B, Fig. 1(*a*)}; calc. mass shift = 326.4].[11] When increased ratios (>5 : 1) of PMSF to PPE were employed, a series of peaks corresponding to the formation of multiple salt adducts were observed in the ESMS spectrum. As precedented,[12] an increase in the cone voltage resulted in a decrease in the relative intensities of the proposed salt adducts, until at 70 V they were not observed.



Scheme 1 Proposed modes of inactivation of serine proteases by peptide chloromethyl ketones[7,8]



Scheme 2 Possible intermediates in the mechanism of inhibition of serine proteases by 3,4-dichloroisocoumarin **4**

When PPE was incubated with PMSF for more than 30 min, formation of greater than 90% of the sulfonated enzyme was observed. Control experiments were then conducted (at a cone voltage of 50 V) in which this material was further reacted with the chloromethyl ketone inhibitor **1**. An additional set of very weak peaks was observed in the ESMS spectrum (mass shift *ca.* 502). These peaks were absent when the experiment was repeated at higher cone voltages (70–80 V) and may represent non-specific bonding of an intact molecule of the chloromethyl ketone **1** to the PMSF treated enzyme. In contrast the inhibited species **3** produced by the reaction of the chloromethyl ketone **1** with untreated PPE were stable to increased cone voltages, indicating that catalytically active PPE was required for the observation of the mass shifts corresponding to the hemiketal **2** and the alkylated enzyme **3**.

In conclusion, ESMS[13] has proved to be an excellent technique for the study of the interaction of PPE with a range of inhibitors. The results have provided confirmation and clarification of proposed mechanisms of inhibition, providing evidence consistent with formation of several intermediates, including a hemiketal species.

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