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Identification of aprotinin degradation products by the use of high-performance capillary electrophoresis, high-pressure liquid chromatography and mass spectrometry

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ABSTRACT

A preparation of bovine aprotinin, bovine pancreatic trypsin inhibitor, was subjected to high-performance capillary electrophoresis (HPCE) analysis and the purity was calculated to be approximately 80%. The two dominating contaminants were integrated to approximately 7% each as compared to the intact molecule. Characterization by high-pressure liquid chromatographic (HPLC) and mass spectrometric analysis was carried out on digests of the reduced and alkylated molecules. The contaminants were identified as truncated aprotinin, missing one and two amino acids, respectively, at the C-terminus. No such structures were identified in similar amounts in preparations of recombinant aprotinin by HPLC or HPCE.

INTRODUCTION

Aprotinin consists of 58 amino acids in a single polypeptide chain, which is cross-linked by three disulphide bridges[1] (Fig. 1). The molecular mass is 6512 and the isoelectric point close to 10.5. A very compact tertiary structure makes the molecule extremely stable against physical and chemical stress and proteolytic degradation. Aprotinin has a relatively broad inhibitory specificity[1], and has found use as a therapeutic agent based on its inhibition of serine proteases, *e.g.*, in the treatment of acute pancreatitis. It strongly inhibits trypsin as well as chymotrypsin, plasmin and kallikreins of different origin.

Since high-performance capillary electrophoresis (HPCE) was introduced by Mikkers *et al.*[2] and Jorgenson and Lukacs[3] as an instrumental approach to

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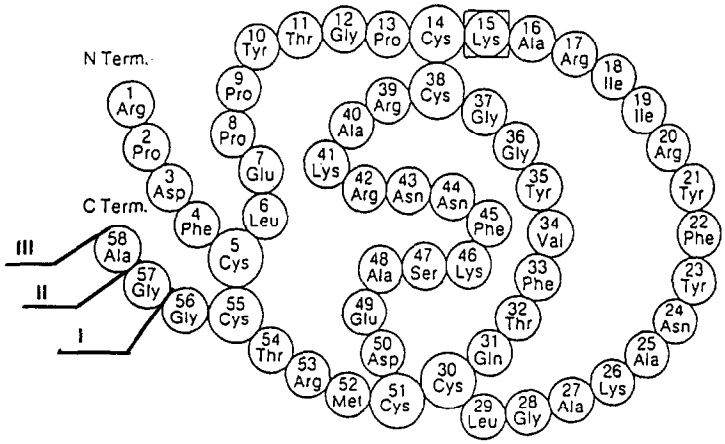

Fig. 1. Primary structure of aprotinin, bovine pancreatic trypsin inhibitor. The inhibitor consists of a single polypeptide chain of 58 amino acids cross-linked by three disulphide bridges. The molecular mass is 6512 and the isoelectric point is close to 10.5.

electrophoresis, the method has shown great promise for the high-resolution separation of charged species. Among the areas of special interest is the characterization of peptides and proteins[4–9], *e.g.*, for purity control.

Polypeptides and proteins may adsorb to the fused-silica capillary column due to electrostatic interactions between the peptide and the negatively charged surface of the capillary. Dependent on the techniques available and the actual peptide preparations of interest, these interactions can be minimized/eliminated in four different ways. First, the peptide is repelled from the negatively charged capillary surface, when electrophoresis is performed at higher pH values than the isoelectric point of the peptide, due to the negative net charge of the peptide[10,11]. Second, physical coating of the capillary wall, by *e.g.*, polyacrylamide[12] or via silane derivatization[13] deactivates the capillary surface. Third, dynamic coating by the addition of ionic species to the buffer solution[14,15] results in a competition between the peptide and the cationic species for the adsorption sites on the capillary surface. Finally, lowering the pH of the running buffer below the isoelectric point of the peptide results in a positively charged ion, but at the same time some of the negative charge will be titrated off the capillary wall, hence lowering the coulombic interactions between the capillary surface and the peptide[11,13]. Due to the high isoelectric point of aprotinin and its high stability at low pH[1], we chose the latter approach by performing the electrophoresis at a pH value of 2.5, where most of the negative charge is titrated off the fused-silica capillary wall[11].

HPCE has a very high resolving power, but contaminants/degradation products are not identified directly with this technique. The standard analytical approach for characterization of the contaminants is fraction collection and further analysis. However, at present the amount of material that can be collected in HPCE is not sufficient for further analysis (*e.g.*, amino acid sequencing). Direct coupling of HPCE and another analytical method, *e.g.*, mass spectrometry[16], is one way to circumvent this problem. Without direct coupling, characterization of the contaminants might be accomplished either by subjecting the samples to enzymatic or chemical treatment

prior to analysis, or by spiking with known structures. In order to identify the two dominating contaminants, bovine aprotinin preparations were spiked with fractions collected from reversed-phase high-performance liquid chromatography (HPLC) of bovine aprotinin and identified by plasma desorption mass spectrometry (PD-MS).

EXPERIMENTAL

Materials

Bovine and recombinant aprotinin were from Novo-Nordisk (Bagsværd, Denmark). *Armillaria mellea* protease was a gift from Lars Thim, Novo-Nordisk, and had been purified according to published procedures[17]. Ammonium sulphate, dithiothreitol (DTT), hydrochloric acid and ammoniumhydrogencarbonate were from Merck (Darmstadt, F.R.G.); acetonitrile was from Rathburn (Walkerburn, U.K.); trifluoroacetic acid (TFA), sequenal grade, was purchased from Pierce (Oud-Beijerland, The Netherlands); 4-vinylpyridine and Tris (Trizma Base) were from Sigma (St. Louis, MO, U.S.A.); guanidinium hydrochloride was from Serva (Heidelberg, F.R.G.); ethanol was from Danisco (Aalborg, Denmark) and citrate buffer for HPCE analysis was from Applied Biosystems (Foster City, CA, U.S.A.).

Methods

HPLC. An analytical Vydac C4 column (code 214TP54, The Separations Group, Hesperia, CA, U.S.A.) was used throughout at 1.5 ml/min with 0.1% TFA and 2% (w/w) ammonium sulphate as eluent A and 0.07% TFA in acetonitrile as eluent B. For HPLC of native aprotinin a linear gradient was run from 5–25% B in 20 min with detection at 220 nm. Three fractions of material were collected and desalted by use of the same system except that ammonium sulphate was omitted. For HPLC of pyridylethylated material a linear gradient was run from 5–45% B in 20 min with detection at 254 nm. For peptide mapping an isocratic elution with eluent A for 5 min was followed by elution with a linear gradient from 0–25% B in 25 min and detection at 214 nm.

Vinylpyridylethylation. Salt-free samples of approximately 50 nmol aprotinin were concentrated to nearly dryness in a vacuum centrifuge and 120 μl of water, 40 μl of 2 *M* Tris–HCl pH 7.5, 100 mg guanidinium hydrochloride and 2.2 mg of DTT were added. Reduction was carried out under nitrogen at 37°C for 2 h. Alkylation with 6 μl of 4-vinylpyridine was then performed at room temperature in the dark. The reaction was stopped after 30 min with the addition of 5 mg solid DTT. The pyridylethyl derivatives were purified by HPLC.

Proteolytic digestion. The HPLC-purified pyridylethyl derivatives were concentrated in a vacuum centrifuge and 400 μl of 0.1 *M* ammoniomhydrogencarbonate pH 7.9 was added. Half of the material (approximately 25 nmol) was incubated at 37°C for 4 h together with 1 nmol *Armillaria mellea* protease.

PD-MS. Fractions from HPLC containing peptides of the proteolytic digestions were dried by vacuum centrifugation and redissolved in 200 μl 0.1% TFA. Aliquots were mixed with an equal volume of ethanol and 5 μl samples, approximately 100 pmol peptide, were applied nitrocellulose coated targets (BIO-ION Nordic, Uppsala, Sweden) for mass analysis[18]. PD-MS analysis was carried out in positive mode on a ^{252}Californium time of flight mass spectrometer (BIO-ION Nordic). PD-MS spectra were collected for 1 million fission events.

HPCE. Analysis was performed on Applied Biosystems Model 270A analytical capillary electrophoresis system. The fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A.) had an internal diameter of 50 μm, with a total length of 100 cm, and a distance of 75 cm to the detector. By applying a vacuum at the detector end of the capillary tube for 1 s, samples were introduced. The samples were diluted with destilled water to a concentration of 0.5 mg/ml. A 20 m*M* citrate buffer (pH 2.5) was used as running buffer. The applied potential was 20 kV and the temperature 27°C. The electropherograms were obtained at 200 nm with a risetime of 1.0 s.

RESULTS AND DISCUSSION

Fig. 2a and b shows the electropherograms of bovine and recombinant aprotinin, respectively. While the dominating peak could be integrated to more than 99% of the total area in the electropherogram of the recombinant aprotinin, the electropherogram of the bovine aprotinin revealed several contaminants/degradation products, and the full-length molecule, 1–58 (III, Fig. 2a) was integrated to approximately 80% of the total area. The two dominating contaminants (I and II, Fig. 2a) eluted non-resolved in front of the main peak. An area of 5–9% could be calculated for each of the peaks I and II. Areas were normalized to migration time to account for variable rates of movement through the detector. None of these two contaminants were present in similar amounts in the electropherogram of the recombinant aprotinin (Fig. 2b).

The running buffer had a pH value of 2.5. Electrophoresis at higher pH values, but below the isoelectric point of aprotinin, resulted in adsorption of the aprotinin

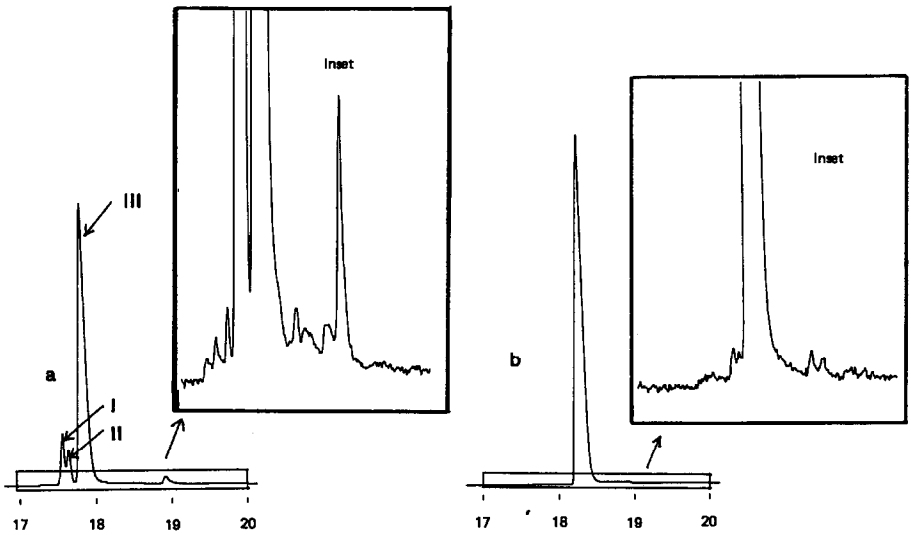

Fig. 2. HPCE electropherograms, obtained at 200 nm, of bovine and recombinant aprotinin. Samples were introduced by applying a vacuum for 1 s. A citrate buffer (pH 2.5) was used as running buffer. The field strength was 200 V/cm and the temperature 27°C. (a) Bovine aprotinin. I–III corresponds to HPLC fractions I–III (Fig. 3). (b) Recombinant aprotinin. All retention times are in minutes. The insets display the electropherograms at higher sensitivity for 17–20 min as indicated by the boxes in the figures.

molecules to the capillary wall. Hence, the results in Fig. 2 show that the charge density of the capillary surface is a very important factor in performing electrophoresis below the isoelectric point of the highly alkaline aprotinin peptide. At very low pH values, most of the negative surface charge is titrated off the fused-silica capillary wall, and the coulombic interactions between the positively charged aprotinin molecules and the capillary surface are minimized, thus avoiding aprotinin adsorption.

Analysis of bovine aprotinin by reversed-phase HPLC showed major resemblance with the corresponding HPCE electropherogram as shown in Fig. 3. Again the main peak was integrated to approximately 80% and preceded by the two dominating contaminants, which also in this analysis eluted as non-resolved peaks. Fractions I–III were collected for further analysis.

HPLC fractions I and II analyzed by HPCE indicated that they were heterogenous by being contaminated with material from at least one of the other fractions (Fig. 4b and c). Spiking analysis demonstrated that fractions I and II were detected in the same order in the HPCE analysis as they were eluted from the HPLC column (Fig. 4d and e). The reason for the relative differences in peak height of fractions I and II in Figs. 2a and 3 might be due to the better resolution of the two fractions in HPCE compared to HPLC. In the HPLC chromatogram (Fig. 3), the number of theoretical plates seems to be considerably lower for fraction I compared to fraction II. Hence, due to tailing of fraction I, the HPLC-collected fraction II is more contaminated with fraction I (Fig. 4c), than the opposite (Fig. 4b). The close elution of the material in the three fractions by HPLC and HPCE, two separation principles with high resolving power, indicates that the material is closely related.

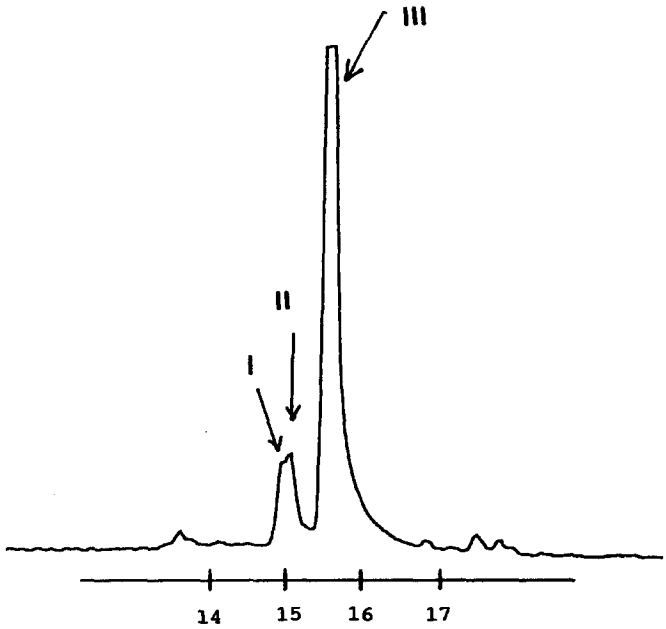

Fig. 3. Reversed-phase HPLC of bovine aprotinin. A linear gradient was run from 5–25% B in 20 min with detection at 220 nm. Eluent A: 0.1% TFA and 2% (w/w) ammonium sulphate, eluent B: 0.07% TFA in acetonitrile. Fractions I–III were collected for further analysis. The *x*-axis scaling is in minutes.

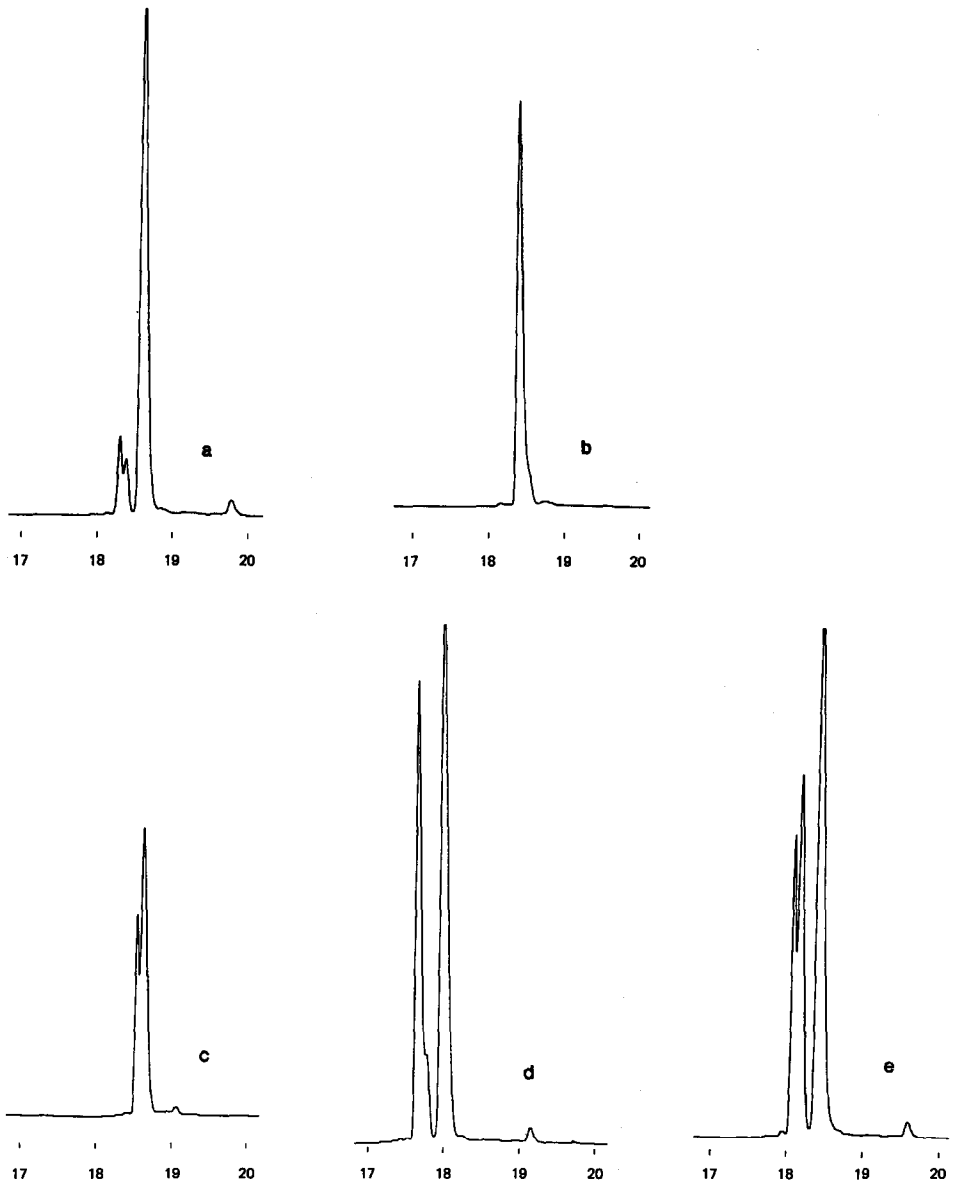

Fig. 4. Electropherograms of HPCE spiking analysis of fractions I and II. The experimental conditions are described in the legend to Fig. 2. a = Bovine aprotinin (1–58), b = fraction I (1–56), c = fraction II (1–57), d = bovine aprotinin spiked with fraction I, e = bovine aprotinin spiked with fraction II. All retention times are in minutes.

Equal amounts of the bovine material, collected as fractions I–III, were reduced and alkylated wih 4-vinylpyridine. The derivatives were separated from reagent surplus by preparative reversed-phase HPLC.

Aliquots of the derivatives were digested with *Armillaria mellea* protease which cleaves selectively at the N-terminal side of the lysine residues. Reversed-phase HPLC of the digested, pyridylethylated material, originating from fractions I–III, gave

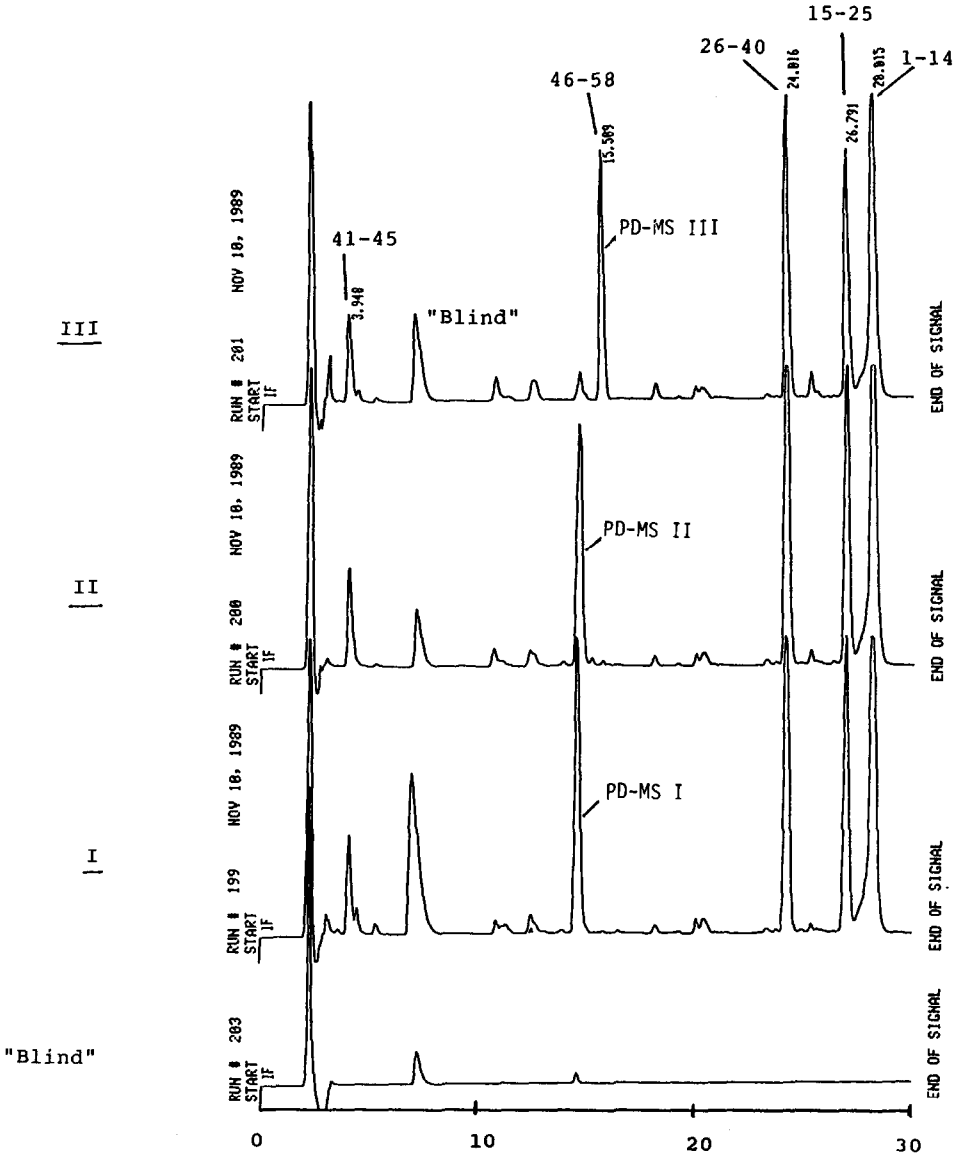

Fig. 5. Elution pattern from HPLC of pyridylethylated bovine aprotinin preparations I–III (Fig. 3) digested with *Armillaria mellea* protease. A "blind"-digest is shown for reference. An isocratic elution with eluent A for 5 min preceded elution with a linear gradient from 0–25% B in 25 min and detection at 214 nm. The eluents are described in the legend to Fig. 3. Fractions were collected for mass analysis (PD-MS) as indicated. The five peptides obtained after digestion of the bovine aprotinin are identified in chromatogram III by their amino acid residue numbers in the intact molecule. The four chromatograms are shown with the same *x*-axis scaling shown at the bottom line of the figure. All retention times are in minutes.

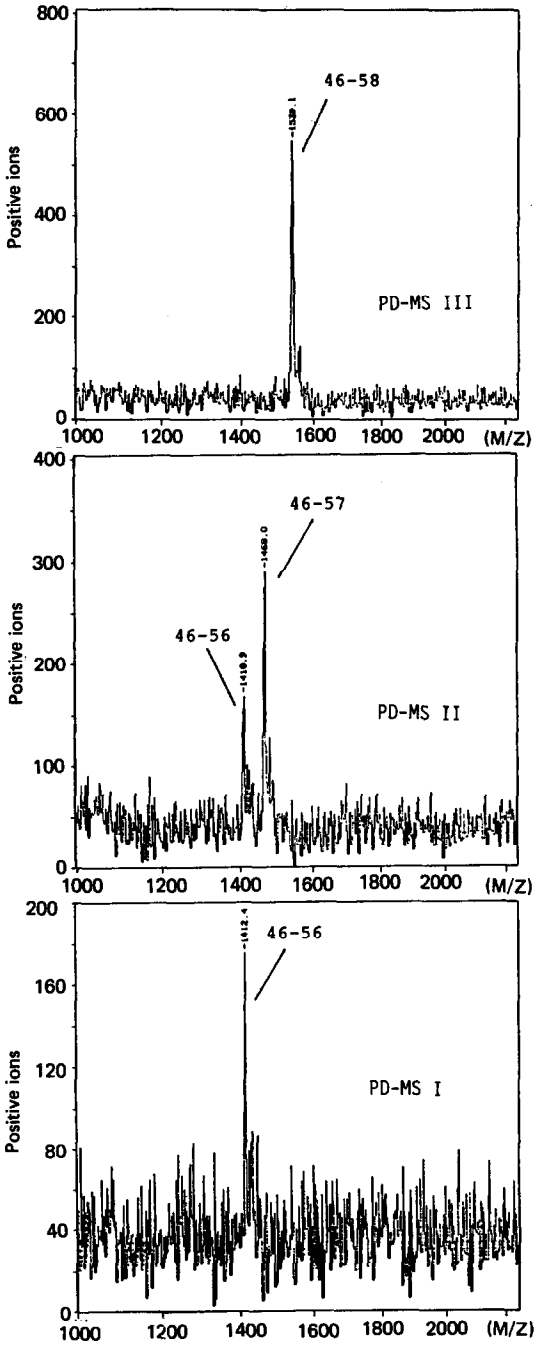

Fig. 6. PD-MS spectra of HPLC-purified carboxy-terminal fragments of pyridylethylated bovine aprotinin preparations I–III after digestion with *Armillaria mellea* protease. PD-MS spectra were collected for 1 million fission events. Fragments: Lys_{46}–Gly_{56}: 1410.7; Lys_{46}–Gly_{57}: 1456.7; Lys_{46}–Ala_{58}: 1538.8 dalton.

almost identical elution patterns (Fig. 5). This strongly indicates that the primary structure of the material collected in fractions I and II is very similar to the primary structure of full-length aprotinin (fraction III).

Primary sequence has unambigously been assigned to the peaks in the corresponding HPLC chromatogram derived from the full-length aprotinin (fraction III). This was obtained by PD-MS analysis and amino acid sequencing. The corresponding chromatogram has been labeled accordingly (Fig. 5). No peak with a retention time corresponding to the C-terminal fragment Lys_{46}–Ala_{58} was identified in the HPLC chromatograms derived from fractions I and II, but in both cases an earlier eluting peak of equal size was observed and the material collected (Fig. 5, PD-MS I and II). This peak was absent in the chromatogram derived from the digest of the derivatized full-length molecule. In addition the fraction containing the C-terminal fragment Lys_{46}–Ala_{58} was collected (Fig. 5, PD-MS III).

PD-MS was used to assess the molecular masses of the fragments collected in fractions PD-MS I–III. Molecular ions, MH^+, were assigned to C-terminal fragments for all three fractions (Fig. 6). As expected the intact C-terminal fragment, Lys_{46}–Ala_{58}, was assigned to the major molecular ion obtained for fraction PD-MS III. The two major molecular ions in the mass spectra of fractions PD-MS II and PD-MS I had lower molecular masses and were assigned to truncated C-terminal fragments of aprotinin missing one (Ala_{58}) and two (Ala_{58}, Gly_{57}) amino acids, respectively. It is observed, that the relative amounts of the two molecular ions in the two fractions (PD-MS I and PD-MS II) obtained in Fig. 6 are in accordance with the results obtained by HPCE (Fig. 4b and c).

It is most likely that the degradation products [des-Ala(58)-aprotinin and des-Ala-Gly(58,57)-aprotinin] detected in the electropherogram (Fig. 2a) and the chromatogram (Fig. 3) of bovine aprotinin are due to the presence of proteolytic enzymes during purification procedures, *e.g.*, carboxypeptidases.

The resolution of the two truncated aprotinin molecules in these particular HPCE and HPLC systems was better in HPCE (Fig. 2a) than in HPLC (Fig. 3), though baseline separation was not obtained. Hence, HPCE can be used as an alternative to reversed-phase HPLC as a standard analytical procedure for aprotinin purity control.

The results as discussed above show how spiking of the bovine aprotinin starting material with species of known sequence, identified by the combined use of HPLC and PD-MS, can be accomplished as a method for the identification of some of the bovine aprotinin contaminants/degradation products.

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