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Characterization of the cleavage specificity of a subtilisin-like serine proteinase from *Ophiostoma piceae* by liquid chromatography/mass spectrometry and tandem MS

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Abstract A proteinase secreted by the sapstaining fungus *Ophiostoma piceae* is thought to be necessary for the primary retrieval of nitrogen from wood proteins. By using mass spectrometry (MS) techniques, we have established the cleavage specificity of this subtilisin-like serine proteinase. This work demonstrated the potential of MS in determining cleavage specificities of newly isolated proteinases in a relatively short time frame, and determined that the *O. piceae* proteinase showed a substrate specificity similar to that of proteinase K. Primary cleavage of the insulin B-chain occurred between Leu[15] and Tyr[16]. In addition numerous secondary cleavage sites occurred after hydrophobic, polar, and charged amino acids indicating a broad specificity.

Key words: Electrospray ionization mass spectrometry; Tandem mass spectrometry; Subtilisins; Serine proteinases; Sapstain; *Ophiostoma*

1. Introduction

Ophiostoma piceae (Münch) H. and P. Sydow is a fungus which causes wood discoloration, thereby reducing the value of lumber. A proteinase was detected and isolated from *O. piceae* during growth on wood and in protein-supplemented liquid culture. It was classified as a subtilisin class II enzyme on the basis of sequence homology at the N-terminus [1]. This enzyme is important in the physiology of *O. piceae* since it appears to be directly implicated in the breakdown of proteins as a means of obtaining nitrogen for growth [2]. However, the cleavage specificity of this proteinase has not been investigated. This information would be essential for designing specific inhibitors and for ascertaining the potential usefulness of the proteinase for commercial applications.

The oxidized B-chain of insulin has served for 30 years as the substrate of choice for the initial screening of the specificity of a newly discovered proteinase [3]. Much of the previous published work to determine points of cleavage in oxidized insulin B-chain has used high pressure liquid chromatography (HPLC), paper chromatography or thin layer chromatography separation of peptides after digestion, followed by amino acid analysis and/or N-terminal sequencing of the separated peptides. These procedures are both time-consuming and laborious. In this paper, peptides generated by digestion of insulin with the proteinase from *O. piceae* were separated by HPLC coupled to an on-line electrospray ionization mass spectrometer (ESI MS). The cleavage sites were determined by matching the peptide masses with the theoretical cleavage of insulin, and by sequencing the peptides using tandem MS. This approach offers a rapid alternative to conventional techniques used to determine the specificity of a proteinase on insulin.

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2. Materials and methods

2.1. Proteinase preparation and purification

The proteinase used for this study was purified from the sap-staining fungus *O. piceae* strain 387N. *O. piceae* was grown on protein-supplemented medium and the extracellular proteinase was purified to homogeneity by hydrophobic interaction chromatography [1]. The homogeneity was verified using the same LC/MS conditions described for the separation of the insulin B-chain proteinase digest (sections 2.2.1 and 2.2.2). The purified proteinase was stored at −20°C in 0.1 M Tris-Cl (pH 8) buffer containing 1 M ammonium sulfate.

2.2. Insulin B-chain digestion

An aliquot (100 μl) of bovine insulin B-chain (Sigma Chemical Co., St. Louis, MO, USA) prepared at 1 mg/ml in 0.1 M Tris-Cl, pH 8, containing 2 mM $CaCl_2$, and 2 μg *O. piceae* proteinase were incubated at room temperature. Substrate cleavage sites were determined after stopping the reaction at 2 min, 5 min, 1 h and 24 h.

2.2.1. HPLC conditions. At the specified times, a 5 μl aliquot was removed from the reaction mixture and diluted 10 times with HPLC solvent A (pH 2.2) to stop the reaction. Peptides in an aliquot (20 μl) were separated on an Ultrafast Microprotein Analyzer (Michrom BioResources Inc., Auburn, CA, USA) by reverse phase HPLC on a 1 × 150 mm Reliasil C-18 column (5 μm, 300 Å). Chromatography solvents were 2% acetonitrile (MeCN), 0.05% triflouroacetic acid (TFA) (solvent A) and 80% MeCN, 0.045% TFA (solvent B). The column was developed with a linear gradient from 5% to 50% solvent B in 30 min, followed by 50% to 80% solvent B in 2 min. The flow rate was 50 μl/min, and the UV absorbance was measured at 214 nm in a 300-nl flow cell with a pathlength of 2 mm. A post-column flow split diverted 85% of the column eluate for fraction collection, and 15% to an ion spray mass spectrometer [4].

For analysis by tandem MS (MS/MS), column fractions containing the peak of interest were each injected onto a concentrator HPLC column (0.5 × 150 mm; Reliasil C-18 column) at a flow rate of 20 μl/min. A 10–50% gradient of solvent B was applied over 12 min, followed by a gradient from 50% to 90% solvent B in 1 min, with all the eluate fed into the MS.

2.2.2. MS conditions. Mass spectra were recorded on a PE-Sciex API III triple quadrupole MS (PE-Sciex, Thornhill, Ont., Canada) equipped with a ion spray ion source. The ion spray voltage was approximately 5000 V and the nebulizer gas pressure was 40 psi. All LC/MS experiments were done in a single quadrupole operating mode using quadrupole 3 of the mass analyzer. The mass range from 175 to 2200 Da was scanned with a step size of 0.5 Da and a dwell time of 1 ms per step.

For tandem MS spectra were obtained by selectively introducing ions

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of single mass-to-charge ratio from the first quadrupole (Q1) into the collision cell (Q2) and observing the daughter ions in the third quadrupole (Q3). Thus, Q1 was locked on a particular m/z ratio, and the Q3 scan range was adjusted to cover the range from 50 to higher than the m/z ratio selected. Conditions: collision gas thickness = 4.3×10^{14} molecules/cm^2 (CGT = 430), collision gas was N_2/Ar mix in 10:90 proportion, step size = 0.5 or 1 Da, orifice energy = 80 V, dwell time = 1 ms.

3. Results and discussion

The digestion of insulin by the *O. piceae* proteinase was analyzed by LC/MS after incubation times ranging from 2 min to 24 h. After 2 min of digestion (data not shown) major peaks were C, D and E, with the last corresponding to the molecular weight of undigested insulin. MS chromatograms (TIC) of 5 min, 1 h, and 24 h digestion times are shown in Fig. 1 as A, B, and C, respectively. Only peaks containing peptide masses that matched various fragments of insulin B-chain, as determined by LC/MS or tandem MS, are labeled. After 5 min of digestion, the major fragments identified as peaks C and D corresponded to the primary cleavage of insulin (peak E) between Leu[15] and Tyr[16]. The remaining peaks labeled in Fig. 1A correspond to peptides resulting from cleavage at secondary sites of fragments C and D. Upon increasing digestion times, the observation made in Fig. 1A was confirmed in Fig. 1B and C which show the disappearance of the labeled peaks C, D and E with time and the relative increase of smaller fragments. The proposed cleavage of insulin is shown in Fig. 2.

The proteinase isolated from *O. piceae* appeared to have broad specificity (Fig. 2) leading to doubts concerning the homogeneity of the proteinase preparation. Therefore, LC/MS was used to conclusively demonstrate the absence of contaminating proteinases. Only one protein species was present upon

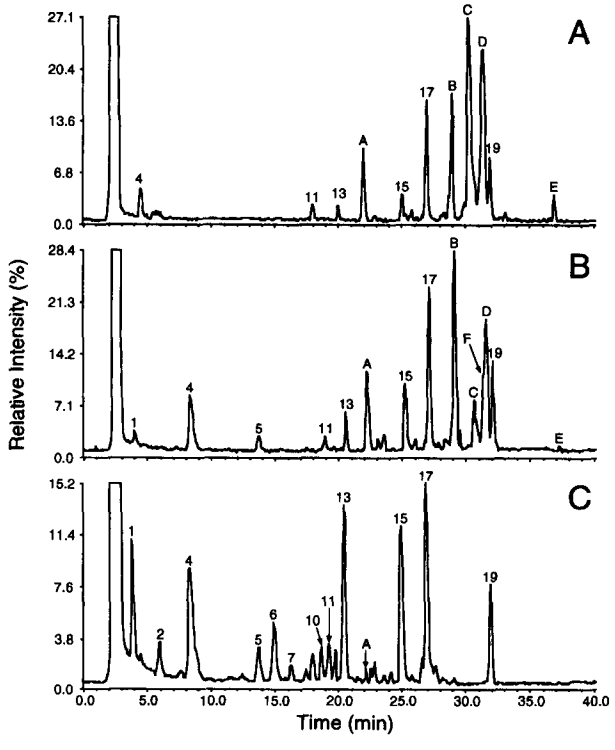

Fig. 1. Mass chromatograms (TIC) of proteolytic digests of insulin B-chain after 5 min (A), one hour (B) and overnight incubations (C). All labeled peaks were identified by LC/MS or tandem MS.

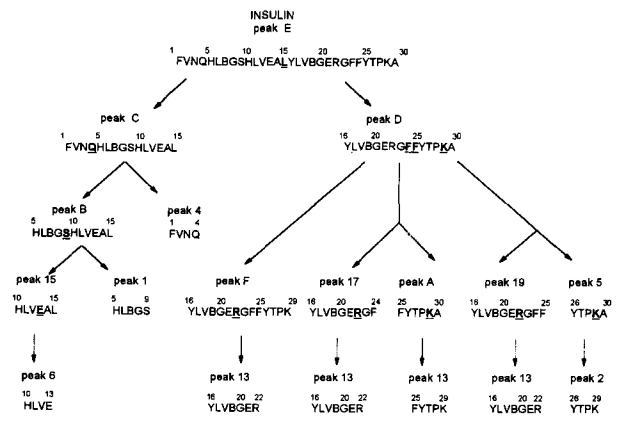

Fig. 2. Digestion of insulin B chain (peak E) by the proteinase isolated from *O. piceae*. All peptides shown were separated by HPLC and their position in the insulin B-chain determined by MS or tandem MS or tandem MS/amino acid analysis. Amino acid residues at the P1 position are underlined and in bold. Dotted and solid arrows represent the partial or complete breakdown of fragments respectively. The minor peaks observed and identified have not been shown on this diagram. B in the amino acid sequences in the diagram represents cysteic acid (R = CH_2SO_3H).

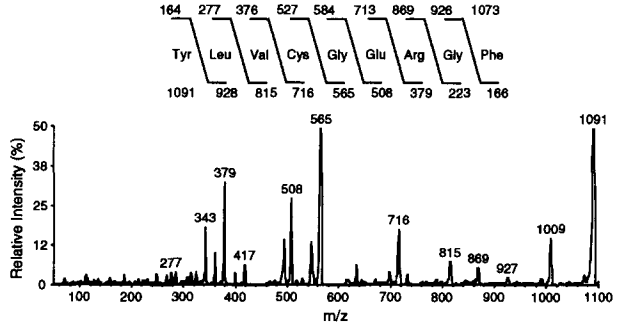

Fig. 3. Tandem MS spectra for the parent ion m/z 1091. The daughter ions generated by the fragmentation process resulted in different classes of fragment ions. Fragment ions of the type-b (top row) and type-y (bottom row) are generated if the charge is retained on the N-terminus and C-terminus of the peptide, respectively.

injection of the proteinase preparation. The only other species detected in the TIC were peptides that are probably due to autolytic degradation of the proteinase. Therefore the observed broad specificity could be attributed to a single proteinase.

The location of the cleavage sites giving rise to the observed peaks was determined by matching the masses of the peptides with masses of various fragments of insulin. Where ambiguities existed, or where more than one potential insulin fragment had the same mass as the observed peptide mass (within ± 2 Da), confirmation of the peptide sequence was resolved by sequencing by tandem MS as shown in Fig. 3 for the analysis of peak 19 in Fig. 1. The daughter ions obtained were mainly type-b ions, where the charge is retained on the amino-terminus of the ion to form an acylium ion; or type-y ions where the charge is retained on the carboxy-terminus [5]. Amino acid composition analyses of selected proteolytic fragments were in agreement with the sequences determined by tandem MS (data not shown).

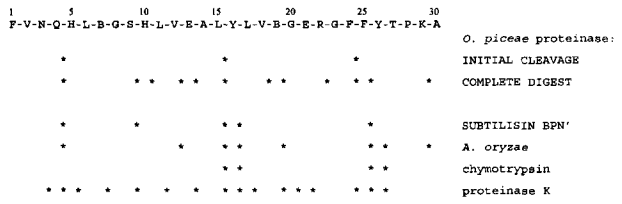

Fig. 4. Sites of cleavage of oxidized insulin B chain by various serine proteinases. Initial cleavage by the *O. piceae* proteinase represents the sites determined after 5 min digests. Complete digests indicate the sites cleaved after extended digests overnight. B in the diagram represents cysteic acid (R = CH_2SO_3H). Splitting sites for the remaining proteinases were taken from Morihara and Tsuzuki [7]; Kraus et al. [6].

The results indicated that fragments C and D resulted from the initial cleavage between Leu^{15} and Tyr^{16}, which happens to be the observed primary cleavage site for proteinase K and subtilisins Carlsberg and Novo [6]. Some of the other cleavage sites (Fig. 2) have previously been identified for other subtilisin-like serine proteinases, including subtilisin [7] and proteinase K [6], however there appears to be unique differences between the digestion patterns (Fig. 4). For example, no evidence was obtained in this study for cleavage after Tyr^{16} or Tyr^{26}, which were common cleavage sites for several other serine proteinases. Cleavage after His^{10}, Val^{18} and Arg^{22} was unusual when compared to the other digestion patterns, and may be unique target sites for the *O. piceae* serine proteinase.

Aromatic residues were hydrolyzed preferentially at the P1 site, using the nomenclature of Schechter and Berger [8]. However, cleavage also occurred after polar, positively charged or negatively charged residues. Interestingly, the first bond to be cleaved had Leu at the P1 site, yet cleavage was not observed after Leu^6, Leu^{11}, or Leu^{17}. This may be influenced by the P4 residue which is hydrophobic for several of the cleavage sites observed. Furthermore, cleavage after Phe^{24} and Phe^{25} was observed, but there was no evidence of cleavage after Phe^1. This suggests the need for more than one amino acid for binding and subsequent cleavage. In fact, work with proteinase K has shown that the smallest peptide hydrolyzable by this subtilisin class II enzyme is a tetrapeptide [6].

A proteinase with broad specificity is consistent with its function when produced by *O. piceae* during growth on wood. Degradation of proteins extracted from the xylem tissue of poplar was observed after incubation with the enzyme for 2 h. Disappearance of proteins bands on SDS-PAGE gels indicated that most proteins in the extract were susceptible to the action of the proteinase. Other proteins hydrolyzed by the proteinase included gelatin, collagen, albumin, edestin, globulins and casein.

In this study, ESI MS offered a rapid, simple and accurate means of obtaining molecular weight information, which was sensitive in the low picomole range. Analysis by tandem MS provided sequence information allowing a direct correlation to sites of cleavage. Using this technique it was possible to follow the digestion of insulin over time and identify the primary and secondary cleavage points in a relatively short time frame. With the development of more sensitive instruments and instruments capable of auto MS/MS, cleavage specificities of new proteinases could be determined with even greater rapidity. This method could be further developed as a general strategy for characterizing sites of cleavage using other enzymes and substrates.

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