PEPTIDE SEQUENCING BY MATRIX-ASSISTED LASER DESORPTION/IONISATION AND POST-SOURCE DECAY MASS SPECTROMETRY: A RAPID METHOD TO DESIGN OLIGONUCLEOTIDE HYBRIDISATION PROBES FOR CLONING cDNA ENCODING PYRANOSE 2-OXIDASE FROM *Trametes multicolor*

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A rapid and elegant method for designing oligonucleotide hybridisation probes for cloning cDNA encoding a biologically and/or biotechnologically important protein is presented. The approach is based on proteolytic digestion of a protein of interest and subsequent matrix/assisted laser desorption/ionisation mass spectrometric (MALDI-MS) analysis of the resulting peptide mixture. The method is demonstrated on the analysis of pyranose 2-oxidase (P2O), a homotetrameric flavoprotein used as a catalyst component in several biotechnologically important carbohydrate conversions. P2O from the fungus *Trametes multicolor* was cleaved directly in the gel by two different proteases and the peptides formed were subjected to MALDI-MS. A comparison of the obtained peptide maps to those theoretically derived from the known sequence of homologous P2O (*Trametes versicolor*) allowed us to select peptide candidates for designing hybridisation probes. The suitable peptides were sequenced by post-source decay (PSD) analysis. The acquired sequence data are aimed at cloning and sequencing of *T. multicolor p2o* cDNA and at production of the recombinant enzyme.

Key words: Mass spectrometry; Matrix-assisted laser desorption/ionisation; MALDI; Proteins; Sequence; Pyranose 2-oxidase; Oligonucleotide probe; Enzymes.

Pyranose 2-oxidase (P2O, EC 1.1.3.10), an FAD sugar oxidoreductase (*ca* 300 kDa, tetrameric form) produced by numerous wood-rot basidiomycete fungi, has been assumed to play a role in lignocellulose degradation by these organisms[1]. Due to its broad substrate specificity, the enzyme has received attention as a biocatalyst for regioselective transformations of unprotected

carbohydrates[2–4] and, importantly, as a diagnostic enzyme for diabetes[5]. It catalyses C-2 oxidation of several aldopyranoses, anhydrosugars and 1→6 disaccharides to the corresponding aldos-2-uloses (2-ketoaldoses), with D-glucose and O_2 as the preferred (co)substrates:

$$\text{D-glucose} + O_2 \xrightarrow{\text{P2O}} \text{D-}\textit{arabino}\text{-hexos-2-ulose} + H_2O_2$$

The dicarbonyl sugars produced can be used as rare chemicals or as intermediates/building blocks in preparative carbohydrate chemistry[6]. Thus, D-*arabino*-hexos-2-ulose (2-ketoglucose) and D-*lyxo*-hexos-2-ulose (2-ketogalactose) have gained potential in food technology as the key intermediates in the production of the modern low-caloric sweeteners D-fructose and D-tagatose, respectively[7–9]. Because chemical syntheses of aldosuloses are laborious, resulting in relatively low yields and a number of byproducts, the enzymatic conversions are the attractive alternative to the chemical routes. In order to make P2O available in higher yields, complementary DNA encoding this enzyme was recently cloned and sequenced from *Trametes (Coriolus) versicolor*[10]. The cDNA was successfully heterologously expressed under control of the *lac*UV5 promoter in *Escherichia coli*.

Our research has been focused on the P2O produced by the related species *T. multicolor*, the biochemical properties of which (especially high stability, high production yield and broad pH optimum)[11] are more suitable for biotechnological applications when compared with *T. versicolor*. The aim of this project was the determination of the sequences of internal peptides applicable to the construction of oligonucleotide probes for cloning *T. multicolor p2o* cDNA. This paper describes the mass spectral part of the recombinant strategy (Fig. 1) used for P2O expression: four internal peptides of P2O were sequenced by PSD-MALDI mass spectrometry and the homology of *T. multicolor* P2O *versus T. versicolor* P2O was roughly assessed.

The peptide sequencing by mass spectrometry for homology searches and cloning of genes was originally introduced by M. Mann *et al.*[12,13]. This approach has gained a significant attention due to its efficiency and sensitivity. Long and accurate amino acid sequences, that are sufficient for PCR-based strategies to clone the corresponding genes, can be obtained within few hours and with low-picomole to femtomole amounts of a sample.

EXPERIMENTAL

Materials, SDS-PAGE, Proteolytic Digestion

Pyranose 2-oxidase from *T. multicolor* was produced as described in detail elsewhere[14]. Multiple-step enzyme purification[9] was completed by SDS-PAGE carried out on 10% gels according to Laemmli[15]. The CBB-stained protein band was cut from the gel and washed several times with Tris-HCl buffer in 50% aqueous acetonitrile (ACN). After complete destaining, the gel was washed with water, shrunk by dehydration in ACN, reswollen again in water and then placed into a tuberculin syringe with a sieve in the bottom. Next, the gel was pressed through the sieve into an Eppendorf tube. The gel was partly dried using a SpeedVac concentrator and then reconstituted with cleavage buffer containing 100 mM Tris-HCl (pH 8.1), 1 mM $CaCl_2$, 10% acetonitrile and sequencing-grade trypsin (Promega, 50 ng/μl). Digestion was carried out overnight at 37 °C, the resulting peptides were extracted with 50% ACN/2% trifluoroacetic acid (TFA) and subjected to mass spectral analysis. A similar digestion procedure was performed in 50 mM ammonium acetate buffer (pH 4.0) using endoproteinase Glu-C (Promega).

Mass Spectrometric Measurements

Positive-ion MALDI mass spectra were measured on a Bruker BIFLEX reflectron time-of-flight mass spectrometer (Bruker–Franzen, Bremen, Germany) equipped with a multiprobe inlet and a gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron (ion mirror) voltage was set to 20 kV. For delayed extraction, a 5 kV potential difference between the probe and the extraction lens was applied with a time delay in the

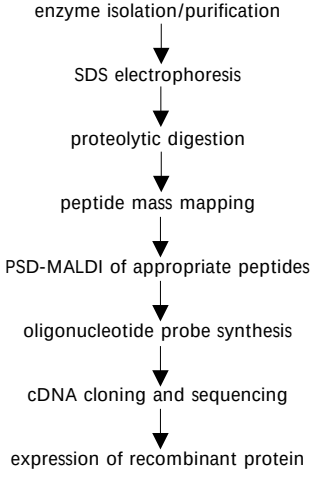

FIG. 1
Complete strategy for the identification of cDNA encoding *T. multicolor* pyranose 2-oxidase and for the expression of the recombinant enzyme

range of 150–200 ns after each laser pulse. Samples were irradiated at a frequency of 5 Hz by 337 nm photons from a pulsed Laser Science (Cambridge (MA), U.S.A.) nitrogen laser. Typically, 20–50 shots were summed in a single mass spectrum. Spectra were calibrated internally using the monoisotopic $[M + H]^+$ ions of peptide standards angiotensin II and bovine insulin (Sigma, Steinheim, Germany). Solutions of 3-(4-hydroxy-3-methoxyphenyl)-propenoic or 2-cyano-3-(4-hydroxyphenyl)propenoic acid in 30% ACN/0.2% TFA were used as MALDI matrices. One µl of the matrix solution was deposited on the target and, after a few seconds, 1 µl of the sample was added. The droplet was allowed to dry at ambient temperature.

Post-source decay spectra were typically recorded in 10–18 segments, with each successive segment with a 20% reduction in the reflector voltage. About 200 shots were averaged per segment. Segments were pasted, calibrated and smoothed under computer control with Bruker XTOF 3.0 software.

RESULTS AND DISCUSSION

Pyranose 2-oxidase from *T. multicolor* was digested by two different proteases and the resulting peptide mixtures were subjected to MALDI analysis. MALDI mass spectrum of tryptic fragments (Fig. 2) of our enzyme showed a high degree of identity with the known sequence of the corresponding P2O from another basidiomycete *T. versicolor*[10]. The total number of analogous peptides was twenty, which covered ≈42% of the sequence of the related

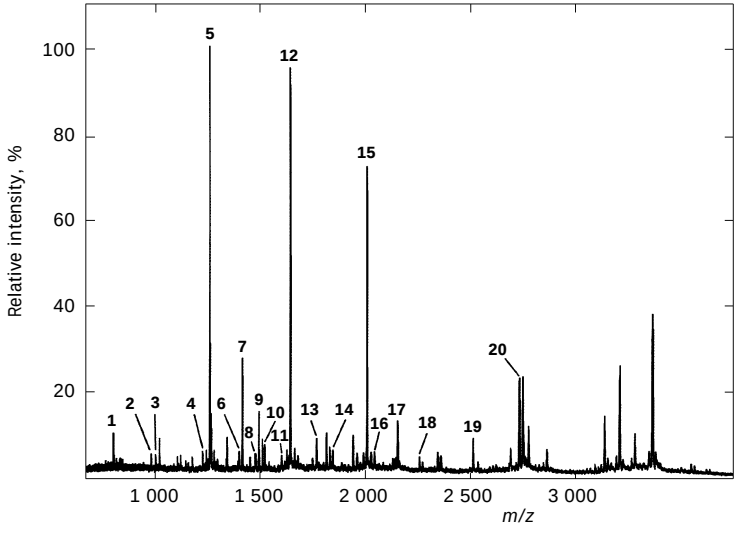

FIG. 2
MALDI mass spectrum of tryptic fragments of pyranose 2-oxidase from *T. multicolor* (for peak numbering, see Table I)

P2O enzyme (*T. versicolor*). The high precision achieved in internally calibrated spectra enabled us to assign tentative sequences and to align the peptides with the *T. versicolor* P2O sequence (Table I).

In addition, Glu-C digestion was also performed in order to obtain more complete information on sequence homology of our enzyme with P2O from *T. versicolor*. Nine peptides were precisely "fished" in this case (Fig. 3, Table I). Compared with tryptic peptides, some of Glu-C peptide fragments covered distinct segments of the *T. versicolor* P2O sequence and thus increased the degree of homology of our enzyme to the related P2O protein (*T. versicolor*). Evaluation of both tryptic and Glu-C peptide mapping data revealed at least 54% sequence coverage of *T. versicolor* P2O. The real value could be in fact higher if one or both of the following is true: a limited number of peptides were recovered during the extraction; ionisation of some peptides was suppressed due to direct complex mixture analysis. Even a single amino acid change present would immediately generate false or "ghost" peptides (non-labelled peaks in Figs 2 and 3).

The sequences of four suitable peptides were verified by post-source decay MALDI analysis (Fig. 4). The candidates were selected with respect to their amino acid sequence and position in the polypeptide chain. These peptides revealed their protonated molecules at *m/z* 1 259.7, 1 643.8 (the most abun-

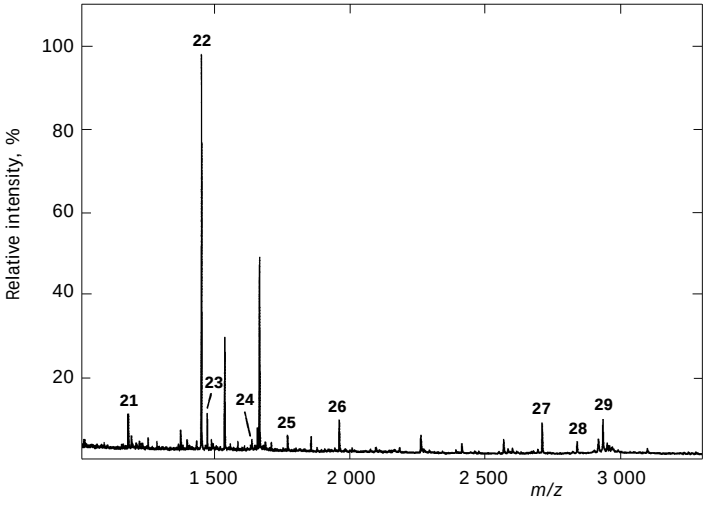

FIG. 3

Peptide map of pyranose 2-oxidase (*T. multicolor*) obtained after Glu-C digestion (for peak numbering, see Table I)

TABLE I

Tryptic and Glu-C fragments of pyranose 2-oxidase (*T. multicolor*) isobaric with the corresponding peptides of *T. versicolor* pyranose 2-oxidase (positions and sequences of the peptides refer to P2O from *T. versicolor*, sequences in bold were confirmed by PSD-MALDI analysis)

No.[a]	MH^+_{meas}	MH^+_{calc}	ΔM	Position	Sequence
1	801.45	801.46	–0.01	467–472	LIVDWR
2	945.44	945.51	–0.07	151–159	NLSGQAVTR
3	981.46	981.51	–0.05	473–480	FFGRTEPK
4	1 226.61	1 226.57	0.04	140–150	NGSNPEQDPLR
5	1 259.67	1 259.67	0.00	235–245	DFQQIPLAATR
6	1 400.76	1 400.80	–0.04	175–185	FDREQRPLLVK
7	1 415.76	1 415.78	–0.02	235–246	DFQQIPLAATRR
8	1 479.81	1 479.78	0.03	91–102	KNTVEYQKNIDK
9	1 494.76	1 494.75	0.01	72–85	VAMFDIGEIDSGLK
10	1 521.66	1 521.72	–0.06	202–214	AESYFKTGTDQFK
11	1 602.76	1 602.75	0.01	160–174	VVGGMSTHWTCATPR
12	1 643.80	1 643.77	0.03	452–466	DAFSYGAVQQSIDSR
13	1 769.98	1 769.89	0.09	47–63	YDVVIVGSGPIGCTYAR
14	1 845.97	1 845.85	0.12	491–505	ITDTYNMPQPTFDFR
15	2 006.95	2 006.98	–0.03	202–218	AESYFKTGTDQFKESIR
16	2 026.84	2 027.01	–0.17	198–214	LYTKAESYFKTGTDQFK
17	2 153.09	2 153.07	0.02	140–159	NGSNPEQDPLRNLSGQAVTR
18	2 258.05	2 258.13	–0.08	473–490	FFGRTEPKEENKLWFSDK
19	2 512.22	2 512.27	–0.05	198–218	LYTKAESYFKTGTDQFKESIR
20	2 730.44	2 730.46	–0.02	529–554	IGGFLPGSLPQFMEPGLVLHLGGTHR
21	1 181.66	1 181.59	0.07	195–203	WDRLYTKAE
22	1 450.67	1 450.68	–0.01	204–215	SYFKTGTDQFKE
23	1 472.73	1 472.68	0.05	359–371	QSLVFCQTVMSTE
24	1 638.84	1 638.82	0.02	297–310	SLHIHDLISGDRFE
25	1 709.97	1 709.96	0.01	80–95	IDSGLKIGAHKKNTVE
26	1 960.94	1 961.06	–0.12	236–252	FQQIPLAATRRSPTFVE
27	2 708.29	2 708.43	–0.14	230–252	YKGQRDFQQIPLAATRRSPTFVE
28	2 837.34	2 837.47	–0.13	229–252	EYKGQRDFQQIPLAATRRSPTFVE
29	2 933.31	2 933.39	–0.08	407–429	WWNEKVKNHMMQHQEDPLPIPFE

[a] **1**–**20** tryptic peptides, **21**–**29** Glu-C peptide fragments.

dant species) and *m/z* 801.5 (the lowest value) in the tryptic map and *m/z* 1 450.7 (the most abundant species) in the Glu-C map. Although the PSD sequencing was done directly from the whole peptide mixture without any separation, we obtained good quality b- and y-type fragment ions (for the ion nomenclature, see ref.[16]) confirming the proposed structures.

The true *de novo* sequencing was not required in this particular case because the sequence of a homologous protein was available. We conclude that the mass spectral approach used in this application paper (Fig. 1) is a rapid and straightforward strategy to determine sequence segments suitable for designing PCR primers for the consequent cloning and sequencing of cDNA, *e.g.*, that which corresponds to *p2o* gene (*T. multicolor*). Contrary to Edman sequencing, the reported approach works at the low-picomole to femtomole level.

FIG. 4

Fragment ions observed in PSD-MALDI spectra of four peptides selected as the most promising candidates for designing PCR primers (positions of the peptides refer to P2O from *T. versicolor*)

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