Novel peroxidases of *Marasmius scorodonius* degrade β-carotene

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Abstract

Two extracellular enzymes (MsP1 and MsP2) capable of efficient β-carotene degradation were purified from culture supernatants of the basidiomycete *Marasmius scorodonius* (garlic mushroom). Under native conditions, the enzymes exhibited molecular masses of ~150 kDa and ~120 kDa, respectively. SDS-PAGE and mass spectrometric data suggested a composition of two identical subunits for both enzymes. Biochemical characterisation of the purified proteins showed isoelectric points of 3.7 and 3.5, and the presence of heme groups in the active enzymes. Partial amino acid sequences were derived from N-terminal Edman degradation and from mass spectrometric *ab initio* sequencing of internal peptides. cDNAs of 1604 to 1923 bp, containing open reading frames (ORF) of 508 to 513 amino acids, respectively, were cloned from a cDNA library of *M. scorodonius*. These data suggest glycosylation degrees of ~23% for MsP1 and 8% for MsP2. Databank homology searches revealed sequence homologies of MsP1 and MsP2 to unusual peroxidases of the fungi *Thanatephorus cucumeris* (DyP) and *Termitomyces albuminosus* (TAP).

Keywords: basidiomycetes; carotenoid degradation; DyP-type peroxidase

Introduction

The degradation of carotenoids has been subject of intense research for several decades. From the perspective of human physiology and nutritional sciences, the centric, symmetric cleavage of carotenoids yielding retinoids has attracted utmost attention. Retinoids act as vitamins, signalling molecules, and visual pigments. A wealth of different apocarotenoids is derived from the excentric cleavage of the carotenoids' polyene chain in various plant and animal species, with the plant hormone abscisic acid being the best-investigated example (Schwartz *et al.* 1997). Many of these apocarotenoids (norisoprenoids) act as potent flavour compounds. Prominent representatives include α- and β-ionone, geraniol, and β-damascenone (Winterhalter and Rouseff 2002).

Apart from the generation of retinoids, plant hormones, or flavour compounds, there is a strong interest of the detergent and food industries in carotenoid degradation for bleaching purposes. In bakery, for example, carotenoids are degraded by the so-called co-oxidation system: Carotenoids are oxidized by free radical species generated from linolenic acid by lipoxygenase catalysis (Wache *et al.* 2003). Mainly soybean extracts containing lipoxygenase isoforms are used to meet consumers' demands for a white crumb.

Though some 100 million tons of carotenoids are biosynthesized and subsequently degraded naturally every year, surprisingly few data have become known on the biotic carotenoid degradation by microorganisms (Rodríguez-Bustamante and Sánchez 2007). Mixed cultures of *Trichosporon asahii* and *Paenibacillus amylolyticus* degraded lutein derived from marigold flowers (*Tagetes erecta*), but the enzymes involved in the degradation pathways have not yet been characterized (Rodriguez-Bustamante *et al.* 2005).

Marasco *et al.* (2006) recently identified and cloned gene homologues of eukaryotic carotenoid cleavage dioxygenases in the genomes of different cyanobacteria.

An extra-cellular versatile peroxidase of the edible mushroom *Pleurotus eryngii* (previously erroneously classified as *Lepista irina*) was found to efficiently degrade β-carotene (Zorn *et al.* 2003a). Fungal versatile peroxidases are key enzymes of natural lignin degradation, which have been reported to share catalytic properties of lignin peroxidases and manganese peroxidases. In a previous study (Zorn *et al.* 2003b), numerous filamentous fungi and yeasts, which were known for *de novo* synthesis or bio-transformation of mono-, sesqui-, tri-, or tetraterpenes, were screened for their capability to degrade β-carotene. Some strains discolored a β-carotene containing growth agar, indicating an efficient carotenoid degradation. Using a photometric bleaching assay, the β-carotene cleaving enzyme activities of *Marasmius scorodonius* were partially characterized. *Marasmius scorodonius* (CBS 137.83) („garlic mushroom“) is a small edible species, which grows on wood and further lignified plant materials (Ainsworth *et al.* 1973). Due to its intense garlic-like flavour, it is used as a spice. In the present study, the enzymes catalyzing the carotenoid degradation were purified to electrophoretic homogeneity, and the encoding genes were cloned from cDNA and genomic DNA.

Materials and Methods

General

The *Marasmius scorodonius* strain (CBS 137.86) was obtained from the Dutch “Centraalbureau voor Schimmelcultures”, Baarn. Due to the heat and light sensitivity of carotenoids, β-carotene containing solutions were prepared freshly before use. The cultivations were performed in the absence of light, and standard sterile

techniques were applied. Quantitative data represent average values of at least duplicate analyses.

Chemicals

The constituents of nutrient media were purchased from Merck (Darmstadt, Germany). β-Carotene and Tween 80 (free of peroxides) were obtained from Fluka/Sigma (Taufkirchen, Germany). Solvents were provided by BASF (Ludwigshafen, Germany) and Baker (Deventer, Netherlands). All solvents were distilled before use.

Biochemical Enzyme Characterisation

An enzyme assay (Ben Aziz *et al.* 1971) was modified according to Zorn *et al.* (2003b). Shortly, the time dependent decrease of absorbance of an aqueous β-carotene emulsion was monitored at 450 nm using a tempered spectral photometer. Applicability of the test was checked by diluting aliquots of the enzyme sample with buffer solution (50 mM sodium acetate buffer, pH 3.5, 27 °C). A linear correlation between activity and sample amount was found.

Protein Concentration

The protein concentration was estimated by the method of Lowry *et al.* (1951) using the *DC*-Protein-Assay (Biorad, Hercules, USA) and bovine serum albumin as a standard.

Electrophoresis

SDS-PAGE was performed by the method of Laemmli (1979) with 4% (w/v) polyacrylamide in the stacking gels and 16% (w/v) polyacrylamide in the resolving gels. Proteins were stained with 0.1% (m/v) Coomassie Brillant Blue G/R-250 (Serva, Heidelberg, Germany) or immunostained using alkaline phosphatase conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA). Polyclonal antibodies were produced in

rabbits using standard protocols (BioGenes, Berlin, Germany). For immunization, MsP1 and MsP2 were purified, pooled, and concentrated from 4 l of *M. scorodonius* culture supernatant as described below.

An electrophoresis calibration kit (14.5 – 200 kDa; Roth, Karlsruhe, Germany) was used for the preparation of a calibration curve for determination of molecular masses. For heme staining with a solution of 3,3',5,5'-tetramethylbenzidine (TMBZ) in methanol according to Thomas *et al.* (1976) and Henne *et al.* (2001), gels were run at 4°C under non-denaturing conditions.

Isoelectric Focussing

Samples were concentrated and desalted using Vivaspin 15R ultra-filtration units (MWCO 10 kDa, Vivascience, Göttingen, Germany) and subjected to isoelectric focussing polyacrylamide gel electrophoresis (IEF-PAGE) with immobilised pH gradient. Electrophoresis conditions: gel dimensions 12.5 cm × 12.5 cm × 0.3 mm; pH range 3 – 6 (Serva, Heidelberg, Germany); voltage 2000 V, 6 mA, 12 W, 3500 Vh; sample volume 12 µl.

The samples were applied twice, laterally reversed on both sides of the IEF gel. For detection, the gel was cut concentric and one half was subjected to Coomassie staining, the second to activity de-staining. 50 ml of β-carotene solution (0.01% m/v + Tween 80™ 1% m/v), 15 ml buffer solution (50 mM sodium acetate, pH 6.0), 100 µl trace element solution (containing Fe-, Cu-, Zn-, and Mn-ions), and 0.7 g agarose were mixed to give an orange coloured agarose gel of 2 mm thickness for the de-staining test. One half of the IEF gel was covered with this carotene agar, pinned down and incubated at 27°C for 2-4 hours. To ensure the correct assignment of protein bands, the decoloured spots were marked on the IEF gel by a pinprick and,

after removal of the carotene agar, the gel was additionally stained by Coomassie blue.

Enzyme Purification by Fast Protein Liquid Chromatography (FPLC)

All purification steps were performed in a cooling chamber at 6 °C. A Biologic Duoflow™ FPLC system (Biorad) was employed for the chromatographic protein separation. The protein concentration of the eluate was monitored at λ = 280 nm, and enzyme activity was determined in all protein containing fractions.

M. scorodonius was maintained on a β-carotene containing growth agar as described previously (Zorn *et al.* 2003b). For preparation of precultures, 14 mm diameter agar plugs from the leading mycelial edge were transferred into 100 ml of standard nutrition solution (30 g l^{-1} glucose $\times$ 1 H_2O; 4.5 g l^{-1} asparagine $\times$ 1 H_2O; 1.5 g l^{-1} KH_2PO_4; 0.5 g l^{-1} $MgSO_4$; 3.0 g l^{-1} yeast extract; 15 g l^{-1} agar agar; 1 ml l^{-1} trace element solution containing Cu, Fe, Zn, Mn, and EDTA; pH adjusted to 6.0) and homogenized using an Ultra Turrax (IKA, Staufen, Germany). After cultivation for seven days at 24°C and 150 rpm, the cultures were homogenized and 20 ml of the pre-cultures were transferred into 500 ml Erlenmeyer flasks containing 250 ml of fresh standard nutrition solution.

Supernatant was collected from the submerged cultures after 4 days, separated from the mycelium by filtration or centrifugation (4,000 g, 1 h, 4 °C), and concentrated by tangential flow filtration (Vivaflow 200, 10 kDa MWCO; Vivascience). The retentate was subjected to ion exchange chromatography (IEC).

Ion Exchange Chromatography

A Q-Sepharose High performance column (25 ml, Pharmacia Biotech, Uppsala, Sweden) was used for ion exchange chromatography. Sodium acetate (50 mM, pH 6.0) served as start buffer, and the enzymes were eluted with a linear gradient of 0-

100% sodium acetate buffer containing 1 M sodium chloride. The flow rate was 3 ml min^{-1}, fractions were collected every minute. The active fractions were pooled and concentrated by ultra filtration (see above).

Size Exclusion Chromatography

A Superdex 200 HR 10/30 column (Pharmacia Biotech) with a bed volume of 24 ml and a separation range of 10 to 600 kDa was used. The elution buffer consisted of sodium acetate (50 mM, pH 6.0; flow rate 0.5 ml min^{-1}). Fractions were collected every two minutes. A calibration curve was prepared with reference proteins (HMW and LMW Gel Filtration Calibration Kit, Pharmcia Biotech) for calculating the molecular mass.

Electrospray-Ionization Tandem Mass Spectrometry

ESI-MS/MS analyses were performed as reported previously (Zorn *et al.* 2005). Protein spots were excised from Coomassie stained SDS-PAGE gels and digested with trypsin. The resulting peptides were extracted and purified according to standard protocols. A QTof II mass spectrometer (Micromass, Manchester, England) equipped with a nanospray ion source and gold coated capillaries (Protana, Odense, Denmark) was used for electrospray MS of peptides. For collision induced dissociation experiments, multiple charged parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell (collision energy 25-30 eV for optimal fragmentation). The resulting daughter ions were separated by an orthogonal time-of-flight mass analyser. The acquired MS-MS spectra were enhanced (Max. Ent. 3, Micromass) and used for the *de novo* sequencing of tryptic peptides. The fasts3 algorithm, which compares linked peptides to a protein databank was used for homology queries.

MALDI-TOF-MS

Mass spectra were recorded on a Bruker ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) at an acceleration voltage of 20 kV in a linear mode. In a dried-droplet application, sinapic acid served as matrix.

UV/Vis-Spectroscopy

Absorption spectra were recorded using a Perkin Elmer Lambda 12 (Überlingen, Germany) spectral photometer equipped with a tempered cell holder and magnetic stirrer.

cDNA Synthesis and PCR-Screening

For cloning of the MsP1 and MsP2 encoding cDNA sequences, a cDNA library of *M. scorodonius* was constructed and screened by polymerase chain reaction (PCR). Mycelium of *M. scorodonius* was harvested at an enzyme activity of ~0.5 mU ml^{-1} on the 4^{th} culture day. Cell disruption was achieved by grinding the mycelium (210 mg) under liquid nitrogen. For isolation of total RNA, a silica gel based membrane (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany) was applied. Integrity of the RNA was checked by denaturing formaldehyde agarose gel electrophoresis and ethidium bromide staining. cDNA was synthesised with the SMART™ cDNA library construction kit (BD Biosciences, Heidelberg, Germany) according to the manufacture's instructions. Super Script II RNase H^- reverse transcriptase (GIBCO BRL, Life Technologies, Paisley, Scotland) was used for first-strand synthesis. Primer construction was performed with the assistance of the primer3 algorithm (Rozen and Skaletsky 2000), and PCR primers were synthesized by Roth (Karlsruhe, Germany). For PCR reactions, ~20 ng of genomic DNA or cDNA, respectively, were used as template in 20-µl reaction mixtures containing 1x PCR Buffer (QIAGEN), 0.2 mM dNTPs, 3.0 mM $MgCl_2$, 0.5 µM of each primer, and 0.5 U HotStarTaq DNA polymerase (QIAGEN). Amplification experiments were performed in a Master Cycler

gradient (Eppendorf, Hamburg, Germany). The following primers were used for amplification of full length cDNAs of MsP1 and MsP2: MsP1 forward (5'>ATG AAG CTT TTT TCT GCC TCC<3'); MsP1 reverse (5'>CTA GAC TGA AAG CAC AGT CCT GAT CG<3'); MsP2 forward (5'>AGT ATG CGG CTC ACT TAC CTT CC<3'); MsP2 reverse (5'>TCA AAC AGA AAG CGT GTT CTG GAT CG<3').

Cloning and sequencing of PCR products

PCR products were cloned into pCR2.1TOPO using the TOPO TA cloning kit (Invitrogen). Sequencing, initiated by M13 reverse and forward primers, was performed at GATCBiotech (Konstanz, Germany) using an ABI automated DNA sequencer. The NetOGly 3.1 algorithm (Julenius *et al.* 2005) was used to identify potential glycosylation sites.

Isolation of genomic DNA and molecular strain identification

To identify the *Marasmius scorodonius* strain used for the production, isolation, and cloning of β-carotene degrading enzymes on a molecular level, DNA was isolated from the fungal mycelium. Fungal mycelium (300 mg) was ground under liquid nitrogen, and the „genomic DNA from plant" kit (Macherey & Nagel, Düren, Germany) was used for the isolation of genomic DNA according the manufacturer's instructions. The PCR primers 5'>ACT GCG AGT GAA GAG GGA AA<3' (forward) and 5'>TTG CAC GTC AGA ATC GCT AC<3' (reverse) were used to amplify a fragment of the DNA encoding the 25S large subunit of ribosomal RNA, and the obtained PCR product was cloned into *E. coli*. A 100% sequence identity of the amplified fragment to the strain JEJ.586 (accession number AF261331) undoubtlessly identified the species under investigation as a *Marasmius scorodonius* strain.

Enzyme Production

For enzyme production, *M. scorodonius* was grown in submerged cultures in standard nutrition solution (Zorn *et al.* 2003b). At the time of highest β-carotene degrading enzyme activity (4th culture day), the culture supernatants were separated from the mycelia by centrifugation and concentrated by means of tangential flow filtration.

Nucleotide sequence accession numbers

cDNA sequences of MsP1 and MsP2 were deposited at the EMBL Nucleotide Sequence Database under the accession numbers CS490657, CS490659, CS490661, CS490662, and CS490663.

Results

Purification and characterization of β-carotene degrading enzymes of *M. scorodonius*

Ion exchange chromatography (IEC) on a strong anion exchanger column was employed as a first chromatographic purification step, which removed 97% of the non-target proteins. The fractions containing enzyme activity were pooled, concentrated by ultra-filtration, and subjected to size exclusion chromatography. β-Carotene degrading enzyme activity was detected in the SEC fractions 16-19 (Figure 1). An overall enrichment factor of 291 (Table 1) was calculated for the purified protein sample.

From the retention indices of the two peaks (mean of 11 independent analyses) eluting in the active fractions 16–19, molecular masses of ~150 and ~120 kDa, respectively, were calculated.

SDS-PAGE analysis confirmed the presence of two proteins in these fractions, while electrophoretically homogeneous proteins were present in fraction 16 (*Marasmius scorodonius* Peroxidase 1; MsP1) and 19 (MsP2) (Figure 2).

Molecular masses of ~75 kDa (MsP1) and ~55 kDa (MsP2) were assigned to the two target proteins under the denaturing conditions of SDS-PAGE analysis. Mass spectrometric analyses by means of MALDI-TOF-MS resulted in molecular masses of 64.2 kDa for MsP1 and 52.4 kDa for MsP2, respectively. Thus, the active enzymes presumably represent homo-dimeric enzymes under native conditions.

UV-VIS spectroscopy revealed absorption maxima of 406 nm (MsP1) and 405 nm (MsP2) (Figure 3a), respectively, indicating the presence of heme groups in both enzymes. This was further confirmed by running SDS-PAGE gels under non-denaturing conditions and using a heme specific staining procedure (Figure 3b).

The isoelectric points of MsP1 and MsP2 were determined by means of IEF electrophoresis using Coomassie staining and activity-destaining. Covering one half of the IEF gel after focussing with a β-carotene containing agarose gel resulted in the formation of distinct bleaching zones in the orange gel. By comparison to the Coomassie stained second gel half and reference proteins, pIs of ~3.7 (MsP1) and ~3.5 (MsP2) were assigned to the two β-carotene degrading enzymes. These results were further confirmed by excising the regions corresponding to the bleaching zones from another IEF gel and transferring the gel slices onto an SDS-gel.

Amino acid sequence data of the purified carotenoid cleaving enzymes from *M. scorodonius* were obtained from Edman sequencing (MsP1: **A**SF?**AGL**?**LTDIQGDIL**?T**MKKNKELFF;** MsP2**: A**P?**L**?L**TDIQG**DI**LI**G**MKKNKE**?**FF;** bold letters: reliable identification), and by electrospray-tandem-MS experiments (Table 2).

Data bank homology searches (EMBL-EBI, programme Fastf3) using the Edman and the internal peptide sequences returned unusual peroxidases of the fungi *Thanatephorus cucumeris* (DyP) and *Termitomyces albuminosus* (TAP) as the best hits for both enzymes.

cDNA Cloning of MsP1/MsP2

Deriving PCR primers from the degenerated translated sequences of the internal peptides and additional primers from the adaptor regions of the cDNA construction kit allowed for identification of the entire cDNA sequences of MsP1 and MsP2 by primer walking. Based on the obtained sequence information, three different full-length cDNAs were amplified for MsP1, and two for MsP2.

The cDNAs of MsP1 (1604 - 1682 bp) contained open reading frames (ORF) of 508 or 513 amino acids, respectively, while the mature proteins consisted of 453 and 458 amino acids. A molecular mass of ~ 49 kDa was calculated for the mature proteins. All of the tryptic peptides of MsP1 sequenced by ESI-tandem mass spectrometry were present in the translated protein sequences. The amino acid sequences contained eight potential N-glycosylation sites and one potential O-glycosylation site.

The cDNAs of MsP2 (1857 - 1923 bp) comprised an open reading frame (ORF) of 1533 bp, corresponding to a 510 amino acid protein. 3' non-coding regions of 279-345 bp were identified in the MsP2 cDNAs. For the mature proteins (453 AA), a molecular mass of ~ 48 kDa was calculated. Four potential N-glycosylation and three potential O-glycosylation sites were detected in the amino acid sequences. The translated amino acid sequences contained all of the peptides derived from sequencing of MsP2 by ESI-tandem mass spectrometry. The overall amino acid sequence homologies of MsP1 and MsP2 to the enzymes DyP (accession number Q8WZK8) and TAP (Q8NKF3) produced by *Thanatephorus cucumeris* and

Termitomyces albuminosus, respectively, were 48%, and the sequence identity between the MsP1 and MsP2 enzymes was 63%.

Cloning of MsP1 and MsP2 from genomic DNA

Amplificates of 2093 and 2135 bp were obtained for MsP1 and MsP2, respectively, when genomic DNA of *M. scorodonius* was used as a template in the PCR reactions. Both genes contained 10 introns of 50 to 83 bp lenght.

Discussion

Based on the overall sequence homologies of ~50% on the amino acid level, the novel peroxidases MsP1 and MsP2 may be assigned to the group of "DyP-type" peroxidases ("dye decolorizing peroxidases"). DyP represents a secreted, iron containing enzyme of the class II plant peroxidases (classification according to Welinder 1992), which was isolated from the fungus *Thanatephorus cucumeris* Dec1 (Sugano *et al*. 1999). As DyP did not reveal sequence homologies to other class II enzymes, it was regarded as the prototype of a new peroxidase family (Sugano *et al.* 2000). This family comprises currently about 170 proteins from eukaryotes and prokaryotes. Among the 13 eukaryotic members, nine are fungal proteins (InterPro at EMBL-EBI web site; Faraco *et al.* 2007), including a phenol oxidase from *Termitomyces albuminosus* (TAP) and a peroxidase of a *Polyporaceae sp*. (PoP). The ability to oxidize phenolic compounds, such as 2,6-dimethoxyphenol and guaiacol, in the absence of Mn^{2+} distinguishes DyP from manganese peroxidases. DyP does not degrade non-phenolic compounds, thus differing from lignin peroxidases. Another interesting characteristic of DyP is its ability to degrade synthetic anthraquinone dyes, which are not oxidized by most other peroxidases (Kim and Shoda 1999).

Different from lignin peroxidases, MsP1 and MsP2 do neither contain the conserved tryptophan residue, which is essential for the oxidation of veratryl alcohol and further aromatic substrates (Blodig *et al*. 1998; Choinowski *et al.* 1999), nor the two Ca^{2+} binding sites that typically stabilize secreted peroxidases (Conesa *et al.* 2000; Martínez 2002). Likewise missing is the manganese binding site of manganese and versatile peroxidases (Kishi *et al.* 1996, Whitwam *et al.* 1997, Sundaramoorthy *et al.* 1997).

MsP1 and MsP2, like most other members of the DyP-type family, exhibit one conserved asparagine and four conserved histidine residues, which are presumed to be essential for the functionality of the enzymes (Figure 4). For DyP, His164 and Asp278 have been shown to be involved in the binding of the heme group by x-ray diffraction (Sato *et al.* 2004), and additionally by site-directed mutagenesis (Sugano *et al.* 2004). A recombinant and deglycosylated enzyme expressed in *A. oryzae* was employed in these investigations.

By MALDI-TOF analyses, molecular masses of and 64.2 kDa and 52.4 kDa, respectively, were determined for MsP1 and MsP2. These data suggest glycosylation degrees of ~23% for MsP1 and 8% for MsP2. Similar carbohydrate contents have been reported for DyP (17%) (Sugano *et al.* 1999) and TAP (15 – 28%) (Johjima *et al.* 2003). Carbohydrate side chains are typically not required for the catalytic activity of fungal extracellular peroxidases (Sato *et al.* 2004; Nie *et al.* 1999), but they have been shown to protect the enzymes from degradation by peptidases and to increase the enzymes' thermostability (Lis and Sharon 1993, Rehm 2002; Sugano *et al.* 2000). While characteristic signal peptide cleavage sites were identified in position 20/21 (MsP1) and 19/20 (MsP2) (SignalP 3.0, Bendtsen *et al.* 2004), no typical cleavage sites were identified before the N-termini of mature MsP1 and MsP2, DyP, and TAP.

Presumably, the secreted proteins post-translationally undergo a two step processing. As DyP, heterologously expressed in *Aspergillus oryzae*, showed the same N-terminus as the wild-type enzyme, catalysis of the second hydrolysis step by the peroxidase itself was discussed (Sugano *et al.* 2002). However, an experimental proof of this hypothesis is still missing.

An uncommon characteristic of MsP1 and MsP2 is their native occurrence as dimers. An extracellular dimeric peroxidase has so far solely been described for the basidiomycete *Pleurotus ostreatus* (Kang *et al.* 1993). The isolelectric points of MsP1 (3.7) and MsP2 (3.5) are close to the one reported for DyP (3.8) (Kim and Shoda 1999). Though SDS-PAGE analysis indicated electrophoretic homogenous proteins in the active fractions of the final chromatographic purification step (cf. Figure 2), IEF electrophoresis with Coomassie and activity de-staining revealed additional faint protein bands. This observation may either be explained by varying glycosylation patterns (Lis and Sharon 1993), or by the presence of iso-enzymes. The multiple cDNAs encoding MsP1 and MsP2 with sequence identities of up to 98% well explain the protein pattern observed on the IEF gels. The production of several iso-enzymes is a typical characteristic of basidiomycetous fungi. Altogether ten genes encoding lignin peroxidases and five genes encoding manganese peroxidases have been identified in the genome of the white-rot fungus *Phanerochaete chrysosporium* (Hoegger *et al.* 2007; Martínez 2002). The basidiomycete *Pleurotus eryngii* secretes three versatile peroxidase iso-enzymes with high sequence homology (Ruíz Dueñas *et al.* 1999), and up to 16 lignin peroxidases with pIs of 3.1 to 3.7 were formed by *Trametes versicolor* (Johansson and Nyman 1996).

The MsP1 and MsP2 encoding genes both contain ten introns. Similarly, a gene encoding a putative DyP-type enzyme with nine introns has been cloned only

recently from a *Pleurotus ostreatus* genomic library (Faraco *et al.* 2007). The intron-exon structure of MsP1 and MsP2 is rather similar (Figure 5).

All introns of MsP1 and MsP2 are flanked by a GT dinucleotide at the 5', and by AG at the 3' ends. While this organization is typical for 98-99.9% of the introns of the fungi *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Neurospora crassa*, and *Cryptococcus neoformans* (Kupfer *et al.* 2004), two uncommon short exons of only four nucleotides are present in the MsP2 gene.

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Figure legends

Figure 1: Size exclusion chromatography as second chromatographic purification step; β-carotene degrading enzyme activity was detected in the shaded area.

Figure 2: SDS-PAGE analysis of active fractions (16 to 19) isolated by size exclusion chromatography; Ref.: reference proteins; left: silver staining; right: Western blot

Figure 3a: UV-Vis absorption spectrum of purified MsP2; b: SDS-PAGE analysis of MsP2 with heme staining using TMBZ

Figure 4: Alignment of the enzymes TAP (*T. albuminosus*), PoP (*Polyporaceae sp*.), DyP (*T. cucumeris*), MsP1, and MsP2 (*M. scorodonius*); (*: conserved amino acid residue; ■: histidine presumably involved in the binding of the heme group; ▲: asparagine residue presumably involved in the binding of the heme group; ↑: conserved histidine residues); N-terminal amino acids shaded; alignment was performed with CLUSTAL W (Higgins *et al.* 1994).

Figure 5: Comparison of the organisation of the MsP1 and MsP2 genes of *M. scorodonius*; ■: Introns, □: Exons; two uncommon short exons (CCCC, GCCT) marked with arrows.