Purification, kinetics and spectral characterisation of a new versatile peroxidase from a *Bjerkandera* sp. isolate

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Abstract

From the extracellular fluid of a novel strain of *Bjerkandera* sp., it was isolated, purified and identified the main enzyme responsible for Remazol Brilliant Blue R dye decolourisation. Such an enzyme is able to oxidise manganese, as well as veratryl alcohol and 2,6-dimethoxyphenol in manganese-independent reactions; hence, it can be included in the new group of versatile peroxidases. The molecular mass of said enzyme is ca. 45 kDa, and the N-terminal amino acid sequence obtained by Edman degradation is VAXPDGVNTA. The enzyme substrate range for oxidation of several phenolic and non-phenolic aromatic compounds was determined and the corresponding Michaelis–Menten kinetic constants calculated. Furthermore, spectrophotometric assays showing the Soret band and allowing observation of band shifts of the enzyme led to the conclusion that *Bjerkandera* strains may also synthesise at least two different versatile peroxidases, as happens with *Pleurotus eryngii*.

Introduction

During the last decade, research on the lignin-degradation ability of fungi has focused mainly on basidiomycetes commonly known as white-rot fungi. The complexity of the lignin attack mechanisms—which involve a number of different enzymes, the relative importance of which depends on the fungus considered, reveals the importance of the search for novel fungal isolates as a potential source of new enzymes with improved performances considering kinetics and substrate specificity.

Among those studies attention has recently been paid to a novel class of ligninolytic peroxidases, with high affinity for manganese and dyes; these enzymes can also oxidise 2,6-dimethoxyphenol (DMP) and veratryl alcohol (VA) in a manganese-independent reaction, hence combining typical properties of both MnP and LiP enzymes—coupled with a broad substrate range. Another unique feature of these versatile peroxidases (VP) is that they do not require mediators for substrate oxidation, including decolourisation of several dyes—a fact that could be of potential interest in many industrial biotechnological applications. Until now, versatile peroxidase enzymes have been isolated only from a few species: *Pleurotus ostreatus*, *Pleurotus eryngii*, *Bjerkandera adusta* and *Bjerkandera* sp. strain BOS55 [1–9].

Not surprisingly, the search for new ligninolytic fungal strains exhibiting high decolourisation activity on Poly R-478 and Remazol Brilliant Blue R (RBBR) dyes, revealed a new type of *Bjerkandera* sp. strain B33/3 [10]. Analyses of peroxidase activities in the extracellular fluid of this strain demonstrated the existence of lignin peroxidase, as well as manganese-dependent and manganese-independent peroxidase activities. In such a strain, RBBR decolourisation occurs via an enzyme-mediated process, which depends on the presence of hydrogen peroxide in the reaction medium.

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For further elucidation of the mechanism of decolourisation it was important to isolate, purify and characterise the peroxidase which was mainly responsible for the RBBR dye decolourising activity of this new *Bjerkandera* sp.

Materials and methods

Culture conditions and enzyme purification

Bjerkandera sp. strain B33/3 was grown in CDBYE medium, as described elsewhere [10]. The extracellular fluid of a 5.5-L culture was separated from the mycelium by filtration through Whatman no. 1 filter paper (from Millipore).

The proteins extracted from that clear extracellular fluid were adsorbed on Q-Sepharose Fast Flow (Pharmacia); elution afterwards was by 10 mM Na cacodylate buffer (pH 6.0), using a linear gradient of NaCl (0–0.6 M); further concentration was provided by ultrafiltration with an Amicon YM 10 membrane (10 kDa MW cut-off), followed by dialysis overnight against 10 mM cacodylate buffer (pH 6.0). The concentrated fractions were applied on a G-100 gel filtration column (Pharmacia), and eluted once again with the aforementioned buffer.

Pooled fractions possessing dye decolourising activity were then applied onto a Q-Sepharose Fast Flow column (Pharmacia), and eluted by 20 mM sodium tartrate buffer (pH 5.0) using a linear gradient of NaCl (0–0.4 M). The fractions of interest were concentrated by ultraflltration with an Amicon YM 10 membrane (10 kDa MW cut-off), and dialysed overnight against 10 mM cacodylate buffer (pH 6.0).

The fractions, thus obtained were further resolved by ion exchange chromatography on a Mono Q column (type HR5/5, from Pharmacia), using a 0–0.4 M linear gradient of NaCl, with an Akta FPLC system (Amersham-Pharmacia Biotech). The pooled fractions were once again concentrated, dialysed overnight against 10 mM cacodylate buffer (pH 6.0), and stored at 4 °C before enzymatic assays were in order.

Enzyme activity characterisation

The activity of manganese peroxidase (MnP, EC 1.11.1.13) was determined using 3-methyl-2-benzithiazolinone hydrazone (MBTH) and dimethylaminobenzoic acid (DMAB) as substrates, following the method by Castillo et al. [11]. The manganese-independent peroxidase (MiP) activity was estimated using DMP as substrate, according to Mester and Field [12]. The activity of lignin-peroxidase (LiP, EC 1.11.1.14) was determined using VA as substrate, following the procedures reported by Linko and Haapala [13]. The RBBR decolourising activity was assayed spectrophotometrically by measuring the decrease in absorbance at 595 nm (30 °C), as described elsewhere [10]. The Mn(II) activity was assayed spectrophotometrically by measuring the decrease in absorbance at 238 nm (30 °C) [3]. The enzymatic standard reaction mixture consisted of 0.1 mM $MnSO_4$ and 100 mM sodium tartrate buffer (pH 5.0); the reaction was initiated via addition of 0.1 mM H_2O_2 ($\varepsilon_{238} = 6500\ M^{-1}\ cm^{-1}$). The 2,2′-azinobis(3-ethylbenzothiazolme-6-sulphonic acid) (ABTS) oxidising activity was assayed spectrophotometrically by measuring the decrease in absorbance at 420 nm (25 °C) [14]. The standard reaction mixture consisted of 0.5 mM ABTS and 100 mM sodium tartrate buffer (pH 4.5). The reaction was initiated via addition of 0.1 mM H_2O_2 ($\varepsilon_{420} = 36{,}000\ M^{-1}\ cm^{-1}$). Kinetic constants for selected substrates in 100 mM sodium tartrate, at pH 3.0, 3.5 and 5.0, were derived from the linear phases of reaction. Activities were calculated from the molar absorbance of the reaction products produced from catechol ($\varepsilon_{238} = 6500\ M^{-1}\ cm^{-1}$), hydroquinone ($\varepsilon_{247} = 21{,}000\ M^{-1}\ cm^{-1}$), 4-methoxyphenol ($\varepsilon_{253} = 4990\ M^{-1}\ cm^{-1}$), methylhydroquinone ($\varepsilon_{250} = 21{,}120\ M^{-1}\ cm^{-1}$), *p*-aminophenol ($\varepsilon_{246} = 15{,}627\ M^{-1}\ cm^{-1}$), guaiacol ($\varepsilon_{456} = 12{,}100\ M^{-1}\ cm^{-1}$), ferulic acid ($\varepsilon_{310} = 8680\ M^{-1}\ cm^{-1}$), α-naftol ($\varepsilon_{255} = 12{,}800\ M^{-1}\ cm^{-1}$) and Reactive Black 5 ($\varepsilon_{598} = 50{,}000\ M^{-1}\ cm^{-1}$). The physicochemical data were from Heinfling et al. [1]. Apparent K_m and V_{max} values were estimated from Hanes plots.

The pH dependence and stability of the enzyme in terms of substrate oxidation reaction were measured under standard assay conditions, using 100 mM sodium tartrate buffer. All assays were performed in triplicate; the data values considered hereafter are means of the replicates that exhibit a standard error below 10%.

Proteinaceous feature determination

During the sequential purification steps, the protein concentration was estimated spectrophotometrically at 280 nm. The final protein concentration of the fractions with interest was also determined via Bradford Protein Assay (Bio-Rad), using bovine serum albumin (Fluka) as standard.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of 20 μg of the native protein was performed in a 10% (w/w) polyacrylamide gel, and protein bands were stained with Coomassie Blue R-250. For molecular weight determination, the gels were calibrated using the 10 kDa protein ladder (Gibco BRL) as standard.

The N-terminal sequence of the mature peroxidase was obtained by automated Edman degradation of ca. 5 μg of protein, using a Procise Protein Sequencing System (Applied Biosystems).

Spectral characterisation

Purified protein ($0.08\ g\ L^{-1}$) in 10 mM cacodylate buffer (pH 6.0) was spectrophotometrically scanned from 300 up to 700 nm, so as to identify the Soret band; H_2O_2 (0.4 mM, 230 molar equivalents) was then added to oxidise the enzyme and hence allow observation of the corresponding band shifts.

Table 1
Comparison of N-terminal sequences of RBP with those encoding novel versatile peroxidases recently described for other strains

Strain	Enzyme	N-terminal sequence	Reference
Bjerkandera sp. B33/3	RBP	VAXPDGVNTA	This work
Bjerkandera sp. BOS55	Mnp-LiP hybrid	VACPDGVNTATNAACCALFAVRDDI	[2]
Bjerkandera sp. BOS55	BOS1 BOS2	VAXPDGVNTATNAAXXXLFAVRDDI VAXPDGVNTATNAAXXALFAVRDDI	[5]
Bjerkandera adusta DSM11310	MnP1	VAXPDGVNTATNAAXXALFAVRDDI	[1]
Bjerkandera adusta UAMH8258	MnP	VAXPDGVNTATNAAXXALFA	[22]
Pleurotus ostreatus	MnP2	V**T**C**AT**G**QT**TANEACCALFPILED	[23]
Pleurotus eryngii	MnPL1 MnPL2	**AT**C**D**DG**RT**TA-NAACCILFPILDDI **AT**C**A**DG**RT**TA-NAACCVLFPILDDI	[9]
Pleurotus eryngii	PS1 PS3	V**T**C**ATGQT**TANEAXXALFPI V**T**C**A**DG**NTV**	[7]

Note: Mismatches are denoted in bold, undetermined are denoted in underlined.

Results

Enzyme features

The efficiency of the purification steps was monitored by the RBBR dye-decolourising activity. The aforementioned activity pertaining to the extracellular fluid from a 5.5-L culture was 254 IU, and the associated specific activity was 5.4×10^{-3} IU mg^{-1}. One unit of enzyme activity (IU) was defined as the amount of enzyme that transforms 1 μmol of RBBR per minute.

Several peaks were obtained following resolution by Mono-Q ion exchange chromatography, which were characterised by distinct enzyme activities; fractions were collected and divided into four main pools, according to their RBBR decolourising activity and ligninolytic activity detected. One of the pools was found to contain a pure protein with high RBBR decolourising activity—as it produced a single band in SDS-PAGE (Fig. 1). This enzyme will hereafter be denoted as RBP (RBBR *Bjerkandera* Peroxidase), for short. Its RBBR decolourising and ligninolytic specific activities were: 11 IU mg^{-1}, for the RBBR decolourising activity; 12 IU mg^{-1}, for the LiP activity; 8.7[1] and 16[2] IU mg^{-1}, for the MnP activity and 3.7 IU.mg^{-1}, for the MIP activity (1 IU = 16.67 nKatal). The RBP exhibited also properties characteristic of the novel class of peroxidases that are able to oxidise manganese, as well as VA and DMP in manganese-independent reactions.

The molecular mass of RBP, estimated by 10% SDS-PAGE, was ca. 45 kDa (Fig. 1). The N-terminal amino acid sequence of RBP was VAXPDGVNTA. Comparison of the N-terminal sequence of said enzyme with those encoding other versatile peroxidases, shown in Table 1, reveals striking similarities, especially with regard to those enzymes obtained from *Bjerkandera* sp.

The absorption spectrum of the RBP protein exhibits a large Soret band at 407 nm, and two small peaks at 498 and 640 nm, which are indicative of a heme protein (Fig. 2). The A_{407}/A_{280} (RZ) value—which reflects the purity and spectral characteristics of the purified enzyme, was ca. 3.2 in 10 mM cacodylate buffer (pH 6.0). The estimated molar extinction coefficient at 407 nm was ca. 203 mM^{-1} cm^{-1}.

When hydrogen peroxide (225 molar equivalents) was added to the enzyme, the peak at 407 nm became broader and smaller, the peak at 498 nm shifted to 550 nm and the peak at 640 nm shifted to 655 nm. The A_{407}/A_{280} value changed to 1.3.

Enzyme kinetics

The effect of pH upon RBBR and DMP oxidation activities was duly studied. The optimum pH for RBBR oxidation was ca. 4.0 in 100 mM sodium tartrate buffer. The optimum

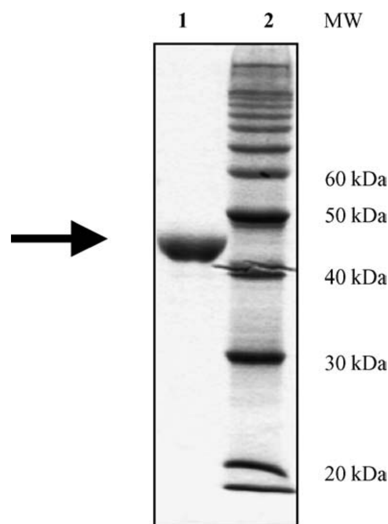

Fig. 1. SDS-PAGE electrophoregram of RBP. Lane 1, purified enzyme (indicated by an arrow) and lane 2, standard molecular weight markers.

[1] Specific activity for MnP, calculated using MBTH/DMAB as substrates.
[2] Specific activity for MnP, calculated using DMP as substrate.

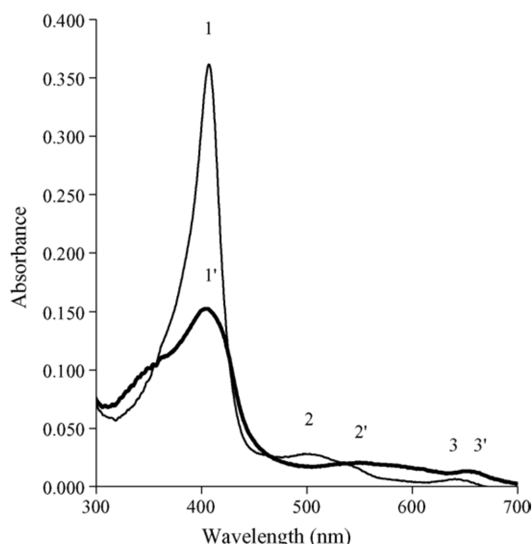

Fig. 2. Absorption spectrum of plain purified RBP 10 mM cacodylate buffer (pH 6.0) (—), and of RBP after addition of 0.4 mM hydrogen peroxide (▬); 1, 2 and 3 denote major peaks in plain RBP; 1′, 2′ and 3′ denote major peaks in RBP after addition of hydrogen peroxide.

pH range for oxidation of DMP in the presence of Mn(II) was between 4.5 and 5.0 on the same buffer. In the absence of Mn(II), but in the presence of EDTA (a chelating agent thereof), the optimum pH range was 3.0–3.5.

The stability of RBP, at various pH values, was studied as the oxidation activity on RBBR and DMP remaining after overnight incubation at 4 °C in 100 mM sodium tartrate buffer (pH range 2.0–5.5). For both substrates, the enzyme was stable at pH values above 3.5.

In order to ascertain the substrate specificity of this enzyme, the oxidation abilities towards a variety of potential substrates—including the aforementioned two dyes, were determined (Tables 2 and 3). RBP was able to oxidise all compounds tested, hence confirming its high versatility and broad specificity. RBP was also able to oxidise Poly-R 478 (50.00 ΔAbs min^{-1} mg^{-1} at 420 nm), *o*-anisidin (95.00 ΔAbs min^{-1} mg^{-1} at 460 nm) and *p*-anisidin (70.00 ΔAbs min^{-1} mg^{-1} at 460 nm) in the absence of mediators-although actual activity values were not determined.

The kinetic constants for selected substrates at given pH values, were duly calculated; the apparent values of K_m and V_{max}, are tabulated in Table 2.

Table 2
Kinetic constants for RBP oxidation of selected substrates

Substrate	pH	K_m (μM)	V_{max} (IU mg^{-1})
H_2O_2 (0.1 mM Mn(II))	5.0	7	250
H_2O_2 (0.05 mM RBBR)	5.0	3	25
H_2O_2 (1.0 mM DMP; 1.0 mM EDTA)	3.0	72	25
H_2O_2 (0 5 mM VA)	3.0	182	28
Mn(II)	5.0	86	4
Mn(II) (1.0 mM DMP)	5.0	55	125
DMP (1.0 mM Mn(II))	5.0	23	83
DMP (1.0 mM EDTA)	5.0	100	13
DMP(1.0 mM Mn(II))	3.0	79	25
DMP (1.0 mM EDTA)	3.0	48	13
RBBR	5.0	1	13
VA	5.0	3670	83
VA	3.0	1500	13
ABTS	5.0	5	25
ABTS	3.0	5	25

Table 3
Specific activity of RBP for the oxidation of selected aromatic substrates[a]

Substrate	Wavelength (nm)	Specific activity (IU mg^{-1})
Cathecol	238	168
Hydroquinone	247	30
4-Methoxyphenol	253	47
Methylhydroquinone	250	28
p-Aminophenol	246	85
Guaiacol	456	8
Ferulic acid	310	43
α-Naftol	255	21
Reactive Black 5	598	1

[a] Measured in 100 mM sodium tartrate (pH 3.5).

Discussion

The homogeneous preparation of RBP was a brownish-red solution, hence suggesting the presence of a heme group. The spectral characteristics of RBP are similar to those of typical peroxidases; the Soret band representative of the absorption peak of peroxidases [15] was observed at 407 nm for the native RBP; the shifts in the smaller peaks observed are also similar to those reported in literature [15,16] for peroxidases containing a protoheme as prosthetic group. The molar extinction coefficient obtained at 407 nm is of the same magnitude of those reported for ligninolytic peroxidases [15–17]. The absorption spectrum is similar, but not identical to the one reported elsewhere [18] pertaining to a versatile peroxidase from *B. adusta* which shows a Soret band at 409 nm, and two charge transfer bands at 501 nm and 632 nm.

It has been suggested that, in LiP and MnP, the heme iron atom is predominantly in the hexacoordinated high spin state, with a water molecule bound at the sixth coordination position [19]. The similarity of the spectra obtained pertaining to RBP and to those of LiP and MnP made available from other authors [20,21] suggests that the major heme state of RBP is also a hexacoordinated high spin state.

It is usually claimed that changes in absorbance peaks, such as those observed in RBP following addition of hydrogen peroxide, arise because of formation of compound I; however, this transformation of native peroxidase is usually completed with the addition of one molar equivalent of the aforementioned compound [17]. It was also reported [17] for manganese peroxidase from *P. chrysosporium* that the addition of two equivalents of hydrogen peroxide produced compound II, and that the addition of 250 equivalents

(considered as an excess of hydrogen peroxide) produced compound III. In fact, the amount of such a reagent used in this assay (225 molar equivalents) is very close to this value but the spectrum produced is still similar to that observed for compound II of MnP and HRP [17]. The amount of hydrogen peroxide required, at pH 4.5, to produce MnP compound III from *P. chrysosporium* (ca. 250 equivalents) is similar to that required to produce HRP compound III [17], yet much greater than the amount needed to obtain LiP compound I from *P. chrysosporium* (25 equivalents) at pH 6.0 [16]. As the pH used in the RBP assay was 6.0, this fact might reflect structural and functional differences between LiP and the RBP versatile enzyme. Differences between RBP and MnPs could in fact arise, partially because of the pH used in the assay. According to Renganathan and Gold [16], the formation of LiP compounds I and II is strongly influenced by reaction pH—and so this effect might also be present for other peroxidases. The catalytic differences, between RBP and other manganese peroxidases, observed may also help to explain those values. In general, the presence of such too high an excess of hydrogen peroxide (225 molar equivalents) in the absence of a reducing substrate leads to emergence of compound III, both for LiP and MnP.

The value of 45 kDa estimated for the molecular mass of the enzyme via SDS-polyacrylamide gel electrophoresis is similar to that of the other versatile peroxidases—which, at present, encompasses only *Bjerkandera* and *Pleurotus* sp. strains [1–6,8,9]; it is also of the same order than the molecular masses of typical ligninolytic peroxidases. Comparison of the N-terminal sequence of the purified RBP enzyme to those of the novel versatile peroxidases—recently described to be produced by other strains [2,4,5,7,9], indicates that the highest degree of similarity is associated with enzymes isolated from *Bjerkandera* sp., as expected.

With the exception of VA, all substrates tested exhibit apparent K_m values (at one or more of the conditions tested) similar or lower than those found for Mn(II) oxidation—hence suggesting a high affinity of the RBP for those substrates. This RBP enzyme shows also high affinity for (the oxidising substrate) hydrogen peroxide at pH 5.0, with K_m values (3 and 7 μM) much lower than those found for *B. adusta* LiP isoenzymes (40–60 μM)—but similar to those obtained for the *Pleurotus* and *Bjerkandera* spp. versatile peroxidases [2,3,5,7,9].

Comparison of K_m values for the oxidation of Mn(II) also revealed high similarity with values obtained for the other versatile peroxidases. Smaller values of K_m for DMP oxidation were obtained, as expected, at or near the optimum pH values (as previously determined) for each condition tested.

The smallest K_m values obtained were for oxidation of RBBR and ABTS (1 and 5 μM, respectively); this fact confirms RBP as a versatile peroxidase with high affinity for dye substrates, e.g. anthraquinone-derived, and high redox compounds, e.g. ABTS (which are substrates usually preferred by plant peroxidases).

Oxidation of VA is favoured at pH 3.0, as happens with LiP peroxidases, and has an apparent K_m value of 1500 μM. This value is one half of those reported for other versatile peroxidases (viz. 3000–5330 μM) [3,5,7,9], with the exception of the MnP–LiP hybrid obtained from *Bjerkandera* sp. strain BOS55 (viz. 534–116 μM) [2]. Such small K_m value for VA oxidation is probably the main characteristic that distinguishes this MnP–LiP hybrid peroxidase from others in the same versatile group. The difference found in the K_m values pertaining to VA oxidation suggests that RBP and MnP–LiP hybrid peroxidase could actually be isozymes. *Bjerkandera* strains might produce more than one different versatile peroxidase, as happens with the two versatile peroxidases already demonstrated to be present on *P. eryngii*.

Conclusions

A novel versatile peroxidase (RBP), possessing unique kinetic and spectral characteristics, was isolated and purified from a *Bjerkandera* strain isolate. This enzyme is able to oxidise manganese, as well as VA and DMP in manganese-independent reactions. Despite the differences found in terms of K_m values, namely for VA oxidation, RBP seems closely related to other versatile peroxidases from strains of the genera *Bjerkandera* or *Pleurotus*. However, the differences found between the versatile peroxidases of *Bjerkandera* strains point for the presence of at least two different versatile peroxidases on this genus, as already found within the *Pleurotus* genus.

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