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ORIGINAL ARTICLE

# New insights on the features of the vinyl phenol reductase from the wine-spoilage yeast *Dekkera/Brettanomyces bruxellensis*

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**Abstract** Vinyl phenol reductase activity was assayed in extracts from 19 strains of *Dekkera bruxellensis* isolated from wine. In all strains, vinyl phenol reductase activity was insensitive to the presence/absence of 4-vinyl guaiacol, confirming that expression is not related to the presence of the substrate. *D. bruxellensis* CBS 4481 showed the highest vinyl phenol reductase activity toward 4-vinyl guaiacol. Vinyl phenol reductase from *D. bruxellensis* CBS 4481 was purified to mass spectrometric homogeneity, and sequenced by trypsinolysis and mass spectrometry. The sequence of the purified protein showed convincing homology with a Cu/Zn superoxide dismutase in the *D. bruxellensis* AWRI 1499 genome, and indeed it was found to possess both vinyl phenol reductase and superoxide dismutase activities. A bioinformatics analysis of the sequence of vinyl phenol reductase/superoxide dismutase from *D. bruxellensis* CBS 4481 reveals the presence in this protein of cofactor-binding structural features, that are absent in sequences of superoxide dismutases from related microorganisms, that do not display vinyl phenol reductase activity.

**Keywords** *Dekkera bruxellensis* · Volatile phenols · Vinyl phenol reductase · Mass spectrometry · Superoxide dismutase

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## Introduction

Contamination with *Dekkera bruxellensis* is a major cause of wine spoilage, causing significant economic losses. *D. bruxellensis* produces off-flavours (namely, ethylphenols) from hydroxicinnamic acids in wine, leading to an unpleasant taint described as 'phenolic odour' (Chatonnet et al. 1992), and to other noticeable sensory effects (Licker et al. 1999), that in some cases has been considered to improve complexity (Brandam et al. 2008). Red wines were found to contain up to 6,047 μg/L (perception threshold: 440 μg/L) and 1,561 μg/L (perception threshold: 40 μg/L) of 4-ethyl phenol and 4-ethyl guaiacol, respectively. *D. bruxellensis* has been isolated from aged wines worldwide, vineyards and grapes being important sources of contamination (Agnolucci et al. 2007; Renouf and Lonvaud-Funel 2007).

It appears that *D. bruxellensis* developed the capability to use wood sugars, such as cellobiose, extracted by ethanol from toasted barrels during wine aging (Aguilar Uscanga et al. 2007). *D. bruxellensis* is not considered a natural competitor of *Saccharomyces cerevisiae* and it is detected in wine after the alcoholic fermentation. Due to the harsh conditions in wine (pH, oxygen limitation, starvation, high ethanol and $SO_2$ concentrations), the yeast drastically reduces its specific growth rate and produces off-flavours during exponential growth (Agnolucci et al. 2009).

In the proposed pathways, suitable acids are first decarboxylated to vinylphenols by hydroxycinnamate decarboxylases (HCDs; EC 4.1.1.) and then reduced by vinyl phenol reductases (VPRs; EC 1.3.1.74), that play a key role in *D. bruxellensis* (Chatonnet et al. 1992). Godoy et al. (2008) purified a *p*-coumarate decarboxylase from *D. bruxellensis*, but no conclusive evidence for the expression of a functional VPR was claimed. A complete characterization of VPR has been hampered by the fast enzyme inactivation in its isolated form (Harris et al. 2009). Detailed information on the biological basis that regulate production of ethyl phenols is still missing, although growth rate and off-flavor production appear strain-dependent (Vigentini et al. 2008).

The present study was aimed at characterizing the VPR produced by a "high volatile phenols producer" strain of *D. bruxellensis*, and involved: (1) screening different strains of *D. bruxellensis* for their ability to produce volatile phenols and/or to show a detectable VPR activity; (2) obtaining enzyme of purity appropriate for molecular characterization; and (3) carrying out a bioinformatics-based study of the purified protein.

## Materials and methods

### Strains

Nineteen *D. bruxellensis* strains belonging to the CBS International collection (Vigentini et al. 2012) were screened for VPR activity, namely: CBS 73, CBS 74, CBS 1940, CBS 1941, CBS 1942, CBS 1943, CBS 2336, CBS 2499, CBS 2547, CBS 2796, CBS 2797, CBS 4459, CBS 4601, CBS 4602, CBS 4480, CBS 4481, CBS 4482, CBS 5206, and CBS 5513. All were maintained at −80 °C in YPD medium [1 % yeast extract, 2 % peptone, 2 % glucose (w/v)] with 20 % (v/v) glycerol. Two strains (CBS 4481 and CBS 2797) were used for VPR purification and characterisation.

### Preparation of cell extracts

Cells in exponential phase (48 h culture at 30 °C in YPD medium, 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, pH 5.6) were harvested (10,000 *g*, 30 min, 4 °C) and washed in 100 mM Tris–HCl buffer, pH 8.0. Pellets were suspended in the same buffer, containing 0.1 mM dithiotreitol, and cells broken with 0.45 mm glass beads (6 × 4 min cycles, 1 min ON, 1 min OFF). Extracts were clarified by centrifugation (10,000 *g*, 30 min, 4 °C), and stored at −80 °C. Proteins were measured colorimetrically (Bradford 1976).

### Vinyl phenol reductase (VPR) assay

VPR activity was measured at 23 °C by following spectrophotometrically the NADH-dependent enzymatic reduction of 4-vinyl guaiacol to 4-ethyl guaiacol at 340 nm. The reaction was started by adding 0.3 mM 4-vinyl guaiacol to a 0.2-mM solution of NADH in 50 mM phosphate buffer, pH 7.0. The substrate, 4-vinyl guaiacol, was omitted from controls. One unit is defined as the amount of enzyme oxidizing 1 μmol of NADH per minute under these assay conditions.

Formation of 4-ethyl phenol was confirmed by HPLC analysis after 1 h reaction, using a Waters 2695 Alliance HPLC module, a Waters 2487 double-wavelength UV detector (Waters, Milford, MA, USA), and a Vydac C18 column (3.9 mm ×150 mm; Vydac, Hesperia, CA, USA). HPLC solvents were water/trifluoracetic acid (TFA) 0.1 % (v/v) (solvent A) and acetonitrile/TFA 0.1 % (v/v) (solvent B). Elution was achieved at 1 mL/min flow at increasing B (20 to 35 % in 5 min, and 35 to 80 % in 15 min). Vinyl- and ethyl-guaiacol were detected at 260 and 280 nm, respectively, after injecting 0.1 mL of the reaction mixture, made protein-free by ultrafiltration on Centricon® devices (nominal cut-off, 5 kDa).

### Superoxide dismutase assay

Total SOD activity was measured at 23 °C in 1.0 mL of 50 mM phosphate buffer pH 7.5, containing 0.1 mM EDTA, 0.06 mM nitroblue tetrazolium (NBT), and 0.1 mM NADH. For the assay, an appropriate aliquot of the protein to be tested (either the purified protein or a commercial bovine Cu/Zn SOD, S9697; Sigma-Aldrich, St. Louis, MO, USA) was added just before starting the reaction leading to with 20 μL of freshly prepared phenazine methosulfate (PMS, 0.1 mM in 50 mM phosphate, 0.1 mM EDTA, pH 7.5). The increase in $A_{560}$ nm due to NBT reduction by the superoxide anion were monitored. No changes were observed in the absence of NADH or PMS.

### Purification of VPR

The crude cell extract was concentrated 8-fold by ultrafiltration (5 kDa nominal cut-off membranes; Millipore, Bedford, MA, USA) and diluted to the original volume in 40 mM Tris–HCl, pH 8.0. The extract was applied to an anion exchanger (DEAE-cellulose) equilibrated in the same buffer. Proteins were eluted using a stepwise NaCl gradient (0.1; 0.25; 0.5; 1 M). Fractions with enzymatic activity were pooled, concentrated and diluted in 25 mM phosphate buffer, pH 6.5. After a further concentration step, proteins were applied onto a strong cation exchanger (SP-Sepharose; GE Healthcare Europe, Milan, Italy) pre-equilibrated in 25 mM phosphate buffer, pH 6.5, and eluted using a stepwise NaCl gradient (0.1; 0.25; 0.5 M) in 25 mM MES buffer, pH 6.0. Active fractions were concentrated by ultrafiltration, and separated by size exclusion chromatography (SEC) (Superdex 200, 10/300 GL; GE Healthcare) in 25 mM MES buffer, 150 mM NaCl,

pH 6.0. Active fractions were pooled and concentrated in 25 mM phosphate buffer, pH 6.0.

In another set of experiments, the active protein fractions recovered from the DEAE column were applied to a weak cation exchanger (CMC-cellulose) in acetate buffer (25 mM, pH 5.0). Proteins were eluted with a stepwise NaCl gradient (0.1; 0.25; 0.5; 1 M), and further separated by SEC (Superdex S-75, 10/300; GE Healthcare) in 25 mM acetate, pH 5.0, containing 150 mM NaCl.

### SDS-PAGE and in-gel digestion of proteins

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Miniprotein apparatus (Bio-Rad, Milan, Italy), using 12 % gels and Precision Plus Protein™ All Blue molecular weight markers (Bio-Rad) and Coomassie Brilliant Blue R-250 staining. For in-gel digestions, gels were destained with 50 % acetonitrile (ACN) in 50 mM $NH_4HCO_3$, treated with 10 mM DTT (45 min at 56 °C), and reacted with 50 mM iodoacetamide (45 min, room temperature). Gels were washed with 50 mM $NH_4HCO_3$, dried in 100 % ACN, and vacuum-centrifuged prior to be taken up into 20 μL of 25 mM $NH_4HCO_3$ containing 15 μg/mL of trypsin (Sigma Aldrich, St. Louis, MO, USA). After incubation on ice for 45 min, the excess solution was removed, and another 20 μL of 25 mM $NH_4HCO_3$ were added. Digestion was left to proceed overnight at 37 °C. Peptides were extracted thrice with 50 % aqueous ACN containing 5 % formic acid (FA) with intermittent sonication, and dried in a vacuum centrifuge. The resulting solids were dissolved in 0.1 % (v/v) aqueous trifluoroacetic acid and analyzed by nanoLC-MS/MS.

### Mass spectrometry analysis

Molecular mass of the purified protein was determined using a Q-STAR mass spectrometer with a nanospray interface from Protana (Odense, Denmark). Samples were sprayed from gold-coated 'medium length' borosilicate capillaries (Protana). Capillary voltage was 800 V. Analysis of proteins were carried out by nanoLC-ESI MS/MS using an Ultimate 3000 HPLC (Dionex, Sunnydale, CA, USA) coupled to a Q-STAR spectrometer (Applied BioSystems, Framingham, MA, USA). Eluents were: (A) 5 % ACN in 0.1 % FA, and (B) 80 % ACN in 0.08 % FA. Peptides were loaded onto a $C_{18}$ loading cartridge (LC Packings, Sunnyvale, CA, USA) and separated with a C18 PepMap100 column (15 cm length, 75 μm ID, 300 Å; LC Packings), using a linear gradient (5-40 % B over 60 min) at a flow rate of 300 nL/min.

LC-MS/MS experiments were performed in information dependent acquisition (IDA) mode. Precursor ions were selected using the following MS to MS/MS switch criteria: ions greater than m/z 400; charge states 2 to 4; intensity exceeding 15 counts. Former target ions were excluded for 30 s and ion tolerance was 50.0 mmu. CID was used to fragment multiple charged ions, and nitrogen was used as the collision gas. The raw spectra files were converted to text files in Mascot generic file format (.mgf) for submission to the Mascot v.2.3 (http://www.matrixscience.com) and Batch-tag (Protein Prospector, University of California San Francisco, USA) search engines.

## Results and discussion

### Screening for vinyl-phenol reductase (VPR) activity

Screening for VPR activity was carried out by using extracts of cells grown on YPD liquid medium in the presence and in the absence of 4-vinyl guaiacol (0.7 mM). Presence/absence of the substrate in the medium gave no significant differences in VPR activity (not shown), confirming reports about the constitutive nature of this enzyme in *D. bruxellensis* (Tchobanov et al. 2008).

As reported previously (Tchobanov et al. 2008), the VPR activity measured with NADH was much higher than with NADPH (not shown). Formation of 4-ethyl guaiacol was confirmed by HPLC, giving activity values consistent with the spectrophotometric measurements. Three different ranges of VPR specific activity were observed in the strains tested here (Table 1), and confirm that strains could be discriminated for their aptitude to produce off-flavours (Conterno et al. 2006; Vigentini et al. 2008; Harris et al. 2009). *D. bruxellensis* CBS 4481 gave the highest specific VPR activity (0.159 U/mg total protein). This strain also produced the highest amounts of ethylphenols when grown under model-wine conditions in the presence of cinnamic acids (Vigentini et al. 2008) and was used for carrying out enzyme purification.

### Purification of VPR from *D. bruxellensis* CBS 4481

Previous studies reported that no VPR activity could be detected in cell extracts after 24 h, because of fast enzyme inactivation (Harris et al. 2009). Although VPR activity was highly unstable in fresh cell extracts, a first step of purification and storage in 25 mM acetate buffer pH 5.0 at 4 °C allowed retention of 70–80 % of the original activity after 2 weeks.

Following classical purification protocols (Table 1S), VPR activity was not bound to anion exchangers at pH 8.0. The unbound fraction from the anion exchanger also did not bind to a strong cation exchanger in different buffers at pH 6.5 or 6.0. The active materials loaded on a SEC column gave fractions with VPR activity, that resulted to contain four protein bands (P1, P2, P3 and P4) by SDS-PAGE (Fig. 1S). These bands were cut out and submitted to nano-LC ESI MS/MS protein sequencing. A MASCOT search results gave the

**Table 1** Specific activity of VPR in various *D. bruxellensis* strains

| VPR specific activity (U/mg of total proteins) | CBS designation |
|---|---|
| Low activity (0–0.040) | 73, 74, 1940, 1941, 1942, 1943, 2336, 2499, 2547, 2796, 2797, 4482, 4602, 5206 |
| Medium activity (0.041-0.080) | 4480, 5513 |
| High activity (>0.081) | 4459, 4481, 4601 |

closest match to the purified proteins as present in the database. All four proteins matched sequences found in the genome of *D. bruxellensis* AWRI 1499 (Table 2S). A search in the Universal Protein Resource (Uniprot) database indicated a link to metabolic and/or redox activities (Table 2S). Two additional proteins (5 and 6; Table 2S) were also identified by nano-LC ESI MS/MS sequencing of the active fraction from SEC prior to SDS-PAGE separation, being homologous to carboxypeptidase-y (gi|385301734, 5), and to peroxiredoxin prx1 (gi|385304076, 6) in the *D. bruxellensis* AWRI 1499 genome.

Tchobanov et al. (2008) also obtained a protein fraction that exhibited VPR activity and was made up of three major bands, identified as triosophosphate isomerase, superoxide dismutase and carboxypeptidase-y. These proteins were also detected in our SEC fractions with VPR activity. These findings support the hypothesis that VPR activity should be related to one of them.

To investigate whether the different physiological aptitude of strains to produce off-flavours was related to a different protein expression, the same purification steps described above were carried out on extracts from a strain with low VPR activity (*D. bruxellensis* CBS 2797). SDS PAGE tracings for the fraction obtained from CBS 2797 were almost identical to those reported in Fig. 1S for CBS 4481, highlighting the difficulties of using a comparative proteomic approach for differentiating strains with different VPR activity.

## Optimization of the VPR purification strategy

Purification was improved by using a weak cation exchanger instead of the strong exchanger used above. These substitutions gave a lower losses of activity through the various purification steps, including SEC. Fig. 2S shows the chromatographic and SDS PAGE profile of the fraction showing VPR activity.

The homogeneity of the VPR active fraction was confirmed by nanoESI-MS analysis, which gave a molecular mass of 15674 Da (carboamydomethyl protein derivative) for the SEC-purified protein (Fig. 1). This, along with the difference between the apparent molecular mass of the native protein (≈30 kDa by SEC) and of the denatured protein (≈20 kDa by SDS PAGE) suggests a dimeric enzyme.

An aliquot of the VPR-active protein from the SEC column was digested with trypsin after alkylation with iodoacetamide. NanoLC-MS/MS analysis of peptides in tryptic digests gave a 70 % protein sequence coverage, and the protein was

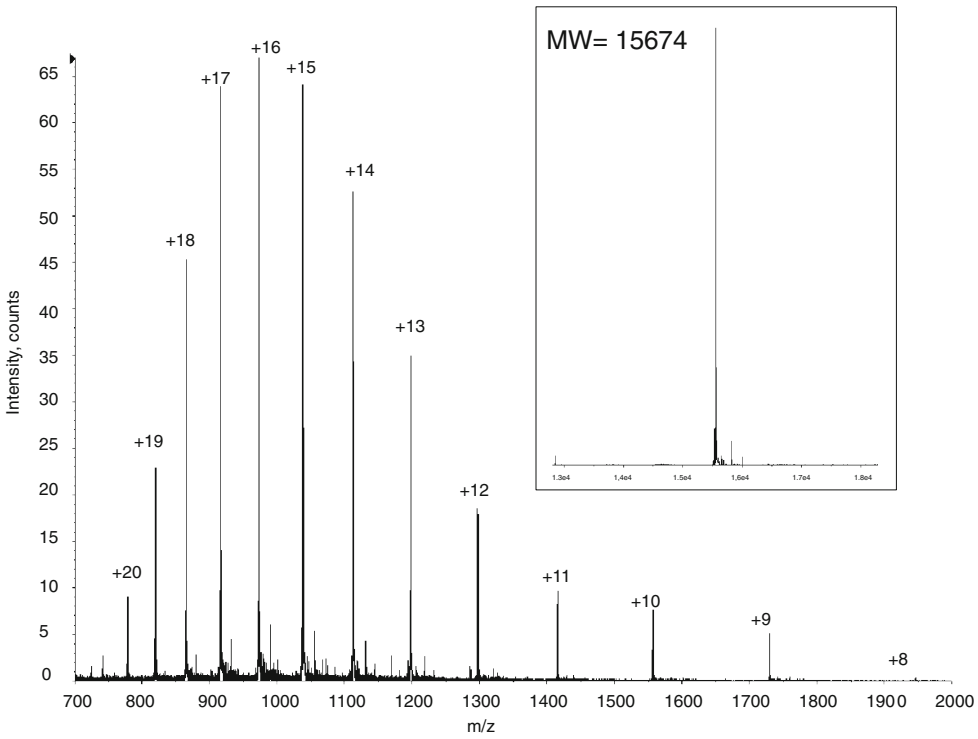


**Fig. 1** NanoESI-MS spectrum of purified and alkylated SOD protein obtained from GPC on a S-75 Superdex column. *Inset* transformed spectra on a real mass scale. Molecular mass was 15,674.0 Da

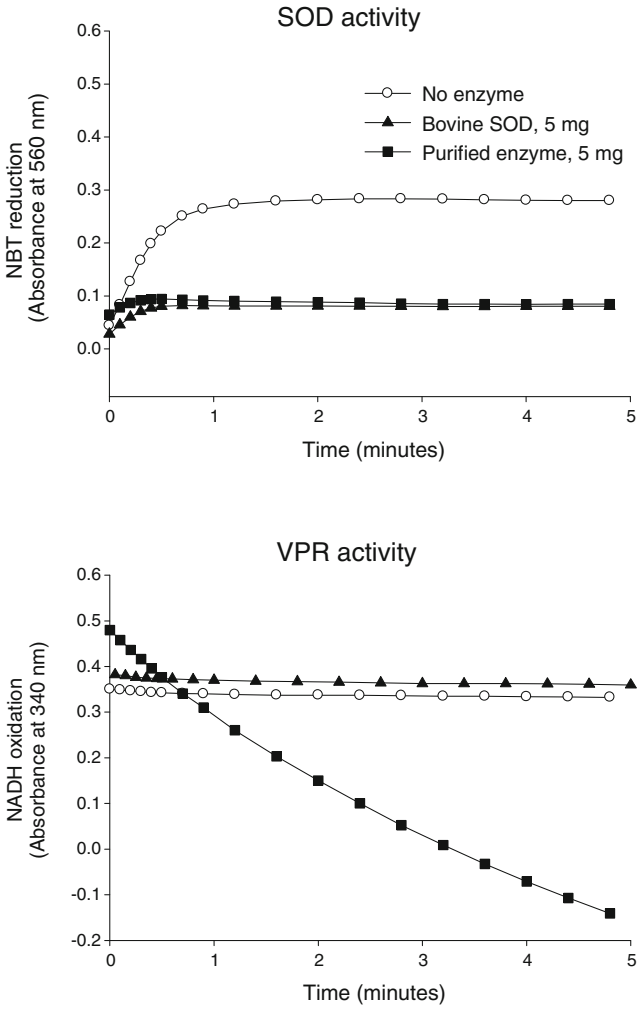


**Fig. 2** SOD (**a**) and VPR (**b**) activities in the protein purified from *D. bruxellensis* CBS 4481

univocally identified as a superoxide dismutase (SOD; EC 1.15.1.1) in the *D. bruxellensis* genome (Uniprot database: I2JWC1; NCBI database: gi|385303182).

Indeed, as shown in Fig. 2, the VPR-active fraction coming from the SEC column was found to have SOD activity, which was comparable (on a protein basis) with that of a SOD standard. This rules out the possibility that minor impurities could be responsible for the simultaneous presence of SOD and VPR activity in the same protein. Indeed, RP-HPLC of the protein fraction from SEC column (Fig. 3S) confirmed the purity of VPR-active protein, as tryptic digestion of each of the three peaks detectable in the RP-HPLC profile in Fig. 3S gave the same peptide pattern as the bona-fide SOD/VPR present as the main component (see Table 2). This, and the MS evidence discussed above, suggest that the minor species separated by RP-HPLC from the SEC-purified could be related to different forms of the same enzyme.

Both the Uniprot (I2JWC1) and the NCBI (gi|385303182) databases return only partial sequences for *D. bruxellensis* SOD, as some amino acid residues (32 and 110, in particular) and the N-terminal sequence of the mature protein have never been determined. Manual exploration of the peaks along the LC chromatogram of tryptic peptides and de-novo sequencing of unassigned MS/MS spectra allowed the identification of a peptide at $MH^+$ 840.55, corresponding to the N-terminal peptide (sequence 2–9; Fig. 3a; Table 2) without the N-terminal Met. Sequencing of peptides 16–44 (Fig. 3b) and 107–116 (Fig. 3c) allowed to define residues 32 (Asn) and 110 (Ala). Thus, considering Val-2 at the mature amino terminus and the previously unassigned amino acids, the theoretical mass of the isolated active VPR matches that reported above for its carbamidomethyl derivative (15,674 Da; Fig. 1).

**Table 2** Identification by nanoLC-MS/MS of the superoxide dismutase tryptic peptides

| Measured mass[a] | Position[b] | Sequence[c] | Note |
|---|---|---|---|
| 840.55 | 2–9 | VKAVAVVR | N-terminal sequence confirmed by ESI-MS/MS (Fig. 3a) |
| 606.30 | 10–15 | GDSTVK | |
| 3,195.50 | 16–44 | GVVTFEQTSESEPTTINYNIEGNDPNALR | Sequence identified by MS/MS-based de novo sequencing (Fig. 3b) |
| 2,817.25 | 45–70 | GFHIHTFGDNTNGCTSAGPHFNPFGK | |
| 1,097.49 | 71–80 | THGAPTDENR | |
| 952.51 | 81–89 | HVGDLGNIK | |
| 775.39 | 90–97 | TDANGVAK | |
| 661.38 | 98–103 | GTIKDK | |
| 358.26 | 104–106 | LVK | |
| 1,013.60 | 107–116 | LIGANSIIGR | Sequence identified by MS/MS-based de novo sequencing (Fig. 3c) |
| 1,311.68 | 117–129 | TVVVHAGTDDLGK | |
| 2,243.08 | 130–154 | GGDAGSLQTGNAGGRPACGVIGLSA | |

[a] $MH^+$ measured by MS experiment.

[b] Numbers indicate the amino acid residues at the extremities of each peptide refers to superoxide dismutase (NCBI, gi|385303182; Uniprot, I2JWC1)

[c] Sequence peptides

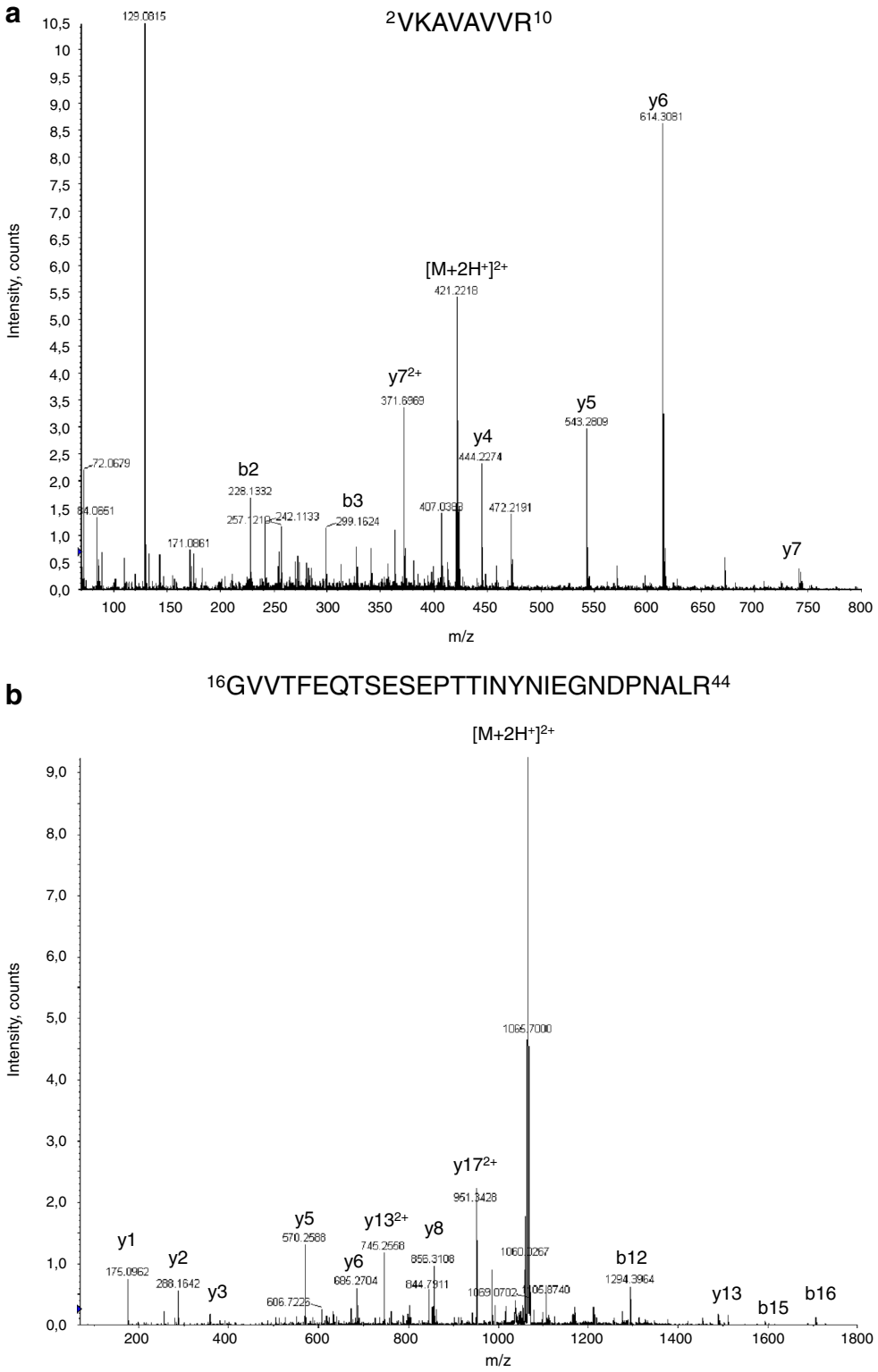


**Fig. 3** MS/MS spectra of tryptic peptides. **a** Double-charged ion at m/z 421.22 Da ($MH^+$ 841.55 Da) for the N-terminal peptide 2–10; **b** MS/MS-based *de novo* sequencing of peptide 16–44 (double-charged ion at m/z 1,065.70 Da ; $MH^+$ 3,195.50 Da); **c** MS/MS-based de novo sequencing of peptide 107–116 (double-charged ion at m/z 507.2393 Da; $MH^+$ 1,013.60 Da). Residues not identified in the previously reported sequence are *underlined*

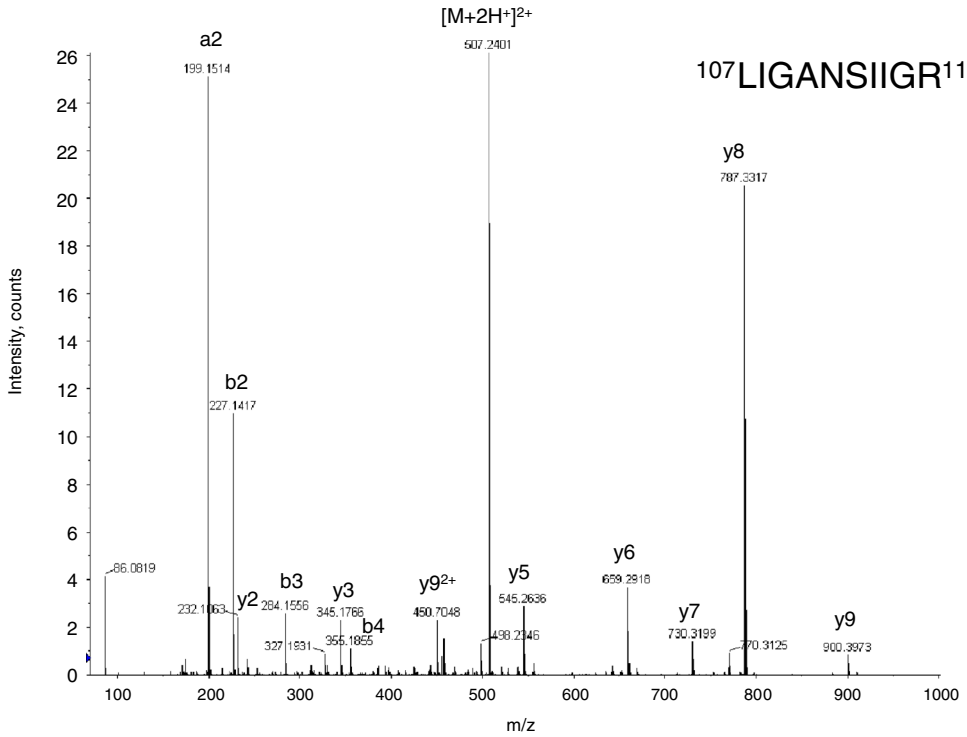


**Fig. 3** continued.

A bioinformatics-based comparison of the VPR/SOD complete sequence as determined in this study is presented in Fig. 4, where the sequence determined in this study is compared with those of other yeast SODs. Proteins with the same reported function and high similarity sequences were found in some yeasts (e.g. *Komagataella pastoris*, 82 % identity), whereas sequence similarities with *S. cerevisiae* SOD were much lower (74 %).

Bioinformatics evidence also indicates that VPR enzymes belong to the short chain dehydrogenases/reductases (SDRs) family, a functionally diverse family of NAD(P)(H)-dependent oxidoreductases that have a single domain with a structurally conserved Rossmann fold, a NAD(P)(H)-binding region, and a structurally diverse C-terminal region. A large variability of structural details is noticed within this enzyme family. Some atypical SDRs have lost catalytic activity and/or have an unusual NAD(P)-binding motif and may be missing active site residues (or have unusual ones in place of the "standard" ones; Oppermann et al. 2003; Jçrnvall et al. 1995).

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                    <------><----><---------------------------><---------><----
D.b. SOD             VKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS 60
gi|254574244        MVKAVAVLRGDSTVGGTVVFEQSSESSPTTITYDIKGNSPNAERGFHIHQFGDNTNGCTS 60
gi|51243303         MVQAVAVLKGDAGVSGVVKFEQASESEPTTVSYEIAGNSPNAERGFHIHEFGDATNGCVS 60
                    **:****::**: * *.* ***:***.***: *:* **.*** ****** *** ****.*

                    ---------><---------><-------><------><--><><-><--><----><---
D.b. SOD            AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV 120
gi|254574244        AGPHFNPFGKTHGAPTDEARHVGDLGNVKTDAEGVAKGVITDNQVKLIGETSILGRTVVI 120
gi|51243303         AGPHFNPFKKTHGAPTDEVRHVGDMGNVKTDENGVAKGSFKDSLIKLIGPTSVVGRSVVI 120
                    ******** ********* *****:**:*** :***** :.*. :**** .*::**:**:

                    <-------><------------------------>
D.b. SOD            HAGTDDLGKGGDAGSLQTGNAGGRPACGVIGLSA 154
gi|254574244        HDGTDDLGKGGHADSLKTGNAGGRPACGVIGLAA 154
gi|51243303         HAGQDDLGKGDTEESLKTGNAGPRPACGVIGLTN 154
                    * * ******.   **:***** *********:
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**Fig. 4** Multiple sequence alignments of *D. Bruxellensis* Cu-Zn SOD (*D.b.* SOD) with other yeast SOD in the NCBI database. The alignment was performed using CLUSTALW2. Identical residues are indicated by *asterisks*. *Colons* and *semicolons* indicate conservative and semi-conservative substitutions, respectively. Sequence of the Cu-Zn SOD from *D. bruxellensis* includes the previously non-identified residues detected in this study. *Arrows* denote the peptides identified by LC/MS/MS. Other proteins are: Cu-Zn SOD from *Komagataella pastoris* (gi|254574244|); Cu-Zn SOD from *Saccharomyces cerevisiae* (gi|254574244|)

The most conserved feature in this family is a structural motif characterized by a highly variable Gly-rich sequence pattern (**GXGXXG**), critical for accommodating and binding the pyrophosphate portion of the nucleotide cofactor. In this sequence, the first 2 glycines participate in NAD(P)-binding, and the third one facilitates close packing of the Rossmann fold (Lesk 1995). A consensus binding pattern for NAD(P)H appears to be present in the sequence of *D. bruxellensis* VPR/SOD (128–134, **GKGGDAG**), This specific sequence is not present in the sequence of either *S. cerevisiae* or *K. pastoris* SODs, where Gly-134 is substituted by a Glu and an Asp, respectively. The negative charge carried by either residue is absent in the *D. bruxellensis* VPR/SOD sequence, and this may concur to conferring NAD(H)-binding capability to the *D. bruxellensis* enzyme.

SDRs also typically contain another cofactor binding motif (**TGXXX**[**AG**]**XG**) most often located in the highly variable C-terminal region (Oppermann et al. 2003; Jçrnvall et al. 1995). A similar sequence could be detected in the *D. bruxellensis* VPR/SOD sequence (residues 136–141, **TGNAGG**), and is a located near the protein C-terminus. This cofactor binding motif is not present in the *S. cerevisiae* SOD, where Gly-141 is replaced by a structure-breaking Pro residue, thus impairing the binding of pyridine dinucleotides to the *S. cerevisiae* protein.

## Conclusion

Our purification procedure allowed us to obtain a highly purified protein with VPR activity, that also displayed significant SOD activity. The sequence of the purified protein was found to match that of a reported SOD in the *D. bruxellensis* genome. A band corresponding to SOD was observed previously (and in this study) in VPR-active fractions obtained by using less performing purification procedures (Tchobanov et al. 2008). Presence of similar SOD's in yeast different from *S. cerevisiae* may indicate that the moonlighting activity of *D. bruxellensis* SOD as a VPR is related to its capacity of catalyzing NADH-dependent reduction of vinyl phenols. This capacity may be related to specific $NAD(P)^+$/NAD(P)H binding sequences that are present in the *D. bruxellensis* SOD/VPR, but are severely altered in SODs from other wine-relevant yeasts.

The fact that this particular SOD belongs to the class of the Zn/Cu SODs (SOD1) may also explain the rapid inactivation of VPR activity (as a consequence of alteration in the metal ligation or of metal loss/substitution) during enzyme isolation. This should be considered when cloning/overexpressing this protein for functional/structural studies which seem worth pursuing in consideration of the novelty of these findings and of their practical implications and relevance.

Other SODs were found to display additional activities, including a nonspecific peroxidase activity (Hodgson and Fridovich 1975a, b; Liochev and Fridovich 2004), and a weak GSH-dependent denitrosylase activity (Jourd'heuil et al. 1999; Okado-Matsumoto and Fridovich 2007).

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