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SHORT COMMUNICATION

# Identification and Localization of *β*-D-Glucosidase from Two Typical *Oenococcus oeni* Strains

YAHUI LI, YANHONG MA*, KAIHONG HUANG and HONGZHI ZHANG

Institute of Agro-Product Processing, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu, China

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Abstract

β-D-glucosidase (βG) gene from *Oenococcus oeni* SD-2a and 31MBR was cloned, sequenced and analyzed, also intracellular βG of the two strains was further localized. The results showed that βG gene of the two strains was in high homology (> 99%) to reported βG gene, confirming both strains possess βG activity at the molecular level. Intracellular βG of SD-2a is a mainly soluble protein, existing mostly in the cytoplasm and to some extent in the periplasm. While for 31MBR, intracellular βG is mainly insoluble protein existing in the cytoplasmic membrane. This study provides basic information for further study of the metabolic mechanism of βG from *O. oeni* SD-2a and 31MBR.

Key words: *Oenococcus oeni*, β-D-glucosidase, βG cloned, gene localization

Wine fermentation is a complex process driven by microorganisms such as yeasts and lactic acid bacteria (LAB). Malolactic fermentation (MLF), taking place after alcoholic fermentation, is the bacterially driven decarboxylation of L-malic acid to L-lactic acid and carbon dioxide. This process improves the stability and quality of wines (Alexandre *et al.*, 2004; Sumby *et al.*, 2013). *Oenococcus oeni* is the most important LAB to conduct MLF in virtually all red wine and an increasing number of white wines (Bartowsky and Borneman, 2011; Olguin *et al.*, 2011). Although commercial *O. oeni* starters are used to induce MLF in the practice of winemaking at present, the majority of wineries prefer MLF to occur spontaneously. Thus, now more attention is being focused on the isolation and study of native LAB starters possessing important oenological characteristics (Ruiz *et al.*, 2008; 2010; Izquierdo *et al.*, 2009; Capozzi *et al.*, 2010; Mesas *et al.*, 2011; Dong *et al.*, 2014).

Several oenological characteristics, such as the presence of glycosidase activity, are considered as criteria for the selection of qualified MLF starters (Grimaldi *et al.*, 2005; Michlmayr *et al.*, 2012a). It has been well established that apart from free flavor compounds, a significant part of flavors remain in newly made wine as odorless non-volatile glycosides (Maicas and Mateo, 2005; Michlmayr *et al.*, 2010). The odorless glycosides containing aroma and flavor aglycones are not directly accessible to the olfactory mucosa and may affect wine quality greatly after hydrolysis (Mesas *et al.*, 2012; Michlmayr *et al.*, 2012b). β-D-glucosidase (βG) is one of the most important glycosidases to catalyze glycosylated precursors, releasing active aroma and flavor compounds during winemaking. βG activity in *O. oeni*, the main bacterial species conducting MLF, was confirmed more than 20 years ago (Guilloux-Benatier *et al.*, 1993). Over the past decades, numerous investigations have been conducted, providing evidence for the potential βG activity of *O. oeni* strains for flavor enhancement in wines (Spano *et al.*, 2005; Michlmayr *et al.*, 2010; Gagné *et al.*, 2011). It has been reported that the possession of glycosidic activities is widespread and strain dependent among *O. oeni* strains (Barbagallo *et al.*, 2004; Grimaldi *et al.*, 2005; Saguir *et al.*, 2009).

SD-2a and 31MBR are two important *O. oeni* strains widely used during winemaking in China. SD-2a is a patented strain screened from spontaneous MLF of wines in Yantai, Shandong Province, China, while 31MBR is a commercial strain prevalent in China with an excellent performance during MLF. The profile of βG activity for the two strains has been reported previously (Li *et al.*, 2012a; 2012b). It showed that both strains possess βG activity and the enzyme exists mainly in intracellular form. However, up to now, no information about its molecular basis is available. To better understand the nature of βG activity of the two strains, in the present study, βG gene of *O. oeni* SD-2a and 31MBR

* Corresponding author: Y. Ma, Institute of Agro-product Processing, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu, China; **e-mail: liqianhao217@126.com**

was cloned, sequenced and analyzed through bioinformatics, also the intracellular βG of the two strains was further localized.

*O. oeni* strains SD-2a and 31MBR, stored in our laboratory, were cultivated as described before (Li *et al.*, 2012a). Bacterial growth was monitored by measuring the $OD_{600}$nm until the mid-log phase (about 40 h and 20 h for SD-2a and 31MBR respectively). Genomic DNA was extracted with Genomic DNA isolation Kit (TaKaRa, Shiga, Japan) as recommended by the manufacturer and verified on a 1% (w/v) agarose gel. Primers 5'TTGTCTAAGATTACTT CAATTATT TCA 3' and 5' TTAACTTTGATTGGCGA GTTTA3', deduced from the nucleotide sequences of βG gene previously identified in *O. oeni* PSU-1 (Makarova *et al.*, 2006), were used. For the PCR experiments, 25 ng of genomic DNA isolated from *O. oeni* SD-2a or 31MBR was added to a 25 μl PCR mixture containing 0.5 U of ExTaq polymerase, 0.2 mM of dNTP mix, 1 × PCR buffer (TaKaRa, Shiga, Japan), and 0.25 mM of each primer. The reaction was carried out at the following temperature profile: 94°C, 4 min; 94°C, 1 min; 58°C, 40 s; 72°C, 1.5 min – 35 cycles (using the icycler PCR Bio-Rad). The PCR reaction was terminated at 72°C for 10 min. PCR fragments were analysed on gel electrophoresis by applying 5 μl of sample to 1.0% agarose gel and a 2000 bp ladder (TaKaRa, Shiga, Japan) was used as the standard marker. The amplified fragments were purified with PCR Clean-up Kit (TaKaRa, Shiga, Japan), connected with vector PMD18-T (TaKaRa, Shiga, Japan), and transformed into *Escherichia coli* DH5α. Plasmid was extracted using Plasmid Extraction Kit (TaKaRa, Shiga, Japan) and sequenced by Takara Biotechnology Co., Ltd. Dalian, China.

The alignment of the deduced protein sequences against that of *O. oeni* PSU-1 was carried out with software Clustalx 1.81. Physical and chemical parameters of deduced amino acid sequences were computed with ProtParam (Gasteiger *et al.*, 2005). The amino acid scale of hydropathicity was defined following the ProtScale (Gasteiger *et al.*, 2005). Transmembrane helices were predicted with TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMH MM-2.0/). The presence and location of signal peptide cleavage sites in amino acid sequence was predicted with SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). Finally, the subcellular localization prediction was conducted with PSORTb v. 3.0.2 (Yu *et al.*, 2010).

*β*G localization of SD-2a and 31MBR was conducted as described by de Cort *et al.* (1994) with some modifications. Bacterial growth was monitored by measuring the $OD_{600}$nm until the end of exponential growth phase (about 80 h and 40 h for SD-2a and 31MBR respectively). Then 10 ml cultures were centrifuged (5.000 g, 10 min, 4°C), washed with 0.5 M sodium malate buffer (pH 6.0), and recentrifuged to obtain whole cells.

The whole cells were resuspended in 10 ml 0.5 M sodium malate buffer (pH 6.0), then treated with lysozyme (Sigma) in a final concentration of 75 μg/ml and incubated for 2 h at 37°C until spheroplast formation was completed as confirmed by phase-contrast microscopic observation. Spheroplasts were harvested by slow-speed centrifugation (7.000 g, 20 min, 4°C), washed with 0.5 M sodium malate buffer (pH 6), and recentrifuged. The latter supernatant was mixed with the first supernatant as supernatant A (containing cell wall fragments and periplasmic-compounds). Spheroplasts were resuspended in a hypotonic solution (10 mM sodium malate buffer, pH 6), and lysed spheroplasts were centrifuged (24.000 g, 1.5 h, 4°C) to separate the particulate fraction (cell membrane) from the soluble fraction. Supernatant A was recentrifuged (24.000 g, 1.5 h, 4°C) to get the cell wall fragments and supernatant (containing periplasmic-compounds). The particulate fraction and cell wall fragments were resuspended in a minimum amount of 0.5 mM sodium malate buffer (pH 6) for enzyme assay along with other four parts prepared (spheroplasts, supernatant A, soluble fraction and periplasmic-compounds). βG activity assay was conducted following the method described previously (Li *et al.*, 2012b)

PCR amplification resulted in single gene products of *ca* 2200 bp on the genomic DNA from both *O. oeni* SD-2a and 31MBR (Fig. 1). Purified PCR fragments were then transformed in *E. coli* DH5α and subsequently sequenced. The alignment of protein sequence (Fig. 2) showed deduced amino acid sequences of gene

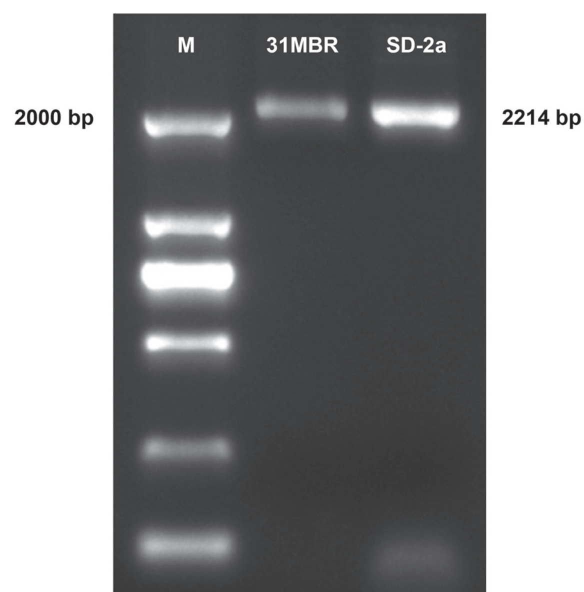


Fig. 1. PCR fragments with primers designed from β-D-glucosidase gene of *O. oeni* PSU-1 on genomic DNA of *O. oeni* SD-2a and 31MBR.

Lane 1: (M), molecular size marker (200 bp ladder). Lane 2: (31MBR), PCR fragment on genomic DNA of *O. oeni* 31MBR. Lane 3: (SD-2a), PCR fragment on genomic DNA of *O. oeni* SD-2a.

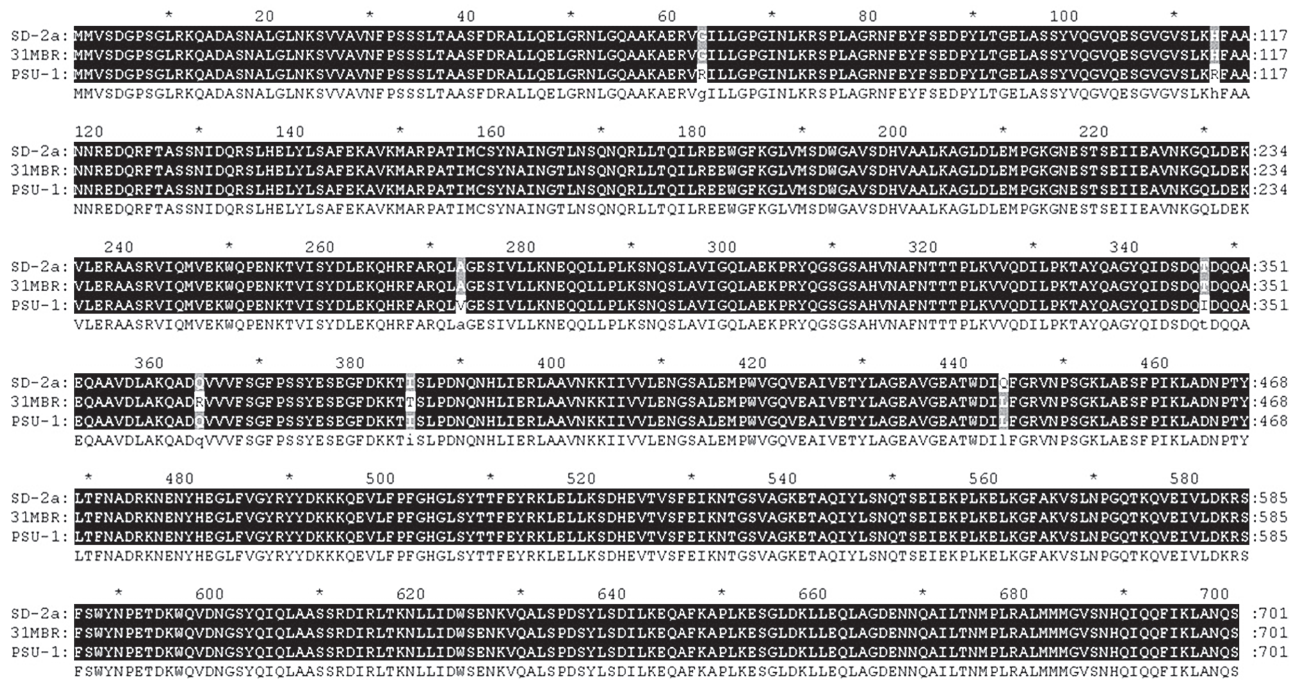

Fig. 2. Alignment of β-D-glucosidase amino acid sequences from *O. oeni* SD-2a and 31MBR with that of *O. oeni* PSU-1. Identical residues are indicated by black background and similar residues by gray background.

cloned from the two strains exhibit high degree of homology (> 99%) to the deduced protein sequence of *β*G gene identified in *O. oeni* PSU-1 (Makarova *et al*., 2006), with five amino acid changes for SD-2a and six for 31MBR respectively, confirming βG gene was virtually cloned from the two strains. While for SD-2a and 31MBR, only three amino acids were observed to be different between them, indicating the βG gene is highly conserved. The coding region of βG gene cloned from the two strains was 2214 nucleotides long and initiates with the rare start codon TTG. While there is no possible start codon (ATG) upstream of this position, expression of the gene initiated with the next ATG codon in frame downstream resulted in inactive protein (Michlmayr *et al*., 2010). Besides, a ribosomal binding site (AAGGAG) was located upstream of the TTG codon. Analysis of the predicted protein sequence showed that βG of the two strains belongs to glycoside hydrolase family 3, which comprises enzymes with a broad specificity toward glycosylated plant metabolites. The βG with 701 amino acids, has a calculated molecular mass of 77.71 kDa and theoretical pI of 5.70. The protein with instability index 30.76 is classified as stable protein, and with a grand average of hydropathicity –0.395 is less hydrophilic. The Kyte-Doolittle hydropathy plot of the deduced protein sequence (data not shown) also showed the two similar protein is probably hydrophobic. Furthermore, no transmembrane domains or signal peptide cleavage sites was predicted within the two protein, indicating βG of the two strains is not secretory protein. The prediction of subcellular localization showed that βG of the two strains exists mainly in cytoplasm, some in cytoplasmic membrane and little in cell wall and extracellular.

In order to confirm the localization prediction, enzyme localization assay was also conducted for the two strains and the results are shown in Fig. 3. As for SD-2a (Fig. 3A), the highest βG activity occurred in the soluble fraction and some in the membrane, suggesting the enzyme exists mainly in the cytoplasm as a soluble protein. This is consistent with the localization prediction. The activity was also detected in the periplasm, as high as that in supernatant A, while low activity was observed in the cell wall. Furthermore, the spheroplast also exhibited high activity, but lower than the total activity of the soluble fraction and membrane, demonstrating the substrate may be transformed into the cells and then hydrolysed and the spheroplast may make the access difficult for substrate and intra-spheroplast βG. As for 31MBR (Fig. 3B), some differences from that of SD-2a were observed. Otherwise than for SD-2a, the highest activity was observed in the membrane for 31MBR, much higher than that in the soluble fraction, indicating that the enzyme exists mainly in the cytoplasmic membrane as an insoluble protein and some in the cytoplasm as a soluble form. In addition, it should be noted that the spheroplast activity kept the same level with the soluble activity , indicating the intra-spheroplast soluble βG may be responsible for the spheroplast activity.

Recently, wine consumption, especially red wine, shows an increasing trend in China. However, wines

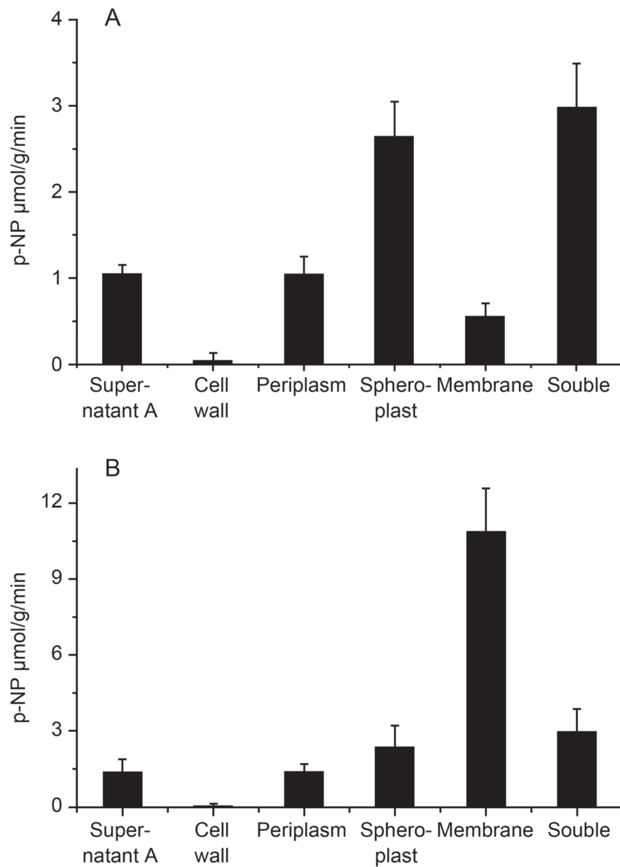


Fig. 3. β-D-glucosidase localization of *O. oeni* SD-2a (A) and 31MBR (B).

Supernatant A, containing cell wall fragments and periplasmic-compounds. Cell wall, sediment fraction from centrifugation of supernatant A. Periplasm, supernatant from centrifugation of supernatant A. Spheroplast, whole cells treated with lysozyme without cell wall. Membrane, particulate fraction from centrifugation of lysed spheroplast. Soluble, supernatant from centrifugation of lysed spheroplast.

made in China are negatively affected by low pH and bland flavor due to the climate and cultivars. Thus MLF is necessary during winemaking and the selection of starter cultures possessing important oenological characteristics is significant for the wine industry. SD-2a and 31MBR are two important *O. oeni* strains widely used during winemaking in China. The two strains possessing βG activity have been reported (Li *et al.*, 2012a; 2012b), whereas in this study, the βG of the two strains was further identified on a molecular level and characterized through bioinformatic analysis.

The βG gene cloned is probably widespread and highly conserved in *O. oeni* strains, since great identity of protein sequence (> 99%) was observed in this study. Through the analysis of amino acid composition, βG of *O. oeni* SD-2a and 31MBR was probably hydrophobic, since many hydrophobic amino acids were included. However, the subcellular localization prediction showed the enzyme exists mainly in the cytoplasm as a soluble protein, which may be due to the secondary and tertiary structure of the protein that alters the hydrophobicity of βG. βG of the two strains was predicted as a non-secretory protein which mirrors the previous report that low βG activity was observed in the culture supernatant for both strains (Li *et al.*, 2012b), as well as the subcellular localization prediction that little of the enzyme was extracellular.

As for location of βG, intracellular enzyme has been reported for different *O. oeni* strains (Barbagallo *et al.*, 2004; Michlmayr *et al.*, 2010; Perez-Martin *et al.*, 2012). The same results have also been observed for *O. oeni* SD-2a and 31MBR (Li *et al.*, 2012b). However, up to now it's not quite clear where the metabolic process of these intracellular enzymes lies and no further localization has been reported. In this study, intracellular βG of SD-2a and 31MBR was further localized through bioinformatic analysis and localization assay. For SD-2a, the results by two methods are coincident. While for 31MBR, the result of localization assay is opposite to the prediction that βG activity was observed mainly in the cytoplasmic membrane instead of the cytoplasm. This could be explained by intra-specific phenotypic diversity. Intracellular βG of SD-2a is mainly a soluble protein, existing most in the cytoplasm and some in the periplasm. While for 31MBR, intracellular βG is mainly an insoluble protein existing in the cytoplasmic membrane, and some soluble enzyme existing in the cytoplasm and periplasm. Thus it may be concluded that the hydrolysis of glycosides may occur in the cytoplasm for SD-2a and on the cytoplasmic membrane for 31MBR.

Although SD-2a and 31MBR were verified to possess βG gene with potential to act on specific substrates, it remains imperative to understand how the gene is regulated under winemaking conditions, and to evaluate whether the expressed enzymes are active in wine. It is also interesting to compare the expression level of βG gene and phospho-βG gene of the two strains in glucose rich and depleted media as well to achieve a better understanding of their metabolic role, since the mechanism of whole cells of both strains possessing high βG activity has not been elucidated. In addition, it is also worth considering the possibility to use other hosts like *Saccharomyces* cerevisiae and *Lactobacillus* plantarum for over-expression of this gene. Related work is being done in our lab and this study is therefore the basis of all further research.

In conclusion, βG gene from *O. oeni* SD-2a and 31MBR was cloned, sequenced and analyzed. Deduced amino acid sequences showed high homology (> 99%) to the βG sequences reported, thus βG activity for the two strains was confirmed on a molecular level. Prediction of enzyme localization by bioinformatic analysis was not totally consistent with the result of localization assay, so to some extent bioinformatic analysis can only be considered as a reference. Intracellular βG of SD-2a was mainly a soluble protein existing in the cytoplasm

and periplasm. In the case of 31MBR, intracellular βG was mainly an insoluble protein present in the cytoplasmic membrane. This study provides basic information for further studies on the metabolic mechanism of βG from *O. oeni* SD-2a and 31MBR.

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