Cloning and Characterization of an Intracellular Esterase from the Wine-Associated Lactic Acid Bacterium *Oenococcus oeni*<sup>†</sup>

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Received 3 July 2009/Accepted 26 August 2009

We report the cloning and characterization of EstB28, the first esterase to be so characterized from the wine-associated lactic acid bacterium, *Oenococcus oeni*. The published sequence for *O. oeni* strain PSU-1 was used to identify putative esterase genes and design PCR primers in order to amplify the corresponding region from strain Oenii28, an isolate intended for inoculation of wines. In this way a 912-bp open reading frame (ORF) encoding a putative esterase of 34.5 kDa was obtained. The amino acid sequence indicated that EstB28 is a member of family IV of lipolytic enzymes and contains the GDSAG motif common to other lactic acid bacteria. This ORF was cloned into *Escherichia coli* using an appropriate expression system, and the recombinant esterase was purified. Characterization of EstB28 revealed that the optimum temperature, pH, and ethanol concentration were 40°C, pH 5.0, and 28% (vol/vol), respectively. EstB28 also retained marked activity under conditions relevant to winemaking (10 to 20°C, pH 3.5, 14% [vol/vol] ethanol). Kinetic constants were determined for EstB28 with *p*-nitrophenyl (**p**NP)-linked substrates ranging in chain length from *C*<sub>2</sub> to *C*<sub>18</sub>. EstB28 exhibited greatest specificity for *C*<sub>2</sub> to *C*<sub>4</sub> **p**NP-linked substrates.

The quality of fermented foods and beverages is affected in part by their composition of aroma compounds. In winemaking, the malolactic fermentation is used to deciacidify wine and is typically carried out by *Lactobacillus* spp., *Pediococcus* spp., and particularly *Oenococcus oeni* (10, 7). Numerous reports clearly show that outside of this core function, lactic acid bacteria (LAB) can also bring about significant changes of sensorial importance (2, 5, 12, 32, 40, 49, 55). Such studies typically examined the action of active cultures or whole cells; however, the promise of LAB as a source of purified enzymes for use as additives in winemaking has recently been highlighted (43).

*Oenococcus oeni* is acidophilic and indigenous to wine and similar environments. While the genome of the commercial PSU-1 strain has been sequenced and analyzed (44), there is limited information on the genes or their potential contribution to food and beverage aroma. The only such genes which have been cloned and partially characterized are alic and aild (24), which are thought to be responsible for the production of diacetyl, the principle compound conferring “buttery” aroma and flavor in wine (reviewed in reference 4). Analogous characterization of other flavor-related genes and enzymes not only may have practical implications for processes using LAB but also may be of fundamental interest.

As a group, esters are a quantitatively significant constituent of beverages such as wine (total of >100 mg · liter<sup>−1</sup>) (15). Included in this group are the C<sub>4</sub> to C<sub>10</sub> ethyl esters of organic acids, ethyl esters of straight-chain fatty acids (and branched-chain fatty acids to a lesser degree), and acetates of higher alcohols which are largely, if not exclusively, responsible for the fruity aroma of wine (13, 14). Some volatile esters are frequently found in fermented beverages in only trace amounts, often below threshold concentrations (2, 21, 25, 29, 50). However, they are extremely important for the flavor profile of these products, with different esters often having a synergistic effect to collectively affect aroma when their individual threshold concentrations are not exceeded. The fact that most esters are present in wine at concentrations around the threshold value implies that minor concentration changes might have a dramatic effect on the wine’s flavor (3, 21, 25, 29, 50). For this reason, an understanding of the hydrolysis and synthesis of esters in winemaking and how these may be manipulated is essential.

A large amount of esters is formed during the primary fermentation by yeast; after this, LAB can contribute by increasing and decreasing the ester concentration (2, 5, 12, 40, 49, 55). Ester hydrolysis and synthesis can be catalyzed by esterases (6, 35, 38, 54). These enzymes commonly contain a catalytic triad composed of Ser, His, and Asp/Glu residues and a nucleophilic elbow structural motif (GXSXG), which contains the active-site serine residue (1, 31, 36, 48). They also contain an oxyanion hole, of which two residues donate their backbone amide protons to stabilize the substrate in the transition state. The oxyanion hole residues (in bold) have been divided into two groups termed GX and GGGX, with the glycine and a hydrophobic residue (X) being highly conserved (48).

While extensive research has been carried out on the enzymes responsible for ester formation by wine strains of *Saccharomyces cerevisiae* (22, 23, 45, 51), esterase activity for wine-related LAB is not well documented. Most characterization of esterases in LAB has focused on dairy isolates (9, 16–18, 20). Parallel work in a wine context is limited despite general acceptance of the importance of esters in wine. Until recently, most evidence that wine LAB possess esterase activity came from wine volatile profiling studies which investigated the...
changes in concentration of individual esters during malolactic fermentation (12, 40, 55). Such changes in ester concentration were strain specific and had the potential to greatly affect the final aroma of wine.

Our survey of the esterase activities of whole LAB cells found variations within species and even greater variation between the genera (42), with O. oeni showing greatest activity toward the p-nitrophenyl (pNP)-linked substrates tested. More recently (41), the esterase activities of whole O. oeni, lactobacillus, and pediococcus cells was determined under conditions with some relevance to wine. At least partial resistance to the harsh conditions used was observed, thereby demonstrating a necessary requirement of any enzyme intended for application in analogous environments. To more completely characterize esterases of LAB, the enzymes and their structural genes must be fully investigated. This study represents an effort to dissect the complex array of ester synthesis and hydrolysis activities in whole cells by cloning, heterologous expression, partial purification, and biochemical characterization of a single esterase from O. oeni. With a view to applying such an esterase under conditions found in wine and perhaps other industrial settings, enzyme function under the harsh physicochemical conditions frequently encountered in wine was examined.

MATERIALS AND METHODS

Strains, media, and plasmids. Oenococcus oeni strain Oen28 was isolated from freeze-dried LAB starter cultures for use in commercial winemaking and originates from Lallemand nuov Ceppi Oo2 (42). In preparation for experiments, Oen28 was cultured from the stocks in the Man-Rogosa-Sharp (MRS) medium (Amyl Media) supplemented with 20% (vol/vol) tomato juice. Escherichia coli DH5α was used as a host strain for cloning and maintenance of plasmids. E. coli BL21(DE3) was used as a host for expression of the estB gene under the control of the T7 promoter. E. coli transformants were grown at 37°C in either Luria-Bertani broth (LB) or Terrific broth (for expression only), which contained 100 μg/ml of ampicillin when required. All bacteria were stored at –80°C in 30% (vol/vol) glycerol.

DNA isolation and manipulations. Isolation of DNA from Oen28 was carried out using the UltraClean microbial DNA isolation kit (MoBi). Primers targeting the open reading frame (ORF) of the putative esterase gene were designed based on the PSU-1 O. oeni genome sequence (CP000411) (44). PCR was performed using the T7 Fusion HS DNA polymerase (Stratagene) in an Eppendorf Mastercycler gradient according to the parameters accompanying the plasmid construct were confirmed by nucleotide sequencing, with labeling and capillary separation on the AB 3730xl performed by the Australian Genome Research Facility (Brisbane). The T7 promoter and terminator primers were used for this purpose. Nucleotide sequencing results were analyzed using Chromas Lite version 2.0 (Technelysium Pty. Ltd., Australia), BioEdit (50), and NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST).

Purification of the recombinant esterase. E. coli BL21(DE3) harboring the pET14b(estB28) plasmid was used as the source of the recombinant enzyme. Cultivated cells were induced at an optical density at 600 nm of 0.6 with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) and were then incubated at 14°C with shaking at 180 rpm for 48 h before harvest. The cell pellet was disrupted using EasyLyse bacterial protein extraction solution according to the manufacturer’s instructions (Epicentre Biotechnologies). The crude extract was separated from cell debris by centrifugation at 11,000 × g for 30 min at 4°C. The recombinant esterase with a His tag was purified by using affinity chromatography and the BioLogic LP low-pressure chromatography system (Bio-Rad). The cell lysate was loaded onto a column containing Talon metal affinity resin (Clontech) and washed for 1 h at 1 ml·min⁻¹ with wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, pH 7.0). The recombinant enzyme was eluted at 0.5 ml·min⁻¹ using Mcllvaine buffer (pH 5.0). Chromatography data were recorded using LP Data View software (Bio-Rad). The molecular mass of the purified recombinant esterase was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with a 5% (wt/vol) stacking gel and either a 12% or 18% (wt/vol) separating gel, which were made and run according to the manufacturer’s protocols (Bio-Rad). The His tag was cleaved using a thrombin CleanCleave kit (Sigma). Determination of enzyme activity (see below) revealed no difference between cleaved and uncleaved recombinant enzyme (data not shown). For this reason and because thrombin was lost to the enzyme preparation, we elected to conduct all characterization work with the uncleaved purified recombinant enzyme.

Determination and characterization of esterase activity. Esterase assays were conducted using pNP butanoate substrate, with the exception of the substrate specificity trials, in which eight different pNP-linked ester substrates were used, as described below. Reaction conditions were as follows: 100 μl of recombinant enzyme was incubated in 96-well microplates (Sarstedt) as detailed in each section below and incubated at 37°C (for convenience) for 1 h, with the exception of the temperature optimum experiments. The liberated p-nitrophenol was quantified immediately by measuring the absorbance at 410 nm using a microplate spectrophotometer (μQuant; Bio-Tek Instruments). The data were then corrected for nonenzymatic degradation of the ester substrate using an enzyme-free control of the same reaction mixture that was treated in the same manner. All assays and controls were performed in triplicate, and activities were quantified by comparison with p-nitrophenol standards.

Determination of substrate specificity. To determine the specificity of EstB28 for different ester substrates, eight pNP-linked esters with different carbon chain lengths were used: pNP-acetate, -butanoate, -octanoate, and -decanoate (Sigma); pNP-propionate (Sigma); pNP-tetradecanoate; and pNP-hexadecanote (Fluka). The method of Pencreac’h and Baratti (47) was used with some modifications. Stock solutions (25 mM) of each substrate were prepared in ethanol (pNP-acetate, -butyrate, -hexanoate, and -octanoate) or propan-2-ol (pNP-decanoate, -dodecanoate, -tetradecanoate, and -octadecanoate) and stored at –20°C until required. Assays were carried out as described for the pH optimum study, but for all samples the reaction mixtures (final volume, 200 μl) contained a modified 0.1 M Mcllvaine buffer (pH 5.0, with the addition of 5 μl Triton X-100 and 100 μl of 1% [wt/vol] gum arabic [Sigma] solution per ml). Following incubation for 1 h at 37°C, the absorbance was measured at 410 nm. 

Influence of pH, temperature, or ethanol on esterase activity. The influence of pH on esterase activity was determined by varying the pH of the reaction mixture across the range from 3.0 to 8.0. The buffers used were 0.1 M Mcllvaine buffer (pH 3.0 to 7.5) and Na2HPO4-NaH2PO4 buffer (pH 8.0) (11). Briefly, 200-μl reaction mixtures were prepared by combining 187 μl of the appropriate buffer, 5 μl of diluted enzyme (final amount in the reaction, 1 μg), and 8 μl of a 25 mM stock pNP-butanoate solution in ethanol, to yield a final substrate concentration of 0.1 mM. Following incubation, 40 μl of 0.5 M NaOH was then added to the pH 3.0 to 3.5 samples and 10 μl of 0.5 M NaOH was added to the pH 4.0 and 4.5 samples for alkalization of samples prior to absorbance measurements being taken as described above. Results were quantified by comparison with standard p-nitrophenol solutions prepared under the same conditions.
Esterase activity was studied at six temperatures across the range of 10 to 60°C in dry heating blocks or incubators. The accuracy of temperature settings was confirmed with a thermometer immediately prior to initiation of the experiment. Assays were carried out as described for the pH optimum study, but for all samples, 0.1 M McIlvaine buffer at pH 5.0 was used. Reaction mixtures containing only buffer and substrate were equilibrated at the appropriate temperature for 10 min before the enzyme was added to initiate the reaction. Following incubation, all samples were transferred to a 96-well microplate and absorbances were measured.

The effect of the presence of ethanol on esterase activity was studied at 17 ethanol concentrations ranging from 0 to 50% (vol/vol) and at an extreme concentration of up to 20 g liter⁻¹ and at an extreme concentration of up to 20 g liter⁻¹. Reaction mixtures were prepared as described for the temperature optimum experiments, but different volumes of ethanol were added, from 4 µl (2%, vol/vol) up to 100 µl (50%, vol/vol), and the volume of buffer was adjusted accordingly to maintain a final reaction volume of 200 µl in a 96-well microplate. Reaction mixtures were incubated for 1 h at 37°C, and absorbance was measured.

**Inhibition studies.** The effect of sulfite was determined by adding sodium metabisulfite at concentrations ranging from 0 to 1.5 g liter⁻¹ and at an extreme concentration of up to 10 g liter⁻¹. Reaction mixtures were prepared as described for the temperature optimum experiments, but 0.1 M McIlvaine buffer at pH 3.5 was used and different volumes of a 100 g liter⁻¹ stock solution of sodium metabisulfite were added. The volume of buffer was adjusted accordingly to maintain a final reaction volume of 200 µl in a 96-well microplate. Reaction mixtures were incubated for 1 h at 37°C, and absorbance was measured.

The effects of diethyl pyrocarbonate (DEPC), phenylmethylsulfonyl fluoride (PMSF), phenylarsine oxide (PAO), phenylglyoxal (PGO), and β-mercaptoethanol (β-ME) were also tested using 5 mM of each inhibitor. Reaction mixtures were prepared as described for the temperature optimum experiments, but inhibitors were added and the volume of buffer was adjusted accordingly to maintain a final reaction volume of 200 µl in a 96-well microplate. Reaction mixtures were incubated for 60 min at 37°C, and enzyme activity was measured at 15 min intervals and compared to that of controls without addition of inhibitors.

**Statistical analysis.** Analysis of variance of the data was conducted using Microsoft Excel and GraphPad Prism, version 5.01. Kin and kcat values were calculated using nonlinear regression and GraphPad Prism, version 5.01.

**RESULTS**

**DNA sequencing and sequence analysis.** Based on the published sequences of LAB lipolytic enzymes, an ORF encoding a putative esterase gene in *O. oeni* was identified and successfully amplified from strain Ooeni28. Nucleotide sequencing of the putative esterase ORF was conducted in triplicate. The ORF consisted of 912 nucleotides encoding a deduced protein of 303 amino acids and was designated estB28 (Fig. 1). The sequence was aligned with the *O. oeni* strain PSU-1 genome sequence using NCBI BLAST. The results indicate differences in the nucleotide sequence in single-letter code. An asterisk indicates the stop codon. The GGGX motif containing the N-terminal oxyanion hole residue and the putative active-site serine consensus sequence are underlined. The putative residues of the catalytic triad are marked by a box. Sequence changes from strain PSU-1 are highlighted; in order they are C→T, T→C, T→G, T→A, and V→A. Amino acid changes are also highlighted; in order they are P→S, H→R, and V→A.

**Cloning of the *Oenococcus oeni* esterase gene into *E. coli*.** The putative esterase gene was cloned into pET14.b, and once positive clones had been sequenced, one was chosen for further experiments. The *E. coli* [BL21(ADE3)] cells containing the plasmid pET14.b/estB28 were induced and cell extracts obtained for analysis by SDS-polyacrylamide gel electrophoresis. The induced BL21(ADE3) cells containing pET14.b/estB28 overexpressed a protein of approximately 34.3 kDa (Fig. 2). A faint band at 34.4 kDa was observed in the uninduced sample but not in either of the samples containing induced BL21(ADE3) cells with only the pET14.b plasmid (Fig. 2). Crude cell extracts were assayed for activity (data not shown), and BL21(ADE3) containing pET14.b/estB28 had 10-fold greater activity than BL21(ADE3) containing pET14.b. EstB28 was purified from the whole-cell lysate to electrophoretic homogeneous band sequence identified as GDSAG (Fig. 1) starting at residue 141. This also fits the GXSSG motif found in most esterases (see the supplemental material). The sequence GDSAG in the Ooeni28 esterase is also found in the characterized esterase (EstB) of *Lactobacillus casei* LILA (18). Based on alignment with other esterase sequences (data not shown) His-269 is most likely the catalytic histidine and the catalytic aspartic acid is tentatively identified as Asp-235 (Fig. 1).
The highest activity was observed with longer chain lengths. The highest activity was observed with other inhibitors (Fig. 4) showed that the enzyme was strongly inhibited by DEPC, a histidine modifier, and PMSF, a serine protease inhibitor. PGO, which targets arginine residues, decreased activity by 82%. Inhibition by β-ME or PAO, a cysteine modifier, caused activity to decrease by 71% in each case.

**DISCUSSION**

This research focused on the cloning and biochemical characterization of an esterase identified by sequence homology using the published nucleotide sequence for *O. oeni* PSU-1. Nucleotide sequencing of the esterase from *O. oeni* Ooeni28, designated estB28, revealed a 912-bp ORF which could encode a protein of 34.5 kDa. This is differentiated from the ORFs determined for *Lactobacillus casei* LILA *estB* and *estC* (17, 18), which were 777 bp and 954 bp and encoded proteins of 35.7 kDa and 28.8 kDa, respectively. According to the system proposed by Arpigny and Jaeger (1), the amino acid sequence of EstB28 reveals it to be a member of family IV of lipolytic enzymes. This group of enzymes shows sequence similarity to the mammalian hormone-sensitive lipase. Holm et al. conducted site-directed mutagenesis of the serine at the center of the GXSGx nucleophilic elbow of mammalian hormone-sensitive lipase, resulting in complete absence of enzymatic activity (34). The deduced amino acid sequence of EstB28 was analyzed for both grape juice and wine and are sensitive lipase, resulting in complete absence of enzymatic activity. Possible oxygen hole residues, nucleophilic elbow pentapeptides, and catalytic triad resides (48) were identified. EstB28 showed no potential signal sequence (46), suggesting that this enzyme is located in the cytoplasm. This observation concurs with those made for *Lactobacillus helveticus* CNRZ32 *EstA* (16), *L. lactis* B1014 *EstA* (20), *Lactobacillus casei* LILA *EstB* (18), and *Lactobacillus casei* LILA *EstC* (17).

The esterase of *O. oeni* Ooeni28 (EstB28) was overexpressed in *E. coli* and purified using affinity chromatography. The purified recombinant enzyme was then characterized under conditions relevant to winemaking. The potential role of EstB28, when used as an additive in wine, in aroma development is dependent on the sensitivity of the enzyme to environmental conditions encountered therein. Low pH values, in the range 3.0 to 3.6, are sought for both grape juice and wine and are monitored throughout the winemaking process. At these values EstB28 was stable, and it retained 30 to 40% of the activity inhibited.

**TABLE 1. Hydrolysis of pNP esters by purified heterologously expressed esterase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chain length</th>
<th>Relative activity (%)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (s⁻¹ · μM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNP-acetate</td>
<td>C₂</td>
<td>100</td>
<td>11.54</td>
<td>942</td>
<td>81.6</td>
</tr>
<tr>
<td>pNP-butanate</td>
<td>C₄</td>
<td>92</td>
<td>14.44</td>
<td>890</td>
<td>61.8</td>
</tr>
<tr>
<td>pNP-hexanoate</td>
<td>C₆</td>
<td>56</td>
<td>36.05</td>
<td>901</td>
<td>25.0</td>
</tr>
<tr>
<td>pNP-octanoate</td>
<td>C₈</td>
<td>50</td>
<td>47.67</td>
<td>527</td>
<td>11.0</td>
</tr>
<tr>
<td>pNP-decanoate</td>
<td>C₁₀</td>
<td>24</td>
<td>70.10</td>
<td>322</td>
<td>4.6</td>
</tr>
<tr>
<td>pNP-dodecanoate</td>
<td>C₁₂</td>
<td>2</td>
<td>168.73</td>
<td>153</td>
<td>0.9</td>
</tr>
<tr>
<td>pNP-tetradecanoate</td>
<td>C₁₄</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>pNP-octadecanoate</td>
<td>C₁₈</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Enzyme activity, determined at 37°C in 0.1 M McIlvaine buffer (pH 5.5) containing Triton X-100 and gum arabic, is the mean from triplicate experiments with a standard deviation of less than 8%. Values are shown relative to the highest observed activity, which was arbitrarily designated 100%.

—, not detected.
under optimal conditions at pH 5.0 to 5.5 (Fig. 3A). Based on
other esterases that have been purified from LAB and charac-
terized, only EstC from Lactobacillus casei LILA (17) had an
optimum activity in the same pH range. EstB28 and EstC show
only 11% homology. Because EstC was from a dairy isolate,
Fenster et al. characterized it under conditions relevant to
cheese ripening, and no other aspects relevant to wine were
assessed (17).

Maximum esterase activity of EstB28 was observed at 40°C,
which is a temperature higher than those considered suitable
for wine production and storage. Wine is usually stored at
temperatures below 20°C. EstB28 retained 33% of its activity
at 20°C and 21% at 10°C relative to that observed under op-
timal conditions. This suggests that the enzyme has the poten-
tial to affect the ester profile of wine even at these low tem-
peratures. EstB28 might also be of value as an addition to
ripening cheese, given that the cheese is stored at 10°C and its
pH (5.0 to 5.5) (18, 19) corresponds to the optimum for
EstB28. While the effects of NaCl levels, usually 4% in ripen-
ing cheese, were not considered in this study, it can be sug-
gested that EstB28 will retain at least 20% activity if used at
temperatures at which cheese is ripened. As with wine, high
concentrations of some esters can produce undesirable fruity
off-flavors in some cheeses (39), and investigation of the effect
that addition of EstB28 has in ripening cheese could prove
interesting.

A further potentially inhibitory enological parameter to be
considered was ethanol. This alcohol in fact stimulated ester-
ase activity with 14% (vol/vol) ethanol, producing 149% activ-
ity relative to the 0% ethanol control. EstB28 retained 79% of
its activity at 50% (vol/vol) ethanol. Other reports from our
group on the esterase activity (41) and β-glucosidase activity
(26–28) of LAB isolates describe a similar enhancement of
activity by ethanol when assaying whole cells. While the need
for ethanol to enhance ester formation in cheese has been
reported, only levels of 6% (vol/vol) have been assessed (33).
There are no reports in the literature of LAB esterases retain-
ing activity at 50% ethanol, although an esterase from Bacillus

![FIG. 3. Characterization of EstB28. Values are the means of triplicates ± standard deviations. Values are shown relative to the highest observed activity, which was arbitrarily designated 100%, except for panel C, where values are shown relative to the activity observed in the absence of ethanol, which was arbitrarily designated 100%. (A) Influence of pH on esterase activity of EstB28. (B) Influence of temperature on esterase activity of EstB28. (C) Influence of ethanol on esterase activity of EstB28. (D) Influence of sulfite on esterase activity of EstB28. Free SO₂ includes the concentrations of sulfur dioxide forms which are in equilibrium and are not bound to any compound in solution. This is defined as [SO₂⁻] + [HSO₃⁻] ([SO₃²⁻] is minimal, i.e., 0.02%) at pH 3.5, where 98% of the sulfur dioxide exists as bisulfite ion (HSO₃⁻).](image)

![FIG. 4. Effects of inhibitors on the activity of recombinant EstB28 esterase. Inhibitors at 5 mM were incubated in triplicate with the enzyme for 60 min at 37°C. Readings were taken every 15 min using a microplate reader. Error bars indicate standard deviations.](image)
**licheniformis** retained activity in various solvents, including 13% activity in 50% ethanol (54). While a likely application of this characterized esterase might be in floral, white wines, which rarely exceed 13.5% ethanol, the robustness of this enzyme suggests that it might also find applications in beverages of higher ethanol content (i.e., <15%, vol/vol). Outside of wine, the stability and high activity of EstB28 in ethanol suggest a possible use as an industrial biocatalyst. The use of EstB28 in organic synthesis and further characterization in other solvents could be of interest.

Sodium metabisulfite, the final wine-associated inhibitor to be considered here, did not inhibit EstB28 until it was present at high concentrations (10 g · liter⁻¹), higher than those found in wine. During fermentation, yeast can produce 0.01 to 0.03 g · liter⁻¹ of SO₂, and sulfites are often added by the winemaker in the form of potassium metabisulfite, resulting in final levels of 0.1 to 0.2 g · liter⁻¹ SO₂ in the wine. Inactivation of the *O. oeni* Ooeni28 esterase by PMSF and DEPC suggests the involvement of a serine and a histidine at the active site of the enzyme. The serine residue of the pentapeptide GXXG is well known as the active catalytic residue that is modified by PMSF (9). EstB28 sensitivity to PMSF was similar to that of EstB from *Lactobacillus casei* LILIA (18). PGO caused an initially unexpected 82% decrease in activity of EstB28. However, on closer inspection of the deduced amino acid sequence of EstB28 (Fig. 1), it is evident that the arginine residue (R) is close enough to interfere with the binding of the catalytic histidine (H) with the substrate. EstB28 esterase was only close enough to interfere with the binding of the catalytic histidine (H) with the substrate. EstB28 esterase was only partially inhibited by FAO and β-ME, suggesting that an accessible cysteine is not necessary for activity and disulfide bonds are not present. No disulfide bonds were predicted from the EstB28 protein sequence using DISULFIND (8). This correlates with the lower levels of inhibition of the esterase by β-ME and PAO, which would act through disruption of any disulfide bonds.

The substrate specificity of *O. oeni* Ooeni28 esterase revealed greater selectivity for *pNP* esters of short-chain fatty acids and to a lesser extent *pNP* esters of medium-chain fatty acids. Increases in fatty acid chain lengths resulted in an increase in *Kₘ*; however, the effect on catalytic efficiency is seen only when the chain length is ≥C₆. The specificity of the esterase for *pNP*-acetate and *pNP*-butanoate is most similar to that of EstC from *Lactobacillus casei* LILIA (17), which is also mainly specific for short-chain fatty acids. Examples of such esters in wine include ethyl acetate and ethyl butanoate. The removal of these from wine is not necessarily desirable, as they can contribute to the fruity aroma of wine at low concentrations (37, 52a). However the removal of excess ethyl acetate, which at high concentrations (≥100 mg · ml⁻¹) contributes a solvent-like aroma to wine, would be an advantage. The role that EstB28 plays in ester synthesis will also need to be investigated to determine the usefulness of adding whole cells of *L. lactis* Ooeni28 esterase demonstrated to retain activity under conditions relevant to winemaking. Based on the findings reported for the artificial substrates used in this study, it appears that EstB28 will retain at least partial activity under wine-like conditions of pH, temperature, ethanol, and sulfite concentration. The *O. oeni* Ooeni28 esterase is a potential candidate for altering the ester profile of wine and could play an important role in ester profile modifications during the malolactic fermentation. As most esterases are also capable of ester synthesis, investigation of the role that this enzyme might play in wine would be of interest.

**ACKNOWLEDGMENTS**

The research in this paper was undertaken as part of project UA 05/01, supported by Australia’s grape growers and winemakers through their investment body, the Grape and Wine Research Development Corporation, with matching funds from the Australian Government. K.M.S. is supported by a Grape and Wine Research Development Corporation scholarship.

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