

## Characterization of a Grape Class IV Chitinase

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**ABSTRACT:** A chitinase was purified from *Vitis vinifera* Manzoni Bianco grape juice and characterized. On the basis of proteomic analysis of tryptic peptides, a significant match identified the enzyme as a type IV grape chitinase previously found in juices of other *V. vinifera* varieties. The optimal pH and temperature for activity toward colloidal chitin were found to be 6 and 30 °C, respectively. The enzyme was found to hydrolyze chitin and oligomers of N-acetylglucosamine, generating N,N'-diacetylchitobiose and N-acetylglucosamine as products, but was inactive toward N,N'-diacetylchitobiose. The enzyme exhibited both endo- and exochitinase activities. Because yeast contains a small amount of chitin in the cell wall, the possibility of growth inhibition was tested. At a concentration and pH expected in ripe grapes, no inhibition of wine yeast growth by the chitinase was observed.

**KEYWORDS:** grape chitinase, chitinase activity, yeast inhibition

### INTRODUCTION

Chitinases (EC 3.2.1.14) are glycosyl hydrolases that catalyze the degradation of chitin, a β-1,4-linked polymer of N-acetylglucosamine (GlcNAc) found in fungal cell walls among other natural sources.<sup>1</sup> Although higher plants lack chitin, they harbor chitinase genes and express them in response to pathogens or developmental cues in susceptible tissues.<sup>2,3</sup> In *Vitis vinifera*, type IV chitinase activity has been reported to increase dramatically in berries during ripening, presumably to provide protection from potential fungal attack.<sup>4</sup> Type IV chitinases have been detected in the juice of a number of varieties of *V. vinifera* species<sup>4–7</sup> and in wines made thereof.<sup>7–11</sup> Chitinase concentration in bottled white wines has also been found to correlate with the formation of heat-induced turbidity, a recognized quality defect.<sup>12</sup> Chitinase activity was reported to decrease significantly from initial levels detected in Pinot noir berries through the various stages of sparkling wine production to undetectable levels in the final wine.<sup>13</sup>

Chitinases have been classified into two major categories: endochitinases and exochitinases.<sup>14</sup> However, the current nomenclature for chitinolytic enzymes is confusing, due in part to the fact that the former classification scheme has not been abandoned.<sup>15,16</sup> The former scheme distinguished the exochitinases (EC 3.2.1.29), which catalyze progressive release of dimeric chitobiose residues from the nonreducing end, from the N-acetyl-β-D-glucosaminidases (EC 3.2.1.30), which release monomeric GlcNAc residues from chitin oligomers.<sup>14</sup> Currently, the International Union of Biochemistry and Molecular Biology (IUBMB) recognizes a single β-N-acetylhexosaminidase class of enzyme, EC 3.2.1.52, that includes the former EC 3.2.1.29 and EC 3.2.1.30 enzyme classes. Furthermore, some authors have described the enzymes that release small oligomers (including dimers) from the reducing end as endochitinases (EC 3.2.1.14), differentiating

them from even the strictly endotype enzymes.<sup>17</sup> In the present paper, we use the nomenclature proposed by Harman et al., in which the difference between endo- and exochitinases is substrate specificity: endochitinases require at least tetrameric GlcNAc, whereas the minimum substrate for an exochitinase is the GlcNAc trimer. In addition, we refer to the enzyme that catalyzes the release of dimers as chitobiosidase.<sup>18</sup>

Although a number of studies have characterized family 19 chitinases, which include the type IV enzyme,<sup>19,20</sup> to our knowledge, the grape type IV chitinase has been subjected to limited biochemical analysis.<sup>21,22</sup> Here, we determined enzyme activity and substrate specificity and asked whether chitinase activity likely to be present in ripe grapes has the potential to inhibit yeast growth during wine fermentation.

### MATERIALS AND METHODS

**Purification of Grape Chitinase.** Initially, 6 L of grape juice from *V. vinifera* Manzoni Bianco was treated overnight at 4 °C with 4 g/L of polyvinylpolypyrrolidone (PVPP) (Fluka), 2 g/L activated charcoal (Sigma), and 3 g/L of pectolytic enzymes (Pectazina DC, Dal Cin). The juice was decanted, filtered through a GF/A filter (Whatman), adjusted to pH 3.0 with HCl, and then filtered through a 0.2 μm cellulose acetate filter (Sartorius). Chitinase was purified from the filtered juice essentially as described.<sup>5</sup> The only modification was the substitution of the MacroPrep High S resin with an S-Sepharose resin (220 mL). Fractions containing a single band at 31 kDa (corresponding to the molecular weight of grape chitinase) were pooled, dialyzed against water (3500 Da cutoff dialysis membrane), and freeze-dried. The purity of the protein was checked by HPLC as described.<sup>6</sup>

**Received:** March 17, 2014

**Revised:** May 20, 2014

**Accepted:** May 20, 2014

**Published:** May 20, 2014

**Chitinase Identification by LC-MS/MS Analysis of Tryptic Peptides.** The freeze-dried purified 31 kDa fraction was processed for in-solution trypsin (Promega Trypsin Gold) digestion in the presence of a protease enhancer (Promega ProteaseMAX Surfactant) according to the manufacturer's protocol. LC-MS/MS analysis was conducted on an LTQ-FT MS (Thermo Fisher Scientific, San Jose, CA, USA) instrument coupled to a nanoAcquity UPLC system (Waters Corp., Milford, MA, USA). A binary solvent system consisting of solvent A, water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid, was used for the analyses. Tryptic peptides (2  $\mu$ L) were loaded onto a peptide trapping column (Cap Trap, Michrom) and separated using a C18 column (Agilent Zorbax 300SB-C18, 250  $\times$  0.3 mm, 5  $\mu$ m). Peptides were trapped and washed with 3% solvent B for 3 min at a flow rate of 5  $\mu$ L/min. Peptide separation was achieved using a linear gradient from 10 to 30% B at a flow rate of 4  $\mu$ L/min over 102 min. The LTQ-FT mass spectrometer was operated using data-dependent MS/MS acquisition with an MS precursor ion scan, performed in the ICR cell, from  $m/z$  350 to 2000 with the resolving power set to 100,000 at  $m/z$  400, and MS/MS scans performed by the linear ion trap on the five most abundant doubly or triply charged precursor ions detected in the MS scan.

Thermo RAW data files were processed with Proteome Discoverer v1.4.0. Mascot software (v2.3) and were used to search against the *V. vinifera* database downloaded from UniprotKB on July 8, 2013 (<http://www.uniprot.org/>), which included common processing contaminants. The following parameters were used to search the database: the digestion enzyme was set to Trypsin/P, and two missed cleavage sites were allowed. The precursor ion mass tolerance was set to 10 ppm, whereas a fragment ion tolerance of 0.8 Da was used. Dynamic modifications that were considered included carbamido-methyl (+57.02 Da) for cysteine, oxidation (+15.99 Da) for methionine, and deamidination (+0.98 Da) of asparagine and glutamine. Scaffold\_3.3.1 (Proteome Software, Portland, OR, USA) was used for search data compilation and data evaluation with an embedded X!Tandem database searching algorithm. Peptide identifications were accepted if they could be established at >90.0% probability. Peptide Probabilities from X! Tandem were assigned by the Peptide Prophet algorithm<sup>23</sup> with Scaffold delta-mass correction. Peptide Probabilities from Mascot were assigned by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at >90.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.<sup>24</sup> Proteins that contained similar peptides and could not be differentiated on the basis of MS/MS analysis alone were grouped to satisfy the principles of parsimony. Identified MS/MS spectra were manually validated, and ion assignments were made according to the spectral output of the Scaffold program.

**Purification of Chitin Oligomers.** Two grams of crab shell chitin (Fluka 22780) were hydrolyzed by suspension in 32 mL of 12 M HCl initially for 10 min at room temperature followed by an additional 110 min at 40 °C. The solution was neutralized on ice by slow addition of 32 mL of 50% NaOH. Insoluble matter was removed by centrifugation, and the clear supernatant was adjusted to pH 7 and ultrafiltered on an apparatus equipped with a 500 Da cutoff membrane (Amicon) to remove excess salt and to concentrate the oligomers. When a volume of 3 mL was reached, 1.5 mL was loaded onto a Bio-Gel P2 (Bio-Rad Laboratories) column (100  $\times$  1.5 cm) and eluted at a rate of 0.25 mL/min using distilled water. One milliliter fractions were collected, freeze-dried, and resuspended in 20  $\mu$ L of water.

**TLC Analysis.** TLC analysis was performed using silica TLC plates and a butanol/acetic acid/water (2:1:1) solvent<sup>25</sup> to determine oligomer purity in the Biogel P2 fractions and subsequently to determine the mechanism of chitinase action. Two microliters of the Bio-Gel P2 fractions was analyzed, and fractions with the same profile were pooled and freeze-dried. *N*-Acetylglucosamine (GlcNAc) and (GlcNAc)<sub>2-3</sub> standards (Sigma-Aldrich, St. Louis, MO, USA) were used for reference. Higher oligomers, (GlcNAc)<sub>4-6</sub>, that were not commercially available were identified on the basis of mobility relative to the monomer and (GlcNAc)<sub>2-3</sub> oligomers.

Amino sugars were detected by staining with a solution of aniline/diphenylamine (4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone, 30 mL of 85% phosphoric acid), followed by incubation for 3 min at 180 °C.

**Chitinase Activity.** Colloidal chitin used as substrate was prepared essentially as described by Sheng et al. starting with crab shell chitin.<sup>26</sup> For the determination of pH and temperature optima, chitinase activity was measured using an assay that specifically detected GlcNAc by reaction with *p*-dimethylaminobenzaldehyde (DMAB).<sup>27</sup> One unit of activity was defined as the amount of enzyme that catalyzed the release of 1 nmol of GlcNAc per minute at 37 °C and pH 5.5. A different chitinase assay was used to quantify activity when the inhibition of yeast growth was assessed. Specifically, chitinase added to yeast cultures was assayed prior to the addition by determining a linear increase in soluble reducing sugar over 1 h<sup>28</sup> using a commercial kit (Thermo Scientific Pierce Micro BCA Protein Assay Kit 23235) and GlcNAc as standard. Although this Cu-based assay kit is designated for protein quantification, the same reaction measures reducing sugar as well.<sup>29</sup> For this assay, one unit of chitinase activity was defined as that which liberated 1 mg of GlcNAc equivalents per hour at 25 °C.

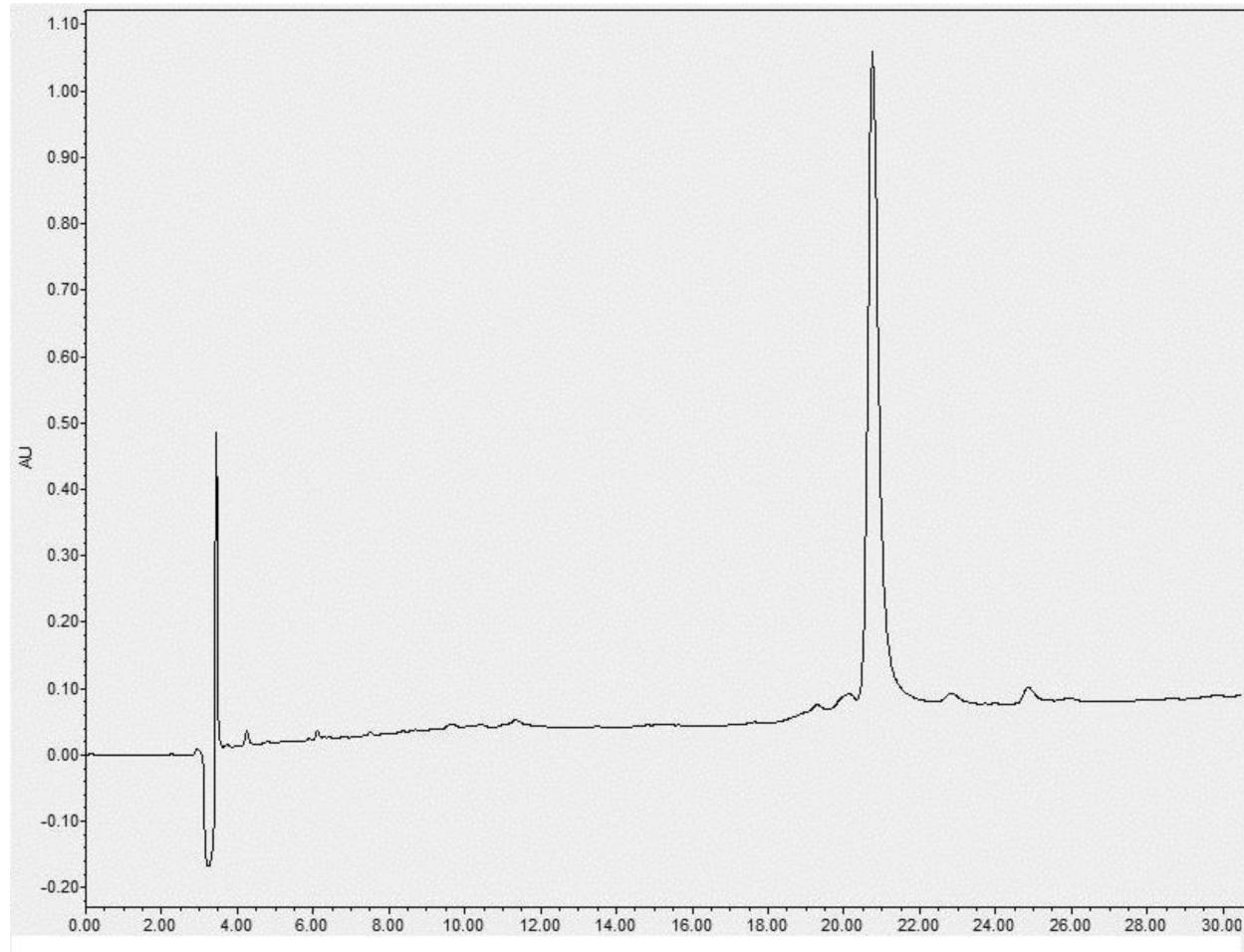
**Activity versus pH Determination.** Two buffers were prepared at 50 mM strength: a citric acid–phosphate McIlvaine buffer, covering the pH range from 2.5 to 8, and a glycine buffer, covering the pH range from 8.5 to 10.5. For each pH tested, 800  $\mu$ L of colloidal chitin was washed three times with 2 volumes of the corresponding buffer and resuspended in a final volume of 1600  $\mu$ L (0.5% w/v final chitin concentration). Half of the solution volume was kept as a control, whereas chitinase was added to the other 800  $\mu$ L of substrate to a final concentration of 50  $\mu$ g/mL. After 2 h at 37 °C, both the sample and the control were centrifuged (12000g, 5 min), and 250  $\mu$ L of clear supernatant was used to quantify GlcNAc. The difference between the absorbance of the sample and that of the control was used to determine the enzyme activity.

**Activity versus Temperature Determination.** Chitinase activity was determined as a function of temperature in 50 mM sodium acetate buffer, pH 6. Colloidal chitin (800  $\mu$ L) was washed three times with 2 volumes of buffer and resuspended in a final volume of 1600  $\mu$ L (0.5% w/v final chitin concentration). After a 30 min equilibration of substrate at each chosen temperature, 50  $\mu$ g/mL of enzyme was added to initiate reactions. After a 1 h incubation, samples were centrifuged (12000g for 5 min) and GlcNAc concentrations were determined in 250  $\mu$ L of clear supernatant.

**Determination of Substrate Specificity.** Activity against *N,N'*-diacetylchitobiose and the oligomers (GlcNAc)<sub>3-6</sub> was assessed under the following conditions. Two microliters of individual substrates at 10 mg/mL in water was diluted into a final volume of 50  $\mu$ L of 50 mM ammonium acetate, pH 5.5, to which 1  $\mu$ L of enzyme (10 mg/mL) was added. Two control reactions were run per substrate: one with substrate but without enzyme and the other with substrate and heat-inactivated enzyme (100 °C for 5 min). After a 1 h incubation at 37 °C, samples were freeze-dried, during which the ammonium acetate was evaporated. The residues were then resuspended in 3  $\mu$ L of water and loaded onto TLC plates.

**Yeast Strains, Growth Media.** Four strains of *Saccharomyces cerevisiae* were used; laboratory strain S288c (*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6*); a brewing strain, 2007-Pilsen Lager (Wyeast Laboratories); and the wine strains EC1118 and RC212 (Lallemand). Strains were grown in yeast nitrogen base (YNB) without amino acids, pH 5.2 (Difco), containing 2% glucose (YNB + glu) or in YNB containing 2% glucose adjusted to pH 3.5 by the addition of 26.6 mM tartaric acid (YNB + glu + tartrate, pH 3.5).

**Yeast Growth Assay.** Growth inhibition was assessed as a reduction in cell yield ( $A_{600}$ ) in treated versus control cells. Treated cells were grown in the presence of grape chitinase, whereas control cells were grown in the presence of the same amount of boiled grape chitinase. Cells were grown overnight in YNB + glu or YNB + glu + tartrate, pH 3.5, washed twice in sterile distilled water, and then diluted to an initial concentration of 10<sup>3</sup> cells/mL in 1 mL aliquots of YNB + glu or YNB + glu + tartrate, pH 3.5, respectively, containing 50  $\mu$ g of chitinase or boiled chitinase (control). In 100 mM sodium



**Figure 1.** HPLC chromatogram of pooled fractions containing the purified 31 kDa protein (elution peak at 21 min).

**Table 1. Identified Peptides with Matches in *V. vinifera* Chitinases Q7XAU6\_VITVI and O24530**

protein	sequence	Mascot ion score <sup>a</sup>	m/z
Q7XAU6_VITVI	AAFLSALNSYSGFGNDGSTDANKR	104.89	821.7239
	TALWFWMNNVHSVIGQGFGATIR	82.87	1303.17
O24530	AINGAVECNGGNTAAVNAR	105.66	929.9467

<sup>a</sup>Mascot ion score =  $[-10 \log(P)]$ , where  $P$  is the calculated probability that the observed MS/MS match between the experimental data and the database sequence is random.

phosphate at pH 6.0, 5.2, and 3.5, 50 µg of grape chitinase was found to contain  $0.0237 \pm 9.7 \times 10^{-4}$ ,  $0.0288 \pm 1.3 \times 10^{-3}$ , and  $0.0249 \pm 1.8 \times 10^{-3}$  units of activity ( $n = 2$ ), respectively. Initial cell concentrations were determined by use of a hemacytometer. Cells were incubated in triplicate for 48 or 72 h at 30 °C and 200 rpm in 1.5 mL screw-capped polypropylene tubes, after which  $A_{600}$  values were measured. Growth inhibition was expressed as the ratio [ $A_{600}$  (treated cells)/ $A_{600}$  (control cells)] × 100. The significance of differences was assessed using Student's two-sided, two-tailed *t* test ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

**Chitinase Purification and Identification.** After the two purification steps, 130 mg of chitinase (97% purity by HPLC, calculated as peak area on the chromatogram) was obtained from 6 L of Manzoni Bianco grape juice (Figure 1), comparable to yields obtained by Van Sluyter et al., 45 and 10 mg/L for Semillon and Sauvignon blanc juices, respectively.<sup>5</sup>

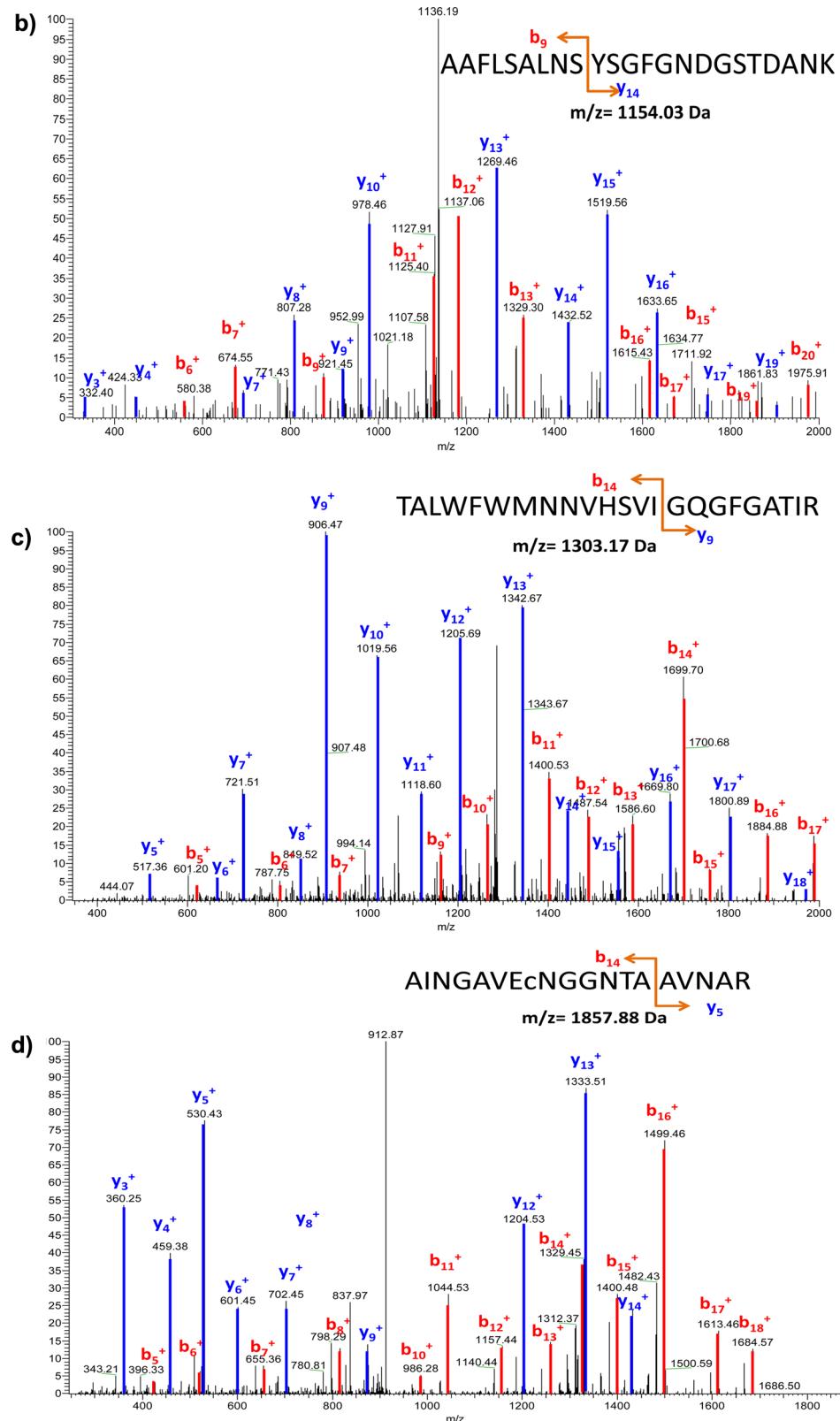
The purified 31 kDa protein was identified as a type IV chitinase on the basis of significant matches with three different

tryptic peptides (Table 1) shared by two enzymes designated Q7XAU6\_VITVI (UniProtKB) based on the deduced amino acid sequence of cDNA VvChi4D (EMBL AAQ10093.1) and the *V. vinifera* chitinase designated O24530 (UniProtKB), the sequence of which was deduced from cDNA VvChi4A.<sup>4</sup> Q7XAU6\_VITVI was previously detected in juice from Semillon and Sauvignon blanc grape varieties.<sup>5,30,31</sup> It is possible that the two proteins are allelic variants encoded by the same gene (Figure 2a). Tandem mass spectra of ion fragments derived from the three identified peptides are listed in Figure 2b–d. Although protein and peptide identification probabilities were set to >90%, the peptides listed in this figure have peptide identification probabilities of 100%.

**Determination of Substrate Specificity.** Because tetramers and larger oligomers of GlcNAc were unavailable commercially, these substrates were purified from a partial chitin hydrolysate by size exclusion chromatography. Figure 3 shows the purified oligomers (GlcNAc)<sub>3–5</sub> separated by TLC. The fractions were freeze-dried and resuspended in water at 10

a)	Q7XA-	1	MAAKLLTVLLVGALFGAAVAQNCGCASGLCCSKYGYCGTGS DYGCGCQSGPCDSSSGSG	60
			MAAKLLTVLLVGALFGAAVAQNCGCASGLCCSKYGYCGTGS DYGCGCQSGPCDSS	G
	0245-	1	MAAKLLTVLLVGALFGAAVAQNCGCASGLCCSKYGYCGTGS DYGCGCQSGPCDSS---G	57
	Q7XA-	61	SSVSDIVTQSFFDGIIINQAASSCAGKNFYTRAFLS ALNSYSFGNDGSTDANKREIAAF	120
			SSVSDIVTQSFFDGII+QAASSCAGKNFYTR <b>AAFLSALNSYSFGNDGSTDANK</b> REIAAF	
	0245-	58	SSVSDIVTQSFFDGIIISQAASSCAGKNFYTRAFLS ALNSYSFGNDGSTDANKREIAAF	117
	Q7XA-	121	FAHVTHETGHFCYIEEINGASHNYCDSSNTQYPCVSG QNYGRGPLQLTWNYNNGAAGNS	180
			FAHVTHETGHFCYIEEINGASHNYCDSSNTQYPCVSG QNYGRGPLQLTWNYNNGAAGNS	
	0245-	118	FAHVTHETGHFCYIEEINGASHNYCDSSNTQYPCVSG QNYGRGPLQLTWNYNNGAAGNS	177
	Q7XA-	181	IGFNGLSNPGIVATDVVTSFKTALWF WMNNVHSVIGQGFATIRAIN GAVECNGGNTAAV	240
			IGFNGLSNPGIVATDVVTSFK <b>TALWF WMNNVHSVIGQGFATIRAI</b> <b>NGAVECNGGNTAAV</b>	
	0245-	178	IGFNGLSNPGIVATDVVTSFKTALWF WMNNVHSVIGQGFATIRAIN GAVECNGGNTAAV	237
	Q7XA-	241	NARVQYYKDYCSQLGVSPGDNLTC	264
			<b>NARVQYYKDYCSQLG</b> SPGDNLTC	
	0245-	238	NARVQYYKDYCSQLGASPGDN LTC	261

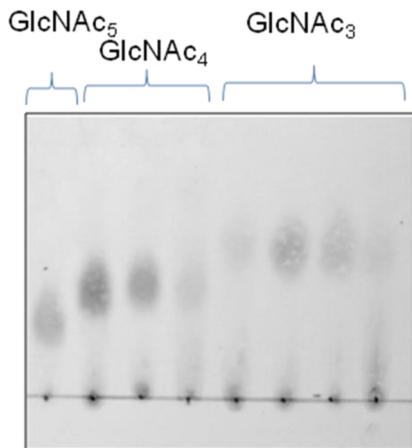
**Figure 2.** continued



**Figure 2.** Sequence alignment of identified *V. vinifera* class IV chitinases and mass spectra for fragment ions derived from the three identified peptides. (a) Aligned sequences of UniProtKB Q7XAU6\_VITVI (Q7XA-) and UniProtKB O24530 (0245-) with the identified peptides indicated in color and in bold font. Because the second and third peptides are contiguous, the third is in italicized bold font. Mass spectra for the major fragment ions (red, b ions; blue, y ions) matched the theoretically predicted fragmentation of (b) peptide AAFLSALNSYSFGNDGSTDANK, (c) peptide TALWFWMNNVHSVIQGQFGATIR, and (d) peptide AINGAVEcNGGNNTAAVNAR.

mg/mL for subsequent analyses. The oligomers ( $\text{GlcNAc}_1 - \text{GlcNAc}_6$ ) were incubated with enzyme or heat-inactivated

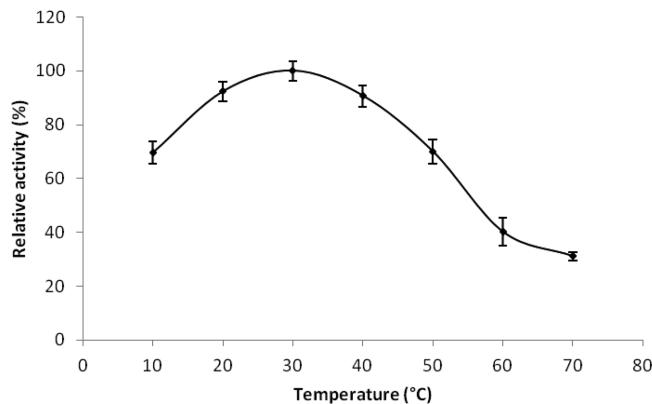
enzyme, and the reaction products were visualized by TLC (Figure 4). In Figure 4, spots are coded by letter and number.



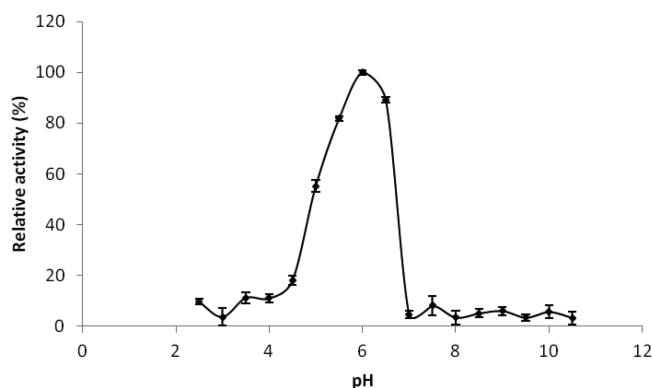
**Figure 3.** TLC analysis of a partial HCl hydrolysate of chitin fractionated by size exclusion chromatography. Lanes: 1, ( $\text{GlcNAc}$ )<sub>5</sub>; 2–4, ( $\text{GlcNAc}$ )<sub>4</sub>; 5–8, ( $\text{GlcNAc}$ )<sub>3</sub>.

Letters A–F indicate substrates  $\text{GlcNAc}_1$ – $\text{GlcNAc}_6$ , respectively. Numbers indicate treatment. Number 1 indicates incubation of substrate in the absence of enzyme. Numbers 2 and 3 indicate incubation with enzyme or heat-inactivated enzyme (100 °C for 5 min), respectively.

As expected, the monomer  $\text{GlcNAc}$  was not affected (Figure 4A1–A3), nor was  $N,N'$ -diacetylchitobiose degraded (Figure 4B1–B3), indicating an absence of 1,4- $\beta$ -N-acetylglucosaminidase activity. On the other hand, chitotriose was split into the monomer  $\text{GlcNAc}$  and dimer  $N,N'$ -diacetylchitobiose (Figure 4C1–C3). Therefore, on the basis of the classification of Harman et al.,<sup>18</sup> this grape enzyme should be considered an exochitinase. However, as noted below, this designation is not consistent with the products generated from degradation of the tetramer ( $\text{GlcNAc}$ )<sub>4</sub>. The degradation of ( $\text{GlcNAc}$ )<sub>4</sub> (Figure 4D1–D3) yielded both a dimer, as would be expected if chitobiosidase activity alone were present, and the monomer. On the basis of the absence of 1,4- $\beta$ -N-acetylglucosaminidase activity (Figure 4B1–B3), we speculate that formation of the monomer was due to transient production of the trimer during cleavage, suggesting an endochitinase-based mechanism. Both monomer and dimer were also obtained from hydrolysis of the

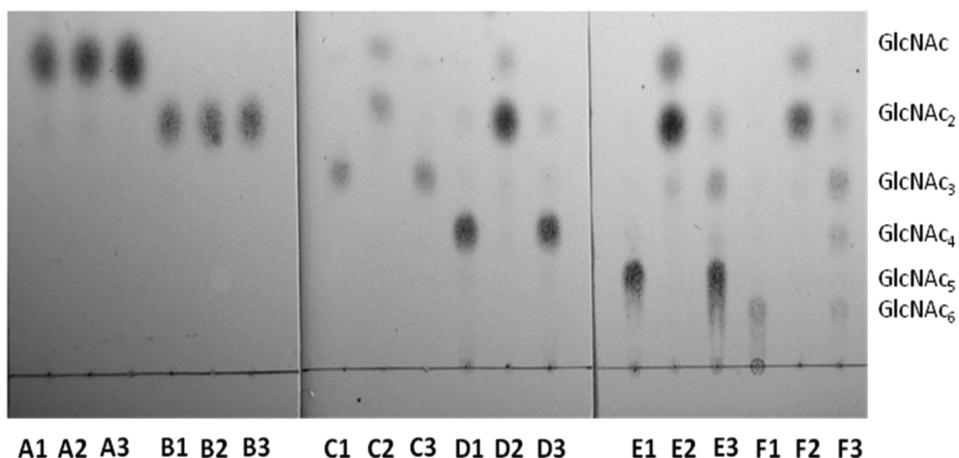


**Figure 5.** Chitinase activity as a function of temperature. Values are means of three replicates, and error bars are standard deviations.

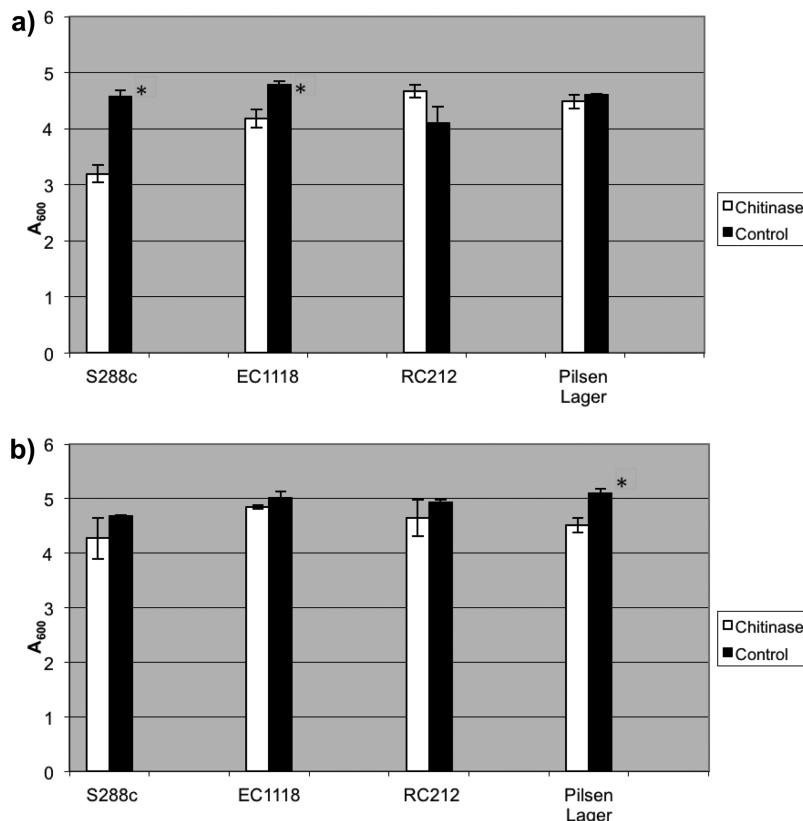


**Figure 6.** Chitinase activity as a function of pH. Values are means of three replicates, and error bars are standard deviations.

pentamer (Figure 4E1–E3) and hexamer (Figure 4F1–F3) as previously observed with other endochitinases.<sup>25</sup> The intermediate oligomers present in lanes E3 and F3 (Figure 4) are likely due to some chitinolytic activity present in the heat-denatured chitinase samples. This can be due to an incomplete denaturation of the catalytic domain or to its refolding upon cooling. Indeed, it was previously demonstrated that the grape chitinase is able to recover its activity after a 5 min treatment at



**Figure 4.** TLC analysis of reaction products generated by incubation of purified chitinase with purified oligomers of  $\text{GlcNAc}$ . Spots are coded by letter and number. Letters A–F indicate substrate  $\text{GlcNAc}_1$  to  $\text{GlcNAc}_6$ , respectively. Number 1 indicates incubation of substrate in the absence of enzyme. Numbers 2 and 3 indicate incubation with enzyme or heat-inactivated enzyme (100 °C for 5 min), respectively.



**Figure 7.** Inhibition of yeast growth: (A) inhibition after 48 h in YNB + glu assessed as a reduction in cell yield ( $A_{600}$ ) in the presence of 50  $\mu$ g of chitinase or boiled chitinase (control) starting with an initial inoculum of  $10^3$  cells/mL; (B) inhibition after 72 h in YNB + glu + tartrate, pH 3.5 assessed as a reduction in cell yield ( $A_{600}$ ) in the presence of 50  $\mu$ g of chitinase or boiled chitinase (control) starting with an initial inoculum of  $10^3$  cells/mL. Data are means of triplicate assays. An asterisk indicates a significant difference between treatment and control (Student's two-sided, two-tailed  $t$  test,  $p < 0.05$ ).

100 °C followed by SDS-PAGE migration.<sup>32</sup> The presence of oligomers is consistent with endochitinase activity. These results are in agreement with a previous study that found the major grape chitinases to be class IV endochitinases on the basis of protein sequence.<sup>4</sup> However, a chitinase purified from *Bacillus brevis* characterized as an endosplitting enzyme produced mostly oligomers much larger than (GlcNAc)<sub>4</sub> from a substrate consisting of a hydrolysate of colloidal chitin.<sup>26</sup> In addition, it has been reported that to determine chitinase activity from a variety of sources (e.g., soybean seeds<sup>33</sup> or *Streptomyces*<sup>34</sup>), a specific chitobiase is needed to degrade the disaccharide to produce p-DMAB-reactive GlcNAc. In our case, it was possible to quantify enzymatic activity directly using p-DMAB without the addition of an exogenous chitobiase.

The pattern of degradation products observed here from various GlcNAc oligomers is very similar to that reported for an *Aspergillus fumigatus* chitinase, presumed to be a family 18 enzyme,<sup>35</sup> that exhibited exo- and endochitinase and trans-glycosylation activities.<sup>36</sup> On the other hand, a chitinase from *Pyrococcus kodakaraensis* classified as an endochitinase<sup>25</sup> produced (GlcNAc)<sub>2</sub> and GlcNAc from (GlcNAc)<sub>3</sub> and (GlcNAc)<sub>2</sub> along with small amounts of GlcNAc from (GlcNAc)<sub>4–6</sub>.

In summary, both exochitinase activity and the previously described endochitinase activity are suggested for the enzyme purified from grape juice.

#### Determination of Temperature and pH Optima for Chitinase.

Enzyme activity was assessed as a function of temperature and pH. The optimal temperature for chitinase

activity was evaluated between 10 and 70 °C (Figure 5). Highest activity was observed between 30 and 40 °C, consistent with a 42 °C optimum found for grape chitinase isolated from *V. vinifera* Red Globe.<sup>21</sup> At temperatures above 50 °C, activity was found to decrease drastically, in agreement with a reported denaturation temperature of about 55 °C.<sup>37</sup> However, it must be noted that this latter result was obtained at pH 3 and that later it was reported that the melting temperature of the grape chitinase increased from 55 to 65 °C by raising pH from 3 to 4.<sup>31</sup> Therefore, the 30% residual activity found here after heating at 70 °C at pH 6 could be due to the effect of pH. More recently, Saito et al. described three chitinase isoforms in *V. vinifera* Semillon with temperature optima between 50 and 60 °C.<sup>22</sup>

The optimal pH for activity was found to be about 6, with relatively high activity observed in the range pH 5–6.5 (Figure 6). The same optimum was found by Gomes et al., although these researchers also detected a second peak of activity at about pH 3.<sup>21</sup> In our case, outside the pH 5–6.5 range, including the pH of grape juice, 3–3.5, activity was found to decrease sharply. On the other hand, chitinase isoforms purified from Semillon exhibited an optimal pH in the range of 4–4.5.<sup>22</sup>

Experiments performed at a pH and temperature close to those of winemaking conditions (25 °C and pH 3.2) demonstrated that the enzyme retained 12% of its maximal activity (data not shown), suggesting that residual activity could be present in grape must during fermentation.

**Inhibition of Yeast Growth.** Like other pathogenesis-related plant proteins, chitinases are synthesized in response to

biotic or abiotic stresses. However, in grapes, they are also generally expressed in a constitutive manner during ripening.<sup>4</sup> As a consequence, the concentration of chitinases can reach relatively high levels in grape juice.<sup>38,39</sup> The question of whether potential activity in grape juice could affect the major yeast species that mediates the wine fermentation is thus relevant because the cell wall of the wine yeast *S. cerevisiae* contains a small amount of chitin (1–2% dry wt), localized in bud scars.<sup>40</sup> Indeed, a class IV-like chitinase from *Streptomyces* was found to inhibit growth of *S. cerevisiae* at pH 5.8.<sup>41</sup> Because chitinase activity could potentially interfere with yeast growth under winemaking conditions, the effect of chitinase on cell yields of four different strains of *S. cerevisiae* was assessed using very low initial inoculum levels and an amount of chitinase expected in ripe grapes, 50 µg/mL. Although this concentration was found to reduce cell yield of the laboratory strain S288c by 30% and that of the wine strain EC1118 by 12% after 48 h in YNB + glu (Figure 7A), after 72 h, no reduction in cell yield was observed for any of the four strains tested (data not shown). In YNB + glu + tartrate, pH 3.5, a modest reduction in cell yield (12%) was observed only for the Pilsen Lager strain after 72 h (Figure 7B). No reduction was observed for either the laboratory or wine strains (data not shown). It is important to note that the chitinase activity tested here was about 10<sup>4</sup> less than that previously reported to inhibit growth of an unspecified strain of *S. cerevisiae*.<sup>41</sup>

These data suggest that yeast growth in a standard medium (YNB + glu) is slowed transiently if at all by the chitinase treatment and that cells recover if given sufficient time. After 72 h at pH 3.5, only growth of the brewing strain was reduced (modestly). Whether greater inhibition might be observed toward the wine strains during vinification in actual grape must (pH 3–4) when cells are exposed to greater osmotic stress at the higher initial sugar levels (~20–25%) and higher ethanol levels is an open question.

In conclusion, we speculate that chitinase is unlikely to have an inhibitory effect on yeast growth during vinification, presuming the activity tested here is representative of that present in grape must and the fact that winemakers who use starter cultures typically inoculate with at least 10<sup>6</sup> cells/mL, which is about 10<sup>3</sup>-fold higher than the level evaluated in the present study. Even if highly stressed cells might be more susceptible to chitinase-mediated growth inhibition, such cells in a wine fermentation have ceased growth for other reasons but continue to produce a significant amount of ethanol during stationary phase. Thus, growth inhibition per se would be unlikely to arrest fermentation, unless endogenous grape chitinase levels were high enough to inhibit initial growth of the starter culture or that of the naturally occurring yeasts present in uninoculated fermentations. On the other hand, we do not know if chitinase can interfere with fermentation in an indirect manner, unrelated to growth, by inhibiting other cellular processes.

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The proteomic analysis was performed in the Oregon State University Environmental Health Sciences Center mass spectrometry facility, which is supported in part by a grant from the National Institute of Environmental Health Sciences (P30 ES000210).

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