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Evidence for an Extracellular Acid Proteolytic Activity Secreted by Living Cells of *Saccharomyces cerevisiae* PlR1: Impact on Grape Proteins

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Supporting Information

ABSTRACT: In this work, *Saccharomyces cerevisiae* PlR1, a strain isolated from Pinot noir grapes in the Champagne area, was shown to secrete an acid proteolytic activity against bovine serum albumin. This proteolytic activity was detectable in cell-free culture supernatants at the beginning of the exponential growth phase and increased with yeast growth. Using a zymography method, only one protease band with a molecular mass of 72 kDa was observed. This extracellular proteolytic activity was detected in the pH range from 2 to 4 with a maximal value at pH 2.5 and 38 °C and was completely inhibited by pepstatin A. The secretion of this protease did not need any protein inducer and seemed to be insensitive to nitrogen catabolic repression. *S. cerevisiae* PlR1 was also able to secrete this proteolytic activity during alcoholic fermentation, and it was found to be active against grape proteins, with a molecular mass around 25 kDa, at optimal conditions of 38 °C, pH 3.5.

KEYWORDS: *Saccharomyces cerevisiae*, secreted enzyme, acid protease, BSA, grape proteins, wine

INTRODUCTION

Haze or deposit formation in a bottle, due to protein aggregation during storage, is the most common nonmicrobial defect of commercial white wines, making them unacceptable for consumers.[1] In general, the haze does not depend on total protein content but rather on a specific grape protein fraction.[2] Previous works have shown that specific protein fractions with a molecular mass between 13 and 30 kDa and a low isoelectric point (pI = 4.1−5.8)[3,4] are largely responsible for the haze in wine. Most of the wine proteins responsible for haze were identified as grape pathogenesis-related proteins (thaumatin-like proteins and chitinases).[5−7] Recently, authors have found that β-1,3-glucanase and the ripening-related protein grip22 precursor might participate in the wine protein haze.[8]

The prevention of protein instability is generally achieved by adsorption of the protein fraction on natural earths such as bentonite.[9,10] Ultrafiltration[11,12] and zirconium oxide treatment[13] can also be effective, but their use is still experimental. Another mean for rendering wines stable is offered by enzymatic hydrolysis of wine proteins.[14] Some papers looked into the application of proteases in free and immobilized forms for the treatment of wine protein instability.[15,16] An alternative procedure for this treatment may be represented by the production of extracellular acid protease(s) by wine yeast.

Grapes and grape juice carry a diversity of yeast strains in addition to *Saccharomyces* species with predominance of the low ethanol tolerant strains of *Hanseniaspora*, *Kloeckera*, and various species of *Candida*.[17,18] Many of the yeast natural flora associated with grapes secrete acid proteases.[19−22] Some of these proteases have been shown to be active against wine proteins.[19,23] Nevertheless, a decrease in protein content has never been associated with the loss of specific proteins, and none of the proteases liberated by these non-*Saccharomyces* wine yeasts was able to favorably impact the heat/chill haze forming potential of the wines.[21]

The fermentative yeast of the genus *Saccharomyces* does not normally secrete external proteases.[24,25] Nevertheless, liberation of a proteolytic activity by such yeast has been sporadically reported.[26] On the other hand, brewing yeasts are able to secrete acid protease(s) during fermentation.[27,28] These proteases are detrimental to beer foam stability because of their ability to degrade foam-promoting barley proteins such as the lipid transfer protein (LTP1).[29,30] Despite the fact that they can be secreted by living cells, these proteases were not identified as external proteases but as vacuolar proteases such as protease A.[31] Relatively little is known about extracellular acid protease production by *Saccharomyces* wine yeast, and the results obtained are quite controversial. A study on a strain of *Saccharomyces bayanus* has demonstrated the presence of a relevant proteolytic activity of this strain during grape must fermentation.[32] The authors have found a maximal activity at the end of alcoholic fermentation and in the presence of proteins. Moreover, no proteolytic activity was detected when fermentations were performed with pasteurized musts. Following a screening on 94 strains of *Saccharomyces cerevisiae*,[33] it has been observed that only 17 strains produced a relevant proteolytic activity in a synthetic medium supplemented with casein or hemoglobin only at the end of the active phase of growth. However, in both studies,[32,33] proteases were not characterized. No proteolytic activity was detected during alcoholic fermentation of a synthetic must by an enological strain of

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S. cerevisiae even after the fermentation medium had been concentrated 200-fold by ultrafiltration.[34]

In the present work, we report on the study of an acid proteolytic activity secreted by a wild strain of *S. cerevisiae* PlR1 after growth under different nitrogen sources. Parameters required for an optimal activity and some of its properties were determined. The secretion of this activity during alcoholic fermentation, by this strain, on chardonnay grape juice and its impact on must proteins were also studied.

MATERIALS AND METHODS

Yeast Isolation. The fermentative yeast used in this study was isolated in 2006 in our laboratory following spontaneous fermentation of healthy Pinot noir grape juice. The grapes used were sourced from Pargny-les-Reims vineyard (Champagne area, France), and the yeast was isolated for its ability to produce a clear zone on casein agar plates[22] (Supporting Information, Figure S1). Molecular identification, done by polymerase chain reaction and based on the sequence analysis of the rRNA D2 LSU gene, proved that this yeast belongs to *S. cerevisiae* species, and it was named *S. cerevisiae* PlR1.

Media and Yeast Growth. Yeast nitrogen base without amino acids and ammonium sulfate (YNB-N-aa; Difco) supplemented at 50 g/L with glucose and at 600 mg nitrogen/L with a nitrogen source was used as the culture medium. The nitrogen sources used were ammonium chloride (NH_4Cl), urea, glutamine, and proline. Media were inoculated at an initial optical density (O.D.) of 0.1 at 600 nm with cultures previously grown for 48 h in the same media. Cultures were carried out at 18 °C under constant agitation (150 rpm) in Erlenmeyer flasks filled to 50% of their volume and covered with a cotton cap. Growth was followed by measuring O.D. at 600 nm.

A Chardonnay grape juice (elaborated in 2008 from Nogent l'Abbesse, Champagne area, France) was used as a fermentation medium. The composition of the grape juice was as follows: pH, 2.99; free sulfur dioxide (SO_2), 19 mg/L; glucose, 88 g/L; and fructose, 86 g/L. Before use, the juice was sterile filtered through a 0.45 μm membrane (Millipore, United States). It was inoculated at an initial O.D. of 0.2 at 600 nm with yeast previously grown for 48 h in a yeast extract glucose (YEG) medium (yeast extract, 0.5% w/v; glucose, 5% w/v) and washed twice prior to use. Fermentations were carried out at 18 °C without any shaking in 100 mL flasks filled to 95% of their volume. The fermentation rate was measured as weight loss (CO_2 evolution). A flask filled with uninoculated grape juice was used as the control.

Supernatant Preparation. Samples were collected at different stages during grape juice fermentation or shaken cultures on yeast nitrogen base media and centrifuged at 3500*g* for 10 min at 4 °C. The cell pellets were discarded, and the crude supernatants were successively filtered through a 0.45 μm membrane (Millipore), dialyzed against Milli-Q water using regenerated cellulose dialysis membranes with a molecular mass cut off 10 kDa, and concentrated by lyophilization. The concentrated supernatants were stored at −20 °C until used. The cell wall integrity was confirmed by the lactate dehydrogenase (LDH) test.[35] The measurement of LDH activity in the concentrated supernatants was performed as previously described.[36] Ten microliters of concentrated supernatant was added to 600 μL of 100 mM phosphate buffer, pH 7, and mixed with 200 μL of 1 mM NADH. The absorbance at 340 nm was measured.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis. Vertical SDS-PAGE was performed in a Mini-Protean III electrophoresis cell (Bio-Rad, United States) according to the patented method,[37] using a 4% stacking gel (pH 6.8) and a 12% resolving gel (pH 8.8). Samples were diluted with Laemmli buffer (4×), and 25 μL was loaded into the wells. Gels were run under a constant voltage of 150 V. After migration, proteins were stained with colloidal Coomassie Blue G-250[38] when using BSA (bovine serum albumin, fraction V, essentially fatty acid free; Sigma Chemical Co., United States) as a substrate and with silver nitrate [39] for the grape juice proteins. Computerized SDS-PAGE analysis, including band detection and quantification, was performed using GS-800 scanner and Quantity One 4.6.2 software (Bio-Rad). The average density of each band of interest was computed; means ± standard deviations (n = 3) were calculated for each assay.

Measurement of Proteolytic Activity. The proteolytic activity of the concentrated supernatants was assayed using BSA as the protein substrate. Twenty microliters of each concentrated supernatant obtained from shaken cultures or grape juice fermentation was added to 980 μL of McIlvaine's buffer (0.1 M citric acid, 0.2 M disodium phosphate), pH 3.5, containing BSA. The reaction mixture was incubated at 38 °C, and then, it was stopped by diluting the reaction mixture three times with Sorensen's glycine II buffer (0.1 M glycine in 0.1 N NaCl, 0.1 N NaOH, pH 10) or by freezing at −20 °C; BSA hydrolysis was followed by SDS-PAGE. The proteolytic activity was expressed as μg of BSA hydrolyzed per hour under the experimental conditions, either by μg of protein (specific activity) or by mL of unconcentrated culture supernatant (global activity).

Zymogram Gels. The proteolytic activity of the concentrated supernatant was visualized in 12% SDS-PAGE using the zymography method. After migration, the SDS gel was incubated for 1 h at room temperature and under constant agitation in 2.5% w/v Triton X-100 solution, to remove SDS. Subsequently, the gel was incubated for 18 h at 38 °C in MacIlvaine's buffer (pH 3.5) supplemented with 0.1% w/v BSA. Then, the gel was washed with distilled water, stained for 1−2 h with 0.02% w/v Coomassie Brilliant Blue G-250 in methanol−acetic acid−water (45/10/45, v/v/v) solution, and then destained using methanol−acetic acid−water (45/10/45, v/v/v) solution. The proteolytic activity appeared as clear bands (area of BSA digestion) against blue background. The molecular mass (kDa) of the protease was determined using prestained molecular weight standards (Bio-Rad).

Effect of Physicochemical and Enological Parameters on Proteolytic Activity. For each parameter, the proteolytic activity of the concentrated culture supernatant at the beginning of the stationary growth phase was assayed against BSA for 24 h. To determine the effect of pH, the proteolytic activity was measured at different pH values ranging from 2 to 6 using the following buffers: from pH 2.0 to 2.5, Sorensen's glycine I buffer (0.1 M glycine in 0.1 N NaCl, 0.1 N HCl); from pH 3.0 to 6.0, MacIlvaine's buffer (0.1 M citric acid, 0.2 M disodium phosphate). To study the effect of temperature, enzymatic assays were conducted at different temperatures (10−60 °C) at pH 3.5 in a thermostatically controlled water bath. The proteolytic activity was also determined in the presence of 50, 100, and 150 mg/L of SO_2 and with the ethanol concentration ranging from 4 to 14% v/v.

Effect of Protease Inhibitors. The concentrated culture supernatant of *S. cerevisiae* PlR1 at the beginning of the stationary growth phase was incubated with BSA for 24 h at 38 °C and pH 3.5 in the absence (control) or in the presence of different protease inhibitors (Sigma Chemical Co.): 17 μM pepstatine A, 1.2 mM 1,10-phenanthroline, 1.2 mM phenylmethylsulfonyfluoride (PMSF), 0.8 mM iodoacetamide, and 0.8 mM *N*-α-tosyl-L-lysine-chloromethyl-ketone (TLCK). The 100% of the enzyme activity was the activity of enzyme without any inhibitor.

In-Gel Digestion and Nano-LC-Tandem Mass Spectrometry (Nano-LC-MS/MS) Analysis. SDS-PAGE gel of must proteins was stained with colloidal Coomassie Blue G-250 stain, and the band of interest was cut out manually. Nano-LC-MS/MS analysis was performed after trypsin digestion as described in ref 40. Searches were performed against the National Center for Biotechnology Information nonredundant (NCBInr) database without any taxonomic, molecular weight, or isoelectric point restrictions. The tolerance on mass measurement was 0.25 Da in both MS and MS/MS modes. One missed cleavage per

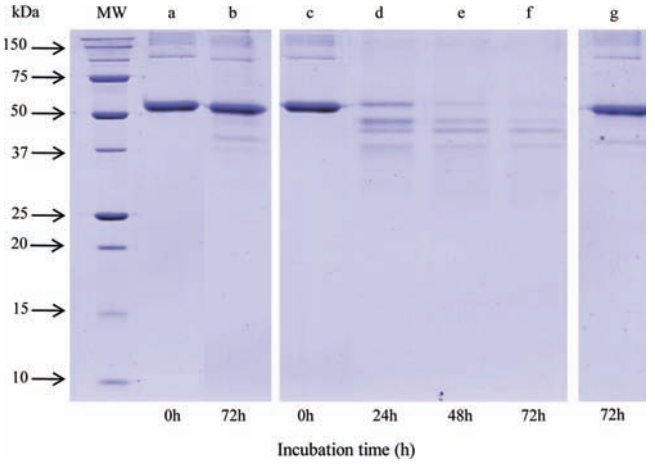

Figure 1. SDS-PAGE analysis monitoring extracellular acid proteolytic activity on BSA of concentrated culture supernatant of *S. cerevisiae* PlR1 at the beginning of stationary growth phase. Incubation was performed at 38 °C and pH 3.5. Lanes a and b, blank sample (BSA without concentrated supernatant) before and after 72 h of incubation, respectively; lane c, BSA with concentrated supernatant before incubation; lanes d−f, BSA after 24, 48, and 72 h of incubation with concentrated supernatant, respectively; and lane g, BSA with concentrated supernatant previously heated (100 °C, 10 min) after 72 h of incubation. Molecular weight standard proteins are given on the left side of the gel.

peptide was allowed, and some variable modifications were taken into account such as carbamidomethylation for cysteine and oxidation for methionine.

Analysis. Concentrations of glucose, fructose, ethanol, glycerol, acetic acid, and malic acid were measured using enzymatic test kits (Raiso Diagnostics, Italy). Proteins were quantified according to Bradford method.[41] BSA was used as a standard. A single-factor analysis of variance [Statistical Package for the Social Sciences (SPSS), IBM] was used to evaluate the differences between the assays.

RESULTS AND DISCUSSION

Evidence for a Secreted Proteolytic Activity. The ability of *S. cerevisiae* PlR1 to secrete an acid extracellular protease activity was studied using a culture medium with proline as the nitrogen source. The concentrated supernatant was obtained from cells collected at the beginning of the stationary growth phase. BSA (400 μg) was incubated with the concentrated supernatant during 72 h at 38 °C, pH 3.5. The reaction was stopped by Sorensen's glycine II buffer, pH 10, and proteolysis was monitored by SDS-PAGE analysis (Figure 1). The first blank sample shows the profile of a commercially available BSA alone before incubation (Figure 1, lane a). The main band, with a molecular mass around 59 kDa, corresponds to BSA. The second blank sample shows the profile of BSA alone after 72 h of incubation (Figure 1, lane b). No important degradation of BSA is observed, indicating that the protein is stable under the conditions used and that the BSA preparation does not seem to contain any proteolytic activity. Profiles corresponding to *S. cerevisiae*-treated samples are illustrated from lane c to lane g. Proteins from *S. cerevisiae* supernatant do not interfere with BSA analysis because the concentrated supernatant does not contribute to observable bands at the concentration at which it is used in these experiments (Figure 1, lane c). *S. cerevisiae*-treated samples show an evident degradation of BSA. The intensity of the major band

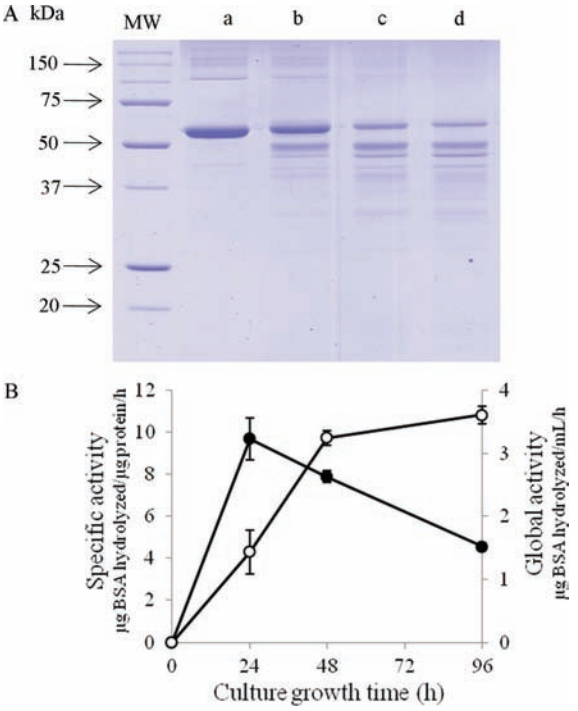

Figure 2. Extracellular acid proteolytic activity of *S. cerevisiae* PlR1 during growth kinetic. (A) SDS-PAGE analysis monitoring BSA hydrolysis after 24 h of incubation at 38 °C and pH 3.5 without concentrated supernatant. Lane a, with concentrated supernatants corresponding to cells harvested; lane b, beginning of the exponential growth phase; lane c, middle of the exponential growth phase; and lane d, beginning of the stationary growth phase. Molecular weight standard proteins are given on the left side of the gel. (B) Quantification of the proteolytic activity during yeast growth. Activity is expressed in specific proteolytic activity in μg BSA hydrolyzed/μg protein/h (●) and global proteolytic activity in μg BSA hydrolyzed/mL/h (○). Error bars indicate the standard deviations of three analyses.

at 59 kDa continuously decreases with the incubation time (24 and 48 h) (Figure 1, lanes d and e, respectively), and this band is no more detectable after 72 h (Figure 1, lane f) of incubation. Simultaneously, bands of lower molecular masses appear, which are in turn hydrolyzed. BSA hydrolysis is not observed when the *S. cerevisiae* concentrated supernatant is previously heated to 100 °C for 10 min (Figure 1, lane g). Thus, BSA hydrolysis in *S. cerevisiae*-treated samples does not result from a chemical or a physical phenomenon but more evidently from an enzymatic one. *S. cerevisiae* cells, in stationary growth phase, seem to be able to secrete a proteolytic activity against BSA at pH 3.5.

Secreted Proteolytic Activity during Growth. BSA hydrolysis in relation with yeast growth kinetic was also studied. *S. cerevisiae* PlR1 was inoculated in the same medium with proline as the nitrogen source, and cells were collected by centrifugation after 24 h (beginning of the exponential growth phase), 48 h (middle of the exponential growth phase), and 96 h (beginning of the stationary growth phase). Each concentrated supernatant was submitted to the LDH test, and the results obtained confirmed that none of the supernatants used was contaminated by intracellular proteolytic activity (Supporting Information, Table S1). The concentrated supernatants were then incubated with 400 μg of BSA for 24 h at 38 °C in MacIlvaine's buffer, pH 3.5. The reaction was stopped as already mentioned, and proteolysis was monitored by SDS-PAGE analysis (Figure 2A).

The proteolytic activity was already detectable in the concentrated culture supernatant of cells harvested at the beginning of the exponential growth phase (Figure 2A, lane a) and increased with yeast growth (Figure 2A, lanes b and c). The proteolytic activity of the supernatants has been quantified following densitometric analysis of the BSA band on SDS-PAGE (Figure 2B). While the global proteolytic activity of the supernatant (expressed as μg of BSA hydrolyzed per hour per mL) increases until the beginning of the stationary growth phase, the specific proteolytic activity (expressed as μg of BSA hydrolyzed per hour per μg of protein) reaches its maximum level when cells are at the beginning of the exponential growth phase and then decreases during growth phases (Figure 2B).

Secretion of protease is not an ability commonly attributed to fermentative yeast belonging to the genus *Saccharomyces*, and none of the available data, which are rather controversial, completely agrees with our results. Some of the published data indicated the presence of acid proteolytic activity released only during autolysis and considered it as a vacuolar protease and more particularly to protease A, which is the only vacuolar acid protease found in yeast.[28,34] Others indicated the presence of proteolytic activity released by living cells before completion of the exponential growth phase, as for *S. cerevisiae* PlR1, but only when growth was performed in the presence of proteins as nitrogen source.[31,32] According to another work,[42] living cells of *S. cerevisiae* are able to secrete several proteases without any protein inducer, but because proteolytic activity has only been quantified at pH 7.5, these data can not allow us to conclude about the secretion of acid proteases.

Characterization of Proteolytic Activity by BSA Zymography. To better characterize the extracellular protease profile of *S. cerevisiae* PlR1, a BSA zymography was performed (Figure 3). Analysis of the gel reveals the presence of only one acid proteolytic activity corresponding to a molecular mass of 72 kDa (Figure 3, lane b). This activity is not observed when the concentrated supernatant was previously heated to 100 °C for 10 min (Figure 3, lane a). Because the molecular mass of the protease A of *S. cerevisiae* is in the range 40−42 kDa[43,44] and the yapsin 1 (yeast aspartyl protease) is in the range 150−180 and 90 kDa,[45] one can then suggest that the proteolytic activity liberated by growing cells of *S. cerevisiae* PlR1 is not protease A or yapsin 1.

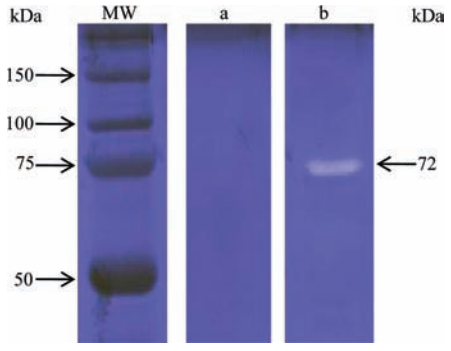

Figure 3. Analysis of extracellular acid proteolytic activity of *S. cerevisiae* PlR1 by BSA zymography. Lane a, concentrated supernatant previously heated (100 °C, 10 min); and lane b, nonheated concentrated supernatant. Molecular weight standard proteins are given on the left side of the gel.

Effect of pH and Temperature on Proteolytic Activity. The activity of the extracellular acid protease of *S. cerevisiae* PlR1 at different pH values is shown in Table 1. The proteolytic activity of the concentrated supernatant is observed in extreme acidic conditions. Only one peak of activity at pH 2.5 was achieved, and 58.3−70.94% of the maximum activity is maintained in the pH range from 2 to 3.5, making this acid proteolytic activity probably suitable for use in low pH media such as must or wine. No proteolytic activity is observed in the pH range from 4.5 to 6. The effect of temperature was also studied. The maximum activity was found to be at 38 °C (Table 1). 46.08% of the maximum activity found at 28 °C and 77.66% at 50 °C. No proteolytic activity was obtained at 60 °C. Although it was unquantifiable by densitometry analysis, a very low activity was found at 18 °C. These results for optimum pH and temperature confirm that this proteolytic activity does not correspond to the yapsin 1.[45]

Effect of Enological Parameters. SO_2 is usually added (50−100 mg/L) during winemaking as an antioxidant and antimicrobial agent. The proteolytic activity of the concentrated supernatant increased more than 2-fold in the presence of 50 and 100 mg/L SO_2 and 3-fold in the presence of 150 mg/L SO_2 when compared with the incubation mixture without SO_2 (Figure 4). This result is in agreement with other studies of the effect of SO_2 on the proteolytic activity of *B. cinerea*[46] and of pepsin.[47] In the presence of low concentrations of ethanol, the proteolytic activity decreased as the percentage of ethanol increased until 72% of inhibition for 14% v/v of ethanol (Figure 4). Another test was done by incubating the concentrated supernatant with the same concentrations of ethanol for 24 h at 18 °C, and then, the proteolytic activity on BSA was tested at pH 3.5 and 38 °C without ethanol. No decrease of the proteolytic activity was observed; thus, ethanol has no denaturation effect on this proteolytic activity (data not shown).

Effect of Protease Inhibitors. Proteolytic enzymes can be classified into four major groups as aspartic, cysteine, metallo, and serine proteases, depending on the nature of the active site. Selective inhibitors can be used to distinguish among these classes of proteases.[48] The 72 kDa acid protease of the concentrated culture supernatant of *S. cerevisiae* PlR1 has its activity completely inhibited by 17 μM pepstatin A, a specific aspartyl protease inhibitor (Supporting Information, Figure S2). On the other hand, PMSF (a serine protease inhibitor) did not alter the activity of the protease. Other protease inhibitors were less effective. 1,10-phenanthroline (a metallo protease inhibitor), iodoacetamide (a cysteine protease inhibitor), and TLCK (a serine protease inhibitor) were tested, and residual 88, 71 and 89% activities were,

Table 1. Effect of Temperature and pH on the Extracellular Acid Proteolytic Activity of *S. cerevisiae* PlR1[a]

temperature (°C)	10	18	28	38	50	60
relative proteolytic activity (%)	0.0 ± 0	2.88 ± 4.08 d	46.08 ± 3.37 c	100 a	77.66 ± 7.30 b	0.0 ± 0
pH	2	2.5	3	3.5	4	4.5
relative proteolytic activity (%)	58.3 ± 7.47 d	100 a	88.10 ± 1.08 b	70.94 ± 1.65 c	28.19 ± 4.57 e	0.0 ± 0

[a] Values are the mean of three determinations. The maximum activity was set as 100% relative activity. Values with different letters within the same line are significantly different by Duncan's multiple range tests at $P < 0.05$. The relative proteolytic activity (%) at pH 5, 5.5, and 6 was equal to 0.

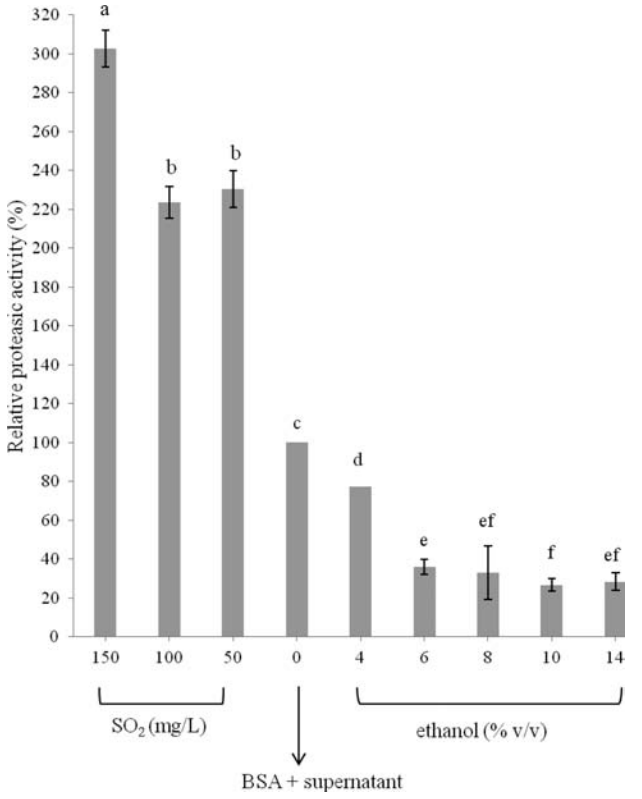

Figure 4. Effect of SO_2 and ethanol on the acid proteolytic activity of the concentrated culture supernatant at the beginning of the stationary growth phase of *S. cerevisiae* PlR1. BSA was incubated with the supernatant alone or mixed with 150, 100, or 50 mg/L SO_2 or with 4, 6, 8, 10, or 14% v/v ethanol. Activity without SO_2 and ethanol was set as 100% relative activity. Values are the mean of three determinations. Values with different letters are significantly different by Duncan's multiple range tests at $P < 0.05$.

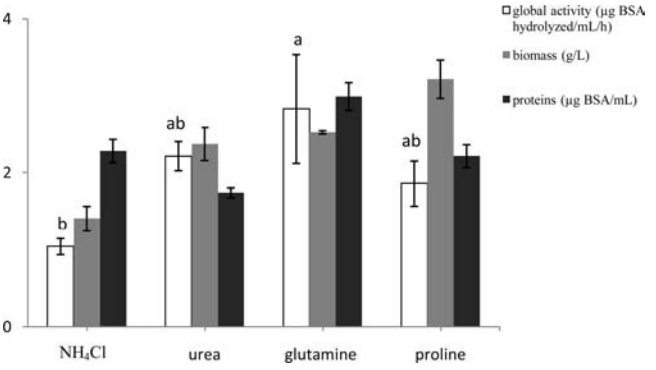

Figure 5. Effect of different nitrogen sources on the proteolytic activity of the concentrated culture supernatants at the beginning of the stationary growth phase of *S. cerevisiae* PlR1. Global proteolytic activity in μg BSA hydrolyzed/mL/h (white box), Biomass in g/L (gray box), and protein concentration in μg/mL (black box). Error bars indicate the standard deviations of three full biological replicates. Values with different letters are significantly different by Duncan's multiple range tests at $P < 0.05$.

respectively, obtained. Consequently, this acid proteolytic activity was considered as an aspartic proteolytic activity.

Effect of Different Nitrogen Sources on Proteolytic Activity. The effect of nitrogen on the secretion of proteolytic activity by *S. cerevisiae* PlR1 was studied using concentrated supernatants obtained from cultures made with NH_4Cl, urea, glutamine, or proline as sole nitrogen sources. All nitrogen sources were used at 600 mg nitrogen/L final concentration, and cultures were stopped at the beginning of the stationary growth phase.

All of the concentrated supernatants were active against BSA, indicating that a protein inducer is not necessary for *S. cerevisiae* PlR1 to secrete an acid proteolytic activity (Figure 5). Such a result is not consistent with previously published data.[31,32] Nevertheless, *S. cerevisiae* can liberate small amounts of proteases without any protein inducer, although proteins induce their secretion.[42]

Biomass, protein production, and proteolytic activity were modified depending on the nitrogen source used (Figure 5). The proteolytic activity of the concentrated supernatants does not seem to be biomass associated, and its secretion does not seem to be sensitive to nitrogen catabolite repression. As far as we know, this phenomenon has not been reported previously.

Evidence for a Secreted Proteolytic Activity during Grape Juice Fermentation. Chardonnay grape juice fermentation by *S. cerevisiae* PlR1 took place for 557 h, and five samples were taken at different stages of the CO_2 release. Although it was slow, the fermentation was complete, and the analysis of the obtained wine indicates that the yeast used does not generate any fermentative problem (Table 2). Dialyzed and concentrated supernatants obtained from samples taken during alcoholic fermentation (numbered from 0 for the uninoculated control sample to 5 for the wine sample) were incubated with 250 μg of BSA at pH 3.5 and 38 °C as mentioned in the Materials and Methods. Reactions were stopped by freezing at −20 °C, and proteolysis was monitored by SDS-PAGE analysis. A proteolytic activity against BSA was detected in these concentrated supernatants. Results obtained within 30 min of incubation (Supporting Information, Figure S3) indicate that samples 3 and 4 were the most active ones (Figure 6), and because no proteolytic activity was detected in the control sample after this incubation period, we can assume that the proteolytic activity detected in these two samples (3 and 4) originates from *S. cerevisiae* PlR1. Moreover, it is noticeable that increasing the incubation time leads to the appearance of a proteolytic activity in more samples. Thus, after 24 h of incubation, a proteolytic activity is detected in all of the concentrated samples, even in the uninoculated one (control sample) that corresponds to the must proteolytic activity[32] that appeared later during incubation (Figure 6).

The impact of the proteolytic activity on grape juice proteins was also studied. First, SDS-PAGE analysis of the protein fraction from the nonincubated fermentative supernatants was performed to evaluate the impact of *S. cerevisiae* PlR1 during fermentation. The electrophoretic profiles of the samples collected during alcoholic fermentation appear to be all identical and with the exception of a new band with a molecular mass around 40 kDa, corresponding to proteins secreted by *S. cerevisiae* PlR1, they do not differ from the electrophoretic profile of the must (Supporting Information, Figure S4). Although *S. cerevisiae* PlR1 is able to liberate a proteolytic activity during grape juice fermentation, this proteolytic activity does not seem to be active against grape proteins under alcoholic fermentation conditions (Supporting Information, Figure S4). Considering that, the proteolytic activity is optimum at 38 °C and very low at 18 °C, the supernatants were then incubated to 38 °C prior to protein analysis. Because of the heat instability of several grape proteins, mainly the grape proteins fraction with a molecular mass between

Table 2. Sugar Consumption and Metabolite Production by *S. cerevisiae* PlR1 during Alcoholic Fermentation

sample	0	1	2	3	4	5
fermentation time (h)	0	102	191	384	431.25	557.25
glucose (g/L)	87.95 ± 0.00	48.02 ± 1	5.55 ± 0.14	0.14 ± 0.05	0.07 ± 0.00	0.01 ± 0.00
fructose (g/L)	86.28 ± 1.94	59.21 ± 1	19.83 ± 0.23	0.48 ± 0.0	0.43 ± 0.06	0.36 ± 0.05
ethanol (% v/v)	0.00 ± 0.00	4.68 ± 0.01	8.88 ± 0.17	10.40 ± 0.08	10.9 ± 0.02	11.02 ± 0.03
glycerol (g/L)	0.00 ± 0.00	2.84 ± 0.07	3.97 ± 0.09	4.205 ± 0.035	4.275 ± 0.045	4.405 ± 0.005
acetic acid (g/L)	0.03 ± 0.00	0.32 ± 0.00	0.36 ± 0.00	0.33 ± 0.04	0.37 ± 0.01	0.26 ± 0.00
malic acid (g/L)	7.5 ± 0.05	7.02 ± 0.01	6.21 ± 0.64	6.5 ± 0.05	6.22 ± 0.03	6.08 ± 0.05

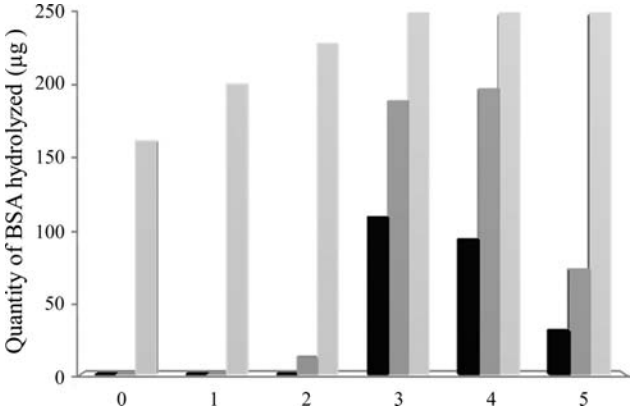

Figure 6. Quantity of BSA, in μg, hydrolyzed by the concentrated samples obtained during alcoholic fermentation for the control (must) and the samples numbered from 1 to 5 and after 30 min (black box), 1 h (dark gray box), and 24 h (light gray box) of incubation at pH 3.5, 38 °C.

12.6 and 30 kDa,[4] incubations were performed with supernatants previously dialyzed against Milli-Q water before concentration. Indeed, dialysis was found to increase the stability of heat-unstable proteins (Supporting Information, Figure S5). This result, which agrees well with those obtained after wine ultrafiltration,[14] can be explained by the loss, during dialysis operation, of compounds of low molecular weights such as factor(s) X,[49] making proteins heat-unstable. The dialyzed and concentrated supernatants (50×) were then incubated at 38 °C and pH 3.5 for 1, 24, 48, and 72 h, and the protein fraction was analyzed by SDS-PAGE. The electrophoretic profiles showed a progressive loss of intensity for only one band with a molecular mass around 25 kDa and corresponding to grape proteins. The disappearance of this protein band was detectable after only 1 h of incubation (data not shown) and increased with the incubation time (Figure 7A). Moreover, this protein band did not disappear similarly in all of the supernatants used. Its disappearance was mainly noticeable in the dialyzed and concentrated fractions of the supernatants 3 and 4 (Figure 7B), which were also the most active supernatants against BSA. Incubation with the concentrated fraction of the supernatant 4 was also performed at lower temperatures. Decreasing the incubation temperature resulted in a decrease in the disappearance of the 25 kDa band, with no disappearance observed at 18 °C even after 6 days of incubation (data not shown). Although other studies have underlined the combined effect of heat and enzyme treatment on the reduction of the grape protein content in wine,[14,19,47] none of them has reported the possibility that *S. cerevisiae* nonvacuolar proteases could hydrolyze grape proteins.

The protein band of interest (25 kDa) was excised from SDS-PAGE gel, hydrolyzed with trypsin, and then analyzed via

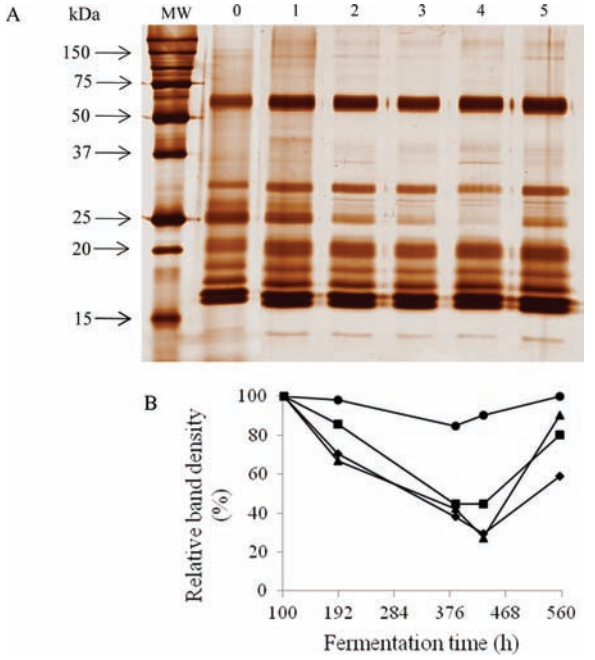

Figure 7. (A) SDS-PAGE analysis monitoring the profile of grape juice proteins after 48 h of incubation at 38 °C and pH 3.5. Sample (0) corresponds to the dialyzed and concentrated control sample. Samples from 1 to 5 correspond to the dialyzed and concentrated samples obtained during alcoholic fermentation with *S. cerevisiae* PlR1. Molecular weight standard proteins are given on the left side of gel. (B) Density evolution of the 25 kDa band in the dialyzed and concentrated supernatants during alcoholic fermentation after 1 h (●), 24 h (■), 48 h (♦), and 72 h (▲) of incubation. Density of the 25 kDa band in the concentrated sample 1 (after 102 h of fermentation) was set as 100% relative density.

Table 3. Proteins from the 25 kDa Band Identified by Nano-LC-MS/MS after SDS-PAGE

protein name	theoretical molecular mass (kDa)	accession number	sequence coverage (%)
class IV endochitinase (*V. vinifera*)	27.2	gi|2306811	60
VVTL1 (*V. vinifera*)	23.9	gi|2213852	54
hypothetical protein (*V. vinifera*)	23.8	gi|225426793	30
vacuolar invertase 1, GIN1(*V. vinifera*)	71.5	gi|1839578	5

Nano-LC-MS/MS. Results obtained using the NCBInr database are listed in Table 3. Four proteins were identified in the band of interest, a class IV endochitinase from *V. vinifera* (gi|2306811),

which belongs to the PR-3 family; a VVTL1 from *V. vinifera* (gi|2213852), which belongs to thaumatin-like family; an hypothetical protein from *V. vinifera* (gi|225426793); and finally a vacuolar invertase 1, encoded by GIN1 from *V. vinifera* (gi|1839578). Although the theoretical molecular mass of grape invertase is around 71.5 kDa, the presence of the vacuolar invertase in bands of 26 and 22 kDa has been reported to be likely the result of a hydrolysis of the grape invertase by the must proteolytic activity.[50] Recently, Falconer and colleagues found that the incubation temperature used in our study is able to partially unfold chitinase.[51] This effect could partially be responsible for observed reduction in the 25 kDa band density, but surely, it is not the only responsible because in this case we would observe a similar band reduction in every sample.

Some of these proteins were identified as pathogenesis-related proteins (class IV endochitinase and VVTL1), which are heat-sensitive proteins. This study has revealed and characterized, for the first time, an extracellular proteolytic activity produced by *S. cerevisiae* PlR1 on these grape proteins. Further research is needed to purify this extracellular yeast protease and apply it to prevent haze in wine.

■ ASSOCIATED CONTENT

Supporting Information. Five additional figures and a table. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

BSA, bovine serum albumin; LDH, lactate dehydrogenase; MS/MS, tandem mass spectrometry; NCBI, National Center for Biotechnology Information; NH_4Cl, ammonium chloride; PMSF, phenylmethylsulfonyfluoride; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SO_2, sulfur dioxide; TLCK, *N*-α-tosyl-L-lysine-chloromethyl-ketone; YEG, yeast extract glucose

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