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Evidence for Protein Degradation by *Botrytis cinerea* and Relationships with Alteration of Synthetic Wine Foaming Properties

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Botrytis cinerea is an important fungal pathogen particularly dreaded in the cool climate vineyard. It is responsible for important damage, especially the decrease in foamability of sparkling wines, such as Champagne. Different studies have shown that proteins are largely involved in the stabilization of Champagne foam despite their low concentration. Other works demonstrated changes in the electrophoretic characteristics of must proteins originating from botrytized grapes, although the cause of such alterations was never explained. In the first part of this study, results showed the release by *B. cinerea* of 3.5 mg/L total proteins in a synthetic liquid medium. Among these proteins, the presence of a protease activity on bovine serum albumin (BSA) and must proteins was demonstrated by using a colorimetric method and sodium dodecyl sulfate−polyacrylamide gel electrophoresis. In the model wine, the Bradford method showed a BSA loss of 66% after 24 h and a loss of 96% after 120 h. In the same model wine, the soluble must protein concentration decreased by 35% after 1 week and by 53% after 2 weeks while the control showed no protein loss. *B. cinerea* proteases were then able to degrade BSA and must proteins and were above all active at must and wine pH and in the presence of ethanol and SO_2. The second part of this work was dedicated to the relationship between the presence of *B. cinerea* proteases and its effects on the synthetic wine foaming properties. The addition of a *B. cinerea* culture medium (1/33 v/v) to the synthetic wine containing 21 mg/L soluble grape proteins induced a decrease in foamability by 60% after 1 week. For BSA in the model wine, the foamability decreased by 32% after 24 h and by 95% after 120 h, as shown by the colorimetric method. These experiments demonstrate for the first time the relationship between *B. cinerea* protease activity and the decrease in wine foaming properties.

KEYWORDS: ***Botrytis cinerea*; protease; foam; grape proteins; BSA; Bradford method; SDS−PAGE; grape juice**

INTRODUCTION

Botrytis cinerea is an important fungal necrotrophic pathogen found in at least 235 plant species (*1*). This agent, responsible for gray rot in Champagne vineyards, is particularly dreaded by winegrowers and winemakers. Indeed, the geographical situation of this wine-growing area and its northern climate are extremely favorable for the development of this fungus at the end of grape maturation.

Different studies have shown that botrytized grape berries resulted in wines of a low quality. Wines are marked by characteristic mushroomy, moldy, and rotten smells and tastes (*2*), and sparkling wine foaming properties are strongly impaired (*3*). The foam, as a result of effervescence, is the first feature that is observed by the consumer after a Champagne has been poured into the glass. The foam behavior is often studied to characterize sparkling wines. The foam must show a persistent collar composed of fine and white bubbles, unceasingly supplied with a continuous effervescence, so that the wine is appreciated. Reliable information on the factors and the components controlling the foamability and the foam stability of Champagne is of considerable interest to the winemaker. These foaming properties are related to those of the champenois base wine (*4*) and also depend on the infection level of grape berries by *B. cinerea* (*3*). Other factors such as the grape variety (*5*) and treatments made during the winemaking process (*6*−*8*) should be considered. The effect of the chemical composition of wine on foam (*9*−*13*) must also be mentioned. Particularly, various studies have shown that proteins are implicated in the stabilization of foam in Champagne wines (*4*, *14*) despite their low concentration, which ranges from 4 to 20 mg/L (*15*). For this reason, a recent study has focused on the must protein fraction (*16*) and

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demonstrated that alterations occurred in the electrophoretic characteristics of proteins following an infection by *B. cinerea*. However, the origin of such alterations is misunderstood, although the role of fungal proteases in these modifications has already been suggested (*16*).

The secretion of proteases by *B. cinerea* has indeed been shown in culture media (*17*) and fruits such as grapes (*18*), apples (*19*), tomatoes (*20*), zucchini (*17*), bell peppers (*17*), and carrots (*21*). Moreover, Marchal et al. (*16*) showed by immunodetection the presence of *B. cinerea* proteins in a must obtained from highly botrytised fruits (80%), with some of them possibly having proteolytic activity that may be responsible for the degradation of grape proteins. Similarly, Modra and Williams (*22*), using commercial enzyme preparations, indicated that both plant and fungal proteases can significantly alter the chromatographic profile of a must protein fraction. Nevertheless, the potential relationship between the must protein degradation by fungal proteases and the decrease in the foaming properties of sparkling wines has never been established. The aim of this study was thus to search whether such a relationship could exist using a model protein, bovine serum albumin (BSA), and grape juice proteins.

The effect of enological conditions on the *B. cinerea* protease enzymatic activity has never been evaluated. The second part of our study was thus dedicated to the influence of physicochemical and enological parameters, particularly the effect of temperature, pH, and the presence of ethanol and SO_2 on *B. cinerea* protease activity.

MATERIALS AND METHODS

Organism and Culture Conditions. The strain used in this study was *B. cinerea* 630 originally collected and isolated from grapevines in the Champagne area by Dr. Brygoo (INRA, Versailles, France). It was maintained on tomato agar medium, adjusted to pH 5.5, containing commercial canned tomato juice and 25 g/L of agar. Conidia of 4 week old cultures were mechanically harvested in sterilized distilled water and immediately transferred into a liquid-based medium (*23*), which contained glucose (31.7 g/L), potassium nitrate (1.5 g/L), $NaH_2PO_4 \cdot H_2O$ (90 mg/L), $CaHPO_4$ (38 mg/L), $MgSO_2 \cdot 7H_2O$ (60 mg/L), $FeSO_4 \cdot 7H_2O$ (20 mg/L), $ZnSO_4 \cdot 7H_2O$ (20 mg/L), $MnSO_4 \cdot H_2O$ (20 mg/L), and peptone (500 mg/L). The conidial suspension was then incubated at room temperature on a rotary shaker at 150 rpm for 3 weeks, with a day−night alternance. Fungal cultures were centrifuged for 10 min at 9500*g* and filtered through a 0.45 μm membrane (Alltech, France). A pool of the filtered supernatants was stored at −20 °C until protease assays.

Isolation and Quantification of *B. cinerea* Proteins. The *B. cinerea* supernatant was concentrated and dialyzed six times against distilled water (0.2 L of concentrate plus 1.6 L of water). A low-protein adsorption polyethersulfone membrane with a 10000 molecular weight cutoff was used (Minisette Omega, screen channel membrane, Pall-Filtron, France). The ultrafiltration module (tangential membrane, manifold, and side plates) was connected to a Filtron pumping system. Ultrafiltration was carried out at room temperature. The ultraconcentrate was lyophilized and stored at −20 °C before being analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and used for protein quantification. The percentage of humidity in the lyophilizate isolated from *B. cinerea* cultures was 9% (w/w) as determined by thermogravimetry (Thermogravimetric Analyzer Hi-Res TGA 2950, TA Instrument, Guyancourt, France). The concentration of proteins in the lyophilizate was estimated using the nitrogen content determination (protein content = nitrogen % × 6.25) by elemental analysis (2400 CHN Elemental Analyzer, Perkin-Elmer). This result was corrected on the basis of the humidity percentage. The *B. cinerea* culture supernatant obtained after 3 weeks contained 3.5 mg/L of total proteins and 37.5 mg/L of polysaccharides (calculated by difference). *Botrytis* proteins and polysaccharides were not separated. When *Botrytis* culture supernatant was solubilized in the model wine (30 mL of *Botrytis* culture supernatant contains 1.12 mg/L polysaccharides and 0.105 mg/L total proteins), the model wine did not present any foaming properties. This control experiment clearly demonstrated that, for this concentration, the fungus macromolecules were absolutely not able to produce foam.

Must Proteins. Must proteins were isolated from a Chardonnay grape juice. It came from sound grape berries hand-harvested in the Champagne area. The must was treated by the same ultrafiltration technique as that used for *B. cinerea* cultures and stored at −20 °C before it was used. In a previous work (*24*), we showed that pectins (400 mg/L) and neutral glucans (500 mg/L) did not present any foaming properties when added to a wine or a model wine because they had no tensioactivity and no surface properties. For this reason, polysaccharides and proteins contained in the lyophilisate were not separated.

It was demonstrated in different studies than the higher the must protein concentration was, the higher the wine foamability was (*4*). This clearly indicated that must proteins largely contributed to the wine foaming properties. In the Champagne area, a really cool climate, proteic haze was never observed and the precipitation of must proteins during alcoholic fermentation was very low when compared with other vineyards. Moreover, the proteins released by the yeast during the two alcoholic fermentations were essentially glycoproteins (proteoglycans) that had very high molecular weights and were essentially composed of glycans (more than 95%). Yeast proteins presented bad surface properties and very low foamability. That is the reason that this study is focused on must proteins.

Protease Assays. The protease activity of *B. cinerea* was assayed spectrophotometrically by following BSA degradation using the Bradford method (*25*). Oligopeptides were produced during the digestion of proteins by endoproteases, and oligopeptides less than 3 kDa appeared to be unable to bind the Coomassie Brilliant Blue (CBB) dye (*26*). Then, the hydrolysis of proteins was followed by determining the loss in the capacity of the protein substrate to bind CBB. On the basis of this principle, a rapid and sensitive procedure was developed to follow proteolytic activities and was applied in this study (*27*, *28*).

To estimate the effect of *B. cinerea* proteases on a standard protein, 25 mg of BSA was dissolved in the supernatant culture (30 mL) containing 1 g/L of tartaric acid, at pH 3.2, and 20 mL of synthetic wine and incubated at 30 °C (**Figure 1**). This mixture constituted the sample to be assayed for different times of incubation (24, 55, and 120 h). A control experiment was assayed in parallel without the *B. cinerea* culture supernatant (25 mg of BSA dissolved in 50 mL of synthetic wine). BSA proteolysis was followed by monitoring the decrease in absorbance at 595 nm. The Bradford method was employed as follows: 1.5 mL of Bio-Rad Protein Assay Reagent (CBB G-250), diluted in MilliQ water (1 + 4, v/v), was added to 50 μL of sample. The blue coloration was measured after 15 min of incubation. Each value corresponded to the average of three measurements. After 24, 55, and 120 h, the mixture was diluted with the synthetic wine to 1 L, which then contained 25 mg/L BSA, 0.105 mg/L of *B. cinerea* proteins, and 1.125 mg/L of *Botrytis* polysaccharides.

In another experiment, 700 mg of lyophilizated must colloids, containing 21 mg of proteins, was dissolved in 20 mL of a synthetic wine and 30 mL of *Botrytis* culture supernatant to estimate the effect of *B. cinerea* proteases on the soluble grape juice proteins present in a wine (**Figure 1**). The mixture was incubated at 30 °C. The synthetic wine compsition was 1 g/L of tartaric acid adjusted at pH 3.2 with KOH and 12% v/v ethanol. After 1 and 2 weeks, the mixture containing the partially hydrolyzed must proteins was diluted to 1 L with the synthetic wine to measure the foaming properties. As for the BSA assay, the synthetic wine, which contained hydrolyzed must proteins, only contained 0.105 mg/L of *B. cinerea* proteins and 1.125 mg/L of *Botrytis* polysaccharides (**Figure 1**).

In parallel, a synthetic control wine containing exclusively must proteins (without the *B. cinerea Botrytis* culture supernatant) was prepared. A third control wine containing exclusively the *B. cinerea* culture supernatant (without must proteins) was also prepared.

Protease Activity Inhibition. A test was made with the addition of 1.5 μM pepstatin (*29*) in the mixture 25 mg of BSA, 30 mL of *Botrytis*

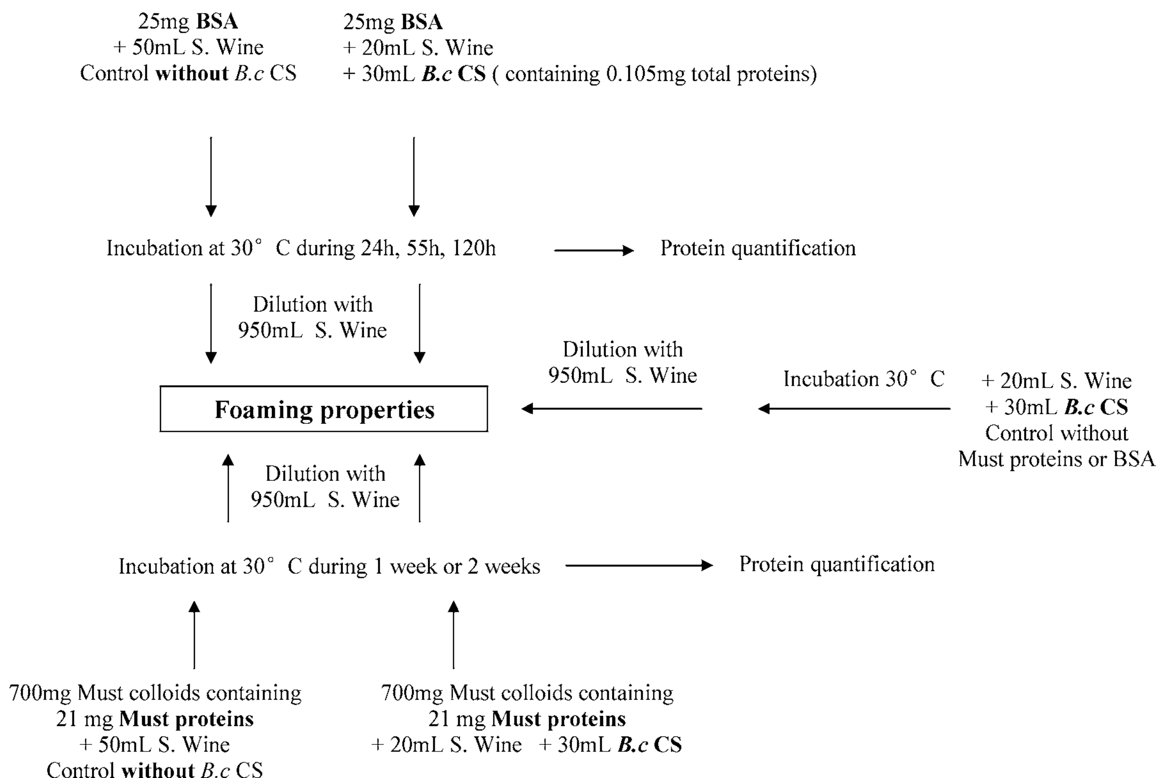

Figure 1. Protocol used to estimate the impact of *Botrytis* proteases on the model wine foaming properties. S.Wine, synthetic wine; and *B.c.* CS, *B. cinerea* culture supernatant.

supernatant culture, and 20 mL of synthetic wine and incubated at 30 °C as for the assay without pepstatin (**Figure 1**).

Foaming Properties Using a Sparging Procedure. Foam measurements were carried out using the Mosalux apparatus (*4*). Samples were filtered (0.45 μm) before foam measurement. A glass cylinder (4 cm in diameter and 40 cm long) placed on a glass frit (pore size = 16−40 μm) was charged with 100 mL of the sample to be analyzed. Carbon dioxide gas was injected into the glass cylinder through the glass frit with a constant rate of gas flow (7 L/h) and under a constant pressure (100 kPa). Foam height (measured in millimeters) was controlled by photoelectric cells (infrared beams). All measurements were conducted in triplicate and made the same day in order to reduce the spread of values. Each value was the average from three determinations. Two foaming parameters were measured as follows: (i) H_m (maximum height, mm) corresponded to the maximum height reached by the column of foam and represented the foamability and (ii) H_s (stability height, mm) represented the persistence of the foam collar during CO_2 injection. Values are expressed as follows: m (mean) ± standard error (standard deviation); m is the average of three measures (n = 3); t is the Student value for 2 degrees of freedom and for a probability of 95%.

Electrophoretic Analysis by SDS−PAGE. Discontinuous SDS−PAGE was performed according to the method of Laemmli (*30*) using slab gels (dimension 8.3 cm × 7.3 cm, 0.75 mm thick). The stacking gels consisted of T = 5% and C = 2.7%, and the separating gels consisted of T = 13.5% and C = 2.7%. A vertical electrophoresis apparatus (Mini-Protean II, Bio-Rad) was used to run the gel at a constant voltage setting of 150 V until the bromophenol blue tracker dye reached the bottom of the gel (usually 65 min at room temperature). Protein samples were diluted with the Laemmli buffer (v/v) and boiled for 4 min, and 24 μL was loaded into the wells for each analysis. Standard proteins from 14.4 to 94 kDa (LMW electrophoresis calibration kit, Amersham Pharmacia Biotech) or from 10 to 250 kDa (Precision Plus Protein Standards, Biorad) were used as molecular mass markers. The lyophilized proteins were resuspended in the Laemmli buffer as notified by the manufacturer. Ten (for CBB stain, see below) and 3 μL (for silver stain, see below) of markers were loaded into the wells. The molecular weights (MWs) of unknown molecules were calculated from the linear regression equation of log MW vs mobility. After migration, the separated proteins were stained with 1.5% CBB R-250 in 50% (v/v) methanol and destained in acetic acid/methanol/water (1:2:7) melange or silver stained according to the protocol described by Rabilloud (*31*).

Effects of Physicochemical and Enological Parameters on the Fungal Protease Activity. For each parameter following, the protease activity was expressed as a percentage of the maximum activity reached. To study the effect of temperature, standard enzymatic assays were conducted at different temperatures (4−60 °C) at pH 3.2. To determine the effect of pH, the protease activity was measured in a citric acid Na_2HPO_4 (0.1 M) buffer at different pH values ranging from 2 to 7 and at 30 °C. The protease activity was also determined in the presence of 10 and 20% (v/v) of ethanol and with 100 mg/L of SO_2.

RESULTS AND DISCUSSION

Evidence for *B. cinerea* Protease Activity. The *B. cinerea* protease activity was checked by using BSA or grape juice proteins, as substrates, in enological conditions, mixed with the *B. cinerea* supernatant and incubated at 30 °C. **Figure 2** shows a rapid, exponential decrease in the absorbance at 595 nm with time. Ninety percent of the absorbance was lost within 24 h of incubation. When BSA was incubated without the *B. cinerea* supernatant in the same conditions (control **Figure 1**), no decrease was observed. These results show the presence of proteases among proteins secreted by *B. cinerea* in the liquid medium. When must proteins were mixed with the *B. cinerea* supernatant, the rate at which the absorbance decreases was less important than that observed with BSA (**Table 1**).

Protease Activity on BSA followed by SDS−PAGE. BSA was added to the *B. cinerea* supernatant during 136 h at 30 °C. Proteolysis was monitored by SDS−PAGE analysis (**Figure 3**). The first blank sample (with the supernatant of *B. cinerea*) showed the profile of commercially available BSA before proteolysis (**Figure 3**, lane A). The main band corresponds to

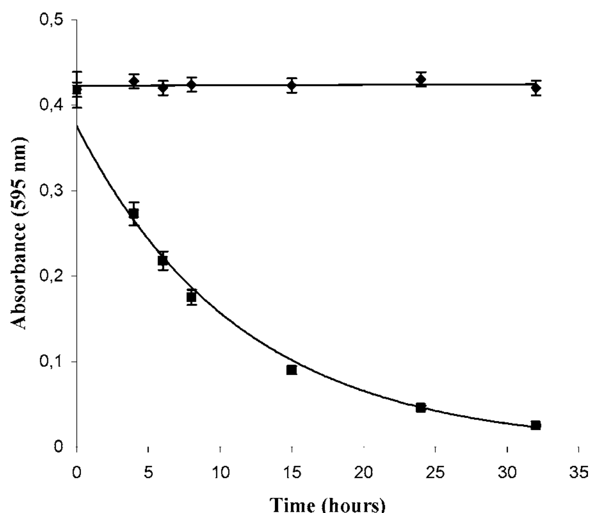

Figure 2. Evidence for *B. cinerea* protease activity by a colorimetric method using CBB (*25*). The degradation of BSA was followed with (■) ($y = 0.3755e^{-0.0872x}$) or without (◆) supernatant of *B. cinerea* cultures. Error bars indicate the standard deviations.

BSA, around 67 kDa. This commercial protein also contained lower molecular mass bands, in very low quantity, presumably corresponding to degradation products of BSA. Proteins from *B. cinerea* did not interfere with BSA analysis because the supernatant did not contribute to observable bands at the concentration at which it was used in these experiments. In the mixture, the ratio between *B. cinerea* proteins and BSA was 1/240 (w/w) (profile of *Botrytis* culture alone not shown because no band was observed with the dilution 1/33 v/v used). The second blank sample profile (**Figure 3**, lane B, BSA without *Botrytis* culture) did not show any degradation even after 136 h, indicating that the BSA preparation does not contain any proteolytic activity for this incubation period. In contrast, *B. cinerea* protease-treated samples showed an evident degradation of BSA. The intensity of the major band (corresponding to BSA) continuously decreased with the incubation time. Simultaneously, bands of lower molecular masses appeared, which finally disappear with time. Waters et al. (*32*) also showed that BSA was hydrolyzed particularly rapidly in wines by a commercial peptidase preparation (which contained a range of different peptidases). The proteolysis of BSA, as analyzed by SDS−PAGE, gave additional information when compared to the colorimetric results obtained by the Bradford method. Then, it would be interesting to know whether the hydrolysis of BSA could have an effect on the foamability of the proteic solution.

Protease Activity on Must Proteins followed by SDS−PAGE. The same experiment was achieved by mixing must total proteins with the *B. cinerea* culture supernatant in the synthetic wine. For this study, we choose to work with grape juice proteins in a synthetic wine in order to free ourselves from all of the yeast proteasic activities present among wine proteins (*33*). Moreover, grape juice proteins are present in the wine (*34*).

In the control experiment sample ($t = 0$ h), about 10 bands were observed, with molecular masses ranging from 12 to 62 kDa (**Figure 4**). With *B. Cinerea* culture, we observe visually for the lanes 0 and 2 a serious decrease of the intensity between 23 and 33 kDa, at 12 kDa and for many minor bands. This is not quantified, but visually, this coresponds to a relatively serious alteration. Proteins at 13 and 29 kDa completely disappeared within 1 week. After 2 weeks, other bands slightly diminished (12, 14, 16, 23, and 33 kDa) while others did not (24, 26, 61, and 62 kDa). The blank sample profile did not show any degradation even after 2 weeks (band at 33kDa, for example), indicating that the must proteins did not contain any proteolytic activity for this incubation period.

Comparison of the electrophoretic profiles (**Figures 3** and **4**) showed that the susceptibility of must proteins to *B. cinerea* proteases was much lower than that of BSA. These different behaviors are certainly linked to the biochemical characteristics of must proteins. Marchal et al. (*16*) observed that juices obtained from grapes highly infected by *B. cinerea* showed reduced protein levels; these authors suggested that proteolytic enzymes from *B. cinerea* were implied in must protein degradation. However, Waters et al. (*32*) indicated that wine proteins from healthy grapes show a remarkable resistance to a highly active commercial peptidase preparation, resulting in no significant alteration of the electrophoretic profile by SDS−PAGE. Such discrepancies can be explained by the experimental conditions used in these studies (origins of enzymes, pH, enzyme concentration, duration of treatment, or temperature) (*16*).

The susceptibility of must proteins to *B. cinerea* proteases thus differs from that of BSA. It is possible that hydrolysis of the more resistant proteins could not be seen because of the duration of the experiment, which was not long enough to observe such changes.

In **Figure 4**, there is an alteration of the electrophoretic profile by SDS−PAGE of must proteins incubated with *Botrytis* culture supernatant and above all a serious decrease of the intensity of the bands. In the same conditions, must protein loss as determined by the colorimetric Bradford method, which gives a quantification expressed in mg total proteins/L, reached −35% after 1 week (**Table 1**). Using the SDS−PAGE method (**Figure 4**), it is not possible to precisely observe the high molecular weight (glyco)proteins (essentially proteoglycans such as arabinogalactan proteins) because the polypeptidic part represents generally 5−10% of the molecule. For these reasons, one can consider that there are no discrepancies between the observations made in the **Table 1** and those of **Figure 4**.

Foam Characteristics of the BSA in a Model Wine. To evaluate whether the BSA degradation by *B. cinerea* proteases could be deleterious to the foaming properties, foam characteristics were followed for 120 h (**Table 1**). The synthetic wine containing the *B. cinerea* culture supernatant (dilution 1/33 v/v) but without BSA or must proteins did not produce any foam. We can then assume that the foaming properties exhibited by the synthetic wine containing (hydrolyzed) BSA or (hydrolyzed) must proteins are exclusively due to the presence of these proteins and not to the extremely low content of *B. cinerea* proteins (0.105 mg/L) added to assess the protease activity. In the same way, the control synthetic wine with BSA and without *B. cinerea* cultures did not show any changes in foamability after 24, 55, and 120 h, demonstrating the absence of BSA autolysis. It was also shown by the stability of the response to the CBB dye reagent with the Bradford method. As for the study including BSA, no decrease in foamability was observed after 1 and even 2 weeks in the synthetic wine containing must proteins without *B. cinerea* cultures, demonstrating once again the absence of must protein autolysis. In the model wine containing BSA at 25 mg/L, the foamability H_m was 73 mm. After 24 h of proteolysis, 66% of the BSA was lost while only a decrease by 30% in foamability was observed. The loss of BSA was not proportional to the decrease in foamability. During BSA degradation, the lower MW protein fragments formed probably kept the capacity to produce foam but at a lower degree

Table 1. Modification of Foam Characteristics of a BSA Solution (25 mg/L) and Must Protein Solution (700 mg/L Colloids Containing 21 mg/L Proteins) in a Synthetic Wine during the Hydrolysis of the Protein(s) by a *B. cinerea* Culture Supernatant (*B.c* CS)[a]

			Hm (mm)		
in the model wine	incubation time	protein loss (%)	with *B.c* CS	without *B.c* CS	Hs (mm) with *B.c* CS
B.c CS	0, 24, 55, and 120 h and 1 and 2 weeks		0 (no foam)	0 (no foam)	0 (no foam)
BSA	0 h	0	73 ± 6	72 ± 4	30 ± 5
	24 h	–66 (±4)	50 ± 0 (–32%)	75 ± 6	14 ± 2 (–53%)
	55 h	–83 (±5)	32 ± 6 (–56%)	71 ± 4	5 ± 0 (–83%)
	120 h	–96 (±7)	4 ± 0 (–95%)	76 ± 5	3 ± 0 (–90%)
must proteins	0 h	0	50 ± 2	49 ± 3	ND
	1 week	–35 (±6)	20 ± 0 (–60%)	47 ± 4	ND
	2 weeks	–53 (±5)	20 ± 6 (–60%)	51 ± 3	ND

[a] ND, not determined. Values are presented as follows: m (mean) ± standard error (standard deviation); m is the average of three measures ($n = 3$); t is the Student value for 2 degrees of freedom and for a probability of 95%.

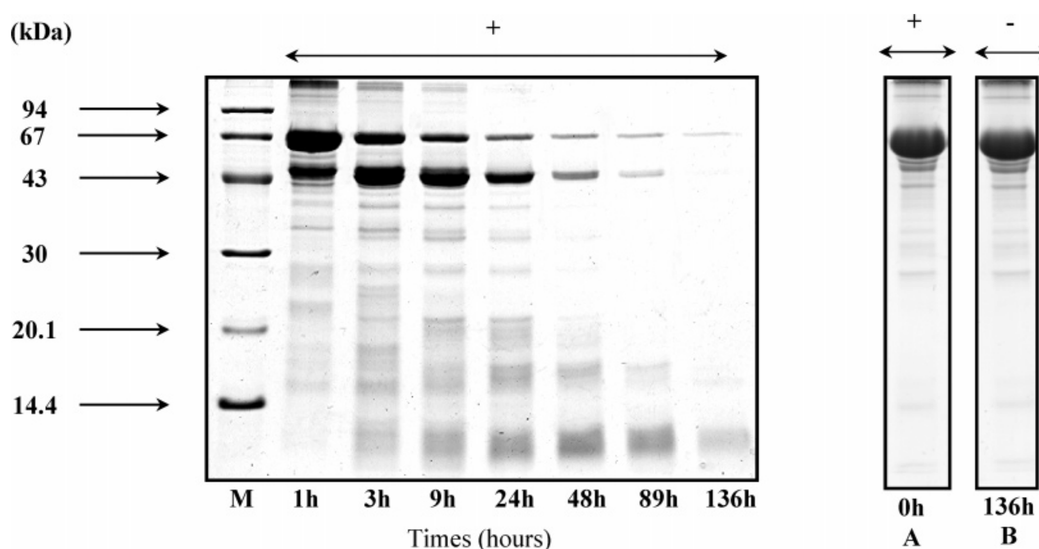

Figure 3. Protease activity on BSA as monitored by SDS–PAGE. BSA was incubated at 30 °C with (+) or without (–) the supernatant of *B. cinerea* cultures. Relative MWs (×10^{-3}) of protein standards are given on the left side of the gel. Proteins were stained by CBB.

when compared to that of the nonhydrolyzed BSA solution. After 120 h, as BSA was almost completely hydrolyzed, there was a decrease by 96% in foamability. These results show that BSA proteolysis dramatically affects the foaming properties of this protein.

Likewise, for the other parameters tested (H_s), values obtained decreased with BSA proteolysis. Before degradation, the H_s value was 30 mm. Within 24 h, 53% of H_s was lost. After 120 h, 100% of H_s was lost.

For the first time, this study underlines the relationships between fungal protease activity, wine protein degradation, and decrease in the foaming properties of a synthetic wine. It would be interesting now to search whether the must protein proteolysis may have an effect on the foamability in the synthetic wine.

Foam Characteristics of the Grape Proteins in a Model Wine. The experiments described here were achieved by using grape juice proteins to evaluate how their degradation by *B. cinerea* proteases could influence the synthetic wine foaming properties. The foam characteristics were followed in parallel for 2 weeks (**Table 1**). In the model wine containing colloids at 700 mg/L (21 mg/L proteins), the foamability H_m was 50 mm. After 1 week of proteolysis, 35% of the protein concentration was lost while a decrease by 60% in the foamability was observed. The loss of must proteins was not proportional to the decrease in foamability. Contrary to what was observed for BSA, a low reduction in the grape juice protein concentration induces a strong reduction in their foaming properties.

After 2 weeks of proteolysis, 53% of the protein concentration was lost while a decrease by 60% in the foamability was observed. During this second week, certain proteins were degraded (**Table 1** and **Figure 4**) whereas the foaming properties were not affected any more.

For BSA, one can observe a relationship between the degradation of this unique protein and the decrease of its foaming properties. In this experiment, there is only one protein with specific biochemical characteristics: pI, MW, hydrophobicity, and surface properties. For must total proteins, it is much more difficult to understand because there are probably more than 80 different proteins, with many different pI values and MW. All of these proteins present very different hydrophobicities and, of course, surface properties. Between 1 and 2 weeks of incubation, there was no decrease in foamability (−60% for the two different times) while the protein content continued to decrease. This seems to indicate that the proteins degraded by *B. cinerea* proteases during the first week participate more to the expression of the foamability than the more resistant proteins

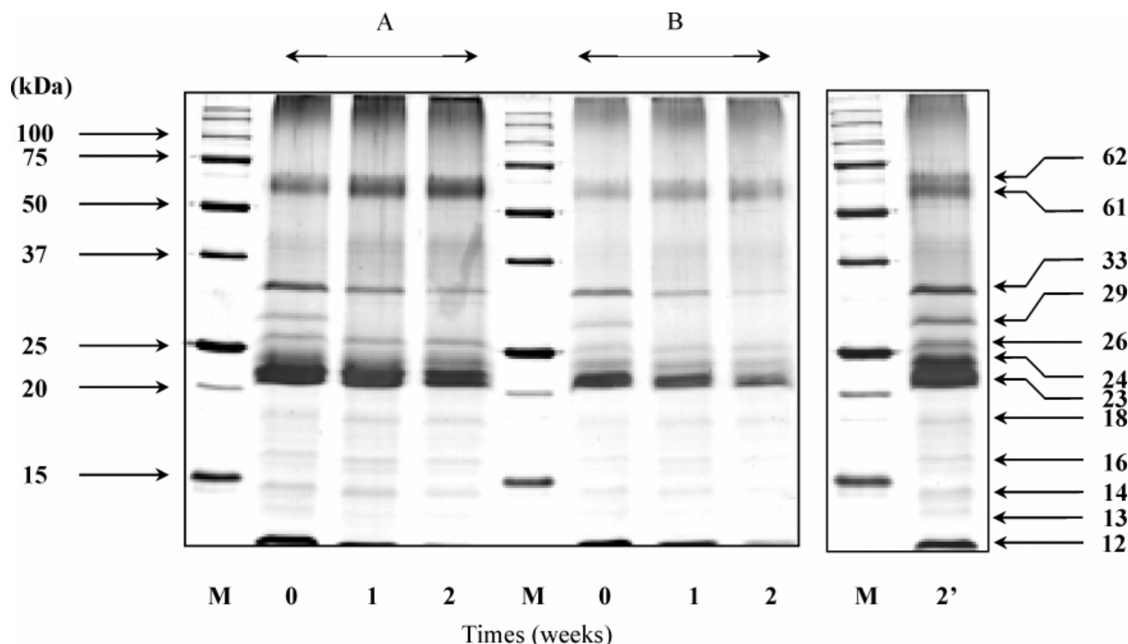

Figure 4. Protease activity on grape juice lyophilized proteins observed by SDS–PAGE. Must protein was incubated at 30 °C with (lanes 0, 1, and 2) or without (lane 2′) *B. cinerea* culture supernatant. Relative MWs ($\times 10^{-3}$) of protein standards (M, molecular weight markers) are given on the left side of the gel. Relative MWs of some must proteins are given on the right side of the gel. Proteins were silver stained. A quantity of proteins two times larger was deposited in track A than in B.

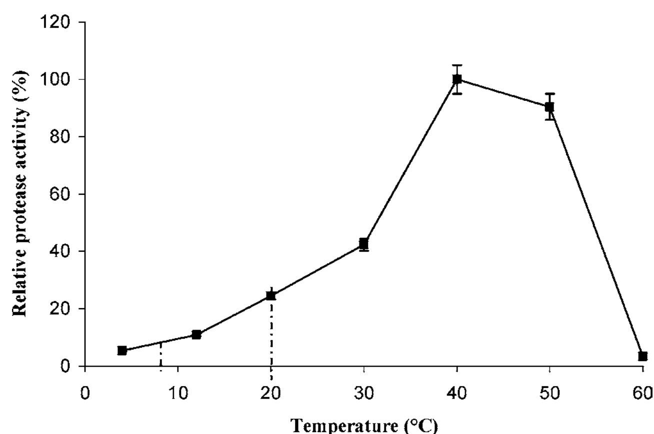

Figure 5. Influence of temperature on protease activity of *B. cinerea*. Mixtures containing *B. cinerea* supernatant and BSA were incubated at temperatures ranging between 4 and 60 °C at pH 3.2. Values are the mean of three determinations. Error bars indicate the standard deviations. The maximum activity was set as 100% relative activity. The dashed bars delimit the temperature range under enological conditions.

degraded during the early stage. These results also suggest that compounds other than protiens could be implicated in the expression of foaming properties.

Effect of Temperature on the *B. cinerea* Protease Activity. To evaluate the effect of temperature on the BSA degradation by *B. cinerea* proteases, relative activities were determined between 4 and 60 °C (**Figure 5**). One hundred percent of the activity represents the maximum enzymatic activity observed for BSA hydrolysis. At 4 and 60 °C, the protease activity was the lowest, i.e., around 5%, of the maximum observed. The optimum activity was reached for temperatures around 40 °C. This does not reflect the conditions observed for a cool climate, such as the Champagne area; the temperature of the grapes during harvest was always below 20 °C. During the alcoholic fermentation, as well, the temperature of the tanks was often maintained around 18 °C. In these conditions, only 25% of the optimal protease activity could be obtained. According to SDS–PAGE results, this activity could be sufficient to efficiently degrade the proteins during the time of the winemaking process: 15 months for a Champagne and between 6 and 12 months for most sparkling wines. This hypothesis has to be tested because yeast can also secrete proteases or inhibitors able to degrade or inhibit *Botrytis* proteases.

Effect of pH on the *B. cinerea* Protease Activity. The pH of musts and wines tested ranged from 3 to 3.7. This range corresponds to the maximum protease activity observed (**Figure 6**). This result typically reflects the activity of acidic proteases. The nature of the protease activity secreted by *B. cinerea* was already identified (*17*, *19*, *21*). These authors reported that the protease activity produced at pH 4, when *B. cinerea* was grown in a liquid medium and during the early stages of the infection process, was inhibited by the addition of pepstatin, which has been well-documented as a specific inhibitor of enzymes belonging to the aspartic proteinase family (*29*). We also found that the *B. cinerea* protease activity, assayed in the presence of 1.5 μM pepstatin, was completely inhibited (data not shown). This result together with the optimum pH observed indicates that exclusively aspartic proteases were detected in culture media of *B. cinerea* (*17*). These last authors, in agreement with the conclusions of Manteau et al. (*35*), suggested that aspartic proteases represent the sole protease activities detected in *B. cinerea* culture media.

Contrary to what was observed with temperature, the conditions of pH observed in musts and wines are optimal for protease activity expression. Thus, we can suggest that the protease of *B. cinerea* could also be active in the wine.

Effects of Ethanol and SO_2 Treatment on the *B. cinerea* Protease Activity. Insolubilization of plant proteins takes place during the alcoholic fermentation and is still observed in the wine for several months. This phenomenon is due to changes

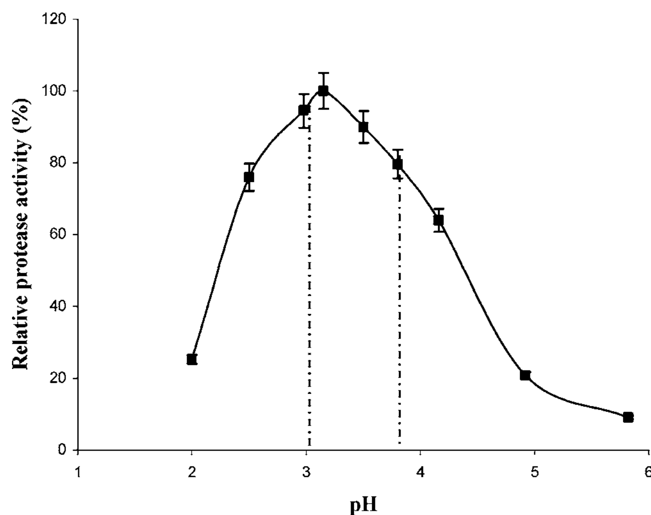

Figure 6. Influence of pH on protease activity of *B. cinerea*. Mixtures containing the *B. cinerea* supernatant and BSA were incubated at pH values ranging from pH 2 to 7 and incubated at 30 °C. Values are the mean of three determinations. Error bars indicate the standard deviations. The maximum activity was set as 100% relative activity. The dashed bars delimit the pH area of musts and wines.

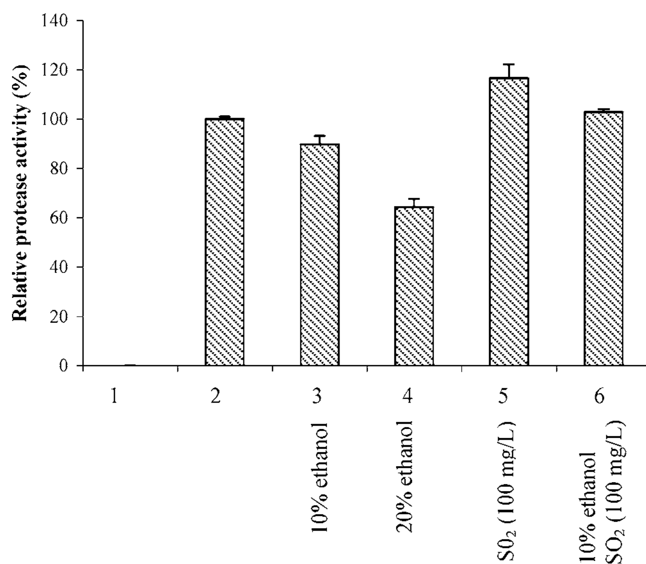

Figure 7. Effect of ethanol and SO_2 on the protease activity of *B. cinerea*. Bars represent relative activity. BSA was incubated (1) alone or (2) with the supernatant culture in the presence of (3) 10% ethanol, (4) 20% ethanol, (5) 100 mg/L SO_2, or (6) a mix of 10% ethanol and 100 mg/L SO_2. Error bars indicate the standard deviations.

of solvatation conditions (production of ethanol) and to pH modifications (precipitation of potassium hydrogen tartrate whose solubility decreases when the alcoholic content increases). For these reasons, we have searched whether a low ethanol concentration (10−20%, v/v) could induce a decrease in the biological activity of *B. cinerea* protease because of its partial denaturation. As shown in **Figure 7**, when BSA was incubated with the *B. cinerea* supernatant in the presence of ethanol, the proteolytic activity decreased by 10−36% when compared with the incubation mixture without alcohol. The enzymatic activity decreased as the percentage of ethanol increased (**Figure 7**). This result is in agreement with the denaturant effect exerted by ethanol on the three-dimensional structure of proteins. Nevertheless, the *B. cinerea* protease activity could remain active in wines but probably at a lower level than that observed in a must.

Proteases originating from grape and yeast can also be present in musts and wines. Cordonnier and Dugal (*36*) have demonstrated that grape juice proteases are active before the alcoholic fermentation takes place and that their activity is reduced as soon as ethanol is produced. Some yeast strains, present in the fermenting must, are able to produce extracellular proteolytic enzymes (*37*, *38*). The occurrence of such a protease activity was reported in still wines by Feuillat et al. (*33*) and by Moreno-Arribas et al. (*39*). However, because of the specific conditions found in wine, only a few proteases are active (*40*). These activities were also observed during yeast autolysis (*41*, *42*). Because yeast protease activities can occur in wine, *B. cinerea* proteases could also be present after completion of the alcoholic fermentation.

To fight the harmful organoleptic effects linked to phenoloxidases essentially, 5−10 g/hL of SO_2 is usually added to the must during pressing. Additional SO_2 treatments are always made after alcoholic fermentation and malolactic fermentation to ensure a constant level of free SO_2. To evaluate the effect of SO_2 on the *B. cinerea* protease activity, BSA was incubated with the *B. cinerea* culture supernatant and 100 mg/L SO_2. Contrary to expectation, the protease activity increased by 17% in the presence of SO_2 (**Figure 7**). Thus, the addition of SO_2 cannot inhibit *B. cinerea* protease activity in musts and wines. Otherwise, a previous study has shown that even with a free SO_2 level of 150 mg/L, only a 20% reduction in polyphenoloxidase activity occurred (*43*).

In another experiment, ethanol and SO_2 were added together to the buffered medium, at a concentration close to that observed in musts and wines. No effect was observed on the protease activity of *B. cinerea*. Then, it seems that the negative effect of ethanol is balanced by the positive effect of SO_2.

In conclusion, for the first time, it is shown that proteases secreted by *B. cinerea* are able to degrade BSA and grape juice protein extracts, inducing the decrease in foamability of a synthetic wine. It was also suggested that the capacity of a protein to foam is linked with its size. In fact, the more the degradation of proteins is, the more their foaming properties will be affected. The foamability was less affected than foam stability.

The alteration of foam parameters, which was observed with botrytized wines in a previous study but never explained, could thus be attributed to the effects of fungal proteases on must and wine proteins. Fungal proteases are active at the pH of musts and wines (between 3 and 3.7) and only slightly decreased in the presence of ethanol and SO_2, a common adjuvant to avoid must and wine oxidation. The temperature during the winemaking process only allows a low protease activity, but it would be interesting to know whether this activity remains sufficient to hydrolyze proteins along the complete winemaking process necessary for Champagne and sparkling wines (6−15 months).

Concerning sparkling wines, this study demonstrates that it is essential to harvest healthy grapes to produce a wine presenting in the glass a beautiful collar and a beautiful mousse during the service/pouring. Champagne's area has a cool climate, and a selection of the best grapes is absolutely necessary. It is alas rarely the case, but we hope that the scientific probes in this article will contribute to better habits/practices.

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