Effects of *Botrytis cinerea* Infection on the Must Protein Electrophoretic Characteristics

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Qualitative effects of *Botrytis cinerea* infection on a must protein fraction were studied by comparing the electrophoretic patterns of musts obtained from healthy grapes or from grapes highly infected by *B. cinerea*. Evidence was obtained that proteins secreted by *B. cinerea* can degrade grape proteins. Most of the proteins present in the healthy must, between 20 and 30 kDa and a major glycoprotein at 62/64 kDa, disappeared in the infected must. Moreover an immunochemical technique, using polyclonal antibodies raised against *B. cinerea*-secreted proteins, was developed to specifically detect proteins originating from *B. cinerea* in the infected must. This is of particular interest when considering that some of the grape proteins participate in the foaming properties of Champagne wines.

Keywords: *Protein; must; Botrytis cinerea; antibodies; electrophoresis; proteolytic activity*

INTRODUCTION

Must and wine proteins are the focus of numerous studies because they are implicated in key enological phenomena such as (1) the formation of haze and deposits during aging or storing (Waters et al., 1992; Ledoux et al., 1992; Dawes et al., 1994), (2) the interaction with aroma compounds (Lubbers et al., 1993a), (3) the tartaric precipitation (Lubbers et al., 1993b), (4) the reduction of heat-induced wine haze (Waters et al., 1994a,b), and (5) the stabilization of foam in Champagne wines (Brissonnet and Maujean, 1991; Malvy et al., 1994). A part of wine proteins originate from the grape berries, while others come from yeasts (Marchal et al., 1996).

Damage caused by *Botrytis cinerea* on grape berries caught the attention of many research laboratories throughout the world (Donèche, 1992). Studies on the enological effects of *B. cinerea* infection mainly concern (1) degradation of plant compounds such as malic acid (Donèche et al., 1985), tartaric acid (Donèche, 1990), and monoterpene alcohols (Bock et al., 1988), (2) concentration of the phytoalexin resveratrol in wine (Siemann and Creasy, 1992; Jeandet et al., 1995), and (3) secretion in grape berries of extracellular D-glucan (Dubourdieu et al., 1980) and fungal proteins. Since some of the proteins secreted by *B. cinerea* possess enzymatic activities, their presence in grape berries may result in alterations of the resulting musts and wines. Particularly, some studies focused upon the role played by fungal laccases on the oxidation of plant phenolics (Marbach et al., 1984).

Karmona et al. (1990) showed that *B. cinerea* secreted an aspartic protease using hemoglobin as a substrate. Secretion of proteases has also been shown on culture media and fruits such as apple (Zalewska et al., 1981)

or tomato (Brown et al., 1983). Nevertheless, the effect of *B. cinerea* proteolytic activities on must proteins is not yet identified.

Enzymatic immunoassays (EIA or ELISA) are employed in enology for pesticide residue quantification such as benomyl (Pilette and Darboret, 1995; Bushway et al., 1993), procymidone (Ferguson et al., 1993; Wynn et al., 1993), or histamine (Rauch et al., 1992) quantification. A rapid immunoassay system was developed for quantifying gray mold in harvested wine grapes (Ricker et al., 1991; Fregoni et al., 1993; Marois et al., 1993). *B. cinerea* antibodies were used to determine the distribution of the fungus in gondolas, but immunotechniques were never used in enology to study proteins. We developed a method to specifically detect proteins originating from *B. cinerea* in the musts.

The objectives of this study are designed to estimate the overall effects of *B. cinerea* grapeberry infection on must protein electrophoretic characteristics. Since must proteins are present in very low amounts (Maujean et al., 1990; Marchal et al., 1997), the high specificity of antibody−antigen binding was assayed to alternatively visualize the proteins originating from grape berries and those produced by *B. cinerea*. To do this, musts were obtained from grapes differing in their level of *B. cinerea* infection.

MATERIALS AND METHODS

Musts. Grape berries of the Pinot noir variety were collected from the Champagne area (France). Grapes were hand-harvested in mid-October 1996 at common commercial maturity [sugar (g/L)/acid (g/L tartaric acid) = 12]. For this study, healthy grapes and grapes affected 80−100% by *B. cinerea* were collected and pressed with a laboratory pneumatic press (pressure between 1.5 and 2 bar). SO_2 (150 mg/L) was added to the free-run juice. After static settling (24 h at 12 °C), the two musts (1 L) were centrifuged (10 min at 8000g). Supernatants were separated and stored at 4 °C.

Must Macromolecule Isolation. After the musts were kept for 2 months at 4 °C, the must obtained from healthy grapes and the botrytized must were centrifuged (10 min at

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8000*g*) and filtered through a 0.45-*μ*m membrane (HA Millipore). The musts were concentrated (eight times) and dialyzed four times against distilled water (0.15 L of concentrate plus 1.35 L of water). A low-protein adsorption poly(ether sulfone) membrane with a 10 000 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. Ultrafiltrations were carried out at room temperature. For the concentration step, musts were diluted with distilled water (v/v) to reduce viscosity and to prevent complexation between proteins and polyphenolic compounds. Dialyzed retentates were freeze-dried (Serail CS 5L) and conserved at −20 °C before being used as must protein antigens (see next section).

Fungal Cultures and Antigen Preparations. The two strains of *B.c-616* (named *B.c-616* and *B.c-630*) used in this study were provided by Dr. Brygoo (INRA Versailles, France). They were isolated from grape berries on the Montagne de Reims (Trépail) and in the Vallée de la Marne (Boursault). Both strains were cultured on an agar-based medium [potato extract (4 g/L) + glucose (20 g/L) + bacteriologic agar (20 g/L), ajusted to pH 5.5]. Conidia were collected from 15-day-old cultures and then incorporated into 3-L conical flasks containing 1 L of Morquer liquid medium (Morquer, 1931) and incubated on a rotary shaker at 150 rpm for 3 weeks at 20 °C, with a 10/14-h day/night photoperiod. Fungal cultures were centrifuged for 10 min at 10000*g* and filtered through a 0.45-*μ*m membrane (HA Millipore), before being ultrafiltered (by the same technique as that used for musts), lyophilized, and stored at −20 °C. The proteic composition of the ultraconcentrate was analyzed by SDS−PAGE immediately after lyophilization or after keeping it respectively for 1 and 4 months at −20 °C. The lyophilisates were used as fungal antigens.

Analytical SDS−PAGE and Western Blotting. Discontinuous SDS−PAGE was performed according to the method of Laemmli (1970) using slab gels (0.75 mm thick). The stacking gels consisted of T = 5% and C = 2.7%, and the separating gels consisted of T = 12% and C = 2.7%. A vertical electrophoresis apparatus (Mini-Protean, 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). Standard proteins from 14 to 94 kDa were used as molecular weight markers (LMW Pharmacia). These proteins were treated like the protein samples (Laemmli buffer v/v), and 24 *μ*L was loaded in the wells for each analysis. The molecular weights (MW) of unknown molecules were calculated from the linear regression equation of log MW vs mobility. After electrophoresis, the separated proteins were either transferred at 4 °C to a nitrocellulose membrane using a Bio-Rad electroblotting apparatus or stained with 1.5% Coomassie brilliant blue in 50% (v/v) methanol and destained in acetic acid/methanol/water (1:2:7). The composition of the transfer buffer was 25 mM Tris, 190 mM glycine, and 20% (v/v) methanol. During Western blotting, a constant current of 100 V was applied for 1 h. Biorad low molecular weight prestained markers (21−112 kDa) were employed during Western blotting studies.

Production of Polyclonal Antisera. Preimmune sera were obtained from two rabbits before immunization. Soluble antigens (*B. cinerea*-secreted proteins) were incorporated in 0.3 mL of 3% polyacrylamide and mixed with 0.9 mL of Freund's complete adjuvant (Sigma-Aldrich, France) (Freund, 1956) to give a final protein concentration of approximately 1 mg/mL. Two rabbits (New Zealand white females) were intradermally immunized on the back at six sites, one rabbit with *B.c*-616- and one rabbit with *B.c*-630-secreted proteins (6 × 0.15 mL of the immunogen). Subsequent injections with the same antigen preparation were made at weeks 3 and 6 in Freund's incomplete adjuvant. Each rabbit was bled at week 5 (first bleed) and week 8 (second and last bleed). Blood samples were left for 1 h at 30 °C before being centrifuged (10 min at 6000*g*). The antisera containing polyclonal antibodies were stored at −20 °C.
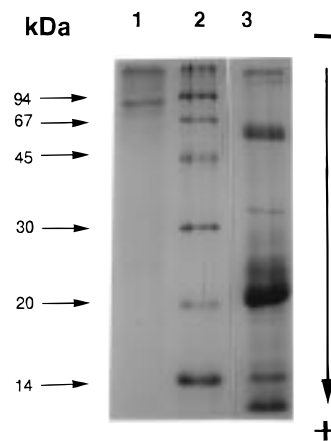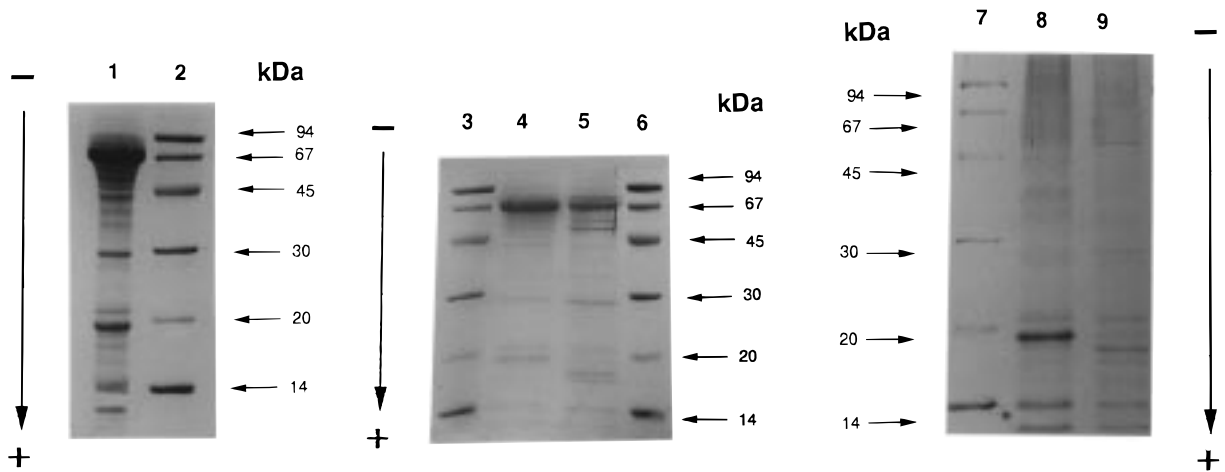

Figure 1. SDS−PAGE analysis of Pinot noir must total proteins isolated by a 10-kDa ultrafiltration and stained with Coomassie brilliant blue: lane 1, botrytized must proteins; lane 2, MW markers; lane 3, healthy must proteins. Relative molecular weights (×10⁻³) of protein standards are given on the left side of the gel.

Cross-Reactivity and Western Blotting Staining. The specificity of the antibodies raised against the proteins secreted by the two strains of *B. cinerea* (*B.c*-616 and *B.c*-630), isolated from grape berries in the Champagne area, was tested using a noncompetitive dot-blot technique. Must proteins and nonimmune sera were spotted (5 *μ*L) in duplicate onto nitrocellulose membranes (Pharmacia Biotech, Upsalla, Sweden) and air-dried at room temperature. Nonspecific binding was blocked with TBS (Tris buffer saline: 25 mM Tris, 0.5 M NaCl, adjusted to pH 5 with HCl) plus 2% nonfat dry milk. The membranes were rinsed three times in TBS and incubated for 3 h in the presence of *B. cinerea* antibodies (antisera, 1/1500 dilution). The membranes were successively washed with TBS, TBS + 0.05% Tween 20, and TBS, before being incubated with goat anti-rabbit IgG (1/1500 dilution in TBS plus 1% nonfat dry milk) during 3 h at room temperature and washed again with TBS, TBS + 0.05% Tween 20, and TBS successively. Goat anti-rabbit horseradish peroxidase-conjugated polyclonal antibodies were obtained from Sigma-Aldrich (France). After membranes were further washed in PBS, peroxidase activity was stained using 4-chloro-1-naphthol (1.2 g/L) in ice-cold methanol and 0.4% H_2O_2 in phosphate buffer saline (PBS). The ability of the antisera to bind to each must protein spot was assessed by visually comparing the color of each spot stained with the antiserum test with that of the nonimmune serum. Western blot membranes were stained as dot-blots.

RESULTS AND DISCUSSION

Comparison of the Protein Fraction of Healthy and Botrytized Musts. To evaluate whether infection of grapes by *B. cinerea* can be the cause of protein alteration in the musts, one aliquot of a must proteins obtained from highly botrytized grapes was compared with the protein fraction of a healthy must by SDS−PAGE. Figure 1, lane 3, shows the protein pattern of a healthy Champagne Pinot noir must after staining with Coomassie brilliant blue. The concentration of macromolecule solution (isolated with a 10-kDa MW cutoff membrane) was 20 g/L (p/v). Most proteins appear in the 20−30-kDa range with a major high molecular weight glycoprotein stained between 62 and 64 kDa as described in Marchal et al. (1996). In contrast, the botrytized must displays a highly altered protein pattern (Figure 1, lane 1) since most of the low molecular weight polypeptides between 20 and 30 kDa disappeared and were replaced by higher molecular weight bands stained between 70 and more than 120 kDa.

Figure 2. SDS−PAGE analysis of total proteins secreted by *B. cinerea* in a Morquer synthetic medium, isolated by a 10-kDa ultrafiltration, and stained with Coomassie brilliant blue: lane 1, *B.c-616* proteins just after lyophilization; lanes 2, 3, 6, and 7, MW markers; lanes 4 and 5, *B.c-616* and *B.c-630* total proteins after keeping the lyophilisate for 1 month at −20 °C; lanes 8 and 9, *B.c-616* and *B.c-630* total proteins after keeping the lyophilisate for 4 months at −20 °C. Relative molecular weights ($\times 10^{-3}$) of protein standards are given on the left and right sides of the gels.

The electrophoretic profile of proteins secreted by *B. cinerea* in a Morquer synthetic medium resulted in a staining pattern (Figure 2, lane 8) which more closely resembles the pattern obtained from a botrytized must (Figure 1, lane 1) than the profile obtained from a healthy must (Figure 1, lane 3). Interestingly, the electrophoretic profile of *B. cinerea*-secreted proteins changed during storage at −20 °C. The major protein of ca. 70 kDa steadily disappeared, probably because of proteolytic activity (Figure 2, lanes 1, 4, 5, 8, 9), though proteins of 14 and 19 kDa, for example, are conserved. Alternatively, the higher molecular weight proteins appearing in the botrytized must, between 75 and more than 100 kDa, could arise from in vitro polymerization of grape proteins. These proteins are poorly stained despite the sample macromolecule concentration (50 g/L = 600 μg of lyophilisate). Loading protein samples at concentrations higher than 50 g/L indeed leads to bad electrophoretic separations, with larger bands and smiles. To precisely identify the origin of protein bands in the botrytized must, antibodies against proteins secreted by *B. cinerea* were constructed to specifically probe for proteins released by this fungus in a must.

Specificity of Antigen−Antibody Recognition in a Must. Figure 3 shows that the *B.c-616* proteins antibodies obtained are able to recognize their antigens (strip 1, spot B) and are also capable of recognizing proteins secreted by another *B. cinerea* strain (called *B.c-630*) isolated from another vineyard in the Champagne area (strip 1, spot C). Antibodies obtained against the strain *B.c-630* yielded similar results as dot-blots obtained with *B.c-630* and *B.c-616* proteins (data not shown). Antibodies can additionally discriminate between *B. cinerea* proteins and proteins synthesized by grape berries since they display no positive reaction when exposed to a healthy must protein fraction (strip 1, spot D). A control experiment using preimmune antisera yielded no positive cross-reaction with proteins secreted by both *B. cinerea* strains (Figure 3, strip 2, spots B and C). The response is also negative with a healthy or botrytized must protein fraction (strip 2, spots D and E, respectively). Finally, by using the same dot-blot technique, *B. cinerea* antibodies recognize proteins present in a botrytized must (Figure 3, strip 1,
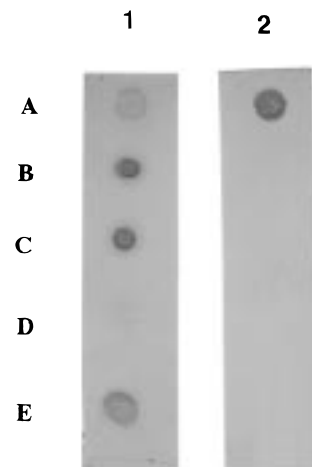

Figure 3. Dot-blot on nitrocellulose strips with color developed using 4-chloronaphthol/peroxidase-labeled goat IgG in PBS buffer: strip 1, rabbit anti-*B. cinerea* protein polyclonal antibodies; strip 2, rabbit preimmune serum; spot A, preimmune serum; spot B, *B.c-616* proteins; spot C, *B.c-630* proteins; spot D, healthy must proteins; spot E, botrytized must proteins.

spot E). Then, some of the botrytized must proteins probably originate from *B. cinerea*.

Recognition of *B. cinerea* Proteins in a Botrytized Must. After separation by SDS−PAGE, many botrytized must proteins yield a positive reaction between ca. 50 and more than 100 kDa (Figure 4, lane 3) with *B. cinerea*-secreted protein polyclonal antibodies. By comparing the staining pattern obtained in Figure 4, lane 3, with the Coomassie brilliant blue staining of a botrytized must protein (Figure 1, lane 1), one can see that grape proteins found in the botrytized must were replaced by *B. cinerea* proteins. This result suggests that the presence of *B. cinerea* proteins in a must can lead to proteolytic degradation of grape proteins by fungal protease, probably. Protein glycosylation confers a certain degree of resistance to proteases (Seymour et al., 1991), and most of must proteins are highly glycosylated or associated with polysaccharides (Marchal et al., 1996). Nevertheless, must protein degradation severely occurred. Williams and Modra (1988) showed that plant and fungal proteases signifi-

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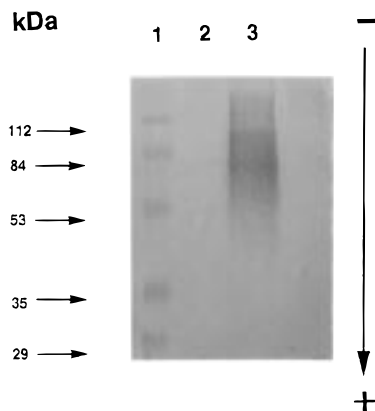
Figure 4. Detection of *B. cinerea* proteins in Pinot noir musts by SDS–PAGE and immunoblotting using rabbit anti-*B.c-616* polyclonal antibodies: lane 1, MW prestained markers; lane 2, healthy must total proteins; lane 3, botrytized must total proteins. Relative molecular weights ($\times 10^{-3}$) of protein standards are given on the left side of the gel.

cantly alter the chromatographic profile of a must protein fraction of Muscat Gordo Blanco after only 7 days. This observation is in good agreement with our data and is consistent with the fact that fungal proteases are active at the pH of the must (about 3.0), at 4 °C and in the presence of 150 mg/L sulfite, a common adjuvant to musts. In contrast, Waters et al. (1992) showed that a 14-day treatment of proteins of a Muscat wine from Alexandria with a commercial peptidase mixture (Vinozym sold by Novo) resulted in no significant alteration of the electrophoretic profile of the wine proteins by SDS–PAGE. According to Waters et al. (1992), the polysaccharides and the polyphenols present in wine are not implicated in protecting wine proteins from proteolytic degradation, these naturally displaying protease resistance. These discrepancies can very likely be explained by the different experimental conditions used in these different studies (e.g., pH, enzyme concentration, duration of treatment, nature of proteases, or temperature). Further investigations conducted on musts and wines obtained with accepted industrial processes are currently under investigation to determine whether commercial wines elaborated from botrytized harvests display an altered protein profile.

Further comparisons of the staining pattern obtained with *B. cinerea* antibodies on botrytized must proteins (Figure 4, lane 3) and proteins secreted by *B. cinerea* on a synthetic medium (Figure 2, lane 8 in particular) show that most of the high molecular weight proteins secreted by *B. cinerea* are present in both samples, while low molecular weight bands were only found when *B. cinerea* was cultured on a Morquer synthetic medium. The synthesis of specific proteins by *B. cinerea* may be controlled by environmental conditions and may thus differ when the fungus is growing on grapes or on a synthetic medium. Alternatively, just a part of grape proteins and proteins secreted by *B. cinerea* in grape tissues is extracted in the musts during pressing (Hsu and Heatherbell, 1987).

CONCLUSION

The results presented in this study show that a botrytized must contains proteins which originate from *B. cinerea*. Moreover, the presence of fungal proteins in a must can result in the complete proteolytic degradation of its protein fraction. Alteration of the must

protein fraction would thus add to the many already characterized must modifications challenged by this fungus. Furthermore, we have described a highly sensitive immunochemical technique to specifically detect *B. cinerea* proteins in a Champagne must. We are now studying the relationship between must degradation by *B. cinerea* and foam properties of Champagne wines.

The modification of protein characteristics is of particular interest when considering that some of the proteins present in grape berries exhibit foaming properties (Maujean et al., 1990; Brissonnet and Maujean, 1991; Malvy et al., 1994).

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