JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of the Grape Invertase Content (Using PTA−ELISA) following Various Fining Treatments versus Changes in the Total Protein Content of Wine. Relationships with Wine Foamability

THIERRY DAMBROUCK, RICHARD MARCHAL, CLARA CILINDRE, MARYLINE PARMENTIER, AND PHILIPPE JEANDET*

Laboratory of Enology and Applied Chemistry, Research Unit number 2069, Faculty of Science, University of Reims, Post Office Box 1039, 51687 Reims Cedex 02 France

Proteins have proven to play a major role in the stabilization of foam in Champagne wines despite their low concentration that ranges from 4 to 20 mg/L. The aim of this study was to evaluate the effect of fining on total protein and grape invertase contents of champenois base wines and their foaming properties. Data showed that fining and especially the use of bentonite at doses ranging from 10 to 50 g/hL leads to a significant decrease in the total protein content of wines together with that of the grape invertase content, with such a decrease being very detrimental to the foaming properties of the treated wines in terms of foam height (HM) and foam stability (HS). Only a slight decrease in the total protein content, in the grape invertase concentration, and in the foam quality of wines was observed when using casein (10 and 20 g/hL) or bentonite combined with casein (both at 20 g/hL). Our study thus clearly establishes the good correlation existing between the wine protein concentration and its foaming properties. A remarkable correlation was observed between the decrease in the grape invertase content and the total protein content of wines, following bentonite treatments, suggesting that the grape invertase (which represents at least 10−20% of the wine proteins) follows a similar behavior upon fining to other proteins of Champagne wines, despite the high molecular mass and the highly glycosylated structure of this particular protein. Moreover, the decrease in total protein and grape invertase contents of wine after fining with bentonite was found to be correlated with a decrease in the foaming properties of the corresponding wines (with respectively $R^2 = 0.89$ and 0.95)

KEYWORDS: Invertase; proteins; fining treaments; foam; ELISA; immunoquantification

INTRODUCTION

Champagne is undoubtedly one of the most prestigious effervescent wines throughout the world. This is linked to the elegance of its sparkling and foaming properties. Consumers appreciate both a regular and durable effervescence, leading to a foam ring composed of small bubbles on the liquid surface, the so-called collar. Before smelling and tasting a Champagne, consumers will pay attention to the appearance of the foam. In light of this, it is important to study both the compounds involved in these phenomena and enological treatments able to modify the quality of foam.

Champagne wine's foaming properties largely depend upon their macromolecule content, that is, mostly unidentified compounds of 10−100-kDa molecular masses (*1*); among them, proteins have proven to play a major role in the stabilization of foam in Champagne wines (*2*−*4*), despite their low concentration that ranges from 4 to 20 mg/L (*5*, *6*). Proteins play an important role as macromolecular surfactants in foam and emulsion-type food products (*7*). All proteins do not contribute equally to the foaming properties of wines (*8*). Protein foamability is indeed governed by three interrelated properties such as their isoelectric point and solubility (*9*, *10*), their hydrophobicity (*11*−*13*), and their flexibility (*14*, *15*). According to other works (*16*, *17*), glycoproteins are more able to stabilize foam than nonglycosylated ones, because hydrophilic glycans reside in the liquid region between bubbles. When the liquid film between two bubbles becomes thinner, the viscosity increases and retards the liquid drainage but the proteic moiety equally acts on the liquid surface tension, which constitutes an essential property for foam stabilization. More recent works developed in our laboratory have demonstrated, indirectly, the adsorption of tensioactive molecules such as proteins at the surface of ascending Champagne bubbles, thus modifying their velocity (*6*, *18*). Owing to this, it is important to study the role played by wine glycoproteins in the foaming properties of Champagne

* To whom correspondence should be addressed. Telephone/fax: +33 (0) 3 26 91 33 41. E-mail: philippe.jeandet@univ-reims.fr.

10.1021/jf051276z CCC: $30.25
Published on Web 09/30/2005

in relation with the effect of enological treatments, particularly fining, on these phenomena.

Champagne wines (generally speaking all white wines) contain a large proportion of proteins and glycoproteins originating from the grape berry (*19*−*24*), while others come from yeast (*19*, *23*−*26*). Most of them have a pI ranging from 2.5 to 4.5 and molecular masses varying from 12 to 65 kDa (*8*). We also have to mention the presence of a grape invertase of 62/64 kDa. This invertase is a N-glycoprotein originating from the plant, as demonstrated by using immunological methods. This enzyme keeps its activity in wine and presents a high hydrophobicity (1050 kcal/100 amino acid residues) and a pI of 3.9 (*21*). More recently, we also characterized grape lectins, showing an hemagglutining activity, which is inhibited by *p*-aminophenyl-*β*-D-glucoside (*27*), although their presence in wines has not yet been demonstrated.

The fact that the grape invertase is believed to be one of the most abundant proteins in wine (from 9 to 14% of the total protein content of a Chardonnay wine) (*4*), together with the fact that this grape protein possesses a pI close to the pH of wine and a high hydrophobicity, potentially confering good surface properties on this protein (*4*), led us to investigate the evolution of this major wine protein following fining on Champagnes. Experiments were thus planned to follow and quantify the grape invertase by using an immunotechnique (PTA−ELISA, plate-trapping antigen−enzyme-linked immunosorbent assay) during the Champagne wine making process. Simultaneously, fining procedures were used to determine their impact on the content of grape invertase and total proteins in wines. All of these data were then correlated to changes in the foaming properties of the treated wines.

MATERIALS AND METHODS

Must. Grape berries of the Chardonnay variety were collected from the Champagne area (France). Grapes were hand-harvested in mid-october 1998 at common commercial maturity [sugar content (g/L)/total acidity acid (g/L of tartaric acid) = 12]. Grapes were pressed with a 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 must (10 L) was centrifuged (10 min at 8000*g*). Supernatants were separated, filtered through a 0.45 μm membrane, and stored at 4 °C.

Wine Samples. The juice was fermented in parallel. The settled Chardonnay must was racked and chaptalized with sucrose (35 g/L). The alcoholic fermentation was achieved by inoculating *Saccharomyces bayanus* at 18 °C. Malolactic fermentation did not take place in this wine. Wines were stabilized with respect to potassium hydrogentartrate, then centrifuged, and filtered through a 0.45 μm membrane and stored at 4 °C.

Fining Agents. Three different types of fining agents were used: (a) Treatment with bentonite. The enological natrium bentonite (Volclay) was soaked in water [50 g/L (w/v)] and allowed to swell 24 h before use. The doses used were, respectively, 10, 20, 40, and 50 g/hL. (b) Treatment with casein. The potassium caseinate was diluted at 10 g/L (w/v) in distilled water. The doses used were, respectively, 10 and 20 g/hL. (c) Treatment with a bento−caseine mixture. The doses used were 20 g/hL for each fining agent. A mixture of potassium caseinate and bentonite (50:50) (w/w) was soaked in water to swell 5 h before being added to the wine. All of the experiments were carried out with 1.5 L of wine. Wines were maintained 1 week along with the fining agents before being racked and centrifuged. Fining was realized before potassium hydrogentartrate stabilization.

Macromolecule Isolation. After centrifugation, the must was concentrated (10 times) and then 4-times dialyzed against distilled water (*21*). A hydrophilic polysulfone membrane of 10 000 molecular weight cutoff was used (Minisette Omega, screen channel membrane, PALL-FILTRON, France). The cross-flow filtration module (1.2 m^2) was connected to the Hi-Flow system (pumping system plus glass tank). Ultrafiltration was carried out at 4 °C under a stream of nitrogen to avoid oxidation. The ultrafiltrate flow was 40 mL/min. For the concentration step, the must was laced with distilled water (v/v) to reduce viscosity and to avoid complexation between proteins and polyphenolic compounds. The dialyzed retentates were freeze-dried (Serail CS 5L) and stored at −20 °C.

Analytical SDS−PAGE and Western Blotting. Discontinuous SDS−PAGE was performed according to the method of Laemmli (*28*) 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 mass markers (LMW Pharmacia). These proteins were treated as the protein samples (Laemmli buffer, v/v), and 24 μL was loaded into the wells for each analysis. The molecular weights (MW) of unknown molecules were calculated from the linear regression equation of log MW versus mobility. After electrophoresis, the separated proteins were either transferred at 4 °C to a nitrocellulose membrane using a Bio-Rad electroblotting apparatus or silver-stained. 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. Bio-Rad low molecular mass prestained markers (19.4−104 kDa) were employed for Western blotting studies.

Purification of the Grape Invertase. The grape invertase was purified from the concentrated must. Samples were subjected to cation-exchange chromatography (SP 40 HR Protein Pak, Waters, Milford, MA). The bound material was eluted with a gradient of NaCl from 0 to 500 mM in 50 mM Tris/HCl buffer (pH 7.5) (invertase was eluted with NaCl concentrations ranging from 80 to 160 mM). The invertase-containing fractions were pooled, brought to 0.3 M with respect to NaCl, and loaded onto a Concanavaline A column (120 × 12 mm) (con A-Sepharose, Sigma), equilibrated with 0.3 M NaCl in Tris/HCl buffer (50 mM, pH 7.5) containing $CaCl_2$, $MnCl_2$, $MgCl_2$ (1 mM each). After extensive washing with saline buffer, invertase was eluted with 25 mL of 5 mM α-methylmannoside. Fractions containing the highest invertase activity were pooled, washed, and concentrated by ultrafiltration using Microcon YM-3 cartridges (Millipore, Bedford, MA). A total of 4 mL of the invertase solution (obtained from the affinity purification step) was loaded onto a model 491 Prep Cell (Bio-Rad) according to the user's operating manual. A 9% T resolving gel was used with a $T = 4\%$ stacking gel, and fractions of 4 mL each were collected after elution of the dye front. Fractions were analyzed by SDS−PAGE using the Protean II system (Bio-Rad) (**Figure 1**). The eluted protein was desalted and concentrated using Microcon YM-10 cartridges (Millipore, Bedford, MA). The purified protein was used for immunization of rabbits. Purification steps of the grape invertase are presented in **Table 1**.

Production of Polyclonal Antisera. Preimmune sera were obtained from a rabbit (New Zealand white female) before immunization. Soluble antigens, that is, the purified grape

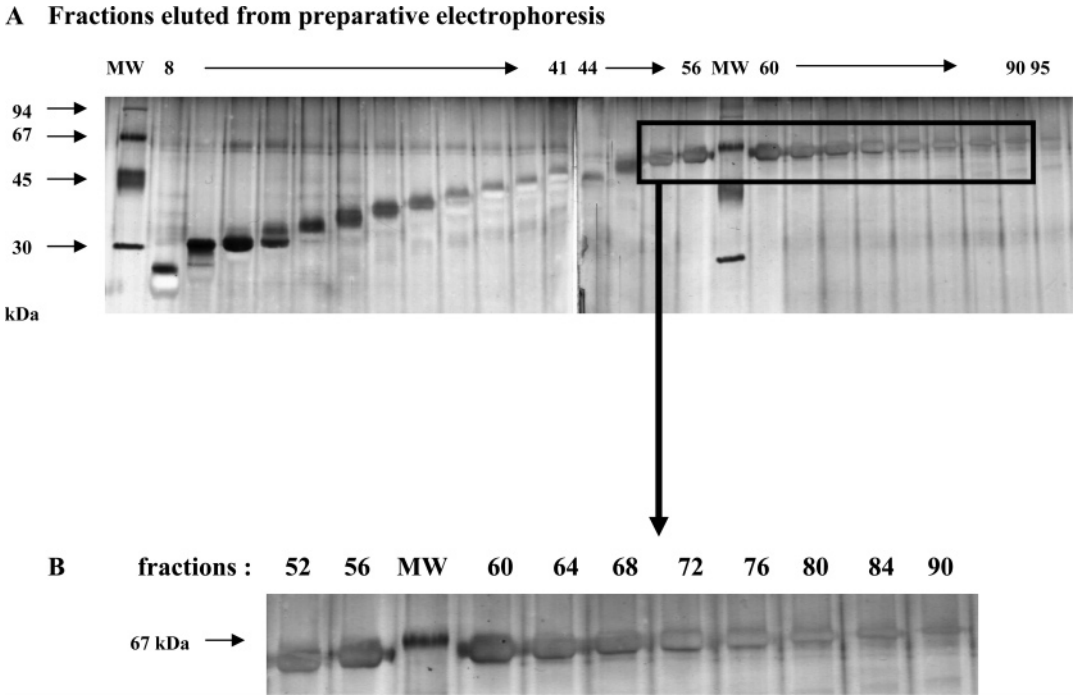

Figure 1. Electrophoretic profiles of the grape invertase fractions isolated from the Prep-Cell. Each numbered line corresponds to one fraction eluted from the Prep-Cell and analyzed by SDS–PAGE (*28*). Fractions were silver-stained. MW, standard molecular weights. (A) Total profiles. (B) Enlargement of fractions numbered 52–90, corresponding to the grape invertase in the 60–67-kDa molecular mass range.

Table 1. Purification Steps of the Grape Invertase

purification steps	vol. (mL)	quantity of total proteins (mg)[a]	total activity (nkat)	yield (%)	specific activity (nkat/mg)[b]	degree of purification
crude extracts (total colloids: 2.75 g/L)	180	270	1340	100	4.96	1
DEAE FR 160 mM	160	67.7	858	64	12.7	2.56
ConA 5 mM	80	9.84	560	41.8	56.91	11.47

[a] As determined using the method of Bradford (*29*) as modified by ref *5*. [b] nkat = 1 nmol of glucose formed/s.

invertase, were incorporated in 0.3 mL of 3% polyacrylamide and mixed with 0.9 mL of Freund's complete adjuvant (Sigma−Aldrich, Saint-Quentin Fallavier, France) to give a final protein concentration of approximately 1 mg/mL. The rabbit was intradermally immunized on the back at six sites (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. The rabbit was bled at week 8. Blood samples were left for 1 h at 30 °C before being centrifuged (10 min at 6000*g*) to obtain the serum. The IgG was purified from serum by using a DEAE Affi-Gel Blue Gel (Bio-Rad) and then stored at −20 °C.

Western Blotting Staining. For nonspecific binding, the nitrocellulose membranes (Sartorius, Göttingen, Germany) were blocked with TBS (Tris buffer saline: 25 mM Tris and 0.5 M NaCl, adjusted to pH 5 with HCl) plus 2% (w/v) nonfat dry milk. The membranes were rinsed 3 times in TBS and incubated for 3 h with invertase polyclonal antibodies (antisera) diluted 1:1500 in TBS containing 1% (w/v) nonfat dry milk. The membranes were successively washed with TBS, TBS and 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 and 0.05% Tween 20, and TBS, successively. Goat anti-rabbit horseradish peroxidase-conjugated polyclonal antibodies were obtained from Sigma−Aldrich (Saint-Quentin Fallavier, France). Membranes were further washed in phosphate buffer saline (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 PBS.

ELISA Procedures. A PTA−ELISA was developed for the grape invertase using 96-well flat-bottom immunoplates (Immulux 2HB, Dynex Technologie). The procedure was as follows. Plates were coated overnight with standards or samples (diluted or not in PBS at pH 7.4 containing 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, and 2.7 mM KCl) (100 μL/well). After the plates were covered and left overnight at 4 °C, they were washed 3 times with PBST [PBS containing 0.05% (v/v) Tween 20]. The plates were then incubated in 3% (w/v) bovine serum albumin (BSA) in cover buffer (200 μL/well) for 2 h at room temperature and washed 3 times with PBST. The plates were subsequently incubated with appropriate dilutions of the antiserum (diluted with PBS containing 1% (w/v) BSA, 100 μL/well) overnight at 4 °C and washed 4 times with PBST. The goat anti-rabbit peroxidase conjugate (diluted with PBS containing 1% (w/v) BSA, 100 μL/well) was added and incubated for 3 h at 37 °C. After the plates were washed 4 times with PBST, the peroxidase substrate was added.

Enzyme Assay. Aliquots (50 μL) of appropriately diluted enzyme solutions were incubated in 50 μL of a sucrose solution for 30 min at 30 °C. The reaction was stopped by addition of 100 μL of DNSA-reagent [3,5-dinitrosalicylic acid 1% (v/v) in 0.5 M KOH and 0.5 M K/Na-tartrate]. This mixture was then kept for 5 min in a boiling water bath. After cooling and addition

of 1 mL of water, the color intensity was determined at 540 nm against an identical assay with 0 reaction time. Invertase activity is expressed as nmol of sucrose hydrolyzed/s (nkat) under the above conditions.

In Gel Activity. Invertase activity was localized after incubation of native gels with gentle shaking for 30−60 min in a 100 mM sodium acetate buffer (pH 4.0) containing 0.6 M sucrose. After a short wash with distilled water, reducing sugars were stained on the gel with 2,3,5-triphenyltetrazolium chloride [0.2% (w/v) in hot 1 M NaOH]. Color development was stopped with 1% acetic acid.

Determination of the Protein Concentration. (a) Protein content of the prepurified or purified fractions. The protein concentration was determined by using the Pierce protein assay kit (Pierce) with BSA as a standard. The concentration of grape invertase used as a standard in the ELISA assay was confirmed by using this kit. (b) Protein content of wines. The wine protein content was determined according to the Bradford method (*29*), modified as to correct interferences because of ethanol and phenolic compounds (*5*). Briefly, the sample protein content is defined as the sample reactivity with the dye reagent (Bio-Rad protein assay, Dye reagent concentrate, Bio-Rad Laboratories GmbH, München, Germany) minus ultrafiltrate reactivity with the dye reagent (using a 3-kDa MWCO membrane; Amicon, Bedford, MA). Results were expressed in mg of equivalent BSA/L. Each value was the average of three measurements. The standard curve coefficient correlation was $R^2 = 0.9924$.

Foaming Properties. Measurements were made after wines have been stabilized with regard to hydrogentartrate. Foam measurements were carried out using the Mosalux apparatus (*30*). Wines were filtered on 0.45 μm membranes before the 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 wine to be tested. Carbon dioxide was injected into the glass cylinder through the glass frit with a constant gas flow (7 L/h) and under a constant pressure (100 kPa), for 8 min. Maximum foam height (mm) was controlled by photoelectric cells (infrared beams). All measurements were conducted in triplicate and done the same day to reduce the dispersion of the values. Two parameters were obtained: (1) foamability, HM, which corresponds to the maximum height reached by the foam column, and (2) foam stability, HS, which represents the persistence of the foam collar.

RESULTS

Specificity of the Polyclonal Antibodies. Purified antibodies have been tested by using the Western Blot technique. Anti-invertase polyclonal antibodies showed a high specificity for the antigen (**Figure 2**). Antibodies recognize the grape invertase as they are placed in contact either with wine or must samples (lanes A and C of **Figure 2**). These antibodies specifically reacted with the must grape invertase (band at 60−67 kDa) (lane D of **Figure 2**) and did not show any cross reaction with other grape must proteins (lane C of **Figure 2**). Moreover, polyclonal antibodies specifically recognized the grape invertase when they were placed in contact with wine colloids and yielded no postive cross reaction with other wine proteins (lane A of **Figure 2**). Finally, no cross reaction was observed between anti-invertase polyclonal antibodies and the yeast invertase obtained from Sigma (lane B of **Figure 2**).

Development of the PTA−ELISA Procedure. To optimize the ELISA procedure, the antiserum (anti-invertase antibodies) titer was tested. The antigen was used at concentrations ranging from 0.1 pg/mL to 10 μg/mL of purified invertase, and the

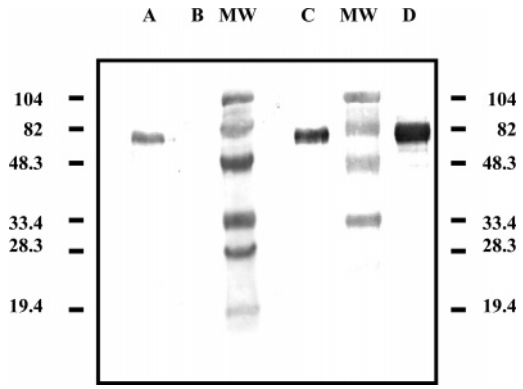

Figure 2. Specificity of the anti-grape invertase polyclonal antibodies as determined using Western blot. Polyclonal antibodies are 1:500 diluted. MW, standard molecular weights; lane A, wine colloid fraction (150 μg); lane B, yeast invertase (Sigma) (10 μg); lane C: must colloid fraction (150 μg); and lane D, purified grape invertase (10 μg).

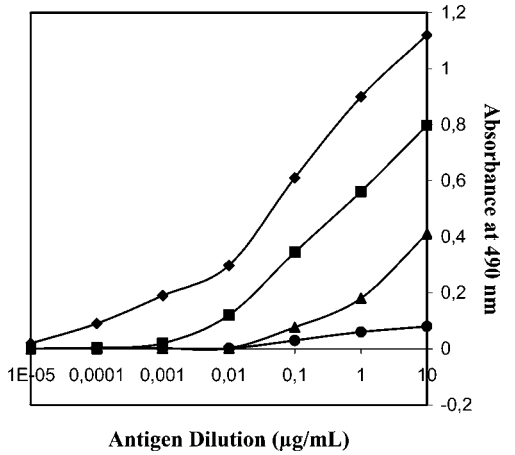

Figure 3. Dilution curves of the antiserum. (◆) 1:1000 diluted serum, (■) 1:10 000 diluted serum, (▲) 1:100 000 diluted serum, and (●) 1:1 000 000 diluted serum.

antiserum was diluted from 1:1000 up to 1:1 000 000. Results are presented in **Figure 3**. A reaction of the antiserum was observed using dilutions lower than 1:10 000, with the most appropriate dilution of the antiserum being fixed to 1:1000. The three lowest dilutions (1:10 000, 1:100 000, and 1:1 000 000) did not show any significant reaction, especially when using low antigen concentrations (**Figure 3**). For goat anti-rabbit IgG horseradish-peroxidase-conjugated polyclonal antibodies (labeling antibodies), the appropriate dilution was fixed to 1:3000.

To quantify invertase, a calibration curve (absorbance at 490 nm versus the invertase concentration) was realized by coating wells of a microtiter plate with different concentrations of the purified grape invertase ranging from 0.1 pg/mL to 100 μg/mL. The antiserum was used at a dilution of 1:1000 and the labeling antibodies at a 1:3000 dilution (**Figure 4**). With this PTA−ELISA procedure, we were able to detect up to 0.1 ng/mL of invertase, with the working range for invertase detection being 1 ng/mL to 4 μg/mL. We also verified that the presence of ethanol in wine samples (12%, v/v) did not interfere with our PTA−ELISA procedure (data not shown).

We have thus at one's disposal a reliable immunochemical method to quantify invertase in wines during the winemaking process and to evaluate changes in the grape invertase content according to various fining treatments.

Influence of Fining on Both the Total Protein and the Grape Invertase Content of Wine Samples. After completion

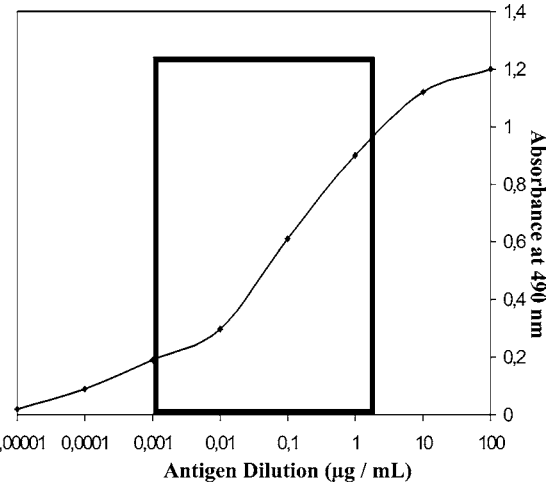

Figure 4. Standard curve for the grape invertase. The box corresponds to the working range for grape invertase detection (between 1 ng/mL and 4 μg/mL).

of the alcoholic fermentation, wine is a colloidal solution and suspension. The particle density, which is close to that of the wine, electrical repulsion forces together with diffusion phenomena lead to very slow and insufficient spontaneous clarification. Organic and mineral fining agents (such as casein or bentonite) are commonly used to clarify white wines but also for limiting oxidation phenomena linked to polyphenoloxidases. Such treatments can considerably affect, qualitatively and quantitatively, the composition of wine in tensio-active compounds, thus decreasing the foaming properties of the resulting sparkling wines (*30*). To search whether fining can modify the protein content of wine and, particularly, the grape invertase content, fining experiments were carried out on wines before stabilization with respect to potassium hydrogentartrate. This included the use of bentonite, casein, and bentonite combined to casein at varying doses.

In the nontreated wines (i.e., without any fining), the total protein content, as determined according to the Bradford method (*29*) modified by Marchal et al. (*5*) was 10.8 μg/mL, while the grape invertase concentration measured by using the PTA−ELISA procedure was 1.9 μg/mL. The grape invertase represents ca. 20% of the total protein content, thus constituting a major protein of wines. Fining treatments with increasing bentonite doses (10, 20, 40, and 50 g/hL) lowered the total protein concentration of the corresponding wines by, respectively, −18, −44, −53, and −72% (**Table 2**). It is remarkable that the grape invertase content is lowered in the same way as wine proteins upon bentonite treatment; that is, losses in grape invertase reach −17, −37, −55, and −73% with the same bentonite doses (see **Table 2**). Changes in the total protein content of wine following bentonite fining versus changes in the grape invertase content were correlated by a linear relationship with $R^2 = 0.9845$. Contrary to the observations of Ledoux et al. (*26*), which showed that wine proteins are removed in different proportions after fining with bentonite, our study suggests that the mechanism of grape invertase adsorption by bentonite (versus total proteins) is not specific and that the grape invertase could thus constitute a "model" protein for studying protein adsorption upon bentonite addition in wines.

Fining treatments using two casein doses (10 and 20 g/hL) only lowered the total protein content of wine by, respectively, −8 and −20% (**Table 2**). The very low decrease (7%) in the protein content of wine, obtained with bentonite combined with casein, both at 20 g/hL, has previously been reported by Maujean et al. (*30*). Here again, the decrease in the grape invertase content was found to be remarkably similar to losses in total wine proteins. Summarizing the results, the diminution in grape invertase reaches, respectively, −11 and −18% with casein fining and only −10% with bentonite combined with casein (**Table 2**). Changes in the total protein content of wine following casein treatments versus changes in the grape invertase concentration were well-correlated with $R^2 = 0.9535$.

Table 2. Influence of Fining Using Varying Doses of Bentonite, Casein, or the Bento–Caseinate Mixture on Both the Total Protein and the Grape Invertase Content of Wine Samples

bentonite doses (g/hL)	proteins[a] (μg/mL)	protein losses (%)	grape invertase[b] (μg/mL)	loss in invertase (%)
control	10.8		1.90	
10	8.87	–18	1.58	–17
20	6.04	–44	1.20	–37
40	5.03	–53	0.85	–55
50	3.05	–72	0.51	–73
casein doses (g/hL)				
control	10.8		1.90	
10	9.90	–8	1.69	–11
20	8.61	–20	1.55	–18
bentocasein mixture (g/hL)				
control	10.8		1.90	
20	10.1	–7	1.71	–10

[a] As determined using the method of Bradford (*29*) as modified by ref *5*. [b] As determined using the PTA–ELISA procedure.

Table 3. Influence of Fining on the Foaming Properties of Wines

	protein losses (%)	HM[a] [$m \pm ts$][b] (mm)	variations of HM (%)	HS[c] (mm)
bentonite doses (g/hL)				
control		113 ± 7		17
10	–18	98 ± 8.6	–13	11
20	–44	96 ± 7	–15	unstable[d]
40	–53	76 ± 8.6	–33	unstable[d]
50	–72	60 ± 9	–47	unstable[d]
casein doses (g/hL)				
10	–8	99 ± 10	–12	13
20	–20	95 ± 3	–16	15
bentocasein mixture (g/hL)				
20	–7	108 ± 12	–5	17

[a] Foamability, HM, which corresponds to the maximum height reached by the foam column. [b] $m \pm ts$, with m = mean of the measurement series; $t = t$ distribution ($p = 95\%$); s = standard error of the mean. [c] Foam stability, HS, which represents the persistence of the foam collar. [d] Describes the variations observed in the foam profile of the wine after the peak HM was obtained.

Influence of Fining of Wines on the Wine-Foaming Properties as Determined with the Mosalux System. The decrease in total protein and grape invertase contents of wine after fining with bentonite is correlated with a decrease in the foaming properties of the corresponding wines (with respectively $R^2 = 0.89$ and 0.95). Treatment with increasing doses of bentonite (ranging from 10 to 50 g/hL) decreases the foamability of wines in a dose-dependent manner. The foam height HM declines by up to 47% with the highest dose of bentonite (**Table 3**). This confirms previous works (*30*), describing a diminution of 40−60% in the foamability of champenois base wines treated with 50 g/hL bentonite doses. Addition of bentonite was also found to be very detrimental to the foam height after 8 min sparging (HS), with the foam showing a high instability (**Table 3**), that is, variations observed in the foam profile of the wine

after the peak HM was obtained, also described in the case of Chardonnay wines originating from lysozyme-treated musts plus bentonite fining (*31*).

Treatments of wines by casein only led to a slight diminution both in the total protein content and the grape invertase concentration (**Table 2**). According to this, a limited decrease in the foaming properties of the corresponding wines was observed, that is, −12 and −16% for HM with 10 and 20 g/hL casein, respectively, and −23% for HS with 10 g/L casein (**Table 3**). Finally, fining using bento−caseinate at 20 g/hL doses did not provoke any significant modification of the foaming properties of wines (−5% for HM and no changes in the HS value) (**Table 3**). Unexpectedly, bentonite combined with casein was found to be less detrimental in terms of protein removal (**Table 2**) and changes in wine-foaming properties than bentonite and casein, separately. These results corroborate the findings of Maujean et al. (*30*) but are not in agreement with those of Puig-Deu et al. (*32*), which showed that the use of a bento−caseinate mixture (both at 50 g/hL doses) leads to a significant decrease in the total protein content of wines as well as in their foaming properties. All together, the data presented in this section confirm that a relationship does really exist between the total protein content of wines and their foam quality parameters.

DISCUSSION

The aim of this study was to evaluate the effect of fining on the total protein content of champenois base wines and their foaming properties. This is, to our knowledge, the first work reporting in a precise manner, the evolution of the protein content of a wine during the winemaking process, namely, with regard to fining treatments, in relation to changes in its foam quality parameters. Moreover, we have determined using an immunochemical procedure the content of a major wine glycoprotein, the grape invertase, following various fining treatments.

Our data show that fining treatments, especially those using bentonite at doses ranging from 10 to 50 g/hL, lead to a significant decrease in the total protein content of wines, as well as in the grape invertase concentration. Such a decrease is very detrimental to the foaming properties of the treated wines in terms of foam height (HM) and foam stability (HS). Only a slight decrease in the total protein content and in the foam quality of wines was observed when using casein or bentonite combined with casein. Thus, our study clearly establishes a correlation between the wine protein content and its foaming properties: a significant decrease in the total protein and in the grape invertase contents, induced by fining, was associated with a diminution of the foam quality parameters. In the same way, a low decrease in wine proteins, consequent upon fining, was accompanied by only a slight modification in the foam properties of the corresponding wine.

Although there is already some evidence that proteins may have a positive effect on wine foams, the question of whether proteins do really play a role in the foaming properties of Sparkling still remains in discussion. Maujean et al. (*30*) were the first to establish a correlation between wine proteins and foamability of champenois base wines. This was further confirmed by Brissonnet and Maujean (*2, 8*), who demonstrated in addition that compounds responsible for the stabilization of foam films in Champagne wines are proteins or glycoproteins with a pI close to the pH of wine and molecular masses ranging from 12 to 65 kDa. In the same way, Péron et al. (*1*) demonstrated that the decrease in the wine-foaming properties and in the surface properties of the adsorption layer of both ultrafiltrated- (i.e., wines in which compounds of a molecular mass higher than 10 kDa are removed) or bentonite-treated wines were linked to their low macromolecular content. The fact that treatments with bentonite lower both the surface and the foaming properties of the corresponding wines indirectly confirms the role of proteins in these phenomena although authors suggested that numerous colloids, different from proteins, with a molecular mass exceeding 10 kDa could also intervene (*1*). Other works have established that a direct relationship exists between the protein content of Sparkling (mainly *Cavas*) and the foam parameters HM and HS (*33*, *34*).

On the other hand, fining treatments, by lowering the total protein content and, simultaneously, the foam quality parameters of wines, indirectly prove that proteins are related to foamability. The effect of the addition of fining compounds, especially bentonite, on the protein composition and foaming properties of Sparkling has already been studied (*30*, *32*, *35*). Whatever the stage of the winemaking process at which fining was realized: (i) prefermentation stage (*32*), (ii) before stabilization of wine with respect to potassium hydrogen tartrate (the present paper), (iii) addition to the *tirage liqueur* (*35*), addition of bentonite always leads to a decrease in the protein content of wines with a simultaneous diminution of their foaming properties.

Some other previously published works have indirectly demonstrated the relationship existing between proteins and foamability and reinforced the role played by these compounds in determining foam quality parameters. Lao et al. (*36*) showed that addition of pectic enzymes (commercial enzyme preparations) to musts decreases their foamability and consequently those of the wines. Authors suggested that the diminution observed in the foaming properties of treated musts and wines were likely related to protein hydrolysis by protease activities present in the commercial enzyme preparation (*37*). In the same way, Marchal et al. (*38*) have linked the deleterious effects of *Botrytis cinerea* infections on the foaming properties of Champagne wines to the secretion, by this microorganism, of fungal proteases. Otherwise, a negative correlation was also reported by Girbau-Solà et al. (*34*) between ethanol concentration, on one hand, and SO_2, on the other hand, and the foaming properties of wines. In the first case, authors suggest that ethanol acts on proteins by modifying the solvent properties, the secondary structure of proteins (and their subsequent adsorption at the gas−liquid interface) and, finally, the structure of the adsorption layer. SO_2 is well-known as a denaturating agent of proteins, thus explaining how this compound can act devafourably on wine-foaming properties (*30*, *39*). Finally, total acidity was found to correlate negatively with foamability (*39*) as the hydrophobicity of proteins and then their surface activity increases when the acidity of musts is low (*8*).

When all of these data are summarized, there is circumstancial evidence that proteins are implied in the foaming properties of musts and wines. In contrast, only a few works reported that not all foam parameters correlate with wine proteins. For Pueyo et al. (*40*), foam stability HS showed a positive correlation with the protein content of Cavas, although the foam height HM was negatively related to the protein concentration. Inversely, Andrés-Lacueva et al. (*41*) observed that a positive correlation exists between the wine protein content and the formation of foam, although no correlation was obtained between proteins and foam stability.

Otherwise, our data clearly indicate that, whatever the fining treatment, the grape invertase concentration shows a behavior very similar to that of the total protein content of wine despite

the high molecular mass (62−64 kDa) and the highly glycosylated structure of this protein. Such a result is not surprising because some works conducted in our laboratory recently demonstrated that, upon protein removal by bentonites, polypeptides are adsorbed on the periphery of the bentonite particles through specific (ionic) interactions between the polypeptide side chains and the silicate sheets (*42*), whereas the protein backbones, for example, the glycosylated moiety of the grape invertase, do not enter the interlayer space (*43*).

LITERATURE CITED

(1) Péron, N.; Cagna, A.; Valade, M.; Marchal, R.; Maujean, A.; Robillard, B.; Aguié-Béghin, V.; Douillard, R. Characterization by drop tensiometry and by ellipsometry of the adsorption layer formed at the air/Champagne wine interface. *Adv. Colloid Interface Sci*. **2000**, *88*, 19−36.

(2) Brissonnet, F.; Maujean, A**.** Identification of some foam-active compounds in champagne base wine. *Am. J. Enol. Vitic*. **1991**, *42*, 97−102.

(3) Malvy, J.; Robillard, B.; Duteurtre, B. Influence des protéines sur le comportement de la mousse des vins de champagne. *Sci. Aliments* **1994**, *14*, 87−98.

(4) Puff, N.; Marchal, R.; Aguié-Béghin, V.; Douillard, R. Is grape invertase a major component of the adsorption layer formed at the air/Champagne interface? *Langmuir* **2001**, *17*, 2206−2212.

(5) Marchal, R.; Seguin, V.; Maujean, A. Quantification of interferences in the direct measurement of proteins in wines from the champagne region using the Bradford method. *Am. J. Enol. Vitic*. **1997**, *48*, 303−309.

(6) Liger-Belair, G.; Marchal, R.; Robillard, B.; Dambrouck, T.; Maujean, A.; Vignes-Adler, M.; Jeandet, P. On the velocity of expanding spherical gas bubbles rising in line in supersaturated hydroalcoholic solutions: Application to bubble trains in carbonated beverages. *Langmuir* **2000**, *16*, 1889−1895.

(7) Damodaran, S. Protein stabilization of emulsions and foams. *J. Food Sci*. **2005**, *70*, 54−66.

(8) Brissonnet, F.; Maujean, A. Characterisation of foaming proteins in a champagne base wine. *Am. J. Enol. Vitic*. **1993**, *44*, 297−301.

(9) Bastiaens, A.; Paquot, M.; Marbaise, M.; Deroanne, C. I. Capacité moussante des isolats protéiques: Influence du potentiel électrocinétique des dispersions aqueuses. *Sci. Aliments* **1990**, *10*, 773−783.

(10) Le Meste, M.; Colas, B.; Simatos, D.; Closs, B.; Courthaudon, J. L.; Lorient, D. Contribution of protein flexibility to the foaming properties of casein. *J. Food Sci*. **1990**, *55*, 1445−1447.

(11) Slack, P. T.; Bamforth, C. W. The fractionation of polypeptides from barley and beer by hydrophobic interaction chromatography: The influence of their hydrophobicity on foam stability. *J. Inst. Brew*. **1983**, *89*, 397−401.

(12) Townsend, A. A.; Nakai, S**.** Relationship between hydrophobicity and foaming characteristics of food proteins. *J. Food Sci*. **1983**, *48*, 588−594.

(13) Yokoi, S.; Maeda, K.; Xiao, R.; Kamada, K.; Kamimura, M. Characterization of beer proteins responsible for the foam of beer. *J. Inst. Brew. Proc. EBC Congr*. **1989**, 593−600.

(14) Graham, D. E.; Philips, M. C. Proteins at liquid interfaces. I: Kinetics of adsorption and surface denaturation. II: Adsorption isotherm. III. Molecular structures of adsorbed films. *J. Colloid Interface Sci.* **1979**, *70*, 403−439.

(15) Damodaran, S.; Song, K. B. Kinetics of adsorption of proteins at interfaces: Role of protein conformation in diffusional adsorption. *Biochim. Biophys. Acta* **1988**, *954*, 253−264.

(16) Roberts, R. T. Glycoproteins and beer foam. *J. Inst. Brew. Proc. EBC Congr.* **1975**, 454−464.

(17) Roberts, R. T. Colloidal aspects of beer foam. *Brew Dig*, **1977**, June, 50−58.

(18) Liger-Belair, G.; Jeandet, P. More on the surface state of expanding Champagne bubbles rising at intermediate Reynolds and high Peclet numbers, *Langmuir* **2003**, *19*, 801−808.

(19) Dambrouck, T.; Marchal, R.; Marchal-Delahaut, L.; Parmentier, M.; Maujean, A.; Jeandet, P. Immunodetection of proteins from grapes and yeast in a white wine. *J. Agric. Food Chem*. **2003**, *51*, 2727−2732.

(20) Hsu, J. C.; Heatherbell, D. A. Isolation and characterization of soluble proteins in grapes, grape juice, and wine. *Am. J. Enol. Vitic*. **1987**, *38*, 6−10.

(21) Marchal, R.; Bouquelet, S.; Maujean, A. Purification and partial biochemical characterization of glycoproteins in a champenois chardonnay wine. *J. Agric. Food Chem*. **1996**, *44*, 1716−1722.

(22) Paetzold, M.; Dulau, L.; Dubourdieu, D. Fractionnement et caractérisation des glycoprotéines dans les moûts de raisins blancs. *J. Int. Sci. Vigne Vin* **1990**, *24*, 13−28.

(23) Waters, E. J.; Pellerin, P.; Brillouet, J.-M. A wine arabinogalactan-protein that reduces heat-induced wine protein haze. *Biosci. Biotechnol. Biochem*. **1994**, *58*, 43−48.

(24) Waters, E. J.; Pellerin, P.; Brillouet, J.-M. A *Saccharomyces* mannoprotein that protects wine from protein haze. *Carbohydr. Polym*. 1994, *23*, 185−191.

(25) Feuillat, M.; Charpentier, C.; Picca, G.; Bernard, P. Production de colloïdes par les levures dans les vins mousseux élaborés selon la méthode champenoise. *Bull. O.I.V*. **1988**, *61*, 36−45.

(26) Ledoux, V.; Dulau, L.; Dubourdieu, D. Interprétation de l'amélioration de la stabilité protéique des vins au cours de l'élevage sur lies. *J. Int. Sci. Vigne Vin* **1992**, *26*, 239−251.

(27) Berthier, L.; Marchal, R.; Debray, H.; Bonnet, E.; Jeandet, P.; Maujean, A. Isolation of isolectins from *Vitis vinifera* L. Cv. Chardonnay grape berries. *J. Agric. Food Chem*. **1999**, *47*, 2193−2197.

(28) Laemmli, U. K. Claevage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(29) Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−254.

(30) Maujean, A.; Poinsaut, P.; Dantan, H.; Brissonnet, F.; Cossiez, E. Etude de la teneur et de la qualité de mousse des vins effervescents. II. Mise au point d'une technique de mesure de la moussabilité, de la tenue et de la stabilité de la mousse des vins effervescents. *Bull. O.I.V.* **1990**, *63*, 405−427.

(31) Marchal, R.; Chaboche, D.; Douillard, R.; Jeandet, P. Influence of lysozyme treatments on Champagne base wine foaming properties. *J. Agric. Food Chem*. **2002**, *50*, 1420−1428.

(32) Puig-Deu, M.; López-Tamames, E.; Buxaderas, S.; Torre-Boronat, M. C. Quality of base and sparkling wines as influenced by the type of fining agent added pre-fermentation. *Food Chem*. **1999**, *66*, 35−42.

(33) Moreno-Arribas, V.; Pueyo, E.; Nieto, F. J.; Martín-Álvarez, P. J.; Polo, M. C. Influence of the polysaccharides and the nitrogen compounds on foaming properties of sparkling wines. *Food Chem.* **2000**, *70*, 309−317.

(34) Girbau-Solà, T.; López-Tamames, E.; Bujan, J.; Buxaderas, S. Foam aptitude of Trepat and Monastrell red varieties in Cava elaboration. 1. Base wine characteristics. *J. Agric. Food Chem*. **2002**, *50*, 5596−5599.

(35) Martínez-Rodriguez, A. J.; Polo, M. C. Effect of the addition of bentonite to the *tirage solution* on the nitrogen composition and sensory quality of sparkling wines. *Food Chem*. **2003**, *81*, 383−388.

(36) Lao, C.; Santamaria, A.; López-Tamames, E.; Bujan, J.; Buxaderas, S.; de la Torre-Boronat, M. C. Effect of grape pectic enzyme treatment on foaming properties of white musts and wines. *Food Chem*. **1999**, *65*, 169−173.

(37) Lao, C.; López-Tamames, E.; Buxaderas, S.; de la Torre-Boronat, M. C. Grape pectic enzyme treatment effect on white musts and wines composition. *J. Food Sci.* **1996**, *61*, 553−556.

(38) Marchal, R.; Valade; M.; Moncomble, D.; Tabary, I.; Viaux, L.; Robillard, B.; Jeandet, P. Effects of *Botrytis cinerea* infections on Champagne wine foaming properties. *J. Sci. Food Agric*. **2001**, *81*, 1371−1378.

(39) López-Barajas, M.; Viu-Marco, A.; López-Tamames, E.; Buxaderas, S.; de la Torre-Boronat, M. C. Foaming in grape juices of white varieties. *J. Agric. Food Chem*. **1997**, *45*, 2526−2529.
(40) Pueyo, E.; Martin-Alvarez, P. J.; Polo, M. C. Relationship between foam characteristics and chemical composition in wines and Cavas (sparkling wines). *Am. J. Enol. Vitic*. **1995**, *46*, 518−524.
(41) Andrés-Lacueva, C.; Gallard, M.; Lopez-Tamames, E.; Lamuela-Raventos, R. M. Influence of variety and ageing on foaming properties of sparkling wine (cava). *J. Agric. Food Chem.* **1996**, *44*, 3826−3829.
(42) Gougeon, R. D.; Reinholdt, M.; Delmotte, L.; Miéhé-Brendlé, J.; Chézeau, J. M.; Le Dred, R.; Marchal, R.; Jeandet, P. Direct observation of polylysine side-chain interaction with smectites interlayer surfaces through ^{1}H-^{27}Al heteronuclear correlation NMR spectroscopy. *Langmuir* **2002**, *18*, 3396−3398.
(43) Gougeon, R. D.; Soulard, M.; Reinholdt, M.; Miéhé-Brendlé, J.; Chézeau, J. M.; Le Dred, R.; Marchal, R.; Jeandet, P. Polypeptide adsorption onto a synthetic montmorillonite: A combined solid-state nuclear magnetic resonance, X-ray diffraction, thermal analysis and N_2 adsorption study. *Eur. J. Inorg. Chem*. **2003**, 1366−1372.

Received for review May 31, 2005. Revised manuscript received August 11, 2005. Accepted August 23, 2005. We thank the Europol'Agro Institute, The Association Recherche Oenologie Champagne et Université, and Champagne Moët and Chandon for financial support of this work.

JF051276Z