Heat-Unstable Proteins in Wine. I. Characterization and Removal by Bentonite Fining and Heat Treatment

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The effect of bentonite fining on the total proteins and the heat-unstable (80°C, 6 h; 4°C, 12 h) proteins in Gewürztraminer, White Riesling, Sauvignon blanc, and Sylvaner wines was investigated. Protein molecular weights (MW), isoelectric points (pI), and glycoproteins were determined by using lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS PAGE) and two-dimensional isoelectric focusing-lithium dodecyl sulfate (IF-LDS) electrophoretic techniques with silver staining as well as protein blotting for glycoprotein detection. Relative concentrations of proteins in stained gels were determined by laser scanning densitometry. Bentonite fining tends to remove the higher pI (5.8 - 8.0) and intermediate MW (32 000 - 45 000) protein fractions first. However, these represent only a small proportion of the soluble proteins. In general, it is necessary to remove the lower pI (4.1 - 5.8), lower MW (12 600 and 20 000 - 30 000) fractions, which contain glycoproteins and represent the major component of the proteins, to protein stabilize wines to heat testing. Protein fractions with MW of 60 000 to 65 000 and having a wide range of pI (4.1 - 8.0) were highly resistant to removal by bentonite fining and remained in protein-stabilized wine. In addition, trace amounts of fractions with MW of 28 000 may remain in Gewürztraminer and White Riesling wines and 25 000 in Sauvignon blanc wines. Unstable proteins precipitated by heat tests were recovered and analyzed. Protein fractions with MW of greater than 14 000 were more heat sensitive than lower MW fractions. The heat-precipitated proteins found in sediments were mainly of low MW (< 30 000) and primarily glycoproteins. It is concluded that the protein fractions of lower MW (12 600 and 20 000 - 30 000) and lower pI (4.1 - 5.8) and glycoproteins are the major and most important fractions contributing to protein instability in wines.

Although there have been numerous investigations on grape juice and wine proteins (9), the nature of the proteins responsible for wine turbidity remains unclear. Protein instability does not correlate well with the total protein content, and there appears to be conflicting information in the literature as to which proteins (protein fractions) are responsible for haze and sediment formation. Koch and Sajak (10), using paper electrophoresis, showed that grapes and wines contained two major protein fractions, both being decreased by heat treatment and addition of bentonite. Moretti and Berg (15) associated specific protein fractions with wine turbidity and concluded that only a part of the protein mixture was responsible for protein stability rather than total protein content. Bayly and Berg (3) further classified protein fractions according to their heat stability and concluded that removal of protein fractions by addition of bentonite does not occur in equal proportions but removes the highly charged protein molecules first. Millies (14), using silica sol/gelatin fining and ultrafiltration to fractionate the wine proteins, claimed that the protein fractions with MW of less than 10 000 do not take part in turbidity formation, fractions between MW 10 000 and 30 000 are only partly involved, and those with MW of greater than 30 000 are the main unstable proteins. Mesrob *et al.* (13) indicated that the protein clouding is mainly caused by the protein fraction with lower pI and lower MW. Recently, using gel electrophoresis and isoelectric focusing, Heatherbell *et al.* (8) and Ngaba and Heatherbell (16) determined the MW and pI of heat-stable and unstable proteins present in Oregon and Washington wines and investigated their removal by bentonite fining, ultrafiltration, and protease enzymes. Protein fractions detected had a MW range of 16 000 to 90 000 daltons and pI from 4.5 to 8.0. With progressive bentonite fining, it was not until the lower MW (*ca* 16 000 - 25 000 daltons) and higher pI (5.6 - 8.0) fractions were removed by bentonite fining that the wines became protein stabilized to heat testing. A more recent report by Lee (12) for Australian wines claimed that the major protein fractions of wine have MW of 40 000 to 200 000 daltons and pI of 4.8 to 5.7 and "these fractions must be largely removed from wine before stability.....is conferred."

In this study, we further investigated the nature of the unstable proteins and their removal by bentonite fining and heat treatment by applying improved sensitive techniques (9) for the detection and characterization of proteins and glycoproteins.

Materials and Methods

Preparation of wines: Gewürztraminer, White Riesling, Sauvignon blanc, and Sylvaner grapes from the Oregon State University experimental vineyards were harvested and processed into wines by conventional procedures in the Oregon State University experimental winery. Young wines (after two rackings) were sampled for this study.

Bentonite fining: For determination of the amount of bentonite required to stabilize wines to heat testing, each wine sample was fined with 5 - 80 g bentonite per hL wine and thoroughly mixed. Bentonite (sodium bentonite, Volclay) was added as a smooth aqueous 3% suspension (1 mL bentonite solution when added to 100 mL wine, corresponding to 30 g bentonite per hL or 2.5 lb bentonite per 1000 gal wine). The wines were held at

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room temperature for at least 48 hours and then filtered through 0.45-μm Millipore membrane before analysis. Filtered wines were prepared for gel electrophoresis as previously described (9). Concentration of soluble protein and total phenols were determined as previously described (9).

Heat stability test: The heat stability of the wines was determined by the procedure recommended by Pocock and Rankine (17). Fifty milliliters of filtered wine was transferred into 65-mL bottles. Bottles were sealed with screw caps and heated in a water bath (80°C) for six hours, held at 4°C for 12 hours, and allowed to warm to room temperature. Formation of turbidity was measured by visual observation under a strong beam of light and by a Hunter model D25P-2 color difference meter (Hunter Associates Lab., Inc.) operated in the transmittance mode for the transmission haze reading:

$$\text{percent haze} = Y_{(\text{Arrangement I})}/Y_{(\text{Arrangement III})} \times 100$$

Filtered distilled water was used as the blank giving a reading of 2 units.

Preparation of haze/sediment from heat stability test: Fifty milliliters of unfined wine was heated at 80°C in a water bath for six hours and then stored at 4°C for 12 hours. Protein haze/sediment was collected by centrifuging at 12 100 *g* for 20 minutes at 4°C in a Sorvall RC-5 centrifuge. The precipitate was collected and suspended in 2 mL sample buffer for gel electrophoresis (9). The supernatant was filtered through 0.45-μm Millipore filter membrane, and the filtrate was prepared for gel electrophoresis as previously described (9).

Gel electrophoresis: Lithium dodecyl sulfate polyacrylamide gel eletrophoresis (LDS PAGE), two-dimensional isoelectric focusing-LDS PAGE (IF-LDS PAGE), silver staining, laser scanning, protein blotting, and glycoprotein detection were performed as previously described (9).

Carboxymethyl cellulose concentration of wine proteins: In some instances for two-dimensional IF-LDS PAGE, it was desirable to concentrate proteins. Proteins were concentrated using a modification of the procedure described by Calbiochem-Behring (1) using carboxymethyl cellulose (CMC) (Aquacide II, Calbiochem, La Jolla, CA). Samples of 20 to 30 mL were placed in dialysis tubing and then packed in CMC for two to three hours until up to 10-fold concentration was achieved.

Results and Discussions

Bentonite fining: The effect of bentonite fining on the protein stability (as determined by heat test), soluble protein, and total phenols of Gewürztraminer, White Riesling, Sauvignon blanc, and Sylvaner wines was investigated (Fig. 1). There was a good agreement between visible observations and Hunter haze readings following heat testing. Whereas heat-induced haze formation progressively decreased with bentonite fining and protein reduction, bentonite addition had no effect on total phenol ($p > 0.05$). In these four wines, protein concentration of *ca* 5 mg/L (mean = 5.4 ± 1.6 mg/L) coincided with wine stability (Fig. 1). This occurred irrespective of

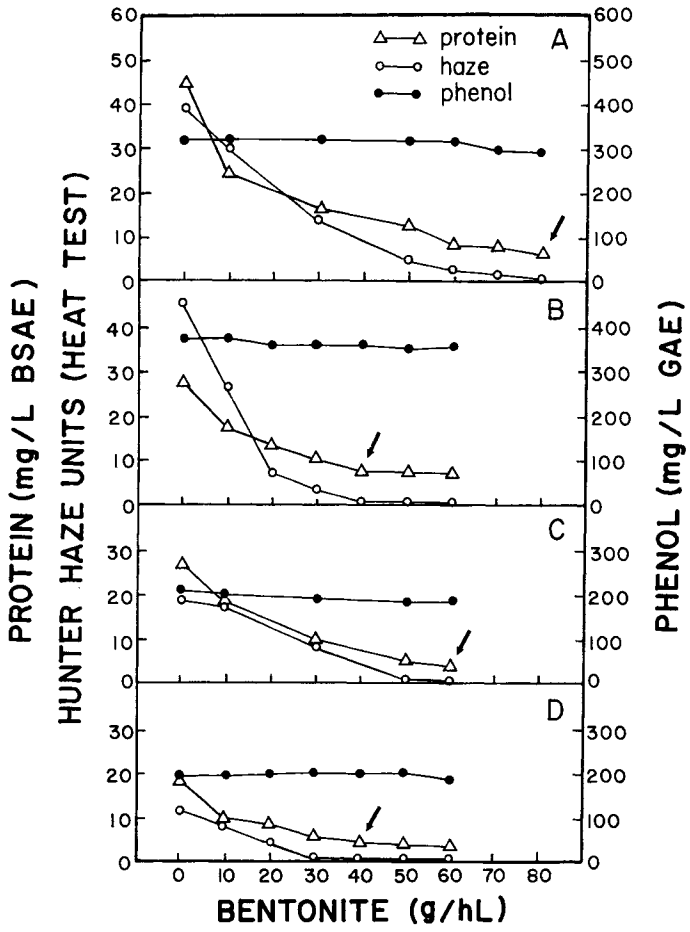

Fig. 1. Effect of bentonite fining on protein stability[1] and concentration of soluble protein and phenol in wines: A - Gewürztraminer (pH 3.67, 12.2% ethanol), B - White Riesling (pH 3.15, 11.7% ethanol), C - Sauvignon blanc (pH 3.20, 11.9% ethanol), D - Sylvaner (pH 3.20, 11.1% ethanol). BSAE - bovine serum albumin equivalent, GAE - gallic acid equivalent. Arrow (←) represents bentonite treatment required to protein stabilize wine as determined by visual assessment.

[1]Determined as Hunter haze formation or by visible observation following heat test.

the initial concentration of protein in the wines which ranged from 19 to 44 mg/L (Fig. 1). However, it should be noted that higher concentrations of proteins have been observed in wine protein stabilized by bentonite fining both in the Department of Food Science and Technology, Oregon State University, Corvallis, (commonly 10 - 20 mg/L) and in the literature (12,17). Relatively large amounts of bentonite were required to remove the proteins which are most resistant to removal by bentonite (Fig. 1). These fractions, which contribute to persistant residual haze, are present in small amounts, as low as 1 to 2 mg/L as previously reported (8). In addition, low concentrations of phenolics may be contributing to persistent residual haze in juices and wines, either independently or in association with the proteins (7,8,18,19).

To obtain further information about the removal of individual protein fractions from wines by bentonite fining, the protein fractions were separated and their MW determined by using LDS PAGE. Although there were differences in the electrograms of these four wines,

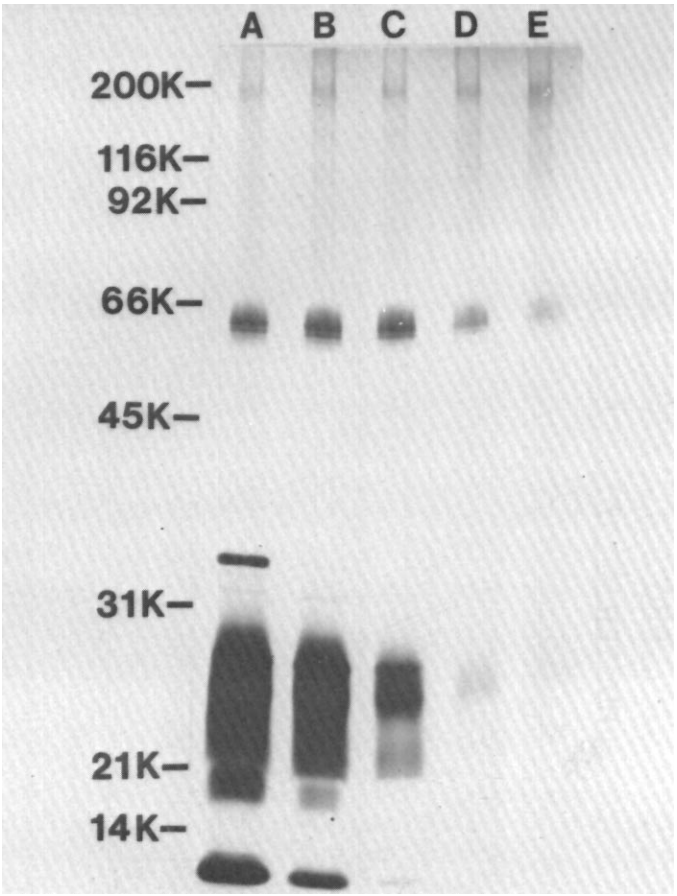

Fig. 2. LDS PAGE of Gewürztraminer wine proteins in wine fined with different levels of bentonite: A - 0 g/hL, B - 20 g/hL, C - 50 g/hL, D - 70 g/hL, E - 80 g/hL (heat stable). 100 μL of each sample was applied to gel. Molecular weights (K = 1000 daltons) of standards are given on left side of gel.

bentonite fining had a similar effect on the removal of the protein fractions present. Protein fractions with MW of 12 600 and between 20 000 and 30 000 are the last fractions to be removed to protein stabilize wine to heat testing. For instance, the last fractions to be removed by bentonite fining in both the Gewürztraminer and White Riesling wines were the 12 600 and 25 000 fractions; in Sauvignon blanc wines, they were the 12 600 and 28 000 fractions.

A typical electrogram for Gewürztraminer wine is shown in Figure 2 (wine A in Fig. 1). Bentonite fining removed intermediate MW fractions (32 000 - 45 000) first, before removing the lower MW fractions (11 200 - 25 000). However, the fractions in the higher MW range of 60 000 to 65 000 were highly resistant to removal by bentonite fining. The same pattern of results was also obtained for White Riesling and Sauvignon blanc wines. The densitometric patterns of the stained gel are shown in Figure 3. These results indicate that the low MW fractions (12 600 and 25 000) are important to protein instability because their removal by bentonite fining coincides with protein stabilization of wines to heat testing (Fig. 2 (E), 3 (E)). In contrast, protein fractions with MW of 60 000 to 65 000 remained in the bentonite-fined protein-stabilized wines (Fig. 2 (E), 3 (E)). In addition, trace amounts (limit of detection 10 ng (20)) of

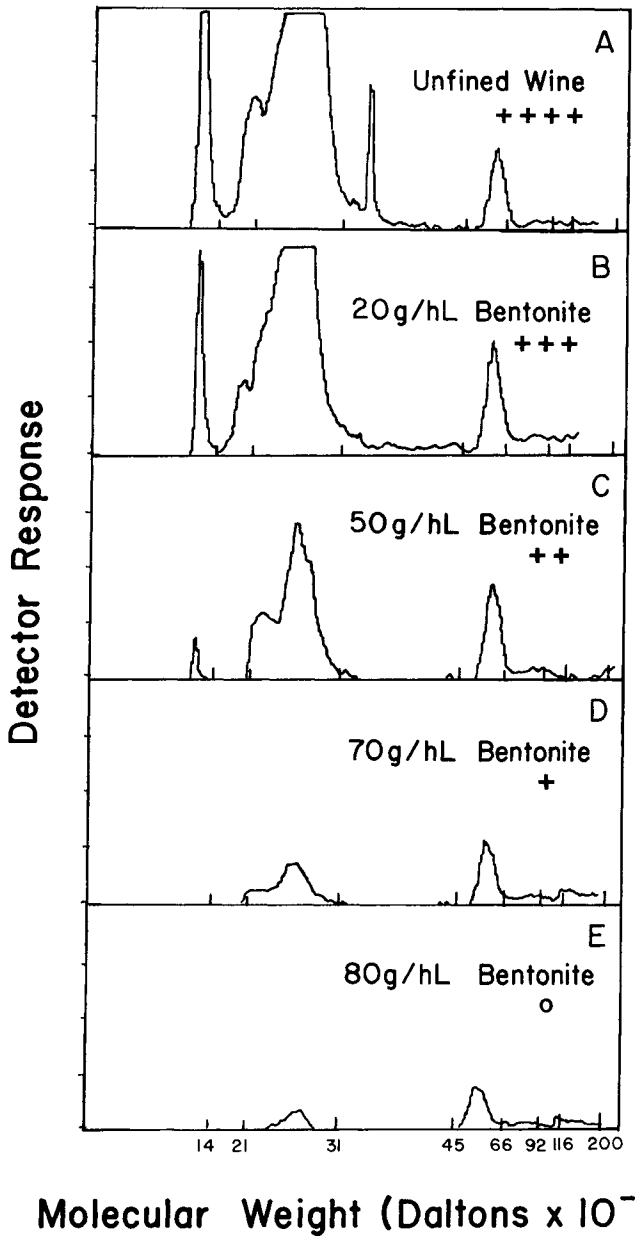

Fig. 3. Densitometric scans of electrophoretic patterns (Fig. 2) of Gewürztraminer wine proteins in wine fined with different levels of bentonite: A - 0 g/hL, B - 20 g/hL, C - 50 g/hL, D - 70 g/hL, E - 80 g/hL. Protein instability indicated by +.

the 28 000-MW fraction was detectable in some instances.

Protein blotting combined with glycoprotein staining (9) was used for the detection of glycoproteins. With this technique, the three protein fractions with MW of 12 600, 25 000, and 28 000 in Figure 2 were identified as containing glycoproteins in Gewürztraminer wine. Only two fractions with MW of 25 000 and 28 000 were detected in White Riesling wine. However, no glycoproteins were detected in the bentonite-stabilized wines.

The effect of bentonite fining on wine proteins was further investigated by subjecting wine to two-dimensional IF-LDS PAGE (9). A typical result for a Gewürztraminer wine is shown in Figure 4 (wine A in Fig. 1)

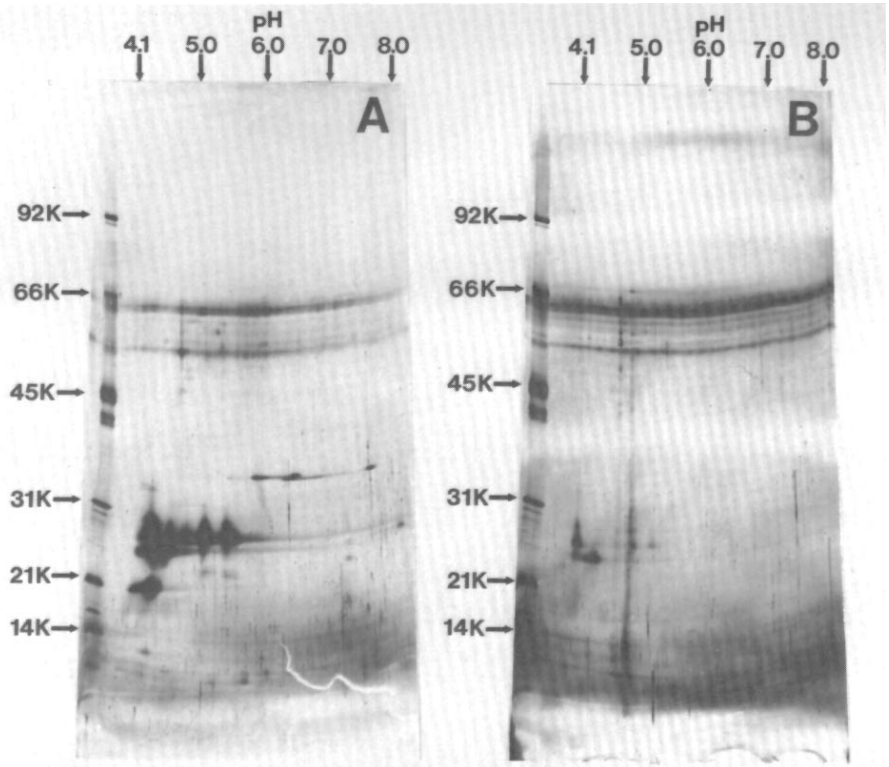

Fig. 4. Two-dimensional IF-LDS PAGE of Gewürztraminer wine proteins followed by silver staining: A - unfined wine concentrated two-fold before electrophoresis, B - wine fined with 80 g/hL bentonite and concentrated 10-fold before electrophoresis. Molecular weights (K = 1000 daltons) of standards are given on left side of each gel. The pH gradient is labeled above each gel.

which shows that the major protein fractions in Gewürztraminer wine are of low MW (20 000 - 30 000) and low pI (4.1 - 5.8). The absence of the major fraction with MW of 12 600 (Fig. 2) in Figure 4 is not accounted for. It is possible that it may have been lost through dialysis tubing during concentration (wine samples applied to gels in Fig. 4 were concentrated as indicated in an attempt to detect low concentrations of some fractions, particularly in the bentonite-fined sample). Isoelectric focusing has further separated each of the protein fractions in Figure 2 (wine A) into several fractions on the basis of their pI (Fig. 4 (A)). For instance, the fraction with MW of 28 000 has been separated in five major fractions with pI from 4.1 to 5.8. When the same two-dimensional technique was applied to bentonite-stabilized wine (wine E, Fig. 2), none of the fractions known to be important contributors to heat instability (8) could be detected (data not shown). However, with sufficient concentration (*ca* 10-fold), trace amounts of these fractions could be demonstrated to remain in the stabilized wine (Fig. 4 (B)). In contrast, considerable amounts of the protein fractions with MW of 60 000 to 65 000 which were highly resistant to removal by bentonite fining and had a wide range of pI (4.1 - 8.0), remained in the bentonite-fined wine (Fig. 4 (B)) and were readily detectable without concentration. In interpreting these results, consideration must be given to the extreme sensitivity of the method; trace amounts of protein (10 ng) are detectable (20). The following interpretations are based upon analysis of single strength (unconcentrated) wine. In general, the application of this technique confirmed and extended previous studies (8). Bentonite fining removed the intermediate MW (32 000 - 45 000) (Fig. 2) and higher pI (5.8 - 8.0) (Fig. 4) fractions first. However, these fractions together only compose a small proportion of the soluble proteins present in these wines, and although they may contribute to instability, their removal did not stabilize wines. It was necessary to remove the lower pI (4.1 - 5.8) and MW (12 600 and 20 000 - 30 000) fractions and glycoproteins to protein stabilize wines.

Specifically, for Gewürztraminer wine, the fractions with MW of 12 600 (containing glycoproteins) and MW of 25 000 (having pI of 4.1 - 4.8 and containing glycoproteins) are the last removed by bentonite fining to protein stabilize wine to heat testing (Fig. 2, 3, 4). In contrast, the protein fractions with MW of 60 000 to 65 000 and trace amounts of the 28 000-MW fraction remained in wine which had been protein stabilized by bentonite fining.

Heat treatment: A standard heat test (80°C, 6 h; 4°C, 12 h) recommended by Pocock and Rankine (17) was used to evaluate the heat stability of wine proteins. In previous studies (6), Heatherbell *et al.* demonstrated that, for Oregon and Washington wines, this one-day test correlated well with the longer five-day test (49°C, 4 days; 5°C, 1 day) proposed by Berg and Akiyoshi (4). To assist in the further characterization of the heat-unstable proteins, proteins precipitated by the heat test (1-day test) were recovered and analyzed. Heat treatment of wines precipitated *ca* 50% (15 - 30 mg/L) of the proteins (Table 1). Similar results were reported by Koch and Sajak (10) who claimed over 60% (20 - 45 mg/L) Voit-N content remained in heat-treated (75°C, 2 min) wine. In contrast, Pocock and Rankine (17) and Lee (12) claimed that heat treatment of wine at 80°C for six hours appeared to completely coagulate the soluble protein present and remove all proteins from the Australian wines tested. A loss ($p < 0.05$) of up to 7.8% of total phenol was observed in heat-treated wine (Table 1) and may be significant in

Table 1. Effect of heat treatment[a] on concentration of soluble protein and phenol in wines.

Sample	Protein (mg/L)[b]	Phenol (mg/L)[b]
Gewürztraminer		
Before treatment	59.2 ± 0.2	302.1 ± 7.7
After treatment	28.6 ± 1.5	282.9 ± 5.4
% Removal	51.7	6.4
White Riesling		
Before treatment	36.1 ± 0.7	371.3 ± 3.1
After treatment	17.8 ± 0.8	342.3 ± 1.3
% Removal	50.7	7.8

[a] Heat test for determining protein stability (6 h. 80 °C; 12 h, 4 °C).
[b] Mean of triplicate determinations ± standard deviation.

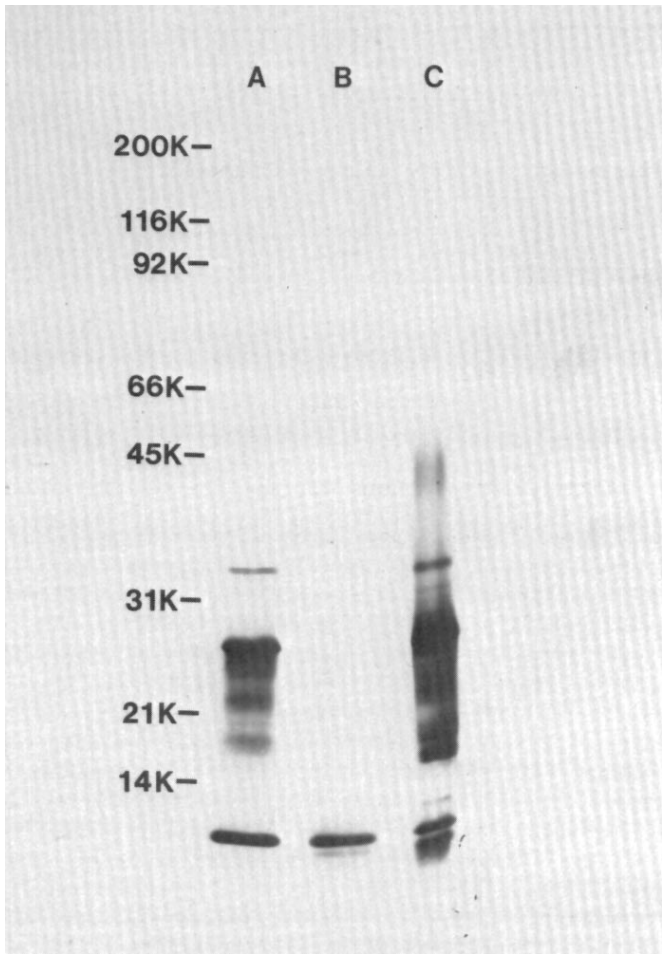

Fig. 5. LDS PAGE of Gewürztraminer wine proteins in wine subjected to heat test: A - before heat treatment, B - after heat treatment, C - sediment. Molecular weights (K = 1000 daltons) of standards are given on left side of gel.

haze formation during heat testing. The combination of phenolics with proteins in hazes and sediments from wines (5,10,18), juices (7,11), and beer (2) has been reported frequently.

The MW of protein fractions, their relative concentrations, and the presence of glycoproteins in initial wine, heat-treated wine, and in recovered sediments were determined (Fig. 5, 6). Protein fractions with MW of greater than 14 000 were heat sensitive and were removed by heat treatment (Fig. 6 (B)). In contrast, protein fractions with

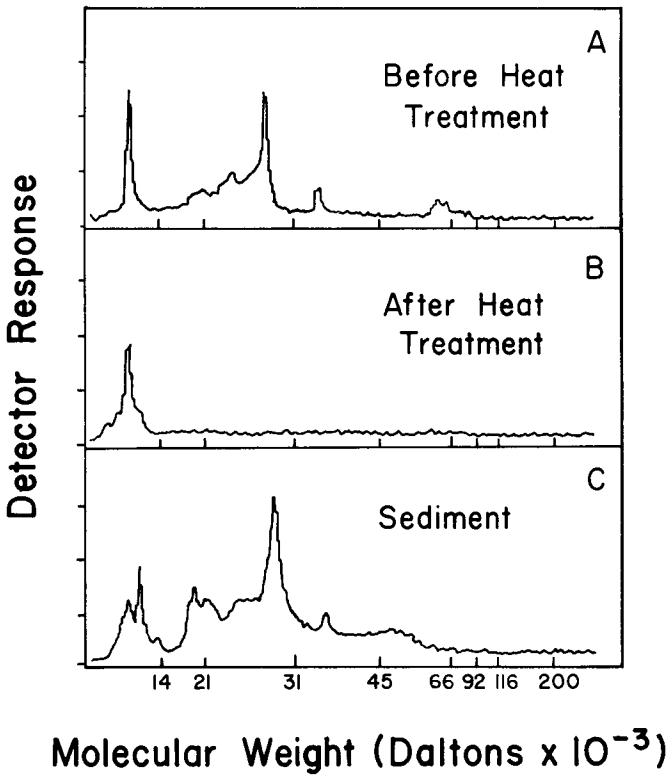

Fig. 6. Densitometric scans of electrophoretic patterns (Fig. 5) of Gewürztraminer wine proteins in wine subjected to heat test: A - before heat treatment, B - after heat treatment, C - sediment.

MW of lower than 14 000, including one major MW fraction (12 600) containing glycoproteins, remained in the wine after heat testing (Fig. 6 (B)). In some wines, trace amounts of fractions of MW 60 000 to 65 000 also remained after heat testing. Protein hydrolysis/dissociation may have occurred during heat treatment, there being an increase in the low MW fractions (< 30 000) present in the sediment (Fig. 6). It is also possible that a higher percentage of the larger MW fractions were heat sensitive/denatured than the lower MW fractions during heat testing. The heat-precipitated proteins found in sediments were mainly of low MW (< 30 000) (Fig. 6 (C)) and primarily glycoproteins. These findings support the conclusions we obtained for characterizing the nature of the heat-unstable proteins removed by bentonite fining.

Conclusions

It is concluded that the protein fractions of lower MW (12 600 and 20 000 - 30 000) and lower pI (4.1 - 5.8) and glycoproteins are the major and most important fractions contributing to protein instability in wines.

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