Soluble proteins and free amino nitrogen content in must and wine of cv. Viura in La Rioja

by

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S u m m a r y : The protein fraction of white musts and wines obtained from grapes (*Vitis vinifera* L. cv. Viura) grown in northern Spain (Rioja) was investigated by SDS-PAGE. Under different fermentation conditions, e.g. aeration, must nitrogen content and yeast strain, SDS-PAGE patterns showed several bands with molecular weights ranging from 16 to 200 kDa. Higher weight proteins were glycosylated, whereas lower weight proteins were not. Under the experimental fermentation conditions none of the proteins showed any chemical modification that would alter the electrophoretical mobility or the covalent binding to their glycosylated moiety.

K e y   w o r d s : wine, soluble proteins, glycoproteins, free amino nitrogen content, grape juice.

Introduction

Soluble proteins in grape juice and wines mainly come from grapes; the protein content increases during grape maturation (Zoecklein *et al.* 1995). It is well established that protein synthesis is activated after veraison and parallels the rapid accumulation of sugar in grapes. Consequently, protein levels in grapes are frequently higher in warmer regions (Zoecklein *et al.* 1995). Proteins in grape musts can cause severe problems affecting the stability of wine due to the generation of amorphous deposits, turbidity or haze in the bottle, known in wineries as "protein casse". Wine instability due to high protein content is more frequent in white wines than in red wines and numerous studies report the presence of protein in wines (Singleton 1974, Hsu and Heatherbell 1987, Paetzold *et al.* 1990, Dorrestein *et al.* 1995, Siebert *et al.* 1996). Nevertheless, only few studies reveal the nature of the proteins and sugar moieties of glycosylated proteins responsible for wine turbidity.

Haze formation is prevented by protein removal, *i.e.* clarification with bentonite (Hsu and Heatherbell 1987). As this procedure is wasteful and flavour denuding, there is much interest in alternative practices. An understanding of the protein nature responsible for turbidity in wines is a prerequisite to develop alternative industrially viable winemaking practices. Glycosylated proteins seem to play an important role in wine turbidity as they interact with tannins, polyphenols and other proteins (Siebert *et al.* 1996). Must protein fractions are subjected to several modifications during winemaking, resulting in the partial loss of solubility and hydrolysis (Murphey 1989, Pueyo *et al.* 1993). During alcoholic fermentation, the amino acid content of grape juice varies due to yeast action and a number of factors affect these variations (Lopez *et al.* 1996). This paper proposes obtaining further knowledge of protein fraction of white wine of cv. Viura. Different yeast strains were investigated and different must nitrogen content and oxidising conditions during fermentation were used in order to establish possible modifications of protein content during alcoholic fermentation.

Material and methods

G r a p e   j u i c e   a n d   f e r m e n t a t i o n : Free running juice of Viura grapes of the 1996 harvest was used and sulphur dioxide (60 mg·l^{-1})was added. The juice was cleared by settling at 10 °C overnight, and then it was divided into 5 equal lots (samples 1-5). Juice data: 20.2 °Brix, pH 3.44, 7.43 g·l^{-1} titratable acidity (g·l^{-1} tartaric acid). Fermentation was carried out in triplicates at 18 °C in 25 l stainless steel tanks for 12-18 d until the residual reducing sugar content reached 2.20 g·l^{-1}. Sample 1 (control) proceeded spontaneously with indigenous yeast. Sample 2 was supplemented with 30 g·hl^{-1} (maximum addition limit in EC countries) of diammonium phosphate (DAP) when the initial sugar content had decreased by 3 °Brix. Sample 3 was aerated with 25 l O$_2$·min^{-1} for 15 min when the initial sugar content had decreased by 3 °Brix. Sample 4 was submitted to both, nitrogen addition and aeration. Sample 5 was inoculated with active dry yeast (*Saccharomyces cerevisiae*, Uvaferm VRB strain, selected in the CIDA, Reg. Nr.: CECT-10840) using 20 g·hl^{-1}. Changes in yeast growth and fermentation rate were determined daily. Samples of wine and juice were frozen and stored at -30 °C for subsequent free amino nitrogen (FAN) analysis. Yeast growth was followed by absorbance at 610 nm and the fermentation rate was determined by °Brix measurements according to the EC official method (Official

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Journal of the EC, ref: 2676/90). FAN analysis was carried out according to AERNY (1996).

Isolation of soluble proteins: In a first experiment, wine samples were concentrated by lyophilization and submitted to SDS-PAGE for detection of total proteins as described below. In subsequent experiments, wine and must samples were submitted to ammonium sulphate fractionated precipitation. 20, 40, 45, 60, 80 and 100 %, saturation of ammonium sulphate were tried; of these 45 % was sufficient for precipitation of all proteins in wines. Wine samples were maintained at 4 °C and ammonium sulphate was added under continuous stirring to 250 ml samples to a 45 % saturation. After mixing (20 min at 4 °C), samples were centrifuged (4 °C, 12,000 g for 15 min) and pellets were collected. They were resuspended in a small volume of dialysis buffer A (acetic acid/sodium acetate 10 mM, pH 3.5), and submitted to dialysis against buffer A. Three changes of dialysis buffer were performed to ensure removal of salts present in precipitates (mainly ammonium sulphate and tartrates). After dialysis, samples were concentrated by lyophilization and resuspended in 1 ml deionized water.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): After isolation of wine soluble proteins, SDS-PAGE analyses were carried out. Samples were mixed in a proportion 1:3 with 4 x Sample Buffer consisting of 0.25 M Tris-HCl pH 6.8, 8 % SDS, 40 % glycerol, 20 % β-mercaptoethanol and 0.08 % bromophenol blue. They were boiled (3 min) and subjected to the standard electrophoretic method of Laemmli (GARFIN 1990). The stacking gel consisted of 3 % (w/v) acrylamide-biscrylamide (37.5:1) and the separating gel consisted of 15 % (w/v) acrylamide-bisacrylamide (37.5:1). Slab gels were prepared in a vertical electrophoresis system (Hoefer, Sturdier SE400 model). The dimensions of the gels were 20 cm x 16 cm x 1.5 mm. Protein molecular mass standards (14.4-200 kDa; BioRad) were included in each electrophoretic run. Protein standards were prepared for electrophoresis as described for wine samples. Electrophoresis was performed at room temperature using a BioRad (1000/500 model) power supply operating constantly at 200 V. Electrophoresis was maintained until the tracking dye was 0.5 cm away from the end of the resolving gel. Gels were stained in Coomassie brilliant blue R-250 and destaining was carried out until neat and clean protein bands were observed. Densitometry of

obtained bands was carried out in a BioRad densitometer (GS-670 model) and protein concentrations were calculated by volume analysis of optical density (OD) (Molecular Analyst ™ program) and comparison with BioRad molecular weight standards (2 μg of each protein/μl).

Glycosidase treatment: 300 μl of protein extracts dissolved in water as described above were denatured at 100 °C for 10 min in a denaturation buffer consisting of 0.5 % SDS, 1 % β-mercaptoethanol (Biolabs). Samples were cooled to room temperature and incubated in reaction buffer (500 mM sodium citrate pH 5.5 with 2,500 units of glycosidase Endo Hf (Biolabs)) at 37 °C for 1 h. Endo Hf is a glycosidase that cleaves the glycosidic core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. Thereafter, samples were boiled in 4 x Sample Buffer and submitted to SDS-PAGE. SDS-PAGE gels were stained with Coomassie brilliant blue R-250 as described above, following the specific method for glycosylated proteins described by DUBRAY and BEZARD (1982); limit of detection is ca. 0.4 ng of bound carbohydrates.

Results and Discussion

Fermentation profiles showed that spontaneous fermentation carried out by indigenous yeast in grape juice reached higher fermentation rates and shorter fermentation times when fermentation tanks were submitted to both, aeration and nitrogen supplementation (data not shown). Inoculation (20 g·hl⁻¹) with the selected *S. cerevisiae* VRB strain led to the same optimal fermentation rates and times under standard conditions. This result was expected, because data obtained during the *S. cerevisiae* VRB selection process (GUTIERREZ 1994) showed a high efficiency in fermentation as compared to other indigenous yeast strains present in grape juices. During fermentation, the FAN content of must and wines underwent severe changes (Table). The FAN content decreased constantly while the yeast cell number increased. The final FAN content was lowest in tanks fermented by *S. cerevisiae* VRB. Nevertheless, the changes in the final FAN content did not correlate with changes in the soluble protein content of final wines. After clarification at 4 °C the concentration of soluble proteins was ca. 6 mg·l⁻¹. All proteins present precipitated at 45 % ammonium sulphate saturation.

Table

Development of assimilable nitrogen (mg·l⁻¹) during and after fermentation. Samples were analysed before fermentation (initial juice), after sugar decrease by 3 and 9 °Brix and in final wines

	Initial juices	Sugar decrease by		Wines
		3 °Brix	9 °Brix	
Control	244.07	125.07	98.00	100.87
+ 30 g·hl⁻¹ DAP	244.07	125.07	121.80	125.07
Oxigenation	244.07	125.07	77.00	61.13
Oxigenation + 30 g·hl⁻¹ DAP	244.07	125.07	89.60	79.30
Inoculated juice	244.07	90.53	49.47	46.67

As shown in Fig. 1, three groups of proteins were found: 1) high molecular weight proteins ranging from 60 to 200 kDa (33 % of the total protein content), 2) medium molecular weight proteins ranging from 24 to 34 kDa (58 % of the total protein content) and 3) a low molecular weight protein of 16 kDa (ca. 4 % of the total protein content). Proteins with medium molecular weight were most abundant as calculated by densitometry after SDS-PAGE and Coomassie blue staining. All 5 wine samples contained the same protein pattern, which was present in the non-clarified grape juice as well (Fig. 1 and 2-A). Proteins of group 1 were highly glycosylated, whereas smaller proteins (16-34 kDa) were not (Fig. 2-B). No change was observed in the electrophoretic

mobility of proteins in all 5 samples and, therefore, molecular weight, glycosylation state and relative concentration of proteins did not change under the fermentation conditions. Similar results were reported by MARCHAL et al. (1996). They isolated 7 glycoproteins in still wine (Chardonnay) which, similar to ours, did not show any modification during the alcoholic fermentation: one protein of 60/64 kDa, 4 proteins ranging from 24 to 30 kDa (the most abundant), and two small proteins of 17 and 14 kDa. In contrast to our results, all 7 proteins isolated from Chardonnay wine were glycosylated. A study by TOLAND et al. (1996) indicated that unstable protein fractions of wines consisted of proteins and glycoproteins of molecular weights between 12.6 and 30 kDa. These were normally less susceptible to removal by bentonite than other intermediate weight proteins (between 32 and 45 kDa), which were not involved in instabilities. Wines from Chardonnay and Verdeca white grapes (SANTORO 1995) contained a protein of 28 kDa as well, that could be responsible for clouding during wine storage. Therefore, proteins with molecular weights between 12 and 30 kDa are most likely responsible for wine instability.

DAWES et al. (1994) reported that the isoelectric point does not influence protein adsorption by bentonite and that the haze character varies in different protein fractions according to many protein properties. In wines of the Viura grape, proteins smaller than 30 kDa could belong to this group of troublesome proteins. Nevertheless, no haze formation was detected in our wines. YOKOTSUKA et al. (1994) reported that the degree of glycosylation of proteins could affect interactions with phenolic components of wines and, therefore, affect the formation of protein turbidity. In our samples, the observed lack of glycosylation of proteins smaller than 34 kDa, which constitute the major protein fraction of wines obtained from Viura grapes, could be the reason for this protein stability, which is a characteristic of Rioja white wines as well.

We summarize that proteins present in white wines obtained under different fermentation conditions (nitrogen
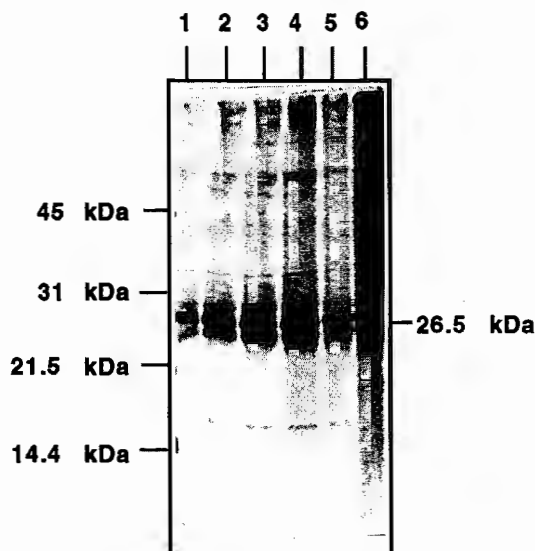

Fig. 1: SDS-PAGE of wine soluble proteins. Molecular weights of standards are given on the left side of the gel. Lane 1: sample inoculated with *Saccharomyces cerevisiae* VRB strain. Lane 2: control sample spontaneously fermented with indigenous yeast. Lane 3: sample supplemented with 30 g·hl⁻¹ DAP. Lane 4: aerated sample. Lane 5: sample submitted to both nitrogen addition and aeration. Lane 6: non-clarified grape juice.
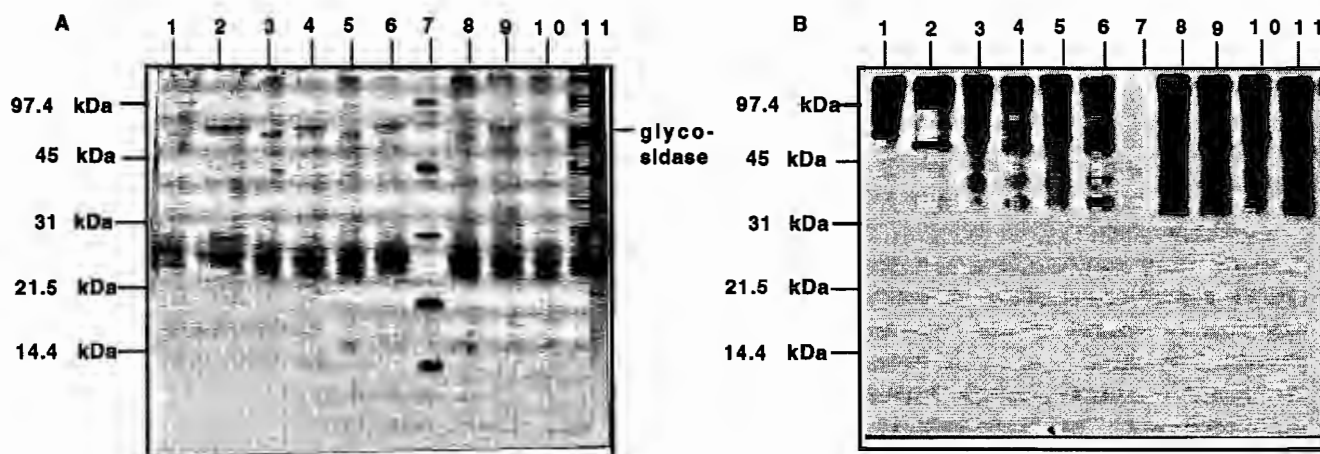

Fig. 2: SDS-PAGE of wine soluble proteins submitted to glycosidase treatment. Lane 1: sample inoculated with *S. cerevisiae* VRB strain. Lane 2: sample inoculated with *S. cerevisiae* VRB strain and submitted to glycosidase treatment. Lane 3: control sample spontaneously fermented with indigenous yeast. Lane 4: control sample spontaneously fermented with indigenous yeast and submitted to glycosidase treatment. Lane 5: sample supplemented with 30 g·hl⁻¹ DAP. Lane 6: sample supplemented with 30 g·hl⁻¹ DAP and submitted to glycosidase treatment. Lane 7: molecular weight standards. Lane 8: aerated sample. Lane 9: aerated sample, submitted to glycosidase treatment. Lane 10: sample submitted to both nitrogen addition and aeration. Lane 11: sample submitted to nitrogen addition, aeration and glycosidase treatment. (**A**) Proteins were visualized by Coomassie blue staining. (**B**) Proteins were overstained. Molecular weights of standards are given on the left side of the gel.

content of the medium, aeration and yeast strain) did not change their concentrations and did not change their state of glycosylation either. Nevertheless, the FAN content of wines varied during fermentation and depended upon fermentation conditions. Aeration stimulated yeast growth and, therefore, FAN content of the medium decreased in accordance. The selected *S. cerevisiae* VRB strain showed a high efficiency in grape juice fermentation, and led to a low FAN content of the final wine. Soluble proteins present in all studied Viura wines originated exclusively from grapes and the proteins of high molecular weight were highly glycosylated, whereas proteins of lower molecular weight (<34 kDa) did not show detectable glycosylation which contributes to wine stability. In further studies analysis and characterisation of the oligosaccharide moieties of higher molecular weight glycosylated proteins will be analysed and characterised in order to achieve a whole understanding of the nature of proteins present in white wines and to find an alternative removal technique to bentonite clarification.

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