

THE INFLUENCE OF YEAST GLYCOSYLATED PROTEINS ON TANNINS AGGREGATION IN MODEL SOLUTION

INFLUENCE DES PROTÉINES DE LEVURES GLYCOSYLÉES SUR L'AGRÉGATION DES TANINS EN SOLUTION MODÈLE

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Résumé : L'incidence des protéines glycosylées de levures sur l'agrégation des tanins dans un vin modèle a été étudiée à l'aide d'une méthode spectrophotométrique (mesure de l'absorption à 700 nm). Plusieurs préparations ont été testées : les protéines glycosylées relarguées par deux souches de levures commerciales (RC212 et BM45) au cours de la fermentation alcoolique d'un milieu synthétique, des protéines glycosylées extraites de biomasses levuriennes par la méthode de Peat, des préparations industrielles de glycoprotéines et polysaccharides de levures purifiées et fractionnées par chromatographie sur gel de concanavaleine A. Les résultats ont montré que l'agrégation des tanins par les glycoprotéines de levures dépend de leur origine et de leur mode de préparation. Les protéines glycosylées libérées par la souche BM45 au cours de la fermentation alcoolique ont des effets plus significatifs vis-à-vis de l'agrégation des tanins. La fraction protéique est la plus active dans le phénomène mais la fraction polysaccharidique joue également un rôle puisque la stabilisation des tanins est maximale pour un rapport manose/glucose voisin de 1.

Abstract : The incidence of glycosylated yeast proteins on tannins aggregation in model solution was investigated using the spectrophotometric method (absorbance 700 nm). Glycosylated proteins released by two commercial *Saccharomyces cerevisiae* strains (RC212 and BM 45) during alcoholic fermentation in synthetic media, glycosylated proteins extracted by Peat's method and industrial glycosylated proteins purified and separated by chromatography on Sepharose Concanavalin A were used to visualize effects on tannins aggregation. Results showed that tannins aggregation was limited by the glycosylated proteins according to their origin and their mode of preparation, glycosylated protein BM45 released during alcoholic fermentation in synthetic media being the most efficient. The use of mannan from yeast indicated that the polysaccharidic fraction of glycosylated proteins was efficient on tannins aggregation but at a lower level than glycosylated proteins. In the stabilisation of tannins, glycosylated proteins with a mannose / glucose ratio close to one were more efficient than the others. The present work suggests i) that the proteic fraction of glycosylated protein is likely a link between tannins and the polysaccharidic fraction of glycosylated protein, ii) that the conformation of a glycosylated protein is more or less likely to stabilise tannins, according to the mannose/glucose ratio.

Key words : tannins, yeasts, glycosylated proteins, stability coefficient, aggregation, precipitation

Mots-clés : tanins, levures, protéines glycolysées, coefficient de stabilité, agrégation, precipitation

INTRODUCTION

Among polyphenolic compounds, flavonoids have important implications for red wine quality. Condensed tannins (proanthocyanidins) are essential grape constituents, responsible for part of the organoleptic properties of wine. They were studied by different scientists such as RIBÉREAU-GAYON (1964), PRIEUR *et al.* (1994), SOUQUET *et al.* (1996). These compounds are polymeric flavan-3-ols, linked by C₄ – C₆ or C₄ – C₈ bonds, sometimes esterified by gallic acid on C₃.

During wine processing, both physical and chemical reactions lead to a modification of the polyphenolic content, colour, astringency and stability of wine. Astringency is defined as the mouth-drying and puckering feeling produced in the mouth by interactions of tannins with salivary proteins and glycoproteins. The phenolic-protein interactions are a subject of continuous research due to their importance in food, but phenolic-glycosylated proteins interactions have been studied to a lesser degree.

Tannins aggregation has been observed in model solution by Dynamic Light Scattering (DLC) by different authors : SAUCIER *et al.* (1997) who studied the colloid behaviour of catechin and the oligomers of procyanidins in model wine and RIOU *et al.* (2002) who studied the ability of grape seed tannins to aggregate into colloidal-size range particles in model wine and the incidence of wine polysaccharides on tannins aggregation.

Glycosylated yeast proteins are implicated in colloidal phenomena, and thus have an influence on wine clarification and stabilization. They are considered as «protective colloids» able to prevent or limit aggregation, flocculation, haze formation and precipitation (PELLERIN et CABANIS, 1998).

In a previous study ESCOT *et al.* (2001) showed that the release of polysaccharides in media differed among yeast strains. Results also suggested that polysaccharides can combine with anthocyanins and tannins in young wines and this combination seems to increase colour stability and decrease astringency. Yeast strains used were *Saccharomyces cerevisiae* BM45 and RC212. They present different characteristics: the BM45 yeast strain released more macromolecules than the RC212 yeast strain in synthetic medium and in must during fermentation. Wines fermented with BM45 yeast strain were characterized by a good mouthfeel at the end of alcoholic fermentation while RC212 wines are more astringent. Nine of twelve tasters from a trained panel considered wines made with yeast strain BM45 less astringent (ESCOT *et al.*, 2003).

Winemakers using this strain confirmed that it may prevent or reduce phenolic compound precipitation and

enable them to obtain wines with more colour and less astringency.

The aims of the present study were:

- to study factors affecting tannins aggregation such as temperature and ethanol percentage in hydroalcoholic solution by the spectrophotometric method.

- to determine the influence of glycosylated protein origin (RC212 or BM45 yeast strain) and of their modes of preparation on tannins aggregation.

- to study the influence on tannins aggregation of such components of glycosylated proteins as carbohydrate and protein.

MATERIALS AND METHODS

I - MATERIAL

Organic solvents were purchased from Carlo Erba (Val de Reuil, France). Glucose, mannose, mannan from yeast, β -cyclodextrine, amino acid and bovine serum albumin (BSA) were supplied by Sigma Chemical Company (Poole, Dorset, UK).

II - GRAPE SEED TANNINS FRACTIONS

Grape seeds from *Vitis Vinifera* v.c Pinot noir were ground and tannins extracted three times with acetone/water (60:40, v/v). Before acetone was evaporated in vacuum, this extract was washed 3 times with hexane, then centrifuged (5,000 g- 15 min) to eliminate particle residues then freeze-dried.

The three oligomeric and polymeric fractions were obtained from the crude extract by liquid-liquid extraction on a CHROMABOND XTR (45 mL, 8.3 g; bed volume 10 mL) cartridge from Macherey – Nagel (Hoerd-France). The crude extract in distilled water was loaded on the cartridge, then the cartridge was washed with six bed volumes of ether to obtain an F1 fraction. Then six bed volumes of ethyl acetate and methanol were eluted to obtain respectively F2 and F3 fractions. The organic solvents were taken to dryness by rotary evaporation and extracts were freeze-dried. Mean degrees of polymerisation (mDP) of the crude extract and of the three fractions were obtained by thiolysis as described by GUYOT *et al.* (2001). Crude extract, F1, F2 and F3 fractions with mDP of 4.4, 1.0, 3.6, 10.0 respectively available in adequate quantities, were used for the experimentation.

III - GLYCOSYLATED PROTEINS

Three type of glycosylated proteins were used in the present study: glycosylated proteins originating from alcoholic fermentation of a synthetic medium by yeast strains *Saccharomyces var. cerevisiae* RC212 and BM45

(Lallemand-Inc., Montreal, Canada), those obtained by Peat's method from yeast strain *Saccharomyces var. cerevisiae* BM45 (Lallemand-Inc., Montreal, Canada) and those coming from industrial preparations (Lallemand Inc.).

1) Production and isolation of glycosylated proteins from synthetic medium

After rehydration, yeasts were added to a synthetic medium without polysaccharides (GENEIX *et al.*, 1983) at 1×10^6 cells/mL. Yeasts were cultivated with stirring (150 t/min) at 28 °C.

Glycosylated proteins were isolated at the end of the alcoholic fermentation. The medium was centrifuged (10,000 g, 20 min, 4 °C) and concentrated with an Amicon cell (Model 202/75 PSI MAX; cut-off 30kDa) to 100 mL. Macromolecules (polysaccharides and glycosylated proteins) were precipitated by the addition of five volumes of 95 % ethanol at -20 °C overnight and centrifuged (8,000 g, 20 min., 4 °C). Ethanol precipitates were redissolved in water and freeze-dried (= BM45 AF and RC212 AF).

2) Isolation of glycosylated proteins by Peat's method (PEAT *et al.*, 1961)

500 g of commercial dried yeast (*Saccharomyces var. cerevisiae* BM45) was suspended in distilled water (40 °C) for 30 min, washed three times with distilled water, centrifuged (5000 g, 10 min, 10 °C), then 50 mL of 20 mM citrate buffer (pH 7) was added, and the mixture was autoclaved at 125 °C for 90 min.

After centrifugation, (10,000 g, 10 min, 10 °C) the supernatant solution was saved. The pellet was resuspended in 200mL of the same buffer and the procedure described above was repeated. The two supernatant extracts were combined. 600 mL of Feeling's solution was stirred in. The white precipitate obtained, which presented a gummy aspect, was dissolved by 100 mL of 3N hydrochloric acid.

The resulting green solution was poured slowly while stirring vigorously into 100 mL of a mixture of methanol and acetic acid (8:1, v/v), and the precipitate of glycosylated proteins was left for several hours to settle. The green supernatant fluid was eliminated, and the precipitate was stirred with fresh methanol-acetic acid mixture. This washing procedure was repeated until the supernatant fluid was colorless. The precipitate was dissolved in 10 mL of distilled water and dialysed (6-8 Kda cut-off) and freeze-dried (= BMp).

3) Industrial glycosylated proteins

Different industrial glycosylated proteins from different companies were tested but their mode of preparation

was unknown. After a preliminary assay, glycosylated protein X was retained. X, a dried glycosylated protein produced by Lallemand (Lallemand-Inc., Montreal, Canada) was used. That glycosylated protein intended for enological applications is produced by confidential process. X was extracted using a physical/chemical method from whole cells of *Saccharomyces var. cerevisiae*. This extracted glycosylated proteins were purified by centrifugation filtration, ultrafiltration, diafiltration and then spray dried.

X was used crude and was also purified to obtain X1. X1 corresponds to X suspended in distilled water and washed 3 times on an Amicon cell (3,500 cut-off), and precipitated by the addition of 5 volumes of 95 % ethanol as described above and freeze-dried.

4) Purification of industrial preparation by concanavalin-A (Con A) affinity chromatography

X was also purified by concanavalin-A (Con A) affinity chromatography. All solutions were degassed before use. 100 mg of industrial lyophilized glycosylated proteins X, was dissolved in 50 mM Tris-HCl, pH 7.4, containing 0.25 M NaCl, and loaded on a Concanavalin A (Con A)-Sepharose (Pharmacia, Sydney, Australia) column (16 x100 mm; 3 ml bed volume/mg protein), equilibrated with the same buffer. The column was washed with 3 bed volumes of 50 M Tris-HCl, pH 7.4, containing 0.5 M NaCl, and the glycosylated proteins were eluted with 2 bed volumes of 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl and 0.1 M-methyl- α -D-mannopyranoside (Sigma). The eluate of the retained fraction and that of the non retained fraction were desalted by dialysis (6-8 Kda) and lyophilized. X2 was the retained fraction and X3 the non-retained fraction.

IV - AGGREGATION MEASUREMENT

Tannins aggregation and glycosylated proteins aggregation with glycosylated proteins were studied in a model wine solution (ethanol (% v/v) determined by preliminary experiments) in distilled water, tartaric acid (5 g/l), pH 3.2.

Preliminary experiments were designed to determine the concentration of tannins and glycosylated proteins necessary to visualize an aggregation and its limitation and to quantify the effect of ethanol and temperature on the aggregation of grape seed tannins.

The different parameters tested were:

- Percentage of ethanol (% v/v) in a model wine solution: 0, 1, 2, 3, 6, 9, 12. The pH of solution (3.2) was adjusted with NaOH.

- Concentration of tannins : 0.5, 1, 2, 5 and 10 g/l.

- Concentration of glycosylated proteins : 0.5 and 1 g/l.
- Temperature: room temperature, 4 °C and 12 °C.

Experiment samples, stored in an optical cell, were shaken before each measurement.

Absorbance at 700 nm and the stability coefficient were determined using a UV-160A Shimadzu spectrophotometer (Japan).

The stability coefficient (SC) is defined as the ratio between the absorbance of tannins alone and absorbance of tannins with glycosylated proteins:

$$SC = \frac{\text{Abs.700nm (tannins)}}{\text{Abs.700nm (tannins + glycosylated proteins)}}$$

After the first measurement, samples were stored at study temperature and absorbance was measured every hour after shaking during the first 24 h then every day during the next 4 days.

V - CHEMICAL ANALYSIS OF GLYCOSYLATED PROTEINS

Neutral sugars were assayed by the phenol sulphuric method of DUBOIS (1956); protein concentration was determined by the procedure of LOWRY *et al.*, (1951). Neutral monosaccharide composition was determined, after hydrolysis with 2 M trifluoroacetic acid (120 °C, 60 min) by GLC of the alditol acetate derivatives as previously described (BLAKENEY *et al.*, 1983).

Phosphorus contents were determined according to the Clare method (1971).

Free amino acids were determined by HPLC using two pumps (Waters™ 626), a system controller (Waters™ 600s), an autosampler (Waters™ 717 plus) combined with a scanning fluorescence detector (Waters™ 474). Samples were submitted to derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate according to the method of STRYDON *et al.* (1994). The separation of amino acids was performed on a Waters Novapack TM C-18 column (pore size : 6.0 nm, particle size: 4 µm, 3.9 x 150 mm) at 37 °C. The precolumn had the same characteristics and was 2 cm long. Eluant A was sodium acetate buffer pH 5.8, eluant B was acetonitrile. The gradient was t=0 : 100 % A, t=35 min. 60 %A and 40 % B, t = 54 min. 100 % B. Detection by fluorescence was employed using wavenlengths of excitation and emission at 250 and 395 nm respectively.

Molecular weight distribution was evaluated by FPLC Superose®6HR 10/30 column (Pharmacia) with a fractionation range of 5 to 103 Kda for dextrans (detected by

refractometry) and 5 to 5.103 Kda for proteins (detected by absorption at 280 nm).

RESULTS AND DISCUSSION

I- AGGREGATION OF GRAPE SEED TANNINS

In model wine, grape seed tannins formed aggregates that could be detected by spectrophotometric measurement at 700 nm.

Preliminary assays were performed in order to determine tannins concentration, percentage of ethanol in model wine solution and temperature allowing visualization of tannins aggregation.

1) Influence of tannins concentration

A mixture of proanthocyanidic polymers extracted from grape seeds : crude tannins with polymerisation degree (Dpm) 4.6 was solubilized in model wine at different concentrations and turbidity was measured. Figure 1A shows the rate of development of cloudiness in the model wine solution with the addition of different concentrations of tannins. The tannins concentration of 5 g/L was selected since it allows for a turbidity value greater than 0.1 after 7 hours. This concentration is also lower than the level of tannins saturation of the model wine (5 g/L). Then, tannins were combined in model systems with different alcoholic degrees and at different temperatures.

2) Influence of temperature on aggregation of grape seed tannins

Results are showed in figure 1B. The level of aggregation of crude tannins increased as the temperature decreased. This phenomenon is due to a modification in the solvation state of molecules by temperature.

3) Influence of ethanol content on aggregation of grape seed tannins

Aggregation of crude tannins decreased when the ethanol content increased, due to a decrease in the solvent polarity that favoured tannins solvation (figure 1C). Tannins aggregation results from the balance between the tendency of molecules to interact with each other and their propensity to be solvated by the surrounding medium.

In conclusion the tannins aggregation was maximal at 4 °C with an ethanol concentration of 1.2 % and a tannins concentration of 5 g/l. In these conditions, aggregation occurred one hour after dissolution. These parameters were used for the following experiments.

II - EFFECT OF GLYCOSYLATED PROTEINS ON TANNINS AGGREGATION

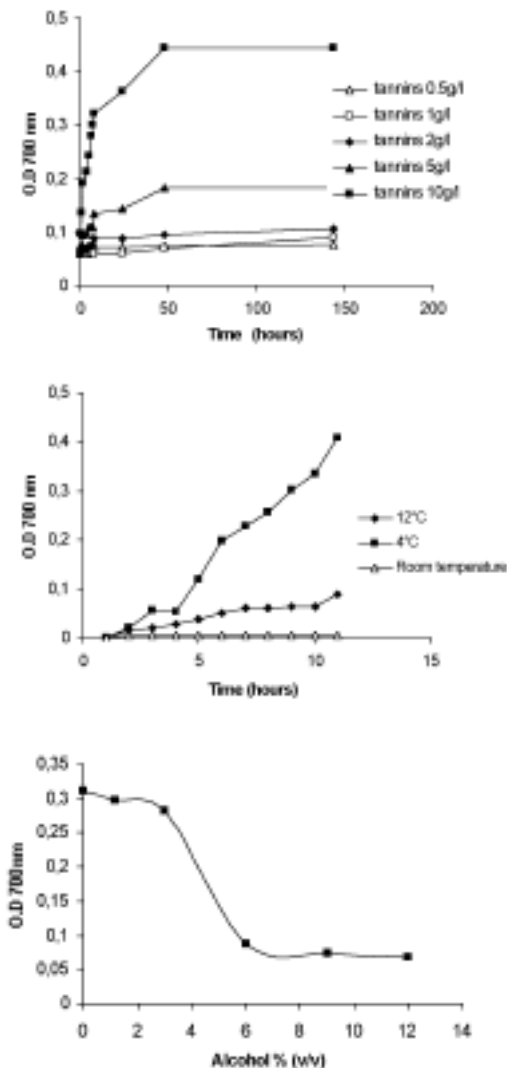


Fig. 1 - Influence of different parameters on aggregation of tannins

A - Tannins concentration, B - Temperature (tannins concentration : 5 g/L), C - Ethanol concentration (tannins concentration : 5 g/L)

Influence de différents paramètres sur l'agrégation des tanins

A - Concentration en tanins, B - Température (la concentration en tanins : est de 5 g/L), C - Concentration de l'alcool (la concentration en tanins est

After verification that glycosylated proteins did not absorb at 700 nm, the effect of five different glycosylated proteins (based on their origin) on crude tannins aggregation was investigated.

1) Influence of glycosylated proteins concentration

Preliminary assays were performed in order to determine the content of glycosylated proteins to assure stable combination with tannins. Different concentrations of glycosylated proteins released by BM45 yeast strain during alcoholic fermentation (BM45 AF) were tested. The absorbance evolution was measured over a period of 48 hours and the stability coefficient was determined (figure 2A).

A

When the stability coefficient was higher than 1, aggregation of tannins was lower; glycosylated proteins stabilized tannins. Tannins alone were used as a control. After 24 h, for concentrations of glycosylated proteins of 0.5 g/l and 1 g/l, the tannins stability was respectively 8.9 and 9.5 fold higher than the stability of tannins alone. SAUCIER (1997) explains that this phenomenon could be due to a colloidal stabilisation by adsorption of polysaccharides around colloid particle of procyanidin.

B

2) Influence of the yeast strain on the effect of glycosylated proteins on tannins aggregation

In the presence of glycosylated proteins BM45 AF, the stabilization of tannins is nine times more important than that of tannins alone and two-fold higher than that in the presence of glycosylated proteins RC212 AF (figure 2B). Although glycosylated proteins differ in their capacity to prevent tannins aggregation, there is no obvious difference in their compositions (table I). The two glycosylated proteins consist of the similar carbohydrate fraction (75-81 %) and a protein fraction (10.9-11.8 %). The percent of amino acid and proline content was not different from one glycosylated protein to another. The main difference between the glycosylated proteins is the mannose/glucose ratio higher for RC212 glycosylated proteins (2.3) than for those of BM45 strain (1.2). A mannose/glucose ratio near one seems to influence tannin stabilization. The nature of spatial conformation or the protein-sugar association can be modified.

C

Results found for tannins aggregation with glycosylated protein from alcoholic fermentation are in agreement with the findings described previously (ESCOT *et al.*, 2001). These two glycosylated proteins were added to a red wine and combination with tannins was estimated by different indexes described by Glories (1984). As shown in figure 3, after addition of BM45 AF and RC212 AF, wines were less astringent (lower gelatine index, higher ethanol index : more tannin/polysaccharide condensation); however the decrease in astringency was less obvious for glycosylated protein RC212 AF. These laboratory results are in accordance with results from tasting (by a trained panel) of the wines made with the RC212 strain which seemed to produce wine of less subtlety when compared with wines fermented with BM45 (figure 4). Indeed, the tannic power of wines fermented with the BM45 strain is lower than wines fermented with the RC212 strain. It was the same at the tasting, the tannins and the mouthfeel were in favour of wines produced with BM45.

3) Influence of the mode of preparation of glycosylated proteins from BM45 on tannin aggregation

The two glycosylated proteins issued from the *Saccharomyces cerevisiae* strain BM45 obtained by fer-

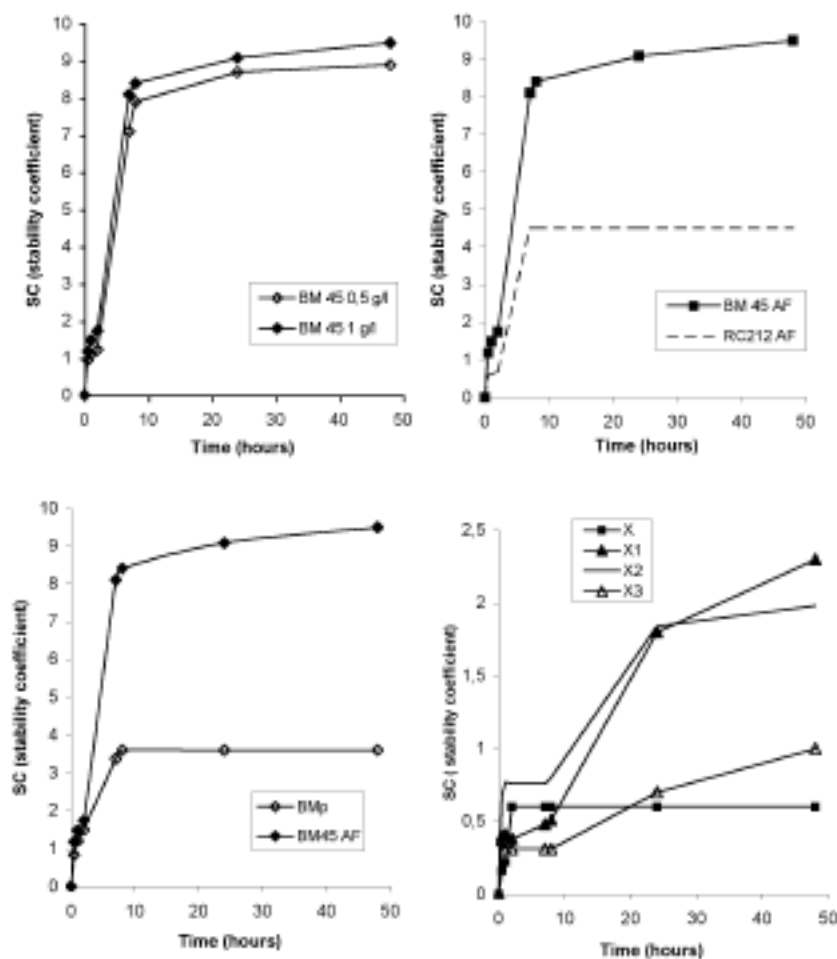


Figure 2 - Influence of glycosylated proteins on stability coefficient of tannins

A- Concentration of glycosylated proteins released by *Saccharomyces* strains BM45 during alcoholic fermentation at concentration :1 g/L
 B- Glycosylated proteins from BM45 and RC212 yeast strains released during alcoholic fermentation (BM45AF and FC212AF) at concentration :1 g/L
 C- Glycosylated proteins released during alcoholic fermentation and glycosylated proteins extracted by Peat's method at concentration :1 g/L
 D- Industrial glycoprotein X and purified fractions obtained by ethanol precipitation (X1) and by affinity chromatography on Sepharose ConA (X2: retained fraction, X3 non-retained fraction) at concentration 1 g/L.

Influence des protéines glycosylées sur le coefficient de stabilité des tanins

A- Influence de la concentration de protéines glycosylées relarguées par la souche de *Saccharomyces* BM45 au cours de la fermentation alcoolique à la concentration de 1 g/l
 B- Influence des protéines glycosylées relarguées par les souches BM45 et RC212 au cours de la fermentation alcoolique à une concentration de 1 g/L
 C- Influence des protéines glycosylées relarguées au cours de la fermentation alcoolique par la souche BM45 et de celles extraites par la méthode de Peat
 D- Influence d'une protéine glycosylée industrielle X et de ses fractions purifiées par précipitation à l'éthanol (X1) ou par chromatographie d'affinité sur Sépharose-conA (X2: fraction retenue, X3 : fraction non retenue).

mentation (BM45 AF) or extraction by Peat's method (BMp) limit the tannins aggregation, at different levels (figure 2C).

Stability appeared at the same time (7 hours) for the two glycosylated proteins, but the effect of BM 45 AF was 2.5 times more important than that of glycosylated proteins isolated by Peat's method from the BM45 yeast strain (BMp). The mode of preparation seems to be impor-

tant in the interaction between tannins and glycosylated proteins. In Peat's method, as yeasts are heated extraction is harsh and the protein fraction may be damaged.

4) Comparison of tannins stabilisation by industrial glycosylated proteins

The various industrial glycosylated proteins differed in their method of preparation. Among the five glycosy-

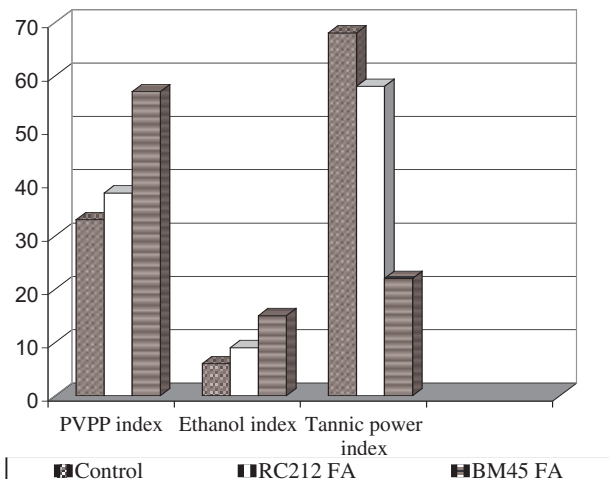


Fig. 3 - Influence of glycosylated proteins released by *S. cerevisiae* BM45 and RC212 (100 mg/L of wine from Pinot noir) during alcoholic fermentation on tannins properties.

Influence des protéines glycosylées relarguées par *S. cerevisiae* BM45 et RC212 (à la concentration de 100 mg/l de vin de Pinot noir) au cours de la fermentation alcoolique sur les propriétés des tanins.

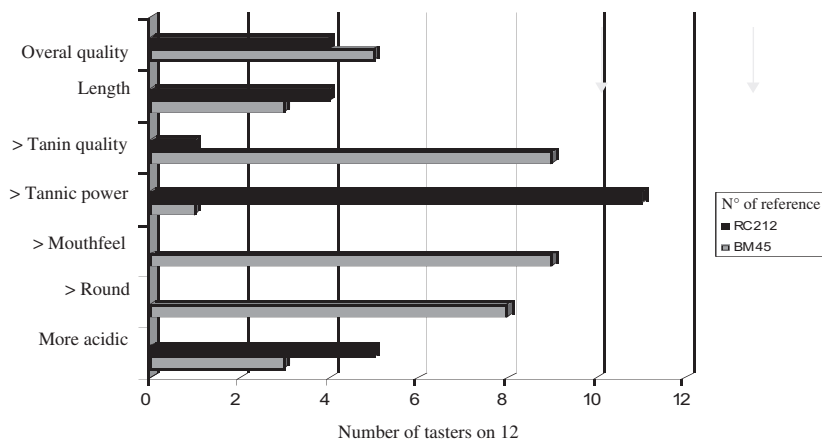
lated proteins tested, two did not modify crude tannins aggregation and three decreased stability coefficient (figure 5).

Glycosylated protein X was retained for further experiment. It was purified by precipitation with ethanol (X1) or affinity on Sepharose concanavalin A (in this case, X2 was the retained fraction and X3 the non-retained fraction). Results obtained for X, X1, X2, X3 are shown in figure 2C. In the presence of X (crude glycosylated pro-

tein) the precipitation of tannins was higher than that observed without. In contrast, in the presence of the purified fraction (X1 and X2), the stability of tannins was two fold higher than that of tannins alone, while X3 had no effect.

The non-purified glycosylated protein X is characterized by a higher level of proteins (26.4 %) and a much lower concentration in oses (35.6 %). As shown by its purification by ethanol precipitation or by chromatography on ConA Sepharose, the product X contains components other than the glycosylated proteins. Moreover, for this same product, the ratio of mannose/glucose is much higher than that of the other glycoproteins (table I). In addition, a heterogeneity is observed for the molecular masses which vary in function of the origin of glycosylated proteins. The mannose/glucose ratio of X1, X2 and X3 obtained by purification of X, respectively of 3.4, 3.8, 4.3 are smaller than that of X : 6.2 and the effect of these glycosylated proteins on tannins stabilisation is increased. The stability coefficients of tannins in their presence are 2.3, 1.98, 1.0 respectively instead of 0.65 for X. In the same way, glycosylated proteins released during alcoholic fermentation by the BM45 yeast strain, with a mannose/glucose ratio of 1.2, stabilised tannins two-fold more than glycosylated proteins released during alcoholic fermentation by the RC212 yeast strain with a mannose/glucose ratio of 2.3.

A mannose/glucose ratio close to one seems to favour tannins stabilization but the proteic fraction also plays a role in tannins stabilisation : X3 containing 2.6 % of protein only is inactive on tannins aggregation.



RC212 : vanilla, empyreumatic
 BM45 : toasted, red riped fruits

Fig. 4 - Evolution of stability coefficient of crude tannins in presence of glycosylated proteins from BM45 and RC212 yeast strains released during alcoholic fermentation (BM45 AF RC212 AF) at concentration 1g/L.

Évolution du coefficient de stabilité des tanins bruts en présence de protéines glycosylées de souches de levures BM45 et RC212 relarguées pendant la fermentation alcoolique (BM45 AF RC212 AF) à une concentration de 1 g/L.

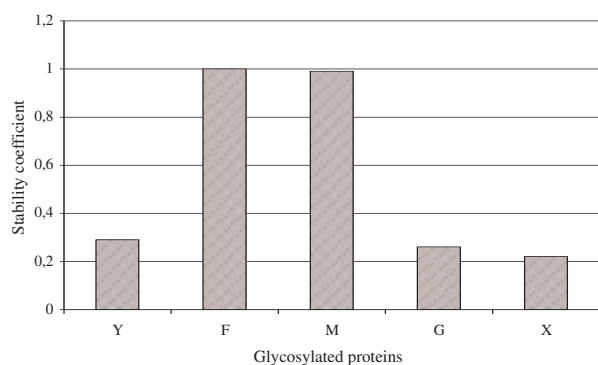


Fig. 5 - Results of wine tasting of Madiran wines elaborated with yeasts strains BM45 and RC212. Profile released by 12 wine trained tasters.

Résultats de la dégustation de vins de Madiran élaborés avec des souches de levures BM45 et RC212, obtenus avec un panel de 12 dégustateurs entraînés.

Two hypothesis can be invoked to explain the mechanism of the «protective effect» of wine polysaccharides on the aggregation of grape seed tannins in model wine: i) molecular association in solution between polysaccharides and polyphenols competing with tannins aggregation, ii) polysaccharide adsorption on particles formed by tannins preventing particle growth. In their studies RIOU *et al.* (2002) showed that polysaccharide addition inhibited the growth of tannins particles but not their formation, indicating that the second hypothesis is the most likely.

III - EFFECT OF CARBOHYDRATES ON TANNIN STABILISATION

The aim of this experiment was to determine if the linkage of crude tannins was possible with monosaccharides or only with polysaccharides. Different polymerised and non- polymerised sugars were tested (cyclodextrin, mannan from yeast, mannose and glucose).

Only glucose and mannose lead to a low stabilization of tannins. The cyclodextrin and especially the mannan caused a major limitation of tannins aggregation but lower than that of glycosylated proteins (figure 6A) However, these results seem to show that the polysaccharidic fraction could be essential in the stabilization of tannins.

IV - EFFECT OF DEGREE OF POLYMERISATION (MDP) OF TANNINS ON TANNINS AGGREGATION

Different mDP tannin fractions (crude tannins, mDP 3.6 or 10.0 and monomer) were used to specify the nature of the interacting fractions. The experiment was carried out with BMp. The aggregation of tannins depends on their molecular size. Turbidity increased with mDP (figure 6B). For tannins with a degree of polymerisation ranging from 3.6 to 10.0, aggregation leading to formation of colloids was detected, but not for monomers. In the presence of glycosylated proteins aggregation was strong, in particular for the mDP 10.0 fraction. Glycosylated proteins seem to interact more with tannins of high molecular size. This result is in keeping with that of RIOU *et al.* (2001) who observed that tannins with mDP (6-9) can form aggregates liable to interact with macromolecules and / or surfaces.

Table I - Composition of glycosylated proteins used to study tannins stabilisation
Composition des protéines glycosylées utilisées pour étudier la stabilisation des tanins

Glycosylated proteins	BM45 AF*	RC212 AF	BMp**	X	X1	X2	X3
% proteins(1) (BSA)	11.8 ± 0.1	10.9 ± 0.1	4.7 ± 0.1	26.4 ± 0.1	6.4 ± 0.2	4.2 ± 0.2	2.6 ± 0.2
% sugar (2)	75 ± 0.6	81.1 ± 0.5	83.2 ± 0.4	35.6 ± 0.5	78.5 ± 0.4	77.2 ± 0.4	64.7 ± 0.5
Ratio mannose/glucose(3)	1.2	2.3	0.98	6.2	3.4	3.8	4.3
% phosphorus (4)	1.6 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	ND	ND
% total amino acid (5)	1.77	2.15	0.98	1.97	0.98	1.20	1.54
Proline %	4.89	4.81	11.95	7.72	8.38	8.3	8.13
	430 000	620 000	68 000	220 000	305 000	ND	ND
Estimation of molecular size (Da)(6)	38 000	59 000	6 100	21 000	3 280		
	2 500	41 000	2 180	2 200	1 700		
	1 100	2 700		1 400			
		1 300					
Stability coefficient at steady state	9.0	4.5	3.6	0.65	2.3	1.98	1

(1) Lowry method, (2) Dubois method, (3) Capillary GC, (4) Clare method, (5) Cohen and Michaud method, (6) estimation by sepharose CL6B,

*AF: from alcoholic fermentation, **p: from Peat method

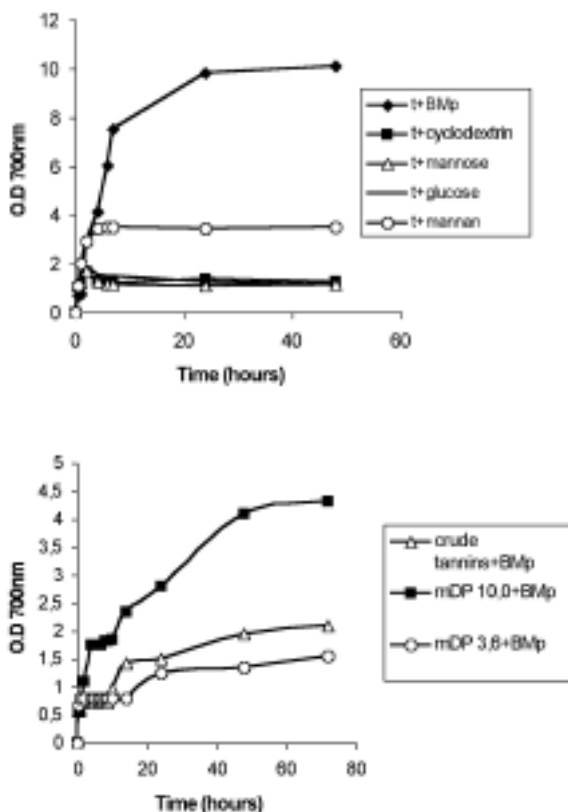


Fig. 6 - Aggregation of tannins

A- Influence of different carbohydrates and BMp (glycosylated protein extracted from strain BM45 by Peat's method) at concentration 1 g/L on crude tannins.

B- Influence of tannins (mDP 3.6, 10.0 and crude tannins) in presence of glycosylated proteins extracted from BM45 by Peat's method (BMp) at concentration 1g/L.

Agrégation des tanins

A - Influence de différents sucres et de la protéine glycosylée BMp (extraite de la souche BM45 par la méthode de Peat) sur les tanins.

B - Influence du degré de polymérisation des tanins (degrés de polymérisation égaux à 3,6 - 10,0 et tanins non fractionnés en présence des protéines glycosylées extraites de la souche BM45 (BMp) par la méthode de Peat à la concentration de 1 g/L.

CONCLUSION

Spectrophotometric measurements showed that grape seed tannins aggregate in a model wine solution. Tannins aggregation increases when ethanol content or temperature decreases due to modification of the solvation state of the molecules. It was demonstrated that intensity of tannins aggregation increased with mDP. At mDP 10.0, tannins could form aggregates liable to interact with macromolecules. The influence of glycosylated proteins, which differed by their origin or their mode of preparation was tested. Among the glycosylated proteins tested, the glycosylated protein BM45 released during alcoholic fermentation was more efficient than the other glycosylated

proteins, especially those extracted by heating. Results obtained with mannan lead us to believe that the stabilisation of tannins in model solution depends on the polysaccharidic fraction of the glycosylated proteins and on its mannose/glucose ratio. When the ratio is close to one, aggregation of tannins decreases.

Glycosylated proteins seem to coat tannins, thereby preventing their precipitation, which leads to improved colour stability and a decrease of astringency.

To specify the nature of the binding involved in glycosylated proteins and tannins interaction, charge densities and conformational study should be investigated.

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REFERENCES

BALLOU C.E., 1988. Organization of the *Saccharomyces cerevisiae* cell wall, 335-360. In : *Self assembling Architecture*. ed Varner, J.E ed, Liss, New York.

BLAKENEY A., HARRY P., HENRI R., STONE B., 1983. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.*, **113**, 291- 299.

CLARE M.C., 1971. An accurate and convenient organic phosphorus assay. *Annal Biochem.*, **39**, 426-428.

ESCOT S, FEULLAT M., DULAU L, CHARPENTIER C., 2001. Release of polysaccharides by yeasts and influence of released polysaccharides on colour stability and wine astringency. *Aust. J. Grape and Wine Res.*, **7**, 153-156.

ESCOT S. 2003. Contribution à l'étude des protéines glycosylées de levure et à leurs interactions avec les polyphénols des vins rouges. Thèse de doctorat, Université de Bourgogne.

DUBOIS M., 1951. A colorimetric method for the determination of sugars. *Nature*, **110**, 167- 168.

GENEIX C, LAFON-LAFOURCADE S., RIBÉREAU-GAYON, 1983. Les causes, la prévention et le traitement des arrêts de la fermentation alcoolique. *Connaissance Vigne Vin*, **3**, 205-217.

GLORIES Y. 1984. La couleur des vins rouges 2 – mesure, origine et interprétation. *Connaissance Vigne Vin*, **18**, 195-217.

GUYOT S., MARNET N., DRILLEAU J.F., 2001. Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agr. Food Chem.*, **49**, 14-20.

LLAUBERES R.M. 1988. Les polysaccharides sécrétés dans les vins par *Saccharomyces cerevisiae* et *Pediococcus* sp. Thèse Doctorat. Université Bordeaux II.

LOWRY O., ROSENBROUGH N.J., FARR A.L., RANDAL A.L., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.

- PEAT S., WHEALEN W.J. EDWARDS T.E., 1961. Polysaccharide of baker's. Part IV. Mannan. *J. Chem. Soc.*, 28-35.
- PELLERIN P. and CABANIS, J.C., 1998. Les glucides et l'œnologie, 41-92. In *Œnologie fondements scientifiques et technologiques*. ed Lavoisier TEC & DOC, Paris.
- PRIEUR C., RIGAUD J., CHEYNIER V., MOUTOUNET M., 1994. Oligomeric and polymeric procyanidins from L grape seeds. *Phytochem.*, **36**, 781- 784.
- RIBEREAU- GAYON P., 1964. Les composés phénoliques du raisin et du vin II. Les flavonoides et les anthocyanosides. *Ann. Physiol. Végét.*, **6**, 211-242.
- RIOU V., VERNHET A. DOCO T., MOUTOUNET M., 2002. Aggregation of grape seed tannins in model wine - effect of wine polysaccharides. *Food Hydrocolloids*, **16**, 17-23.
- SAUCIER C., BOURGEOIS G., VITRY C., ROUX C., GLORIES Y., 1997. Characterization of (+)- catechin- acetaldehyde polymers- a model for colloidal state of wine polyphenols. *J. Agr. Food Chem.*, **45**, 1045-1049.
- SOUQUET J.M., CHEYNIER V., BROSSAUD F., MOUTOUNET M., 1996. Polymeric proanthocyanidins for grape skin. *Phytochem.*, **43**, 509-512.
- STRYDON D.J. and COHEN S.A. 1994. Comparison of amino acid analyses by phenylisothiocyanate and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate precolumn derivatization. *Anal. Biochem.* **1**, 29-33.

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