Study of Interactions between Aroma Compounds and Glycopeptides by a Model System

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Interactions between selected aroma compounds and yeast cell wall peptidomannans, on one hand, and between limonene and a model glycopeptide synthesized by the conjugation between trypsin inhibitor and a dextrin, on the other hand, have been studied using exponential dilution. The neoglycopeptide was purified and its molecular weight determined (66 000 ± 2 450). While exponential dilution did not reveal any interactions between the limonene and the neoglycopeptide at the native state, some interactions were observed after a weak heat treatment. The binding ability of the neoglycopeptide was evaluated by determining the surface hydrophobicity. An attempt was made to interpret the findings obtained using peptidomannans according to the findings obtained using the model system.

Keywords: *Interaction; exponential dilution; aroma compound; peptidomannans; neoglycopeptide; spectrofluorometry; surface hydrophobicity*

INTRODUCTION

To satisfy commercial requirements, it is important that wines are both limpid and stable. Methods such as ultrafiltration or microfiltration give good clarification in a single process but can change the macromolecular equilibrium and affect the organoleptic quality, in particular the typicity of the wine (Feuillat *et al.*, 1987). Wines that are enriched by yeast macromolecules released during yeast autolysis have very good sensorial quality, a good example of this being wines which are aged on their lees (Ferrari and Feuillat, 1988; Feuillat *et al.*, 1989). For these reasons, the hypothesis that yeast cell walls, and in particular peptidomannans, play an important role in aroma retention has been investigated (Geneix *et al.*, 1985; Voilley *et al.*, 1990, 1991; Lubbers *et al.*, 1994a). Voilley *et al.* (1990) have shown that isoamyl acetate, ethyl hexanoate, and β-ionone might be weakly bound to yeast walls during fining or ultrafiltration of a model wine. The influence that peptidomannans released from yeast cell walls during alcoholic fermentation have on the volatility of aroma substances has been previously demonstrated, and the retention of aroma compounds (β-ionone, ethyl hexanoate) has been attributed to peptidomannans having a high protein content (Lubbers *et al.*, 1994b).

In general, aroma compounds are able to interact with different macromolecules. Several authors have given evidence for flavor binding to proteins, probably through hydrophobic interactions, as indicated by a decrease in volatility when the chain length of aldehydes and methyl ketones increases or when denaturation of the protein occurs (Damadoran and Kinsella, 1980, 1981; Mills and Solms, 1984; O'Neill and Kinsella, 1987; Dufour and Haertle, 1990). Langourieux and Crouzet (1995) have shown that aroma compounds are able to interact with such peptides as soybean trypsin inhibitor. With macromolecules such as polysaccharides, there is a variability due to the nature of the molecule. For example, adsorption or inclusion occurs with starches, dextrin, galactomannans, and hydroxypropylcelluloses, whereas a "salting out" phenomenon prevails with dextrans (Rutschmann and Solms, 1989; Langourieux and Crouzet, 1994).

The aim of the present work is first to study the interactions between yeast peptidomannans and certain chosen wine aroma compounds and, second, to study the interactions in a model system constituted of a synthesized glycopeptide, the advantage of this being a known purified system. Because of the presumed relation between the hydrophobicity of both the aroma compounds and the macromolecule, and the fact that there are interactions, the binding ability of the neoglycopeptide was evaluated by determining the surface hydrophobicity using a spectrofluorometric method. This method has been used by several authors to determine the surface hydrophobicity of proteins, by following either the decrease of protein fluorescence when bound to a ligand (Eftink *et al.*, 1977) or the increase of ligand fluorescence when bound to the protein (Wang and Edelman, 1971; Kato and Nakai, 1980; Haque and Kinsella, 1985; Bonomi *et al.*, 1988; Laligant *et al.*, 1991; Iametti *et al.*, 1995).

MATERIALS AND METHODS

Materials. Peptidomannans from yeast cell walls, purified according to the procedure of Peat *et al.* (1961), were kindly donated by the Microbiological Biochemistry Laboratory of Pr. Bonaly (Nancy, France). Corn starch dextrin Tackidex J060K was kindly donated by Roquette Freres (Lestrem, France). Bovine serum albumin, soybean trypsin inhibitor (type II-S), 1-anilinonaphthalene-8-sulfonic acid ammonium salt, and cyanogen bromide were from Sigma Chemical Co. (St. Louis, MO); (+)-limonene, ethyl hexanoate, and isoamyl acetate were from Aldrich Chemical Co. (Milwaukee, WI).

Neoglycopeptide Synthesis: Dextrin−Trypsin Inhibitor Conjugate. This method is based on activating hydroxyl groups of dextrin with cyanogen bromide. Dextrin−trypsin inhibitor conjugate was prepared according to the method of Marshall and Rabinowitz (1976) with a solution of dextrin at 10 g/L and 0.25 g of trypsin inhibitor. The reaction mixture was then lyophilized.

Purification of the Synthesized Neoglycopeptide. The lyophilized product was diluted with water and chromato-

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graphed on a Fractogel TSK HW-55F (Merck) column (30 × 2.6 cm). Elution was performed with distilled water using a Milton-Roy pump at a flow rate of 1.8 mL/min. Four milliliter column fractions were collected and analyzed for carbohydrate and protein content. Fractions between elution volumes of 56 and 92 mL were put together and lyophilized.

Carbohydrate Determination. Carbohydrate was measured according to the phenol−sulfuric acid method (Dubois *et al.*, 1956) calibrated against dextrin.

Protein Measurement. In column fractions protein was usually located qualitatively by measurement at the absorbance at 280 nm.

Neoglycopeptide Molecular Weight Determination. The molecular weight of the neoglycopeptide was determined by high-performance liquid chromatography (HPLC) (Shimadzu LC9A) using two different columns [TSK G5000 PW XL and G3000 SW (Chrompack)] with water as eluant at a flow rate of 1 mL/min. Eluted compounds were detected at 280 nm using a Varian 2550 UV detector. The columns were calibrated using proteins of different molecular weights (Boehringer): tyroglobulin (MW 669 000), apoferritine (MW 443 000), β-amylase (MW 200 000), bovine serum albumin (MW 66 000), ovalbumin (MW 45 000), β-lactoglobulin (MW 14 800), α-lactalbumin (MW 14 000).

Neoglycopeptide Surface Hydrophobicity Determination. For this purpose, anilinonaphthalenesulfonate (ANS) was used due to the ability of this hydrophobic ligand to become fluorescent when bound to the protein. A Jobin-Yvon (Paris) spectrofluorometer operated at room temperature was used to determine the relative fluorescence intensity (FI) of the ANS−protein conjugates. Excitation and emission wavelengths of ANS were, respectively, 370 and 490 nm with slit width of 10 nm. The spectrofluorometer was calibrated with a 1 mg/L solution of quinine sulfate to give a response of 45. Successively increasing concentrations of ANS solution ranging from 9×10^{-6} to 2×10^{-4} M were added in increasing amounts from 10 to 50 μL to 1 mL of the protein solution of known concentration P_0 until fluorescence reached a maximum. After each addition of ANS, the fluorescence intensity was measured after rapid mixing and standing for 1 min. Data were analyzed according to the method of Cogan *et al.* (1976)

$$P_0a = (1/n)[L_0a/(1 - a)] - (K_D/n)$$

where P_0 is the total protein concentration, K_D is the apparent dissociation constant, n is the number of independent binding sites, L_0 is the total ligand concentration, and a is the fraction of binding sites remaining free and assumed to be equal to $(FI_{max} - FI)/(FI_{max} - FI_0)$, where FI is the fluorescence intensity at a given ANS concentration, FI_{max} is the fluorescence intensity when all of the protein molecules are saturated by the ligand, and FI_0 is the initial fluorescence intensity.

Exponential Dilution. Exponential dilution equipment as previously described by Langourieux and Crouzet (1994, 1995) was used. The exponential dilution method enables a reduced infinite dilution activity coefficient γ^{∞}_{ir} to be calculated. It is defined by the ratio $\gamma^{\infty}_{im}/\gamma^{\infty}_{iw}$, where γ^{∞}_{im} and γ^{∞}_{iw} are, respectively, the values of the infinite dilution activity coefficient of the aroma compound (i) in the presence of macromolecules (m) and in pure water (w). A value of 1 for this ratio indicates that there is no interaction between the macromolecule and the aroma compound. A value below 1 indicates retention of the aroma compound by the macromolecule, whereas a value above 1 indicates a salting-out phenomenon.

RESULTS AND DISCUSSION

Study of Interaction with Yeast Cell Wall Peptidomannans. The values of the reduced infinite dilution activity coefficient for different volatile compounds are given Figure 1 as a function of the peptidomannan concentration.

γ^{∞}_r of limonene decreased when the concentration of peptidomannans reached 10 g/L, up to a 36% decrease

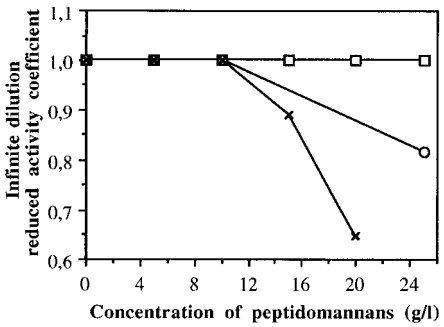

Figure 1. Variations in the infinite relative activity coefficient of isoamyl acetate (□), ethyl hexanoate (○), and limonene (×) as a function of the concentration of yeast cell wall peptidomannans.

for 20 g/L of peptidomannans. Interactions between ethyl hexanoate and peptidomannans were weaker, the decrease in γ^{∞}_r only reaching 20% for 25 g/L of peptidomannans. No variation of the reduced infinite dilution activity coefficient of isoamyl acetate occurred in the presence of peptidomannans. It would appear that this compound does not interact with peptidomannans.

Using a mannoprotein extract (1 g/L) solubilized from yeast cell walls at pH 7−8 at 100 °C during 3 h, Lubbers (1993) did not observe any interaction with ethyl hexanoate. However, for a similar material liberated by the yeast during the alcoholic fermentation of a synthetic must and used in the same proportion, a decrease of 12% of the infinite dilution activity coefficient of ethyl hexanoate was observed (Lubbers *et al.*, 1994b). It would seem, therefore, that extraction and purification conditions of the peptidomannans play a very important role. Using a gentle extraction method, interactions can be observed at low concentrations of peptidomannans in the medium. With more drastic extraction methods such as solubilization in alkaline medium or extraction according to the method of Peat *et al.* (1961), more concentrated solutions of peptidomannans are needed for interactions to occur. To avoid this variability due to nonhomogeneous material, we decided in this study to work on a molecular model system that simulates the peptidomannan/limonene system. Limonene was chosen for this investigation because it is the most retained of the studied aroma compounds by the yeast peptidomannans.

Investigation of a Model System. A soybean trypsin inhibitor was selected because of the similarities of its amino acid composition with the peptidic part of the peptidomannan (Charpentier *et al.*, 1986; Ikenaka *et al.*, 1970). After a screaning study (Langourieux and Crouzet, 1994), a dextrin was chosen for the glucidic part because of its satisfying properties (good retention of the limonene, water solubility, nonviscous in solution). The interactions between limonene and the dextrin, on one hand, and soybean trypsin inhibitor, on the other hand, were already characterized using exponential dilution and dynamic coupled column chromatography (Langourieux and Crouzet, 1995). No interaction occurred between limonene and the dextrin at 10 g/L, but a retention of about 25% of the limonene could be put forward with a dextrin concentration of 25 g/L. At a concentration of 10 g/L in trypsin inhibitor, a retention of about 25% of the limonene occurred.

The formation constants of the dextrin−limonene and the trypsin inhibitor−limonene complexes were, respectively, equal to 13650 ± 250 and 2944 ± 784 M^{-1}.

As a consequence, a neoglycopeptide was synthesized using the trypsin inhibitor and the dextrin.

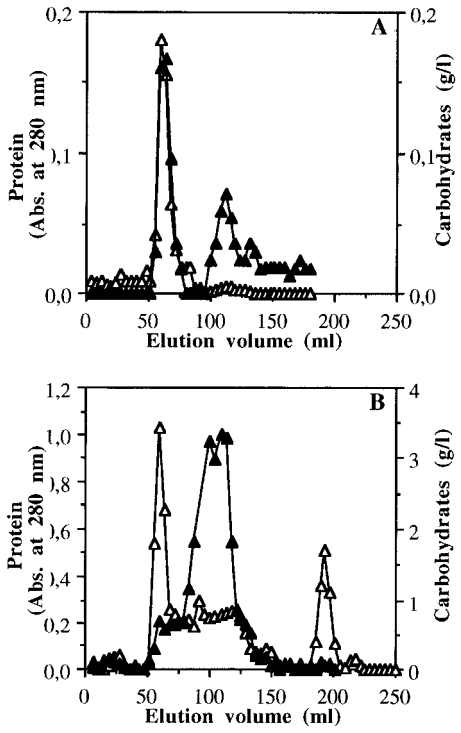

Figure 2. Fractogel TSK HW-55F column chromatography of (A) dextrin−trypsin inhibitor conjugate (9.7 mg/mL) and (B) dextrin (30 mg/mL) and trypsin inhibitor (1 mg/mL) mixture: profiles of protein (△) and carbohydrate (▲) content as a function of volume of water eluted through the column at a flow rate of 1.8 mL/min.

Characterization of the Neoglycopeptide. Protein and carbohydrate measurements have been made for each column fraction after elution of the product of the conjugation between the dextrin and the trypsin inhibitor on a TSK HW-55F column. Results are presented in Figure 2A. The glycoprotein peak eluting between retention volumes of 50 and 75 mL corresponds to the dextrin−trypsin inhibitor conjugate, and the glucidic peak eluting between retention volumes of 100 and 125 mL corresponds to the unreacted dextrin. A control chromatography (Figure 2B), in which a mixture of trypsin inhibitor and dextrin was eluted under the same conditions, was performed. The protein peaks corresponding to the trypsin inhibitor are separated from the glucidic peak corresponding to the dextrin, which shows that the covalent binding between the trypsin inhibitor and the dextrin succeeded.

The elution of the trypsin inhibitor gave retention volumes of 61 and 193 mL. The dextrin has a molecular weight of 37 600 and a retention volume of 113 mL, and an experiment with bovine serum albumin (molecular weight 66 000) gave a retention volume of 60 mL. The two peaks eluted with the trypsin inhibitor have molecular weights of, respectively, 64 000 and 20 000. (The theoretical molecular weight of trypsin inhibitor is 21 000.) This result was reproducible when trypsin inhibitor was taken from a different commercial batch. This is hardly explainable unless trypsin inhibitor would be able to polymerize so that the peak at 61 mL would correspond to a trimer.

HPLC of 1 mg/mL of the purified neoglycopeptide on G3000 SW (Figure 3) and G5000 PW columns gave a molecular weight of 66 000 ± 2 450, which is compatible with the addition of one molecule of dextrin with one molecule of trypsin inhibitor.

Interaction of Limonene with the Synthesized Neoglycopeptide. Exponential dilution was used for this study. The reduced infinite dilution activity coefficient of limonene kept a value of 1 in the presence of a 10 g/L solution of neoglycopeptide. No interaction occurs at this concentration. One can assume that the conjugation of dextrin and trypsin inhibitor induced structural changes of the fixation sites for limonene which were available both on the dextrin and on the trypsin inhibitor. Three hypotheses can be put forward. First, the conjugation could have destroyed the fixation sites for the limonene and no others were created due to the conjugation. Second, the fixation sites on the dextrin and trypsin inhibitor could still be present but could be hidden in the neoglycopeptide molecule or in an unfavorable environment so that the interactions with limonene are no longer possible. Finally, new sites could have been created through the conjugation reaction but they are also either hidden or in an unfavorable environment.

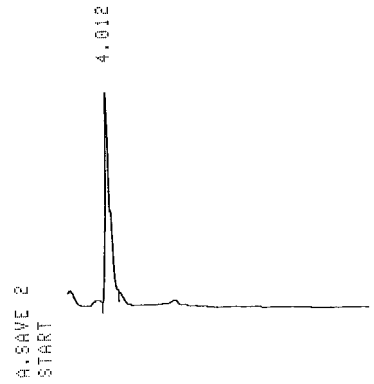

Figure 3. HPLC chromatogram of the neoglycopeptide: G 3000 SW (Chrompack) column; elution with water at a flow rate of 1 mL/min; detection at 280 nm (Varian 2550).

Two weak thermic treatments were applied to the 10 g/L solution of neoglycopeptide in water: the first one at a temperature of 45 °C during 1 h and the second one at 55 °C during 1 h. For the solution heated at 45 °C, the reduced infinite dilution activity coefficient of limonene took a value of 1, indicating no interaction. For the solution heated at 55 °C, this coefficient took a value of 0.74, indicating a decrease of the infinite dilution activity coefficient in water of limonene of about 25%. It can be assumed that this weak heat treatment probably induced conformational changes such as unfolding in the molecule. It seems to indicate that the fixation sites for limonene are hidden in the neoglycopeptide molecule.

Evaluation of the Surface Hydrophobicity of the Neoglycopeptide. To confirm the latter hypothesis, the binding affinity of the native glycopeptide (NG), of the light heat denatured neoglycopeptide (DNG), and of the trypsin inhibitor (TI) for ANS was studied. The fluorescence intensities (FI) of the ANS−NG, ANS−DNG, and ANS−TI conjugates are shown in Figure 4 as a function of ANS concentration for known peptide concentrations.

Only the initial part of the curve can be considered since the absorbance of ANS solutions at 370 nm (λ excitation) exceeds 0.2 absorbance unit at ANS concentrations above 30 μM. Thus, the fluorescence data concerning the neoglycopeptide could not be transformed according to the method of Cogan *et al.* (1976).

Anyway, the values of the maximal fluorescence intensity, 50, 47, and 32 for ANS bound respectively to DNG, TI, and NG, show that the binding capacity of NG is much weaker at the native state than when

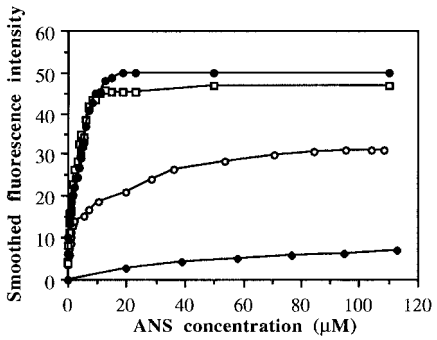

Figure 4. Smoothed fluorescence intensity of ANS−protein conjugate as a function of ANS concentration for the following protein concentrations: (◆) no protein; (□) trypsin inhibitor (1.428 μM); (○) neoglycopeptide (1.106 μM); (●) denatured neoglycopeptide (1.075 μM).

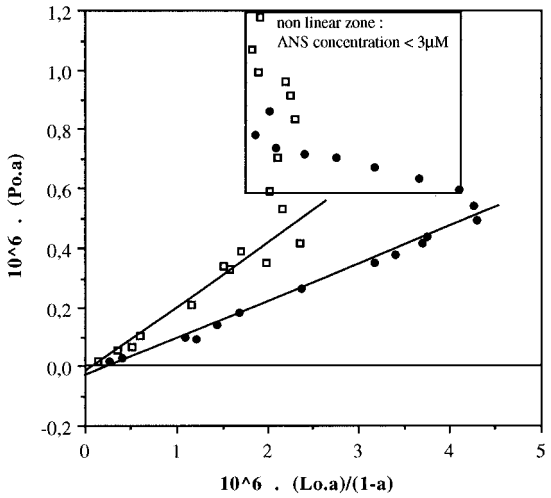

Figure 5. Graphic representation according to Cogan *et al.* (1976) for the titration of (□) trypsin inhibitor (1.428 μM) and (●) denatured neoglycopeptide (1.075 μM) with ANS.

slightly heat denatured. n and K_D values for the ANS−TI and ANS−DNG conjugates were obtained from the representations of Cogan *et al.* (1976) (Figure 5).

Values of $n = 4.6$ (effective number of binding sites between 4 and 5) and $K_D = 9.9 \times 10^{-8}$ M and $n = 8.0$ and $K_D = 26.8 \times 10^{-8}$ M for trypsin inhibitor and denatured neoglycopeptide, respectively, were found. Low K_D and high n values show a good binding capacity. The resulting surface hydrophobicity of the neoglycopeptide after denaturation can be explained by the unfolding of the molecule, confirming the fact that the fixation sites are hidden in the molecule. This explains why no interaction occurred between the limonene, a hydrophobe molecule, and the neoglycopeptide.

Conclusion. If we assume that the yeast cell wall peptidomannans have the same behavior as the neoglycopeptide, we can explain the very weak interactions between limonene and the peptidomannans. This glycopeptide has in aqueous medium or in wine such a conformation that the hydrophobic binding sites are in the core of the molecule, which are not directly accessible for a hydrophobic ligand such as an aroma compound. A small surface hydrophobicity can remain, permitting binding, which is why interactions occurred only for high concentrations of peptidomannans (10 g/L). Such a high concentration in peptidomannans is not found naturally in wine. However, in the concentration polarization layer formed during the ultrafiltration or microfiltration process, both high concentrations and denaturing conditions are found (Lencki, 1994; Meireles *et al.*, 1991; Harris *et al.*, 1989) so that interactions can occur. We can thus explain the aroma losses of ultrafiltrated and microfiltrated wines. It should not be forgotten that the membrane material itself is able to bind aroma compounds as well as the large variety of the macromolecules retained by the membrane.

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