

## STUDY OF WINE PROTEINS BY IMMUNOLOGICAL METHODS. I- PRODUCTION OF HIGHLY SPECIFIC ANTIBODIES

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### INTRODUCTION

With the purpose of studying the proteins present in wines and the reason why they precipitate, during storage, causing a haze which renders these beverages of low commercial value, polyclonal antibodies were raised in rabbits against specific proteins as well as against the whole protein fraction from the Assario wine. Fast protein liquid chromatography (FPLC) cation exchange chromatography was used to isolate the total protein fraction and, when in combination with denaturing electrophoresis, to purify individual wine proteins. The titre of the antisera was measured by the enzyme-linked immunosorbent assay (ELISA) and the specificity of the antibodies detected by immunoblotting. The antibodies produced were shown to be highly specific for the corresponding antigens.

### MATERIALS AND METHODS

**PREPARATION OF WINE:** The white wine used was prepared from the single grape variety, Assario. Ripened Assario grapes were harvested in 1994 in the Dão region, Portugal, and processed into wine by a conventional microvinification procedure, according to the classical white wine technology. Bentonite was not added during fermentation. After opening each bottle, the wine was divided in several aliquots and stored at -70°C until used. To avoid repeated freezing and thawing, a new aliquot was used for each experiment.

**PURIFICATION OF ANTIGENS BY FPLC CATION EXCHANGE CHROMATOGRAPHY:** Wine aliquots were thawed and centrifuged at 15,000g for 5 min., and the supernatant desalted at 4°C on prepacked PD-10 Sephadex G-25M columns

(Pharmacia/LKB, Uppsala, Sweden), previously equilibrated with water (Milli-Q plus, Millipore, Bedford, USA). The protein samples were subsequently lyophilized (Edwards Micro Modulyo freeze drier, Crawley, Sussex, England) and the dried residue solubilized in 20 mM citrate-NaOH buffer, pH 2.5.

A sample containing the wine total proteins was purified or fractionated by cation exchange chromatography on a Mono S HR5/5 column (Pharmacia/LKB) previously equilibrated in 20 mM citrate-NaOH buffer, pH 2.5. The bound proteins were eluted with a step gradient (0/1 M, for the isolation of total proteins) or a continuous gradient (0 to 1 M, for the fractionation of the individual wine proteins) of NaCl.

**ISOLATION OF THE SOLUBLE PROTEINS FROM *LEMNA MINOR*:** *Lemna minor* L. was grown as described by Ferreira & Davies [1986]. *Lemna* fronds were frozen in liquid nitrogen, ground to a fine powder and the total soluble protein extracted in 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was filtered through two layers of cheesecloth, centrifuged for 10 min at 40,000g and 2°C and the supernatant desalted at 2°C on a PD-10 column previously equilibrated with 20 mM Tris-HCl buffer, pH 7.5.

**PRODUCTION OF ANTIBODIES AND PREPARATION OF ANTISERA:** New Zealand female rabbits were immunized with purified antigens (400 µg) in complete Freund's adjuvant. To obtain a high titre, three booster injections of 400 µg antigen each were given every two weeks in complete Freund's diluted 1:10 with incomplete adjuvant. At intervals, blood was collected from the marginal ear vein and the titre determined by the ELISA technique. Total blood was taken from the heart 12 days after the third booster injection. Blood samples were allowed to clot and the serum was collected, centrifuged and stored at -70°C.

**ELISA:** This technique was performed, essentially, by the method described by Morgan *et al.* [1983].

**ELECTROPHORESIS, WESTERN BLOTTING AND IMMUNOBLOTTING:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by a modification, Christy *et al.* [1989], of the method described by Weber & Osborn [1969] and by Laemmli [1970]. The molecular mass polypeptide standards used ranged from the 205 kd subunit of rabbit muscle myosin to the 14.2 kd of bovine milk  $\alpha$ -lactalbumin.

Western blotting was performed using a semi-dry electrophoretic transfer cell (Trans-Blot SD, Bio-Rad, Richmond, USA). The proteins were immobilized on the membranes according to the method described by Swerdlow *et al.* [1986], visualized by incubation in an amido black 10B/methanol/acetic acid solution and destained in isopropanol/acetic acid. The blots destined for immunoblotting were processed as described by Ferreira & Shaw [1989] except that Tween 20 (0.05 % v/v) was included in the antibody containing solutions to reduce unspecific binding and non-fat dried milk was used as a blocking agent to saturate the remaining protein binding sites.

**GENERAL ASSAYS:** Protein content was measured using a modification of the Lowry method, Bensadoun & Weinstein [1976]. Total polysaccharides were deter-

mined by the method of Robyt & White [1987] and Segarra *et al.* [1995]. Acid polysaccharides were estimated according to Tusseau & van Laer [1993] and the neutral polysaccharides by the difference between total polysaccharides and acid polysaccharides, Segarra *et al.* [1995].

## RESULTS AND DISCUSSION

**PREPARATION OF THE ANTIGENS:** The profile depicted in the first chromatogram (Figure 1) refers to the purification of the total protein fraction from the Assario wine.

The wine components with no positive charge at pH 2.5 did not bind to the column and appeared as a large  $A_{280}$  peak (peak 1), whereas the components with positive charge were eluted with a 1 M NaCl step gradient (peak 2).

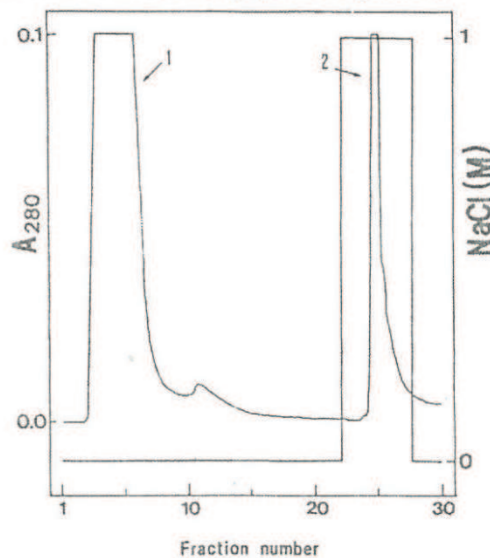


FIGURE 1. Isolation of total wine proteins by FPLC: A wine sample was centrifuged, desalted, lyophilized and subjected to cation exchange chromatography on the Mono S column of the FPLC. The bound proteins were eluted with a 1 M step gradient of NaCl.

Assays for the presence of polysaccharides and protein (Table 1) and SDS-PAGE analysis (results not shown) of the two major  $A_{280}$  peaks obtained revealed the presence of polysaccharides in peak 1 and of the total wine protein in peak 2.

TABLE 1. Polysaccharide and protein content of the Assario wine.

	Assario wine	Peak 1
Total polysaccharides (mg/l of wine)	470.5	446.0
Acid polysaccharides (mg/l of wine)	62.4	37.7
Neutral polysaccharides (mg/l of wine)	408.1	408.3
Protein (mg/l of wine)	111.6	0

A continuous gradient (0 to 1 M) of NaCl was necessary to purify the individual wine proteins (Figure 2). Peak 1, as already mentioned, contained polysaccharides that did not bind to the cation exchanger at pH 2.5. The bound proteins were fractionated into six major fractions (peaks 4, 5, 6, 9, 11 and 12) and a number of minor fractions. Proteins 4 and 5 were selected for the immunological studies. However, because these two proteins were not well resolved when eluted from the Mono S column (Figure 2), before immunization they were further purified by SDS-PAGE.

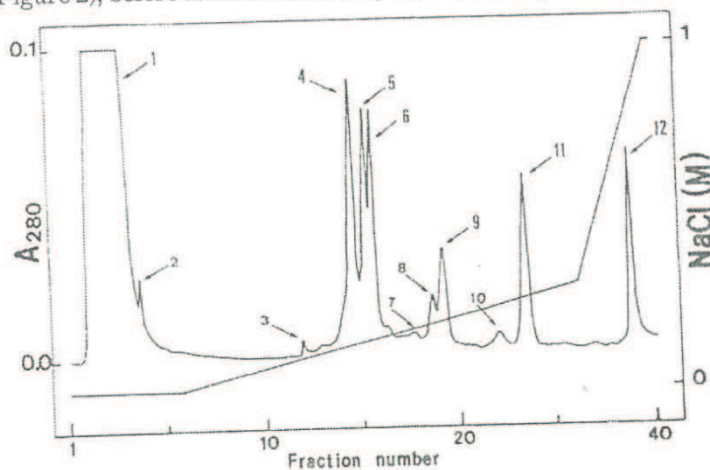


FIGURE 2. Fractionation of the total wine proteins by FPLC: A wine sample was centrifuged, desalted, lyophilized and fractionated by cation exchange chromatography on the Mono S column of the FPLC. The bound proteins were eluted with a continuous gradient of NaCl (0 to 1 M).

**PRODUCTION OF ANTIBODIES:** Three different antigens were used in the immunization of six New Zealand female rabbits (two rabbits for each antigen), the total wine protein, isolated by FPLC (Figure 1, peak 2) and proteins 4 and 5 (Figure 2) purified by FPLC and preparative SDS-PAGE (results not shown). At suitable intervals blood was collected from each rabbit and the titre determined by the ELISA technique (results not shown). These results indicate that a large increment in the titre of all antibodies occurred after the second booster and a subsequent but smaller increment after the third booster. The anti-serum collected after the third booster injection was therefore selected for the subsequent immunological experiments.

**SPECIFICITY OF THE ANTIBODIES:** Highly specific antibodies are of the utmost importance for any immunological method. To adapt the immunoblotting technique to the analysis of wine proteins, a number of preliminary experiments had to be performed (data not shown). Once the conditions were optimized, this technique was used to study the specificity of the antibodies. *Lemna minor* was used as a control not only because the *Lemna* cells contain a very wide range of different proteins but also because two of them are extremely abundant - the large (52 kDd) and small (14.5 kDd) subunits of ribulose biphosphate carboxylase. The SDS-gel presented (Photo 1A) shows the protein patterns of *Lemna minor*, of the total protein and of the specific wine proteins. When the proteins present in the gel were transferred onto a nitrocellulose membrane and the resulting blot stained with

amido black (results not shown) it was observed that the transfer process had been highly efficient. When a similar blot was probed with the anti-total wine protein antibodies (Photo 1B) no signal was produced with the *Lemna* proteins, despite the extremely large number of different structures (proteins) present and the high abundance of some of them. This result clearly indicates that these antibodies against wine proteins possess an extremely high specificity and can be used in various aspects of wine protein science and technology. The anti-total wine protein antibodies produced, as expected, a strong signal with all proteins tested (Photo 1B).

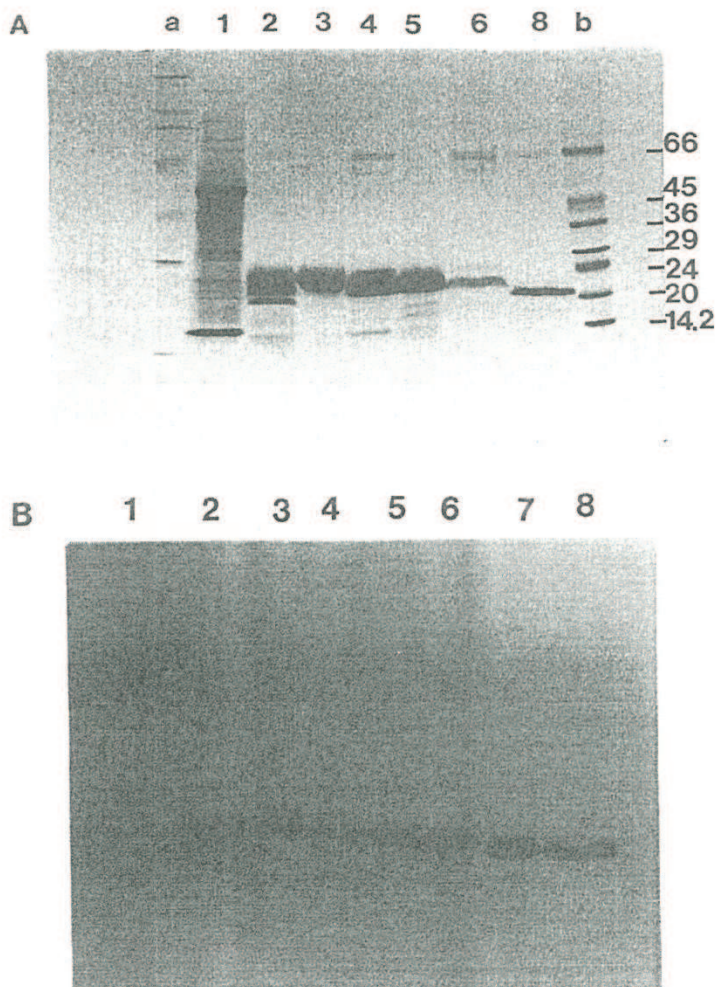


PHOTO 1. Specificity of the antibodies. The protein samples were subjected to SDS-PAGE and the total polypeptides stained with Coomassie Brilliant Blue R (A) or subjected to immunoblotting and probed with anti-total wine protein antibodies (B). Lanes a, b: molecular mass markers (kd); lane 1: *Lemna minor* total soluble protein (40  $\mu$ l extract in A and 20  $\mu$ l in B); lane 2: total wine protein (80  $\mu$ g protein in A and 9  $\mu$ g in B); lanes 3, 4, 5, 6, 7, 8: samples corresponding to peaks 4, 5, 6, 9, 11, 12 (80  $\mu$ g protein in A and 9  $\mu$ g in B), respectively, in Figure 2.

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