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STUDY OF WINE PROTEINS BY IMMUNOLOGICAL METHODS. II-EVIDENCE FOR STRUCTURAL SIMILARITY AMONGST THE MAJOR WINE PROTEINS AND STRUCTURAL DISSIMILARITY WITH CHITINASE AND THAUMATIN

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INTRODUCTION

Highly specific polyclonal antibodies against the whole protein fraction and the individual proteins of a wine prepared from a single grape variety, Assario, were produced. By immunological methods, immunoblotting, it was observed that the antibodies obtained against a highly purified wine polypeptide seem to recognize the other major wine proteins, raising the possibility of structural similarity between the different wine proteins. Grapes from the same region but different, white or red, varieties were, also, found to contain an identical set of proteins. In addition, regardless of the variety, year or region of the grapes used in their production, wines appear to contain a structurally identical or very similar set of proteins. However, neither the anti-total protein antibodies nor the anti-specific wine protein antibodies recognized chitinase or thaumatin.

MATERIALS AND METHODS

GRAPE JUICES AND WINES: Ripened white (Assario, Verdelho, Borrado de Mosca) and red (Tinta Pinheira) grapes were harvested in 1994 in the Dão region, Portugal.

Musts were prepared, under laboratory conditions, from these white and red grape varieties.

Most experiments were performed with the white wine prepared from a single grape variety, Assario. The wine was produced in the Dão region, Portugal, by a conventional microvinification procedure, according to the classical white wine technology. Bentonite was not added during fermentation. Two other Dão wines

were prepared by the same methodology, in 1995, from the single white grape varieties Encruzado and Borrado de Mosca. A fourth wine was prepared by an identical microvinification technique in 1995 and 1996, in Bucelas, Portugal, from the single white grape variety, Arinto. When appropriate, the Arinto wine was treated with bentonite or with bentonite plus casein. Four other single grape variety white wines of commercial origin were also used: Alvarinho (prepared in 1995, in Monção, região demarcada dos vinhos verdes, Portugal), Roupeiro (prepared in 1994, in Alentejo, Portugal), Sauvignon and Chardonnay (prepared in 1994, in Ribatejo, Portugal). 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.

Thaumatin, from *Thaumatococcus daniellii*, and chitinase, from *Serratia marcescens*, were obtained from Sigma (St. Louis, USA).

PREPARATION OF THE PROTEIN SAMPLES FROM GRAPE JUICES AND WINES: Grape juices and wine aliquots were thawed and centrifuged at 15000g for 5 min., and the supernatants 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 residues solubilized in 20 mM citrate-NaOH buffer, pH 2.5, or directly in electrophoresis sample buffer.

PROTEIN CONCENTRATION: Protein content was measured using a modification of the Lowry method, Bensadoun & Weinstein [1976].

PREPARATION OF THE ANTIBODIES: The methods used are described by Monteiro *et al.* [1998].

ELECTROPHORESIS, WESTERN BLOTTING AND IMMUNOBLOTTING: The techniques used are described by Monteiro *et al.* [1998].

RESULTS AND DISCUSSION

STRUCTURAL SIMILARITY OF THE ASSARIO WINE PROTEINS: Monteiro *et al.* [1998] showed the specificity of the antibodies raised against the total protein fraction from the Assario wine. A similar experiment using the anti-protein 4 or the anti-protein 5 antibodies as probes (results not shown) showed that: i) these two antibodies are highly specific, too (no signal was produced with the *Lemna* proteins); ii) the anti-protein 4 antibodies and the anti-protein 5 antibodies produced very strong signals with protein peaks 4, 5, 6 and 9 and weaker signals with peaks 11 and 12 (Figure 2 of Monteiro *et al.* [1998]). Given the very high specificity of the antibodies, these results suggest that proteins 4, 5, 6 and 9 may be structurally similar, whereas proteins 11 and 12 may show a weaker structural similarity when compared with proteins 4 and 5. Similar amino acid sequences have already been reported for distinct protein fractions of a white wine, Waters *et al.* [1996]. These observations could be tentatively explained by the existence of a protein precursor in the Assario grapes, common to all the major wine proteins.

IMMUNODETECTION OF THE ASSARIO WINE PROTEINS IN GRAPE JUICES OF OTHER VARIETIES: Immunological methods were used to determine if different grape varieties possess the same proteins. For that purpose, both white (Assario, Verdelho and Borrado de Mosca) and red (Tinta Pinheira) grape varieties were selected. To minimize the effects of "terroir" the grapes from all varieties were grown in the same year and region (Dão, Portugal). The protein content of the juice prepared from each selected grape variety ranges from 60 μg/g fresh

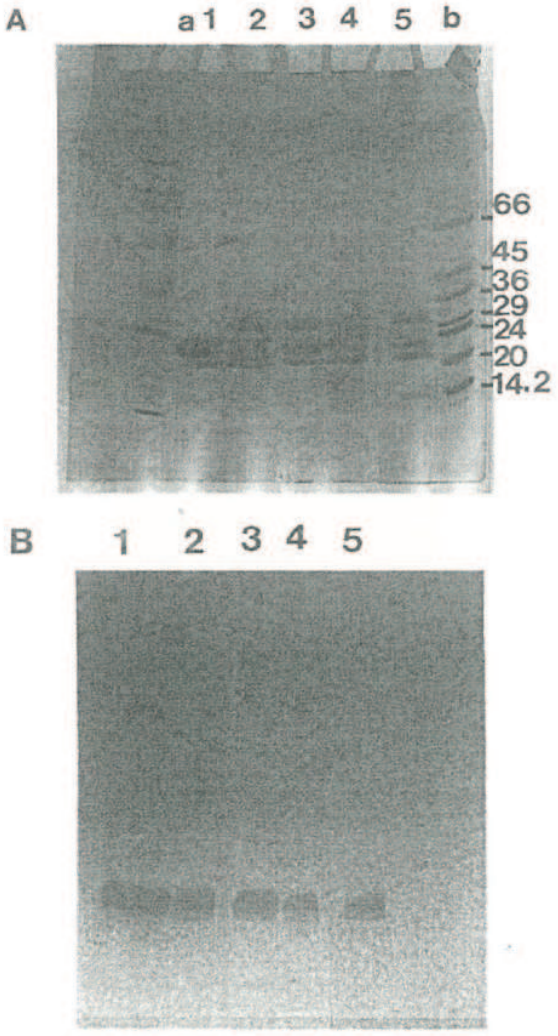

PHOTO 1. Search for identical proteins in different grape varieties. The proteins were isolated, subjected to SDS-PAGE (A) or probed with anti-protein 5 antibodies (B). Lanes a,b: molecular mass standards (kDd); lane 1: total protein from the Assario wine; lanes 2, 3, 4 and 5: total protein from Assario, Verdelho, Borrado de Mosca and Tinta Pinheira juices, respectively. The protein loaded in each lane was: 150 μg (A), 20 μg (B).

weight (Verdelho) to 110 μg/g fresh weight (Tinta Pinheira). The SDS-PAGE gel presented (Photo 1A) shows that the different grape varieties analysed possess proteins with a similar range of molecular masses, regardless of their protein content. Furthermore, a comparison (Photo 1A) between lanes 1 (total soluble protein from the Assario wine) and 2 (total soluble protein from the Assario grape juice) indicates that the protein pattern is markedly altered during vinification. When the proteins of the selected grape varieties were blotted onto a nitrocellulose membrane and the resulting blot probed with appropriate antibodies, the immunoblots obtained (Photo 1B) clearly indicate that grapes from different varieties, white or red, grown under similar conditions contain an identical set of proteins.

IMMUNODETECTION OF THE ASSARIO WINE PROTEINS IN OTHER WINES: Immunological methods were used in the analysis of proteins from a range of different wines. Single grape varieties, white or red, produced in distinct regions of Portugal and prepared by microvinification techniques or of commercial origin were selected. The major characteristics and the protein content of these wines varied widely (data not shown). SDS-PAGE of a fixed amount of protein from each wine (Photo 2A) shows that different wines, produced in different years, in distinct regions of Portugal and from different grape varieties contain proteins of similar size. Furthermore, immunoblot analysis of the blotted wine proteins using anti-protein 5 antibodies (Photo 2B) revealed that all wines tested contain structurally identical or very similar proteins regardless of grape variety, year or region.
Waters *et al.* [1996], studying a white wine, identified proteins which showed homology to pathogenesis-related proteins: the proteins in some fractions had N-terminal amino acid sequences similar to thaumatin, whereas others possessed N-terminal amino acid sequences similar to plant chitinase. To detect the presence of thaumatin-like or chitinase-like proteins in the Assario wine, the total wine protein, thaumatin and plant chitinase were subjected to SDS-PAGE, tranferred to a nitrocellulose membrane and probed with anti-total protein, anti-protein 4 or anti-protein 5 antibodies (data not shown). The results obtained clearly indicate that the antibodies tested recognized neither thaumatin nor chitinase, suggesting the absence of proteins in the Assario wine with sequences similar to the pathogenesis-related proteins. Therefore, the evidence produced by immunological methods did not support the observations reported by Waters *et al.* [1996].

The experiments performed show: (i) the major Assario wine proteins show some degree of structural similarity; (ii) different white or red grape varieties possess an identical set of proteins; (iii) wines prepared by microvinification techniques or of commercial origin, from grapes of distinct varieties, grown under different climate and/or soil conditions also contain an identical set of proteins; (iv) structural dissimilarity between the Assario wine proteins and thaumatin and chitinase.

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