Analysis of the provenience of the grape proteins

S. Monteiro[1], M.A. Piçarra-Pereira[1,2], P.R. Mesquita[1], M.C. Tanganho[1], V.B. Loureiro[1], A. Teixeira[1] and R.B. Ferreira[1,3]

[1]*Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1349-017 Lisboa Codex, Portugal.*
[2]*Escola Superior Agrária, Instituto Politécnico de Castelo Branco, 6001 Castelo Branco Codex, Portugal.*
[3]*Instituto de Técnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal.*

INTRODUCTION

Advances in scientific knowledge are typically closely followed by the development of new technological methods. In the particular case of wine protein research, the application of modern biological techniques has contributed considerably to elucidate the structure of the wine proteins and the problems associated with their presence in wines. Over the last decades, immunology has been an expanding area of biological research. Despite its wide application in most areas of modern biology, this promising tool has never been used to study wine proteins. The use of immunological methods in wine protein research may answer many questions about the structure of these polymers and ultimately contribute to the development of a powerful methodology, capable of removing them specifically from wines.

In the present work the antibodies previously produced (Monteiro *et al.*, 1999) are used to analyse the provenience of the wine proteins.

MATERIALS AND METHODS

Ripened Assario grapes and leaves were harvested in 1994 in the Dão region, Portugal, and used as a source of plant material. *Saccharomyces cerevisiae* yeasts

(Fermol Bouquet strain) was also used.

The Assario wine (Dão region, Portugal, 1994) was prepared according to the classical white wine technology. Bentonite was not added during fermentation. After each bottle was opened, 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.

Isolation and concentration of the soluble proteins from the Assario wine. - As described by Monteiro *et al.* (1999).

Extraction of the total soluble protein from the biological material.

(1) Grape skin – grape skins were frozen in liquid nitrogen, then ground to a fine powder and the total soluble protein extracted (2 ml. g^{-1} fresh weight) in 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was filtered through two layers of cheesecloth, centrifuged at 40,000 g for 10 min at 2°C and the supernatant desalted at 2°C on a Sephadex G-25M prepacked PD-10 column (Pharmacia/LKB, Uppsala, Sweden) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.5.

(2) Pulp – A similar procedure to the one described for the grape skin was used to extract the soluble proteins from the pulp except that the plant material was not frozen in liquid nitrogen and that a different amount of extraction buffer was used (0.5 ml. g^{-1} fresh weight).

(3) Grape seed – Different approaches were tested to optimise the extraction of proteins from the seeds. Grape seeds frozen were in liquid nitrogen and ground to a fine powder. The resulting powder was deffated with n-hexane (8 ml. g^{-1} fresh weight), the mixture allowed to settle and decanted. The residue was air-dried, dissolved in electrophoresis sample buffer (12 ml. g^{-1} fresh weight), incubated at 100°C for 5 min and clarified by centrifugation during 10 min at 20,000 g

(4) Stem – The stems were ground to a fine powder in liquid nitrogen and the soluble protein extracted (4 ml g^{-1} fresh weight) in 100 mM Tris-HCl buffer, pH 7.5, containing 1% (w/v) sodium dodecyl sulphate (SDS) and 1% (w/v) 2-mercaptoethanol. The homogenate was centrifuged at 14,000 g for 15 min at room temperature and the supernatant subsequently desalted on a PD-10 column previously equilibrated with 20 mM Tris-HCl buffer, pH 7.5.

(5) Leaves – The extraction of proteins from vine leaves proved to be a very difficult task, probably due to their high content in secondary metabolites, tannins included. Once again, several procedures were tested. The one which led to better results was developed in this laboratory in the extraction of proteins from *Eucalyptus* leaves. The leaves were initially lyophilised, then ground in liquid nitrogen to a fine powder and washed consecutively, for 10 min at 4°C, with the following three mixtures: methanol -acetic acid – water (10: 1: 9; 60 ml g^{-1} fresh weight); n-hexane (40 ml g^{-1} fresh weight); and acetone (40 ml g^{-1} fresh weight). After each wash, the mixture was centrifuged for 10 min at 20,000 g and 4°C. The proteins were then extracted from the final precipitate with 80 mM Tris-HCl buffer, pH 6.8, containing 0.1 M 2-mercaptoethanol, 2% (w/v) SDS, 15 % (v/v) glycerol and 0.006% (w/v) m-cresol purple. The mixture was subsequently incubated at 100°C for 5 min, centrifuged at 14,000 g for 15 min at room temperature and desalted on a PD-10 column previously equilibrated in 20 mM Tris-HCl buffer, pH 7.5.

(6) Yeast – A mixture containing 75 ml toluene and 0.7 ml 2-mercaptoethanol was added to 16 g of yeast. The suspension was liquefied by incubation at 37°C with agitation. To this mixture, 365 ml of a solution containing 15 mM EDTA and 5 ml 2-mercaptoethanol, pH 7.0, were added. The solution was incubated overnight at room temperature and centrifuged for 40 min at 14,000 g and 4°C. The floating layer of lipids was removed and the supernatant, containing the yeast soluble proteins, was collected.

Protein measurement.–Protein content was determined by a modification of the Lowry method (Bensadoum and Weinstein, 1976)

Preparation of antibodies specific for the total or individual wine proteins.–As described by Monteiro *et al.* (1999).

Electrophoresis.–Sodium dodecyl sulphate-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 kDa subunit of rabbit muscle myosin to the 14.2 kDa of bovine milk .α -lactalbumin.

Western Blotting and Immunoblotting.–The techniques used are described by Monteiro *et al.* (1999).

RESULTS, DISCUSSION, CONCLUSIONS

Immunological study on the provenience of the Assario wine proteins.– The total soluble protein was extracted from each constituent that may potentially contribute to the Assario wine proteins, namely the Assario cluster (including skin, pulp, seed and stem), the Assario leaves and the *S. cerevisiae* yeast. Distinct extraction procedures had to be optimised for each biological material due to the great difficulty encountered in extracting proteins from some of them.

Yeast cells are by far the richest in protein (780.0 μg g^{-1} fresh weight). Assario grape seeds are particularly rich in protein (290.0 μg g^{-1} fresh weight), followed by the leaf (192.0 μg g^{-1} fresh weight). Pulp (95.6 μg g^{-1} fresh weight) and stem (71.0 μg g^{-1} fresh weight) contain low levels of protein, whereas the grape skin, apparently, only contains trace amounts of these polymers (1.6 μg g^{-1} fresh weight).

When the total pattern of polypeptides from each of the biological materials was analysed by SDS-PAGE, (Fig.1A), it was observed that yeasts contain a huge number of different polypeptide chains, covering a wide range of molecular masses, (Fig.1A, lane 2) and pulps present a limited number of polypeptide chains with molecular masses between 20 and 36 kDa (Fig. 1A, lane 3).

It is interesting to note the differences observed in the polypeptide patterns when the total pulp proteins are compared with the total wine proteins (Fig. 1A, lane 1), highlighting that important changes occur during vinification.

The seed contains one major type of polypeptide chain, with a molecular mass of 24 kDa (Fig. 1A, lane 5). Stems (Fig. 1A, lane 6) and leaves (Fig. 1A, lane 5) show poorly resolved SDS-PAGE profiles as a consequence of the extreme difficulty in extracting proteins from these plant tissues, despite the optimized methods used for this purpose.

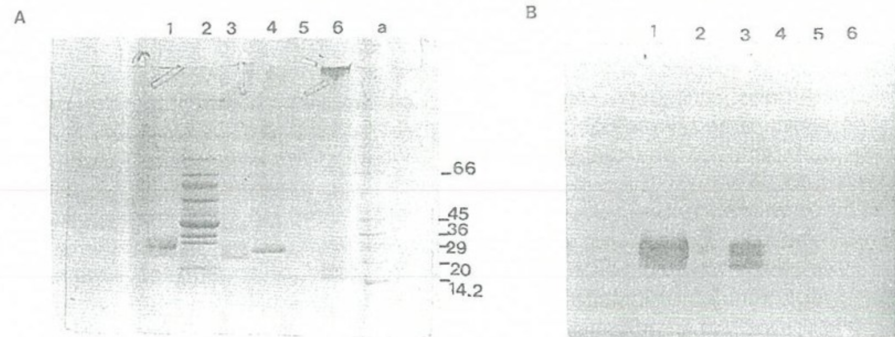

Figure 1.–*Provenience of the Assario wine proteins.* The total soluble protein from the Assario wine (control, lane 1), *S. cerevisiae* (lane 2) and the Assario pulp (lane 3), seed (lane 4), leaf (lane 5) and stem (lane 6) were isolated, subjected to SDS-PAGE (A) or probed with anti-polypeptide 5 antibodies (B), as described in the Material and Methods section. Molecular masses of standards are indicated in kDa.

When the polypeptides present in a gel identical to that shown in Fig. 1A were blotted onto a nitrocellulose membrane and probed with anti-polypeptide 5 antibodies (Fig. 1B), the resulting blot shows a number of features:

a) the antibodies (anti-polypeptide 5) recognised the total pattern of the Assario wine polypeptides, suggesting (Monteiro *et al.* 1999), the existence of a structural similarity among the major wine proteins;

b) the recognition, in all cases, of pulp polypeptides, but not of polypeptides from the yeast, seed, leaf or stem, indicating that the Assario wine proteins are originated from the pulp;

c) the signal produced when the total pulp proteins are probed with the antibodies (Fig. 1B, lane 3) are identical to the signals produced when the total wine proteins are probed with the antibodies (Fig. 1B, lane 1) and not to the pattern of total pulp polypeptides (Fig. 1A, lane 3). This observation suggests not only that all the Assario wine proteins are present in the pulp, but also that the pulp contains other unrelated proteins that do not end up in the wine.

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