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Analysis of the soluble proteins in grape must by two-dimensional electrophoresis

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ABSTRACT

Soluble proteins were separated from grape musts by two-dimensional electrophoresis. Analysis of the electrophoretic patterns obtained yielded information about the structure and composition of these proteins. Structural and/or functional relationships between certain proteins, according to the fractions separated in the two dimensions, were also established.

INTRODUCTION

Previous interesting results from applying different one-dimensional electrophoretic techniques to the analysis of grape must proteins [1–3] encouraged us to study the possibility of applying a two-dimensional separation. Two-dimensional electrophoretic techniques have frequently been applied in the study of complex systems [4,5] and in the characterization of simple proteins [6]. The use of two concurrent separation criteria offers additional information, although the interpretation of the resulting electrophoretic patterns is often complex.

The protein compositions of many foods have been analysed using two-dimensional gel electrophoresis, including milk and dairy products [7,8], beer [9], cereals [10,11], grape must and wine [1,12].

The study of grape must and wine proteins is interesting from a genetic standpoint with a view to using the results in taxonomic classifications [2,13] and also as an indicator of viral infections, which cause changes in the patterns of the isozymes peroxidase and polyphenoloxidase [14].

In this work, two-dimensional gel electrophoresis was used to study the soluble protein fraction of different varieties of grape musts. The proteins were separated by native polyacrylamide gel electrophoresis (PAGE) in the first dimension, based on their relative electrophoretic mobilities (R_F). Sodium dodecyl sulphate (SDS)-PAGE in the second dimension was then used to achieve further separations according to the

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different molecular weights of the protein subunits originally constituting the proteins.

Separation based on the relative mobility (R_F) rather than on the isoelectric point (p*I*) conventionally employed in the first dimension was chosen because the former yields more interesting information for the genetic characterization of the variety of musts. A perfect classification of six different cultivars of Spanish origin was obtained by applying multivariate statistical methods (principal component and cluster analysis) to the R_F values for must proteins [2]. Information useful in genetic characterization according to variety has not been derived from isoelectric focusing (IEF), although the information so obtained has been useful in drawing other conclusions [3].

EXPERIMENTAL

Samples

Six samples of the following varieties were studied: Airen (2), Cabernet Sauvignon (2), Moscatel (1) and Monastrell (1). The corresponding musts were collected in various regions in Spain.

Materials

Analytical-reagent grade chemicals were used and deionized water was employed for all solutions. Polyethylene glycol (PEG) 20 000, glycerol, β-mercaptoethanol, SDS, silver nitrate and N,N,N′,N′-tetramethylenediamine (TEMED) were supplied by Merck (Darmstadt, Germany), acrylamide, N,N′-methylenebisacrylamide and tris(hydroxymethyl)aminomethane (Tris) from Sigma (St. Louis, MO, U.S.A.), Coomassie Brilliant Blue G-250 dye (Serva Blau) from Serva (Heidelberg, Germany) and absolute ethanol and acetic acid from Panreac (Barcelona, Spain).

Sample preparation

The musts were obtained by light pressing. They were centrifuged at 4000 *g* for 15 min and the proteins were separated by dialysis of 100 ml of must against tap water for 18 h using a Spectra POR3 membrane, which retains molecules with molecular weights greater than 3500 dalton. The samples so obtained were concentrated 40-fold by introducing the dialysis bags into 20% (w/v) PEG 20 000 solution.

Two-dimensional gel electrophoresis

Native PAGE was applied in the first dimension to separate the proteins according to their relative electrophoretic mobilities. The first dimension electrophoresis was performed according to the method of Hillier [15] employing polyacrylamide gels (70 × 80 × 0.75 mm) at 9.4% T and C = 4.25[a]. The buffer used in the gels was 0.0625 *M* Tris–HCl buffer (pH 8.8).

Vertical electrophoresis of the samples (10 μl of protein solution concentrated 40-fold, 10 μl of glycerol and 2 μl of bromophenol blue) was performed at I = 2.5 mA per plate for 2 h. The gels were stained with Coomassie Brilliant Blue G-250 as described by Blakesley and Boezi [16].

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[a] C = g N,N′-methylenebisacrylamide (Bis)/%T; T = g acrylamide + g Bis per 100 ml of solution.

SDS-PAGE was applied in the second dimension to determine the molecular weight of the subunits of the proteins separated in the first dimension. Strips of the first-dimension gels were cut and equilibrated for 5 min in a 0.0625 *M* Tris–HCl buffer solution (pH 8.8) containing 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol and 2.5% (w/v) SDS.

It was determined experimentally that equilibration for 5 min sufficed for the denaturation reaction and formation of the SDS–protein complex.

The gel strips were placed on glass plates, following which the second-dimension gel slabs were prepared (Fig. 1). The plate size was 70 × 80 × 0.75 mm. The composition of the gels per 15 ml of solution was as follows: acrylamide–N,N′-methylenebisacrylamide solution (80:0.8), 7.5 ml; 3 *M* Tris–HCl buffer (pH 8.8), 1.9 ml; distilled water, 5.0 ml; ammonium peroxodisulphate, 0.15 ml; SDS, 0.15 ml; and N,N,N′,N′-tetramethylenediamine, 15.0 μl.

The polymerization of the gels provided perfect fusion between the gels, minimizing the possible distortion when the bands enter the new gel. Vertical electrophoresis in the second dimension was run at a constant current of 5 mA per plate for 3 h. The gels were stained with silver nitrate following a modified procedure of

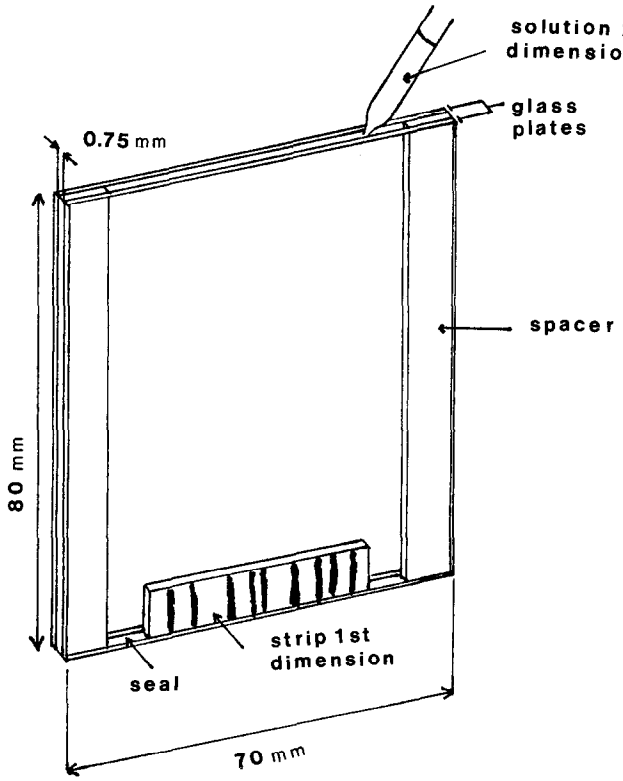

Fig. 1. Preparation of slab gels for the second dimension: the strips of the first-dimension gels are placed in the bottom of the glass plate sandwiches and the second dimension solution is poured from the top in order to avoid the formation of air bubbles; polymerization results in a perfect fusion between the two gels, facilitating migration of the bands.

Blum *et al.* [17], placing the gels in a fixer solution (30% ethanol–10% acetic acid) for 3 h in order to eliminate the excess of SDS, and thereby prevent the gels from blackening too rapidly under the effect of the silver nitrate.

RESULTS AND DISCUSSION

The gel formation technique employed enabled flat gels to be used in the first dimension, as opposed to the traditional use of tubes [4,8,19]. In addition, the two-dimensional electrophoresis with double separation on flat gels permitted the use of techniques other than IEF with high-resolution in the first dimension.

The use of flat gels yielded improved resolution and shortened separation, equilibration, and staining times. Further, polymerization of the second gel on top of a previously equilibrated strip allowed the proteins to pass from one gel to the other without distortion.

Table I gives the R_F data (calculated with respect to bromophenol blue) obtained for the different samples in the first dimension. Twelve bands were obtained.

Complex patterns resulted from the application of the second dimension (Fig. 2). Several of the formerly single bands split into various spots. In order to simplify the study, a general pattern of the separations obtained for the different samples was constructed with all the bands (Fig. 3); to interpret this pattern, the bands of the first dimension for each sample (Table I) are considered and the bands for the other samples disregarded, yielding the pattern for the variety concerned.

The analysis of this pattern showed that the most important fraction of protein subunits was that with a molecular weight of *ca.* 30 000 dalton [1,3,8].

Application of the two-dimensional technique showed that bands V, VI, VII, X and XI were those which contained the subunits with these molecular weights whereas bands III, IV and XII yielded fractions with lower molecular weights.

Some bands (I, II, III, IV and XII) did not split; hence these bands contained proteins with a simple structure, made up of one or more identical subunits.

Bands VIII and IX split into two spots, which may be different subunits of a single protein or of different proteins with the same mobilities that were not separated in the first dimension.

TABLE I

DISTRIBUTION OF THE BANDS SEPARATED IN THE FIRST DIMENSION BY APPLYING NATIVE PAGE TO THE SOLUBLE PROTEINS FROM GRAPE MUST OF DIFFERENT VARIETIES

+ Means present and − means absent in the sample.

Grape	Relative mobility (R_F)											
	0.09 I	0.13 II	0.30 III	0.35 IV	0.39 V	0.43 VI	0.46 VII	0.51 VIII	0.55 IX	0.63 X	0.70 XI	0.85 XII
Airen (2)	−	−	−	+	+	−	+	+	+	−	+	−
Cabernet Sauvignon (2)	+	+	+	+	+	+	+	+	−	−	−	−
Moscatel (1)	+	+	−	+	−	+	+	+	−	+	+	+
Monastrell (1)	+	+	+	+	+	−	+	+	+	+	−	+

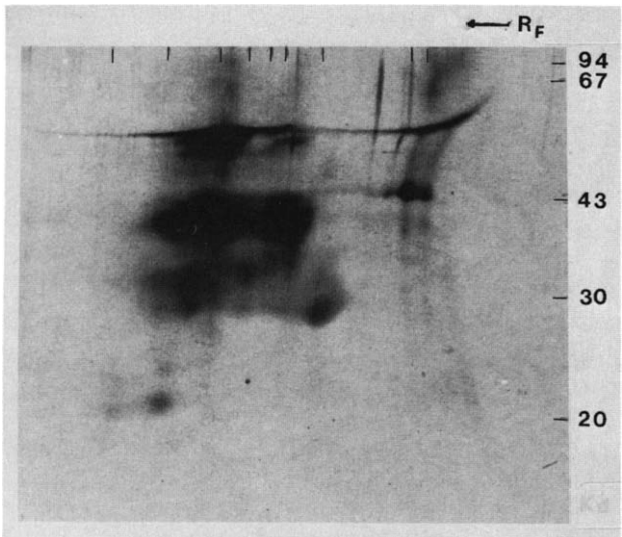

Fig. 2. Must proteins (Moscatel variety) separated by two-dimensional PAGE and stained with silver nitrate. Numbers on the ordinate represent molecular weight in kilodalton.

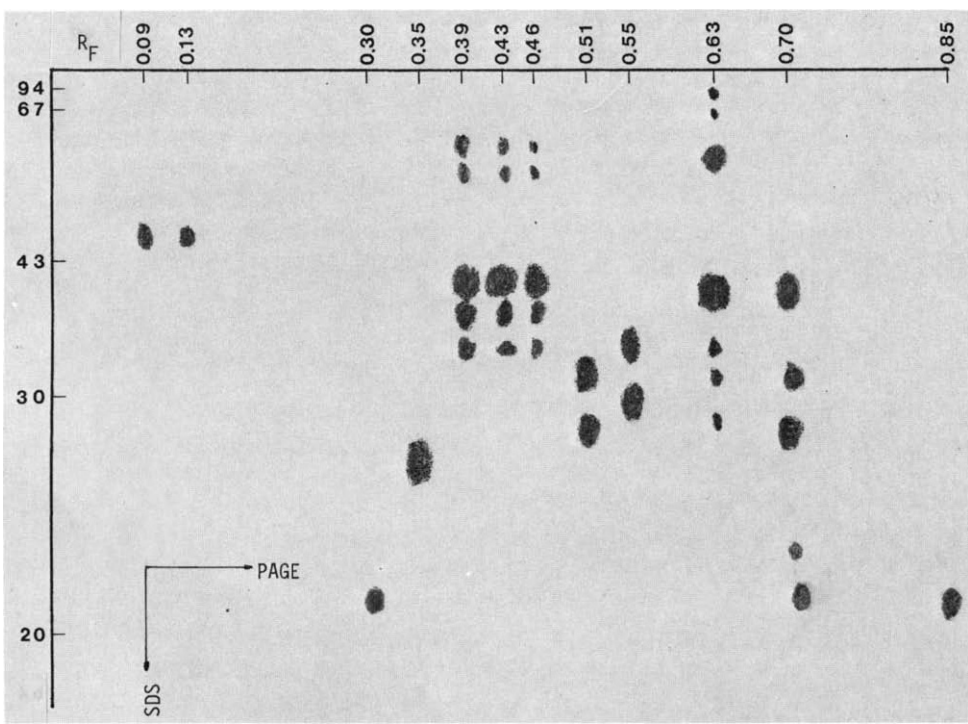

Fig. 3. A general banding pattern obtained by two-dimensional electrophoresis of must proteins of different varieties (see text for interpretation). Numbers on the ordinate represent molecular weight in kilodalton.

Bands V, VI, VII, X and XI split into several spots, indicative of a group of proteins that were incompletely resolved or a complex protein group of enzymic nature formed by unlike subunits with different molecular weights. Enzymic systems such as this have been described by Wolfe [20], Sciancalepore *et al.* [21] and others.

It is interesting that bands V, VI and VII split into the same subunits. This suggests proteins of a similar nature, which is very useful when trying to determine their characteristics and also results in time saving when planning future experiments (on enzymic activity, sequentially, etc.).

CONCLUSIONS

Two-dimensional electrophoresis with double separation on flat gels permits the use of techniques other than IEF in the first dimension without loss of resolution.

Application of native PAGE plus SDS-PAGE to soluble grape must proteins yielded important information about the characteristics of the composition of the subunits and protein structure, and also gave some indication of protein similarity as reflected by their electrophoretic behaviour. This information was in part speculative but nonetheless valuable.

In view of the difficulties attached to the study of proteins, especially because of their molecular complexity, the use of two-dimensional electrophoresis in the study of must proteins can be extremely useful in simplifying analyses and confirming results.

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