

Comparative investigations on soluble proteins and malate dehydrogenase of four grape varieties by isoelectric focusing

by

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Vergleichende Untersuchung über die löslichen Proteine und die Malat-Dehydrogenase von vier Rebsorten mittels isoelektrischer Fokussierung

Zusammenfassung. — Die löslichen Proteine und multiplen Formen der Malat-Dehydrogenase (E.C. 1.1.1.37) sowohl in der Schale als auch im Saft der Beeren von vier Rebsorten wurden in bezug auf Heterogenität und isoelektrischen Punkt verglichen. Die Proteinpräparate ließen sich in 17 bis 28 deutlich unterscheidbare Komponenten auf trennen. Aufgrund übereinstimmender pI-Werte besaßen alle Sorten in der Schale sieben und im Saft acht gleiche Komponenten. Auch wurden in den beiden Teilen der Beere zwei bis sieben charakteristische Proteinbanden beobachtet.

Das Muster der NAD-Malat-Dehydrogenase in der Beere setzt sich aus 9 bis 13 isoelektrischen Formen zusammen, die offensichtlich in drei Gruppen von Isoenzymen angeordnet sind. Die einzelnen Gruppen waren in den Gelen ziemlich übereinstimmend lokalisiert; aber die einzelnen Sorten besaßen sowohl in der Schale als auch im Saft eine unterschiedliche Anzahl von Enzymen eines bestimmten pI-Wertes. In den Schalen wurde eine vergleichsweise größere Anzahl unterschiedlicher Isoenzyme gefunden als in den Säften. In den beiden Beerenteilen einer Sorte wurden zwei bis vier Isomalat-Dehydrogenasen mit übereinstimmendem pI-Wert ermittelt, während die restlichen Isoenzyme anscheinend nicht identisch und somit wohl auf ungleiche Gene zurückzuführen waren.

Introduction

A previous work (PALLAVICINI 1971) using disc electrophoresis has shown the multiplicity of six of the seven grape enzymes tested, and the remarkable difference in the amount of isoamylases in the skins of two varieties of *Vitis vinifera* and of four Seyve Villard hybrids. More recently, RADOLA and RICHTER (1972) using thin-layer isoelectric focusing observed an identical pattern with respect to the major zones of activity for polyphenoloxidase, and a quite different pattern for peroxidase, in the juice of five grape varieties.

This report describes the isoelectric gel patterns of soluble proteins and malate dehydrogenase (MDH) isoenzymes in skins and juices of four *V. vinifera* grapes, the general purpose being to gain data helpful in elucidating taxonomic and phylogenetic relationships in genus *Vitis*. Moreover, a thorough knowledge of the grape proteins could be useful in order to accurately orientate the industrial process of wine production. Information on enzymatic proteins of grape is still scarce (FLANZY 1967, POUX 1967, CORDONNIER and DUGAL 1968, AMERINE and JOSLYN 1970, MARTEAU 1972).

Materials and Methods

Materials

Two black (Nebbiolo, Merlot) and two white (Garganega, Trebbiano) *V. vinifera* varieties were used.

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Separation of skin and juice from berry and their acetone powder preparation

2 kg of technologically ripe berries were harvested and immediately washed and crushed. The juice was separated by filtration on four layers of cheesecloth and stored at -10°C for at least 2 h. After this time, 25 volumes of cold (-20°C) acetone were added and left to stand overnight. The pellet was collected by centrifugation at 37000 g for 20 min and then evaporated to dryness under vacuum. Finally, the resultant residue was lyophilized.

Seeds and pulp residues having been accurately removed by hand, the skins were thoroughly washed with running tap water. The washed skins were homogenized at full speed for four periods of 15 secs, with 2 min intervals for cooling (ice bath), in an "Ultra Turrax homogenizer" in presence of cold acetone, followed by 4 washings also with cold acetone. The pellet was then dried under vacuum and lyophilized.

Extraction and purification procedure

The lyophilized powders of skins (5 g) and juice (2 g) were both homogenized once more, and well dispersed in presence of 25 volumes/g powder of Na-borate 0,2 M, pH 9,0, containing 2 mM dithiothreitol, 2 mM EDTA, 4 mM ascorbic acid and 2% polyclar AT. The slurry was stirred for 2 h and then strained through four layers of cheesecloth to separate cellular debris and polyclar. The homogenate was centrifuged at 12000 g for 15 min and the supernatant recovered. The solid matter was submitted to a second and, finally, to a third extraction. The pooled borate extracts were mixed together and solid ammonium sulphate was added to a 100% saturation by stirring over a 30 min period. This 100% saturation precipitate was collected at 37000 g for 20 min and then resuspended in a small volume (15–20 ml) of Na-borate 0,2 M, pH 8,7. After centrifugation (3000 g for 15 min) of the suspension, the supernatant collected was dialyzed against four changes of distilled water containing 0,005% of β -mercaptoethanol. During 48 h dialysis, a new precipitate appeared, which was removed with another centrifugation at 3000 g for 10 min. The pellet sedimenting of dialysis was discarded, because experiments (not described here) showed that this material had no malate dehydrogenase activity. The supernatant of the latter centrifugation was then lyophilized. Aliquots of these lyophilized extracts were suspended in acrylamide gels for gel electrofocusing. The same general procedure for protein extraction was applied to all varieties. All operations were carried out at $0-4^{\circ}\text{C}$.

Isoelectric focusing

The technique of isoelectric focusing in acrylamide gel was employed largely as described by WRIGLEY (1971), with 2,5% of carrier ampholytes (LKB), pH range 3–10, and lyophilized protein extract cast in $0,6 \times 12$ cm glass tubes. Electrofocusing took place in a Bio-Rad chamber, cooled at about 2°C , with water circulating from a thermostated cooling water bath through the cooling jacket; run time 6 h; potential at the end of runs 240 V; cathode solution 0,4% monoethanolamine; anode solution 0,2% sulphuric acid.

Protein and enzyme stains

The gels, deprived of carrier ampholytes by washing in 10% trichloroacetic acid, were stained for proteins with either Amido Black 10 B or Coomassie R 250. The malate dehydrogenase was coloured at pH 8,5 for 30 min as reported by SHAW and KOEN (1968). A control gel included all constituents except substrate.

Densitometric analysis

Densitometry of the gels was performed with a Servogor (Gelman), fitted at 520 nm.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951).

Estimate of pH gradient and isoelectric points

The pH gradients and the isoelectric points of protein bands and isoenzymes were estimated according RIGHETTI and DRYSDALE (1971). The pH measurements were made with a pH meter (Radiometer, Copenhagen) equipped with a microelectrode unit. The measurements were performed at 22 °C.

Preliminary experiments were performed to determine the most consistent medium to be employed for proteins and malate dehydrogenase extraction, using gel isoelectric focusing. The following buffers were tested: tris-glycine 0,2 M, pH 8, containing 0,5% of triton X-100; Na-acetate 0,2 M, pH 6,5; citrate-phosphate 0,2 M, pH 8,0 and Na-borate 0,2 M, pH 9,0; all containing 2 mM dithiothreitol, 2 mM EDTA, and 4 mM ascorbic acid. The greatest number of proteic and MDH bands was observed with Na-borate buffer extracts. The resolution supplied by the latter was further improved when 2% polyclar AT (w/v) was added (PECH *et al.* 1970). Variations in the pH, i.e. 7,6 (KING 1971), and 8,5, ionic strength, and in the amount of polyclar added (POUX and OURNAC 1972), were also tried. None of these variations led to an increase in the relative amount or in the resolution of proteic or enzymatic components. The protein content of borate-polyclar extracts was essentially uniform. All patterns were reliable, reproducible, and had sharp bands when the amount of protein present in the extracts fell to between 100 and 200 µg.

Results and Discussion

Soluble proteins

Typical patterns from some representative runs of grape proteins are given in a schematic form in Fig. 1. Gel electrofocusing in the pH range 3—10 revealed a considerable extent of heterogeneity of grape proteins, whose complex isoelectric points varied from pH 3,4 and pH 8,6. Despite the great protein heterogeneity, which seriously limited the comparison among the patterns, both similarities and differences could be detected.

The occurrence of seven bands with the same isoelectric point was found in all samples of grape skins examined (Fig. 1, top half). Five of these bands were localized in the more acidic region of the gels (pH 3,4 to 4,8), one at about pH 5,3, and the remaining at pH 6,2. The first two common bands, starting from the anodal side of the gels, were present with a very similar stain intensity. The other five common pI bands were of non-identical stain intensity for all skins of the four varieties. Besides these, there were a few other bands with the same pI in two or three varieties only. These were, however, always of different stain intensity. In addition to these proteins, all varieties showed two (Merlot) to four (Nebbiolo) peculiar bands (Table 1) with intermediate pI values which did not correspond to any of the intermediate forms observed in the other varieties. All of these values were highly reproducible, yielding distinct and perfectly symmetrical peaks, although some of them were faint bands and, thus, presumably minor components of the preparations. Both particular bands of Merlot were markedly basic proteins. In the most cathodic zone of the gel (pH 6,5—8,3) the Merlot extract displayed seven proteic fractions, whereas the same zone of gel belonging to Garganega showed one fraction only.

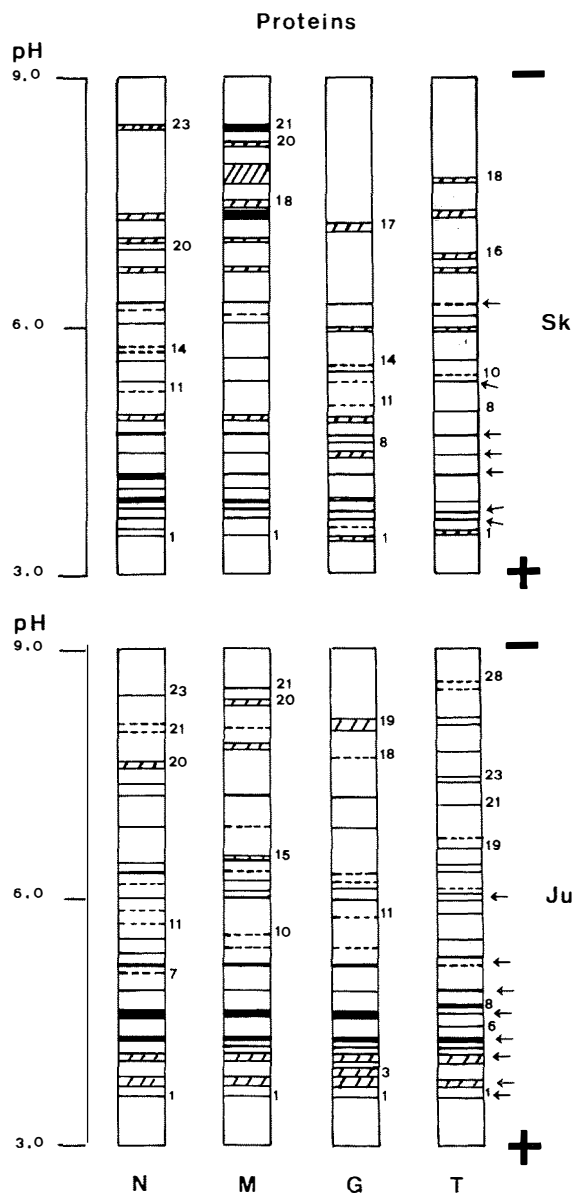


Fig. 1: Analytic isoelectric focusing of soluble proteins of four grapes. From left to right grape varieties: Nebbiolo (N), Merlot (M), Garganega (G), Trebbiano (T). Top half: skins (Sk); bottom half: juices (Ju). pH gradient 3—10. The arrows indicate the bands observed with the same pI value in all varieties.

Analytische isoelektrische Fokussierung löslicher Proteine aus 4 Rebsorten. Von links nach rechts: Nebbiolo (N), Merlot (M), Garganega (G), Trebbiano (T). Oben: Schalen (Sk); unten: Säfte (Ju). pH-Bereich 3—10. Die Pfeile bezeichnen Banden mit gleichem pI-Wert bei allen Sorten.

Table 1
Peculiar bands of the four grape varieties¹⁾
Charakteristische Banden der vier Rebsorten

Nebbiolo	Merlot	Garganega	Trebbiano
Skins			
11 (5,2)	18 (7,5)	8 (4,7)	8 (5,0)
14 (5,7)	20 (8,2)	11 (5,1)	10 (5,4)
15 (5,8)	—	13 (5,4)	16 (6,8)
20 (6,9)	—	—	—
Juices			
7 (5,1)	10 (5,7)	3 (3,9)	6 (4,4)
11 (5,7)	15 (6,5)	11 (5,9)	8 (4,7)
20 (7,6)	20 (8,4)	18 (7,8)	19 (6,6)
21 (8,0)	—	—	20 (6,8)
23 (8,5)	—	—	21 (7,1)
—	—	—	23 (7,3)
—	—	—	28 (8,6)

¹⁾ See Fig. 1 for the location of bands; in brackets their average pI values.

Like the skins, the juices of the four varieties demonstrated both similarities and differences in their patterns (Fig. 1, bottom half). In fact, there were eight common components, seven of which were situated towards the anodal side of the gels. The remaining common component was in the intermediate pH zone of the gels, approximately at pH 6,0. The presence of band pI 4,3 in all samples tested, indicated its similar occurrence among varieties. In the cathodal zones (pH 7,0—8,6), many bands had a specific pI value. Among the juices, that of Trebbiano showed a particularly complex protein mixture, with 28 fractions when the same amount of protein was electrofocused. Of these, seven were particular fractions (Table 1).

The identity of the variety-specific components could be of interest, since, if they are enzymes or isoenzymes, they may prove to be useful biochemical gene markers.

From a comparison between the patterns of skin and juice within each variety, it appeared that the proteic complex of the latter was more heterogeneous in two cases (Trebbiano and Garganega) than that of the former, and in the remaining two (Nebbiolo and Merlot) the proteic complex had the same proteic fraction numbers. The first two are white varieties, whilst the others are black. This may have evolutionary significance. However, to confirm this result, many other varieties remain to be analysed. Most of the bands present in skins differ from those of the respective juices with regard to pI value.

From these results, it may be concluded that the varieties investigated do not form a uniform group. Electrofocusing revealed considerable protein heterogeneity for skins and even more for juices.

Malate dehydrogenase (E.C. 1.1.1.37)

As can be seen from Fig. 2, multiple forms of the enzyme could be revealed for all skins and juices on isoelectric focusing in the ampholytes pH 3 to 10.

The enzyme activity extractable from skins, separated into 13 molecular forms for three of the varieties studied, and into 11 for the remaining one. All of the com-

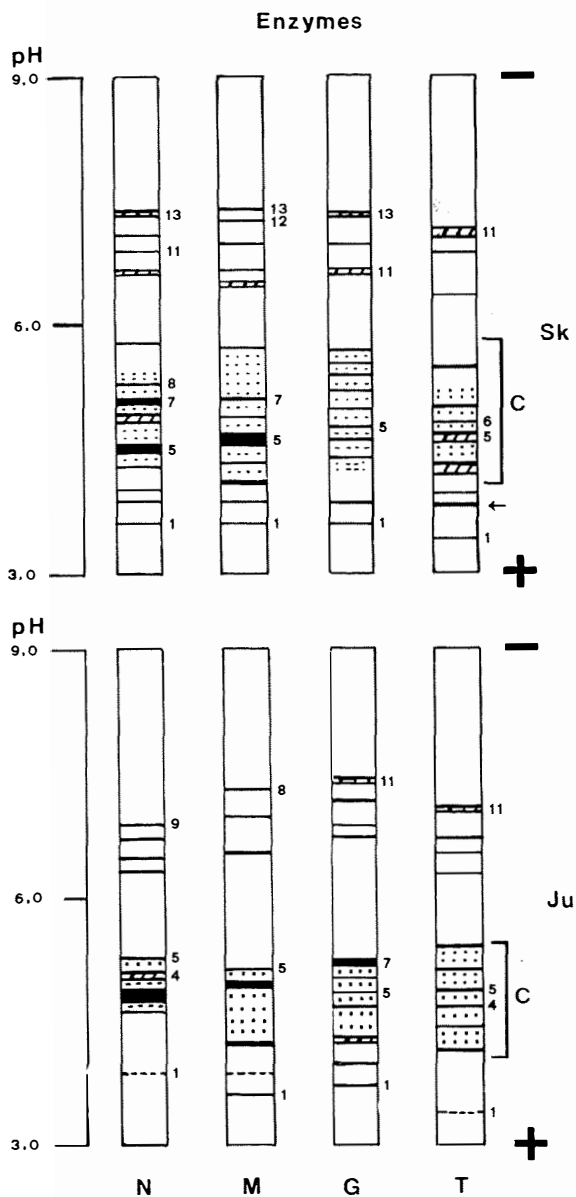


Fig. 2: Diagrams of gel isoelectric focusing profiles of MDH isoenzymes of four grapes. From left to right grape varieties: Nebbiolo (N), Merlot (M), Garganega (G), Trebbiano (T), Top half: skins (Sk); bottom half: juices (Ju). The arrow indicates the band observed with the same pI value in all varieties.

Diagramme der Profile von MDH-Isoenzymen nach isoelektrischer Fokussierung in Gelen. Rebsorten von links nach rechts: Nebbiolo (N), Merlot (M), Garganega (G), Trebbiano (T). Oben: Schalen (Sk); unten: Säfte (Ju). Der Pfeil bezeichnet die Bande mit gleichem pI-Wert bei allen Sorten.

ponents focused in the pH range of about 3,4 to 7,5 (Fig. 2, top half). From the patterns, the separation of skin MDH activity into three definite groups was apparent. They were rather similar in their pI value but differed in their activity level, defined as the staining intensity, and number of isoenzyme bands. One group, the anodal one (pH 3,4—4,0), contained from 2 to 3 bands. The second major group, situated into the zones designed (C), contained from 5 to 8 bands. The third group, the more basic one (pH 6,0—7,5), contained from 3 to 5 bands.

The intermediate (C) zones of the gels were characterized by a rather coloured background; in spite of this, the individual bands could be easily distinguished from the neighbouring ones. In these zones, the most intense sites of MDH activity were localized. About 60 percent of total enzyme activity, in terms of peak areas, were in the (C) zones. Band No. 5 was the predominant variant in two grapes (Nebbiolo and Merlot). A similarly pronounced band of activity was not found in the other two grapes. Only band pI 3,9 was found with the same pI in all skin extracts. The gel zones containing the isoenzyme groups located closest to the electrodes had very weak background colour. All the three sets of MDH activities can be used for comparative purpose, although we think that those of the more extreme pH zones are most favourable.

MDH from the grape juice extracts was rather less heterogeneous compared to that of skin extracts. The former yielded from 8 to 11 isomalates (Fig. 2, bottom half).

The general picture of juice zymograms was basically the same as described above for the skins, since a (C) zone with enough close together isoenzymes, a cathodal, and an anodal band region could be distinguished. In each of these, different isoenzyme numbers together with changes in the relative activity and pI value of each variant, were observed among the varieties. Over 70 percent of the resolved MDH activity in juices were contained in the (C) zones.

Between juice and the correspondent skin of the analyzed grapes, only two to four peaks appear to be proteins with the same net charge, as indicated by their identical value of pI. All bands found with the same pI, between the two parts of berry of each variety, are listed in Table 2. Thus, the skins and the juices behaved differently with regard to their MDH, a fact which suggests functional differences between two parts of the berry. Moreover, the differences found among the four grapes may indicate that the MDH accumulation in different varieties has different physiological bases.

Table 2

MDH isoenzymes of extracts from juice (Ju, number of pairs) and skin (Sk, number of pairs) of the same variety in which an identical pI value could be observed¹⁾

MDH-Isoenzyme aus Extrakten von Beerensaft (Ju; Anzahl der Paare) und Beeren-schale (Sk; Anzahl der Paare) derselben Rebsorte, in welchen ein identischer pI-Wert beobachtet werden konnte.

Nebbiolo			Merlot			Garganega			Trebiano		
Ju	Sk	pI	Ju	Sk	pI	Ju	Sk	pI	Ju	Sk	pI
4	7	5,1	1	1	3,6	5	5	4,8	1	1	3,4
5	8	5,3	2	2	3,8	7	11	6,7	4	5	4,7
9	11	6,9	5	7	5,2				5	6	4,9
			8	12	7,3				11	11	7,2

¹⁾ See Fig. 2 for the location of bands.

The possibility of binding protein-protein, protein-Ampholine was tested by conducting the isoelectric focusing in presence of 3 M urea (MALTHE-SØRENSEN and FONNUM 1972). No change in the pattern of zymograms appeared. In all gels sharply defined areas were present which did not show any background colour, the so-called "anti bands" (HALL *et al.* 1969, HADAČOVÁ 1972).

The shift of isomalate dehydrogenase patterns during the developmental process of the berry is, at present, under investigation.

This study cannot be considered as a taxonomic classification of *V. vinifera* varieties studied; but it should be considered as evidence for the presence of highly reproducible patterns of proteins and MDH activity, and the relationships of these patterns among varieties. By an accumulation of similar data together with chemical and morphological data, a taxonomic classification based on these relationships can be compiled for other *V. vinifera* varieties and other *Vitis* species (especially the American ones).

Summary

Soluble proteins and multiple forms of malate dehydrogenase (E.C. 1.1.1.37) in both skin and juice of four grape varieties, have been compared with respect to heterogeneity and isoelectric point. The protein preparations resolved into 17 to 28 very distinct components. Seven (skins) and eight (juices) of these components were of common occurrence with all varieties, as judged by the same pI value. The presence of two to seven peculiar bands in both parts of berry was also observed.

In the grape the NAD-malate dehydrogenase activity was associated with 9 to 13 isoelectric forms, apparently assembled into three sets of isoenzymes. Each of these showed a rather similar dislocation in the gels; but they contained a different number of isoenzymes of distinct pI value in the skin as well as in the juice of different varieties. A higher number of variant forms was found in the skins in comparison with juices. Between two and four isomalate dehydrogenases of the same net charge were found in both parts of berries of the same variety, whereas the remaining isoenzymes appeared to be non-identical and, thus, presumably the product of distinct genes.

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