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Isoelectric focusing of grapevine peroxidases as a tool for ampelography

by

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Isoelektrische Fokussierung von Peroxidasen bei Reben als Hilfsmittel der Ampelographie

Zusammenfassung: Aus dem Internodialphloem von 71 Arten und Sorten der Gattung *Vitis* wurden enzymatisch aktive Proteine isoliert und mit Hilfe der isoelektrischen Fokussierung auf Dünnschichtplatten analysiert. Von den Peroxidasen (EC 1.11.1.7) wurde ein gut differenzierbares Muster erhalten, das im pH-Bereich zwischen 6 und 11 ausgewertet werden konnte und aus bis zu 8 Banden bestand. Die Zuordnung einzelner Proben zeigte eine gute Übereinstimmung mit ampelographischen Daten. So sind die Muster innerhalb der Burgunder- und der Silvanerfamilie weitgehend identisch, ebenso bei haploiden und tetraploiden Pflanzen derselben Sorte unabhängig von deren Standortbedingungen. Amerikanische und asiatische Wildformen und manche interspezifische Hybriden zeigen gegenüber *V.-vinifera*-Sorten eine geringfügige Verschiebung im isoelektrischen Punkt einer Isoenzymbande.

Key words: enzyme, protein, analysis, phloem, shoot, Vitis, variety of vine, clone, ampelography.

Introduction

There is a growing demand for simple and effective methods to identify plant species, cultivars, and clones (TANKSLEY and ORTON 1983; SIMPSON and WITHERS 1986). Morphometric methods rely on characters which are relatively distant from the genetic material and are thus influenced by environment and growth stages of the plant (for literature see DETTWEILER 1987). The same holds true for the analysis of secondary metabolites (i.e. tertiary gene products, see BACHMANN 1978). On the other hand the prerequisites for an analysis of genes as constant primary characters (e.g., by RFLP tests, for literature see HULBERT and MICHELMORE 1987) are lacking in most laboratories.

Thus the analysis of direct gene products (i.e. proteins) seems more preferable. In grapes (*Vitis* spp.), this has been done by a number of authors (see Table 2) for several organs of the plant. In these cases the availability of the enzyme source is limited to a more or less short period and/or subjected to environmental changes, e.g., in fruits (DRAWERT and MÜLLER 1973; WOLFE 1976) and shoot tips (SCHAEFER 1970, 1971). The collection of pollen for the analysis of esterase isoenzymes (STAVRAKAKIS and LOUKAS 1983) is very tedious and limited to fertile plants and — in the case of isoelectric focusing of pollen proteins (CARGNELLO *et al.* 1988) — the patterns obtained seem too sophisticated for routine applications. The forcing of cuttings (BENIN *et al.* 1985) to obtain fresh and comparable leaf material affords additional equipment and time.

We have tried to combine several techniques to a routine method allowing to gain an additional, readily accessible character for the identification of grapevines throughout a longer period. Peroxidases have been chosen because their pattern in IEF is not

too complicated, their properties and functions in grapevines are well known (SCIANCALEPORE *et al.* 1985; HOOS and BLAICH 1988), the colour reaction is simple and sensitive, and the isoenzyme pattern of horseradish peroxidase may be used as an easily available reference. The phloem of dormant wood proved to be a good enzyme source, because it is available for a longer period, it has a low phenol content and the influence of climate and organ specifity on the enzyme patterns is reduced.

Material and methods

Grapevine material

Canes of the varieties cited in Table 1 were cut in the grapevine collections of the BFAR from November 1987 through April 1988 and processed immediately or stored at 5 °C in a cool chamber. In addition, wood from some cultivars (marked by asterisks in Table 1) was cut from a dry, warm location along a stone wall, where the grapes mature 7 d earlier.

Preparation of extracts

The bark of about 100 g of internode is removed, the phloem layer (3 g) is scratched off with a razor blade, frozen in liquid nitrogen (−178 °C), pulverized in a chilled mortar to a fine powder which is then mixed (Ultra Turrax, 1 min) with 30 ml buffer (phosphate 1 M, pH 7.0, supplied with 500 mg ascorbic acid, 2 g Carbowax 40000, 2 g Dowex-4 Cl-form). After centrifugation of the resulting slurry the clear supernatant is brought to a 80 % ammonium sulfate saturation. The precipitated proteins are centrifuged, and the pellet is dialyzed several times against distilled water which, in the last step, is replaced by a 1 % glycine solution. After sterile filtration the samples are kept at −25 °C. For further analysis all samples are brought to the same overall peroxidase activity (corresponding to 0.5 ng/10 µl of Serva 31942 horseradish peroxidase) by dilution with a 1 % glycine solution or by concentration with Sephadex G-15.

Isoelectric focusing

For IEF experiments, precoated thin layer plates (Serva 42967, pH 3—11) were used, according to WESTERMEIER (1987). Horizontal slab gel electrofocusing was carried out on a LKB Multiphor 2117 apparatus with LKB 2103 powersupply. Sample aliquots of 10 µl were applied to the middle of the gels with an applicator strip (Serva 42914). In preliminary experiments, higher amounts of sample had been applied to be sure to get all enzyme bands: 10 µl proved to be sufficient in all cases tested.

Separation starts at 150 V and about 4 mA; a constant minimal current indicates the end of the run (after 3 h at 1700 V and 1.0 mA, approximately).

Peroxidase activity was revealed by immersing the gel slabs in a solution of 0.005 % benzidine and 0.06 % H_2O_2 in 0.1 M sodium acetate buffer; pH 5.0. Blue bands, turning redbrown, indicate peroxidase activity. After 5—20 min the reaction is stopped by rinsing the slab in tap water.

The isoelectric points of the proteins were determined using marker proteins (Serva 39206), coloured with Serva Blue W (Serva 35053) after fixation with 3 % trichloro-acetic acid. Horseradish peroxidase was normally used as a reference; its isoenzyme pattern is in the same pH range as that of grapevine peroxidases.

Results and discussion

In preliminary experiments peroxidases of different organs of the grapevine, including pollen, were tested. Fig. 1 a shows the organ specific patterns in roots, callus, and leaves of *Vitis riparia*. However, for further tests only phloem enzymes were used, because they produced the most reliable results and are available over a longer period. Cathodic peroxidases show a large number of bands (Fig. 1 a, PK), but will not be included in our evaluations because sometimes there is a cathodic drift (discussed by WESTERMEIER 1987) and we felt that the high number of weak bands would lead to over-interpretations rather than to yield valuable characters. In the following, the anodic peroxidases (Fig. 1 b, pH range 6—9) are referred to as P1 (I.E. point of about 8.6) through P7 (about pH 6; near the application point of the samples). Patterns should never be compared directly, but always relative to the pattern of horseradish peroxidase, because the exact determination of isoelectric points on thin layers is not possible under normal conditions (DELINCÉE and RADOLA 1969).

All separations were repeated several times with identical results. Although a cool storage of the canes of some weeks did not lead to changes, in the pattern of material stored for 2 years at 5 °C, two bands were lacking. The conditions of using stored material are actually being tested.

Typical IEF patterns of some varieties are presented in Fig. 2. For their evaluation the intensities of P1 through P7 were recorded in a dBASE file which then was sorted

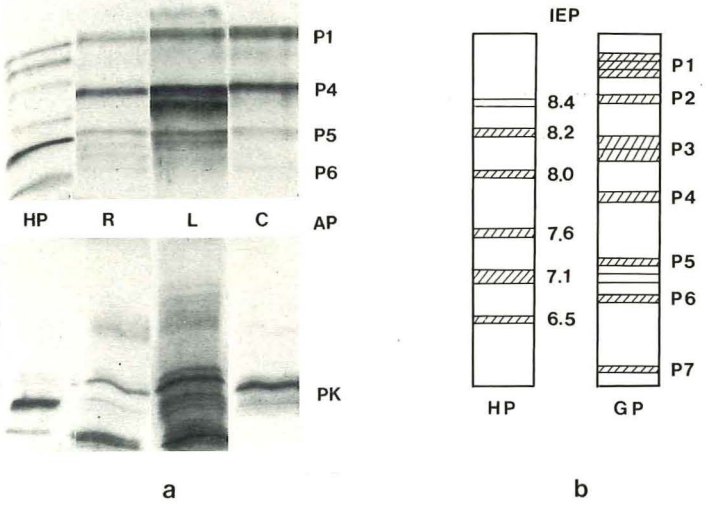

Fig. 1: Isoelectric focusing patterns of horseradish (HP) and grapevine (GP) peroxidases. IEP isoelectric points, P1—P7 names of isoenzyme bands, AP application zone, PK cathodic peroxidase. — a) Anodic and cathodic pattern of peroxidases obtained from different organs of *Vitis riparia*. L leaf, R root, C callus. The bands of horseradish peroxidases are distorted due to a margin effect on the slab. — b) Schematic diagram of the anodic part of peroxidase patterns.

Bandenmuster der Isoelektrischen Fokussierung von Meerrettich- (HP) und Rebperoxidasen (GP). IEP isoelektrische Punkte, P1—P7 Bezeichnung der Peroxidasebanden, AP Auftragezone, PK kathodische Peroxidase. — a) Anodische und kathodische Muster von Peroxidasen aus verschiedenen Geweben von *Vitis riparia*. L Blatt, R Wurzel, C Kallus. Die Banden der Meerrettichperoxidase sind durch Randeffekte auf der Trennplatte verzerrt. — b) Diagramm des anodischen Teils von Peroxidasemustern.

(Table 1)

No.		Cultivar/species	Peroxidase isoenzymes P2	P3	P4	P5	P6
		Vitis vinifera cultivars					
1	**	Diana B	○	+	–	–	–
2	**	Nobling B	+	+	+	–	–
3	**	Helfensteiner	+	+	○	○	○
4	**	Morio-Muskat B	+	–	○	+	+
5		Pinot Noir N	+	–	+	+	+
6		Pinot meunier N	+	–	+	+	+
7		Pinot blanc B	+	–	+	○	+
8		Pinot gris GR (cl. Dunkelgrau)	+	–	+	+	+
9	**	Multaner B	+	–	+	–	–
10		Silvaner blau RG	+	–	+	–	–
11		Silvaner grün B (cl. 75)	+	–	+	–	–
12		Silvaner grün B (cl. 75 4n)	+	–	+	–	–
13	**	Domina N (87/88)	–	+	+	+	○
14		Müller-Thurgau B (4n)	–	+	+	○	+
15	**	Müller-Thurgau B	–	+	+	○	+
16	**	Albalonga B	–	+	+	+	+
17	**	Bacchus B	–	+	+	○	○
18	**	Mariensteiner B	–	+	+	○	+
19	**	Scheurebe B	–	+	+	+	+
20		Chasselas rosé RS	–	+	+	○	○
21		Portugieser blau N	–	+	+	+	+
22	**	Freisamer B	–	+	+	+	–
23	**	Siegerrebe R	–	+	+	○	–
24	**	Dornfelder N	–	+	+	+	–
25	**	Huxelrebe B	–	+	+	–	+
26	**	Kerner B	–	+	+	–	○
27	**	Kanzler B	–	+	+	–	+
28		Gloria B	–	+	+	–	–
29	**	Gutenborner B	–	+	+	–	–
30	**	Heroldrebe N	–	+	○	–	–
31		Schiava grossa N (4n)	–	+	+	–	–
32		Schiava grossa N	–	–	+	–	–
33	**	Rotberger N	–	+	+	–	–
34		Ehrenfelser B	–	–	+	+	+
35		Riesling weiß B (cl. 4)	–	–	+	+	+
36		Riesling weiß B (cl. 4 4n)	–	–	+	+	+
37	**	Faberrebe B	–	–	+	+	–
38	**	Forta B	–	–	+	+	–
39	**	Perle G	–	–	+	○	–
40		Würzer G	–	–	+	○	–
41	**	Optima B	–	–	+	+	–
42		Traminer rot RG	–	–	+	–	+
43		Traminer rot RG (4n)	–	–	+	–	+
44	**	Ortega	–	–	+	–	–

Table 1

Qualitative patterns of anodic peroxidases in grapevine cultivars and species · Asterisks indicate that identical patterns have been obtained from two plants of different provenance · + strong, O weak band, – not present, × double band with slightly different isoelectric point

Qualitative Muster der anodischen Peroxidasen von Rebsorten und -arten · Sternchen bedeuten, daß zwei Pflanzen unterschiedlicher Herkunft identische Muster lieferten · + starke, O schwache Bande, – nicht vorhanden, × Doppelbande mit geringfügig verschobenem isoelektrischem Punkt

No.	Cultivar/species	Peroxidase isoenzymes				
		P2	P3	P4	P5	P6
	Vitis silvestris clones					
45	Guemuelduer 103—64	+	–	+	O	+
46	Guemuelduer 100—64	–	–	+	O	–
47	Ketsch 27 N	–	+	O	+	+
48	Ketsch 27 N (4n)	–	+	O	+	+
49	Ketsch 32	–	+	+	+	+
50	Ketsch 23	–	+	–	O	+
51	Ketsch 6—40	–	+	–	+	+
52	Ketsch 6—40 (4n)	–	+	–	+	+
53	*V. caucasica* VAVILOV N	–	–	+	O	–
54	*V. caucasica* VAVILOV N	–	–	+	O	–
	Vitis species					
55	*V. berlandieri* PLANCHON	–	×	O	–	–
56	*V. coignetiae* PULIAT	+	O	–	–	–
57	*V. davidii* FOEX (male)	+	O	–	–	+
58	B 1668 (female of 57)	+	+	–	–	–
59	Selektion Oppenheim 4	+	+	–	–	–
60	Selektion Oppenheim 4 (4n)	+	+	–	–	–
61	*V. amurensis* RUPRECHT	+	+	–	–	–
	Interspecific cultivars					
62	Chaunac N	+	–	+	–	O
63	Buffalo N	+	–	+	–	–
64	Chelois noir N	–	×	+	–	–
65	Bluestar N	–	+	+	–	–
66	Kyoho N	–	+	+	–	–
67	Kyoho N	–	+	+	–	–
68	Bailey Alicante A N	–	×	–	–	–
69	Cayuga white B	–	–	+	–	–
70	Agawam N	–	–	+	–	–
71	Chambourcin N	–	–	+	–	–

according to the similarity of the patterns. P1 is common to all *Vitis* species and cultivars and will thus not be considered further, although it seems that its 3 sub-bands might be valuable characters (e.g. for Chasselas Rosé, Fig. 2, no. 20), if they could be separated further by suitable ampholine gradients. Qualitative differences will — in view of a method to be used by unskilled personal — not be discussed either but only the distinct presence or absence of a certain band.

Patterns of 25 cultivars planted at two locations at the BFAR under different conditions of microclimate (Table 1, asterisks) proved to be always identical (even qualitatively), although the canes were taken during November and February, respectively. Identical patterns were also obtained from two clones of the interspecific cultivar Kyoho N (66, 67), which come from different collections and differ in berry size, and (except for one plant of doubtful origin) from diploid and haploid plants of the same variety (11/12, 14/15, 35/36, 42/43, 51/52, 59/60).

Within the *V. vinifera* group, homologous bands have identical isoelectric points, whereas in other species and in some interspecific cultivars P3 may differ slightly. Related varieties (e.g. Pinot cvs, Silvaner cvs) are usually grouped together and this is often true for newbred varieties with common ancestors. Further studies on the influence of environmental factors (climate, storage conditions) on the pattern will be necessary, but we feel that the pattern of bands P2, P3, and P4 may already be used as a valid ampelographic character.

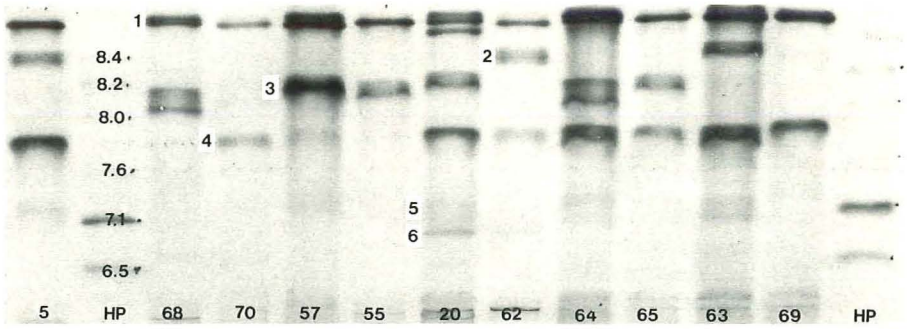

Fig. 2: Anodic patterns of some *Vitis* cultivars, numbers corresponding to Table 1. HP horseradish peroxidase with isoelectric points of bands. Numbers on white background indicate the isoenzymes P1—P6.

Anodische Muster einiger Rebsorten; die Nummern entsprechen Tabelle 1. HP Meerrettichperoxidase mit den isoelektrischen Punkten der Banden. Die Nummern auf weißem Untergrund lokalisieren die Isoenzyme P1—P6.

Concluding remarks

The comparison of electrophoretic patterns presented by most working groups (Table 2) is often difficult or impossible, due to different separation methods (disc, starch gel, acetate strip electrophoresis) and varying conditions used (preparation of gels, buffers, voltage and current).

Isoelectric focusing, however, yields better comparable results: Although the relative distances of bands within a pattern may vary considerably between the runs, they can be identified by their isoelectric points relative to horseradish peroxidase as a reference.

Usually patterns are more complicated in IEF than in other types of electrophoresis, in particular if unspecific proteins are stained. This is often considered an advantage allowing more sophisticated differentiations. We feel, however, that this might not always be so because — although proteins may be considered primary gene products — the expression and formation of isoenzymes is a complicated process, frequently involving epigenetic changes of the proteins. Thus their patterns must be interpreted cautiously, even in a system like the phloem of dormant wood. If too complicated, patterns become very sensitive to influences of the plant's environment and of the preparation and separation procedures (DELINCÉE and RADOLA 1977). In these cases the method cannot be considered a universal tool for ampelography.

Finally it should be pointed out that the stability of enzyme patterns and their suitability as fingerprints cannot be predicted but must be checked out empirically for each plant species and each enzyme. In disc electrophoresis of grapevine extracts, peroxidases show only two bands (HOOS and BLAICH 1988) which are not suitable discriminatory characters; on the other hand the IEF pattern — though not too complicated — is well differentiated and seems to be rather reliable.

Table 2

Literature overview of protein or enzyme pattern analyses in grapevines

Literaturübersicht: Analyse des Protein- oder Enzymmusters bei Reben

	Authors	Grape organs	Separation methods	No. of enzymes	No. of samples
1970	SCHAEFER	Leaves	Disk el.	1	10
1971 a	SCHAEFER	Leaves/tips	Disk el.	Pr [1])	60
1971 b	SCHAEFER	Leaves/tips	Disk el.	1	88
1971 c	SCHAEFER	Leaves	Disk el.	3	20
1973	DRAWERT and MÜLLER	Grapes	IEF	2	10
1974	DRAWERT and GÖRG	Grapes	Disk el.	4	10
1976	WOLFE	Grapes	Starch-gel	4	40
1981	SAMAAN and WALLACE	Pollen	Serology	Pr	5
1981	DAL BELIN PERUFFO *et al.*	Leaves	IEF	Pr	17
1982	SCHWENNESEN *et al.*	Grapes	Starch-gel	10	4
1983	RAJASEKARAN and MUL-	Leaves	Starch-gel	3	1
1983	LOUKAS *et al.*	Pollen	Starch-gel	13	27
1983	STAVRAKAKIS and LOUKAS	Pollen	Starch-gel	13	37
1985	BENIN *et al.*	Leaves	Disk el.	8	130
1985	FALLOT *et al.*	Leaves	SDS-PAGE	Pr	40
1987	SUBDEN *et al.*	Wood	Starch-gel	12	27
1988	CARGNELLO *et al.*	Pollen	IEF	Pr	7

[1]) Pr = Proteins only.

Summary

Enzymatically active proteins have been isolated from the phloem of internodes of 71 *Vitis* species and cultivars and separated by thin layer electrofocusing. Peroxidases (EC 1.11.1.7.) yielded a pattern which could be differentiated into up to 8 bands within the pH range 6 through 11. The patterns obtained from different samples were con-

gruent with ampelographic data, they are virtually identical within the families of Pinot and Silvaner, as well as between haploid and diploid forms of one variety, even under different environmental conditions. American and Asian wild species and some interspecific hybrids show a slight shift of the isoelectric point of one enzyme band as compared to *Vitis vinifera* varieties.

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