Purification and Some Properties of an α-L-Arabinofuranosidase from *Aspergillus niger*. Action on Grape Monoterpenyl Arabinofuranosylglucosides

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An α-L-arabinofuranosidase from *Aspergillus niger* was purified 27-fold from a commercial crude preparation of Hemicellulase REG 2 by gel filtration on Ultrogel AcA 44, ion-exchange chromatography on DEAE Sepharose CL-6B, and affinity chromatography on Concanavalin A–Ultrogel AcA 22. The molecular weight of the enzyme was 61 000; its optimum pH was 3.9 and temperature of maximal activity 60 °C. This arabinosidase was active against monoterpenyl α-L-arabinofuranosylglucosides from grape by liberating monoterpenyl β-D-glucosides and arabinose regardless the structure of the aglycon moiety.

Terpenols are strongly aromatic molecules that occur in berries of Muscat grapes (Bayonove and Cordonnier, 1971; Ribéreau-Gayon et al., 1975; Williams et al., 1980) but also in some fruits such as papaya (Heidlas et al., 1984), mango (Sakho et al., 1985), and passion fruit (Engel and Tressl, 1983). Beside the free volatile (and therefore odorous) forms, a main portion of monoterpenols in grape is linked to disaccharidic moieties (and therefore odorless until hydrolyzed) (Williams et al., 1982a), e.g., 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides and 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides (rutinosides) (Figure 1); traces of β-D-glucopyranosides are also found. Among the glycosidically linked monoterpenols, the arabinofuranosylglucosides of geraniol, nerol, and linalool are the most abundant (Williams et al., 1982a; Gunata et al., 1988). These aromatic aglycons can be released by acid or enzymatic hydrolysis of the carbohydrate moiety (Williams et al., 1982b; Di Stefano, 1982; Gunata et al., 1988; Cordonnier et al., 1989); however, they may undergo rearrangements under the former conditions while enzymatic breakdown of the glycosidic precursors lead to minimal changes in the aglycon moieties.

Since the major terminal nonreducing sugar in these glycosides is α-L-arabinofuranose, it was of interest to purify an α-L-arabinofuranosidase (EC 3.2.1.55) for both use as an analytical tool for the study of the mechanism of hydrolysis of these glycosides and exploration of new ways to enhance odors for the beverage industry. An extensive screening of commercial enzymatic crude preparations (Cordonnier et al., 1989) led to the choice of Hemicellulase REG 2 as the best source of this enzyme of which we now report the purification and some of its properties.

EXPERIMENTAL SECTION

Enzymes and Substrates. The enzyme was a commercial crude powder from *Aspergillus niger* marketed as Hemicellulase REG 2 (Gist-Brocades, Seclin, France).

p-Nitrophenyl α-L-arabinofuranoside (*p*NP-Ara*f*), α-L-arabinopyranoside (*p*NP-Ara*p*), α-L-rhamnopyranoside (*p*NP-Rha), and β-D-glucopyranoside (*p*NP-Glc) were from Sigma. β-D-Glucopyranosides and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides of geraniol (Ger-AraGlc), nerol (Ner-AraGlc), (±)-linalool (Lin-AraGlc), (±)-α-terpineol (Ter-AraGlc), benzyl alcohol (Ben-AraGlc), and 2-phenylethanol (Phe-AraGlc) (Figure 1) were synthetized in our department (Voirin et al., 1989).

Enzyme Assay. α-L-Arabinofuranosidase (or arabinosidase) activity was measured by mixing enzyme solution (0.1 mL, <0.2 nkat) with 0.1 mL of a 4 mM solution of *p*NP-Ara*f* in 0.1 M acetate buffer (pH 4.2) for 20 min at 40 °C. Liberation of *p*-nitrophenol was determined by addition of 0.6 mL of 1 M sodium carbonate and measuring the absorbance at 400 nm. α-L-Rhamnopyranosidase and β-D-glucopyranosidase were measured under same conditions with the corresponding *p*-nitrophenyl glycosides. Enzyme activity was expressed as nanokatals (nkat), 1 nkat being the amount of enzyme that liberates 1 nmol of *p*-nitrophenol/s from appropriate glycosides under standard conditions.

Enzyme Purification. *Gel Permeation Chromatography on Ultrogel AcA 44.* Crude Hemicellulase REG 2 (250 mg) was solubilized in 0.1 M citrate–phosphate buffer (3 mL, pH 7.2), and the clear solution was dialyzed overnight against the same buffer (4 °C). The dialyzed enzyme was then applied to a column (1.6 × 100 cm) of Ultrogel AcA 44 equilibrated with the above citrate–phosphate buffer at a flow rate of 9 mL·h^{-1}. The chromatogram is shown in Figure 2.

Ion-Exchange Chromatography on DEAE Sepharose CL-6B. Fractions containing the α-L-arabinofuranosidase activity were combined (52 mL), dialyzed overnight against 0.025 M imidazole hydrochloride buffer (pH 7.5), and applied to a column (1.6 × 40 cm) of DEAE Sepharose CL-6B in the same buffer at 108 mL·h^{-1}. Bound proteins were then released by application of a linear gradient (0→0.4 M) of sodium chloride in the same buffer (400 mL) (Figure 3).

Affinity Chromatography on Concanavalin A–Ultrogel AcA 22. Fractions eluted in the range (0.34–0.4 M) were combined (69 mL), concentrated through dialysis bag with Sephadex G-200 to 12 mL, dialyzed overnight against 0.05 M Tris-HCl buffer (pH 7.2) containing 0.1 M sodium chloride and 0.1 mM manganese chloride, and applied to a column (1.0 × 10 cm) of Concanavalin A–Ultrogel AcA 22 equilibrated in the same buffer at 30 mL·h^{-1}. A linear gradient (0→0.067 M) of methyl α-D-mannopyranoside in the above buffer (200 mL) was then applied, followed by isocratic elution with 0.15 M methyl mannoside (Figure 4). Appropriate fractions containing the arabinofuranosidase activity were pooled and dialyzed overnight against 0.1 M acetate buffer (pH 4.2).

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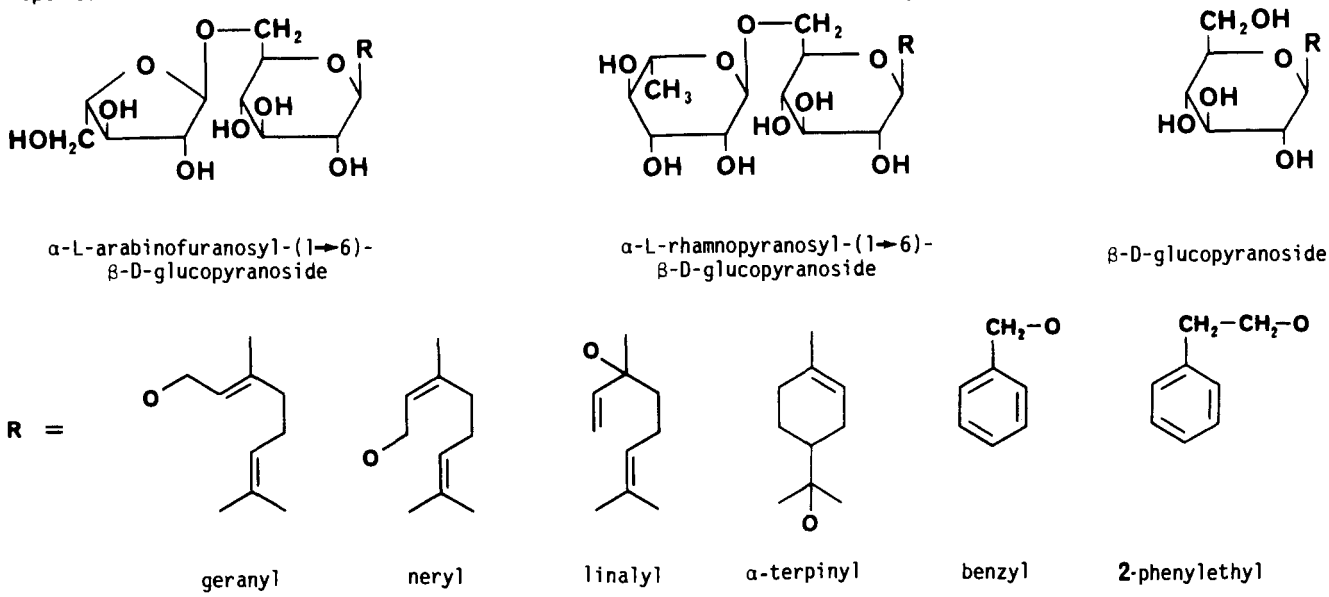

Figure 1. Structure of grape monoterpenyl glycosides.

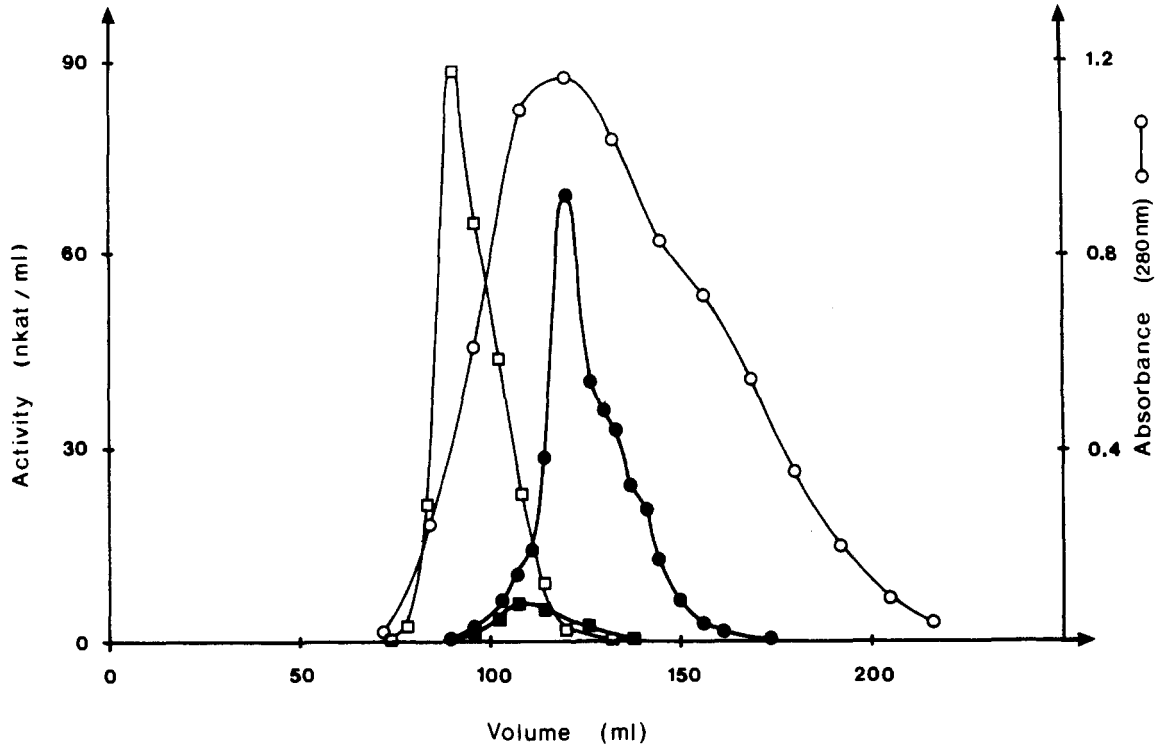

Figure 2. Gel filtration of the *A. niger* α-L-arabinofuranosidase (Hemicellulase REG 2) on Ultrogel AcA 44 (see the Experimental Section). Fractions (1.2 mL) were assayed for protein at 280 nm (○) and for the following activities: α-L-arabinofuranosidase (●), β-D-glucopyranosidase (□), and α-L-rhamnopyranosidase (■).

General Methods. Protein was recorded in column effluents at 280 nm; protein was also measured by the Lowry procedure (Lowry et al., 1951), using bovine serum albumin as reference standard. TLC of glycosides and liberated sugars was performed on silica gel as previously described (Gunata et al., 1988).

Properties of the α-L-Arabinofuranosidase. *Optimum pH.* The arabinofuranosidase activity was measured under standard conditions in a universal citrate–phosphate buffer of various pHs, instead of 0.1 M acetate buffer (pH 4.2) (Figure 5).

pH Stability. The arabinofuranosidase (3.6 nkat·mL^{-1}) was incubated in universal buffer of various pH values at 60 °C for 50 min. The enzyme samples were then dialyzed for 5 h against 0.1 M acetate buffer (pH 4.2), and the residual activity was measured under standard conditions (Figure 5).

Optimum Temperature. The arabinofuranosidase activity was measured under standard conditions in the temperature range 5–80 °C (Figure 6).

Temperature Stability. The enzyme (7.2 nkat·mL^{-1}) in 0.1 M acetate buffer (pH 4.2) was kept at various temperatures (5–80 °C) for 30 min, and then the residual activity was determined under standard conditions (Figure 6).

Molecular Weight. The molecular weight determination was achieved by calibration of the column of Ultrogel AcA 44 with molecular weight markers: cytochrome *c*, 11 700; chymotrypsinogen A, 25 000; ovalbumin, 45 000; bovine serum albumin, 68 000; aldolase, 158 000.

Specificity. Solutions (35 μL) of various monoterpenyl arabinofuranosylglucosides (2 mM in 0.1 M acetate buffer, pH 4.2) were added with 10 μL of arabinofuranosidase (0.19 nkat in 0.1 M acetate buffer, pH 4.2) and incubated for 24 h at 40 °C. Products of hydrolysis (15 μL) were analyzed by TLC (Figure 7).

RESULTS AND DISCUSSION

Purification. Gel filtration of the crude Hemicellu-

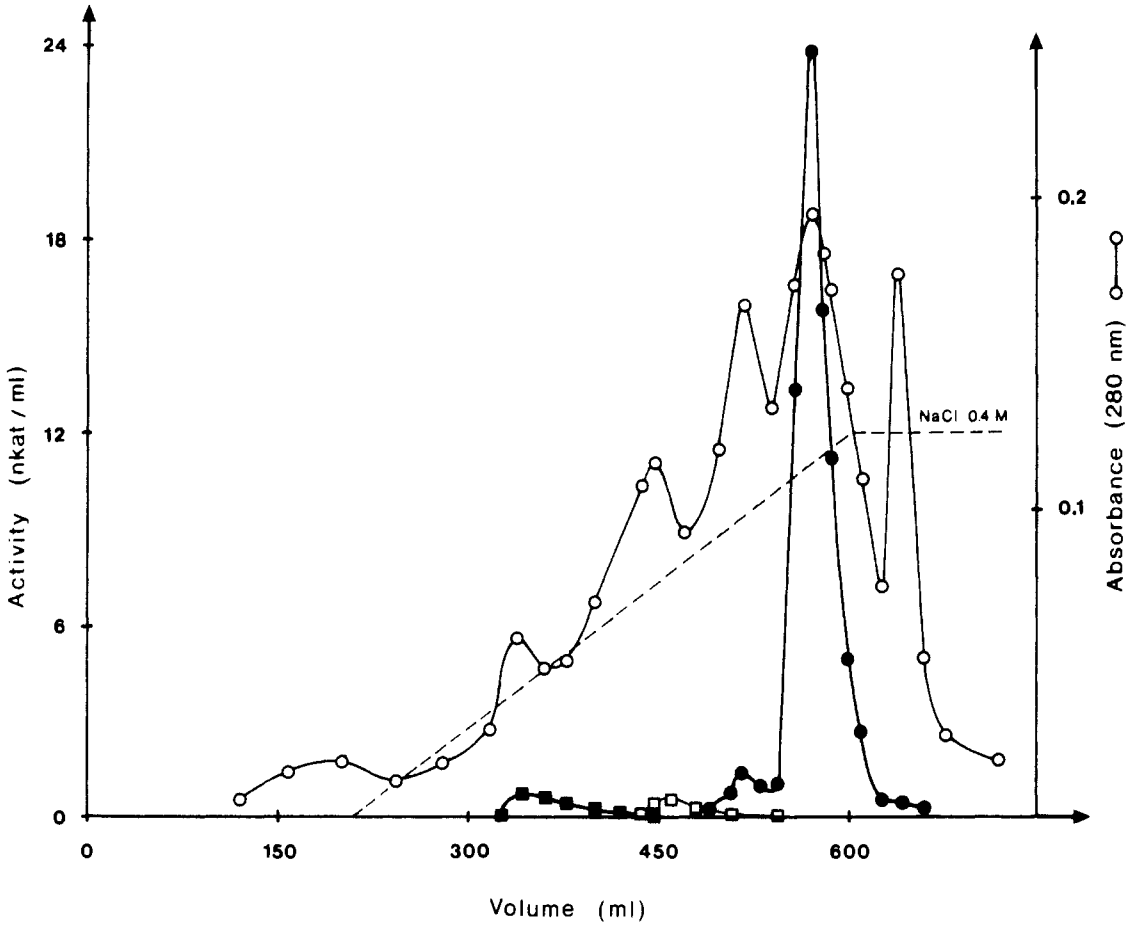

Figure 3. Ion-exchange chromatography of the arabinofuranosidase on DEAE Sepharose CL-6B (see the Experimental Section). Fractions (4 mL) were assayed as in Figure 2.

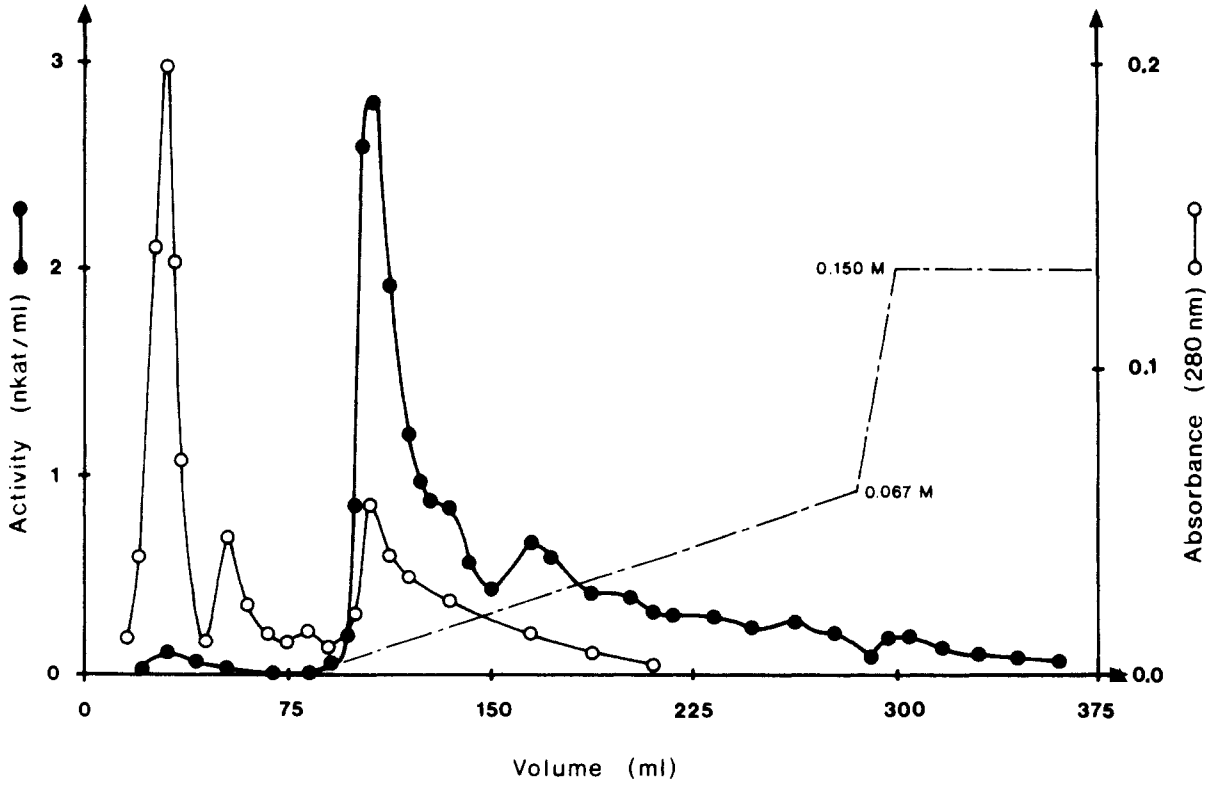

Figure 4. Affinity chromatography of the arabinofuranosidase on Concanavalin A–Ultrogel AcA 22 (see the Experimental Section). Fractions (1.5 mL) were assayed as in Figure 2.

lase REG 2 on Ultrogel AcA 44 (Figure 2) allowed an efficient separation of the two major glycosidases occurring in this mixture, e.g., α-L-arabinofuranosidase (K_{av} 0.45) and β-D-glucosidase (K_{av} 0.18); α-L-rhamnopyranosi-

Table I. Purification of Arabinofuranosidase from *A. niger*

step	vol, mL	total act., nkat	act. yield, %	total protein, mg	protein yield, %	sp act., nkat·mg^{-1}	purifcn
crude enzyme	3	2327	100	42.5	100	54.7	1
gel filtration on Ultrogel AcA 44	52	1759	76	14.7	35	120	2.2
ion-exchange chromatography on DEAE Sepharose CL-6B	69	365	16	0.47	1.1	777	14.2
affinity chromatography on Concanavalin A-Ultrogel AcA 22	34	75	3	0.05	0.1	1500	27.4

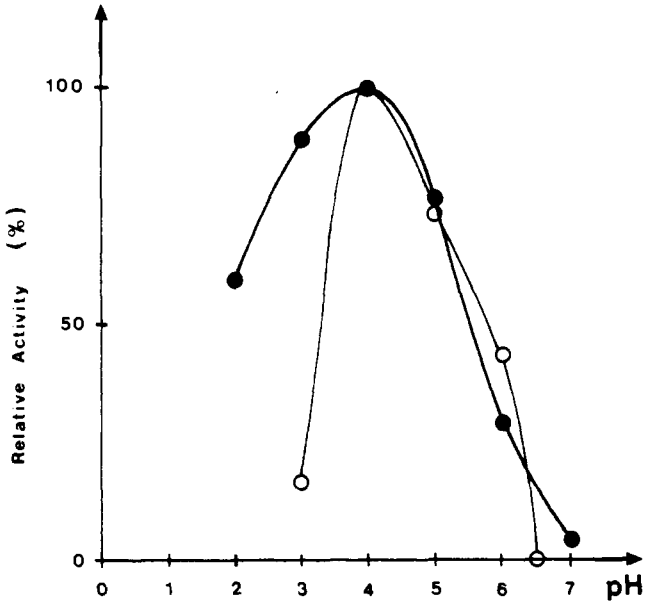

Figure 5. Effect of pH on enzyme activity (●) and stability (○) (see the Experimental Section).

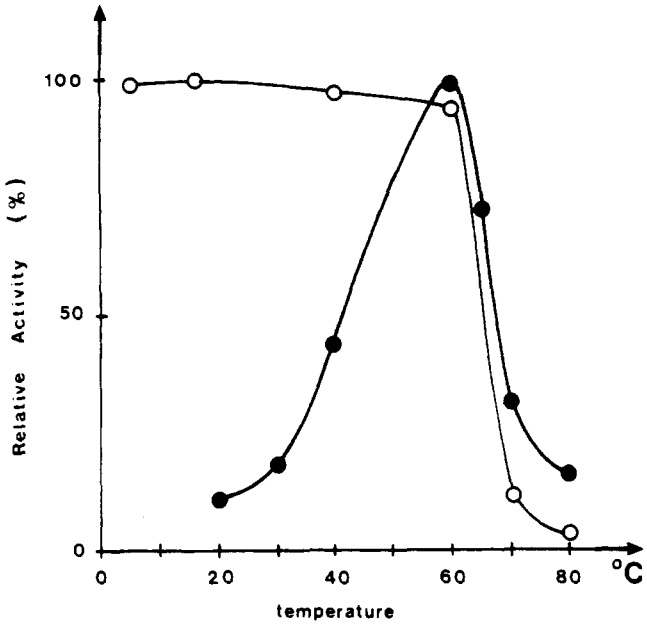

Figure 6. Effect of temperature on enzyme activity (●) and stability (○) (see the Experimental Section).

dase, a minor component of the commercial enzyme, eluted at K_{av} 0.34. Of the starting arabinosidase activity, 76% was recovered, 65% of proteins being eliminated (purification 2.2-fold) (Table I).

Further ion-exchange chromatography on DEAE Sepharose CL-6B at pH 7.5 yielded a unique, sharp arabinosidase peak eluted by 0.37 M sodium chloride (Figure 3) and containing 16% of the original activity and only 3% of the applied proteins (purification 14-fold). Residual contaminating glucosidase and rhamnosidase activities were efficiently eliminated at this step.

Arabinosidase was then bound to Concanavalin A-Ultrogel AcA 22, demonstrating its glycoproteic nature as most fungal glycohydrolases. The enzyme (3% of initial and 20% of applied activity) was released by low concentrations of methyl mannoside (Figure 4); however, some activity was also detected as a tailing of the major arabinosidase peak in the whole inhibitor gradient. Such a strong loss of activity with Con A chromatography has been already observed for glycosidases (Brillouet et al., 1985; Woodward et al., 1986) without any satisfactory explanation. Of the applied proteins 89% was eliminated along with remaining traces of glucosidase and rhamnosidase activities.

The overall purification factor was 27; the enzyme showed no glucosidase, rhamnosidase, and arabinopyranosidase measurable activities in a 40-h incubation time assay (0.2 nkat of arabinosidase).

Properties. The molecular weight of the enzyme determined by gel filtration on Ultrogel AcA 44 was 61 000, a figure similar to already reported values for fungal arabinosidases (Kaji, 1984; Brillouet et al., 1985). Its isoelectric point must be lower than 3.7 since preliminary attempts to purify the enzyme by chromatofocusing (Brillouet et al., 1985) showed that it remained bound to the anionic exchanger at the end of the pH gradient (pH 3.7). Comparable acidic p*I* have been reported for fungal arabinosidases (Kaji, 1984). The arabinosidase exhibited maximum activity in the range pH 3.7–4.0 (Figure 5) and was stable between pH 3.8 and 4.9; its activity rapidly decreased at pH <3.5 and >5.5. This unstability in the neutral and alkaline range is unusual for arabinosidases of fungal origin (Kaji, 1984). The optimum temperature was 60 °C (Figure 6), and the enzyme was inactivated after 30 min at 70 °C.

Specificity. The thin-layer chromatogram (Figure 7) of hydrolysis products of various monoterpenyl arabinofuranosylglucosides by the arabinofuranosidase showed that this enzyme liberated arabinose and the corresponding monoterpenyl β-D-glucosides. So this arabinosidase was able to cleave quantitatively under above conditions the (1→6) linkage between the terminal arabinofuranosyl unit and the intermediate glucose (Figure 1), regardless the structure of the liberated monoterpenyl β-D-glucosides. It should be noted that some plant and fungal β-D-glucosidases are unable to hydrolyze some of the above monoterpenyl glycosides (Gunata et al., 1989) to yield the aromatic volatile terpenols. This apparent difference in specificity could be related to the structure of substrates, the α-L-arabinofuranosidic linkage being farther from the aglycon moiety than the β-D-glucopyranosidic one. This hypothesis is sustained by the ^{1}H and ^{13}C NMR spectra of the substrates (Voirin et al., 1989), which show that the orientation of the α-L-arabinofuranosidic linkage is unaltered whatever might be the monoterpenyl moiety contrary to the β-D-glucopyranosidic linkage, which is affected by the kind of monoterpenyl agly-

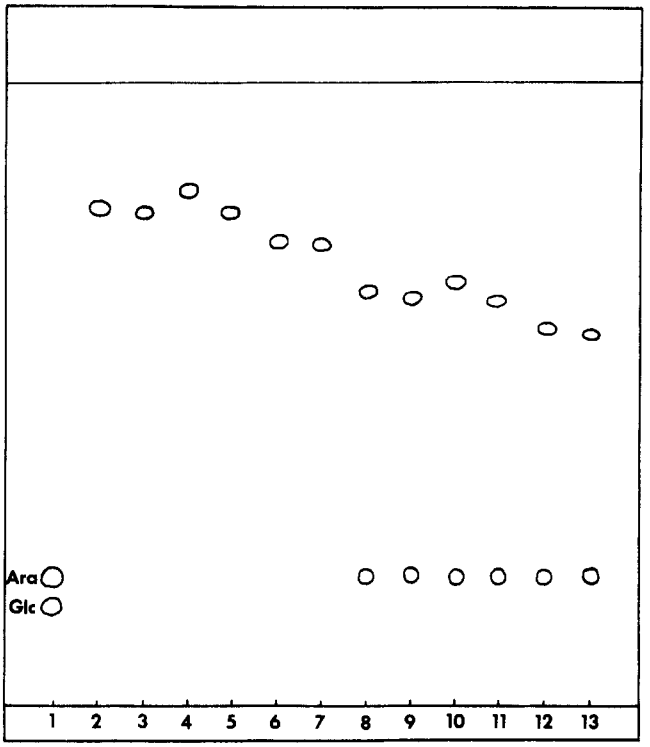

Figure 7. TLC of the products of hydrolysis of synthetic monoterpenyl arabinofuranosylglucosides by the arabinofuranosidase. Key: (1) arabinose and glucose; (2→7) monoterpenyl arabinosylglucosides; (2) Ner-AraGlc; (3) Ger-AraGlc; (4) Lin-AraGlc; (5) Ter-AraGlc; (6) Ben-AraGlc; (7) Phe-AraGlc; (8→13) monoterpenylglucosides; (8) Ner-Glc; (9) Ger-Glc; (10) Lin-Glc; (11) Ter-Glc; (12) Ben-Glc; (13) Phe-Glc.

con. The enzyme is devoid of β-D-glucosidase activity since glucose was not detected even on long incubation periods. Moreover, it was inactive toward monoterpenyl rhamnosylglucosides, indicating the absence of α-L-rhamnosidase (not shown). Thus, this arabinofuranosidase has been used as a powerful analytical tool for the spotting in GLC and HPLC chromatograms (Bitteur et al., 1989) of arabinosylglucosides in a mixture of monoterpenyl glycosides, including the rutinosides and glucosides, and for the elucidation of the sequential mechanism of hydrolysis of these compounds (Gunata et al., 1988).

The wide spectrum of activity of this arabinofuranosidase toward monoterpenyl 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides is interesting for future technological uses of the commercial crude preparation from which this enzyme was obtained for enhancement of the aroma of grape juice and derived beverages since the tested monoterpenyl glycosides are among the most abundant in the juice of Muscat grapes, although the restricted spectrum of action of glucosidases is still a limitation.

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