An Endoglycosidase from Grape Berry Skin of Cv. M. Alexandria Hydrolyzing Potentially Aromatic Disaccharide Glycosides

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We present evidence on the presence of an endoglycosidase in grape skins able to split the heterosidic linkage of disaccharide glycosides, releasing disaccharide and aglycon. Its extraction from grape skins (cv. Muscat of Alexandria) was possible with the use of a detergent (poly(ethylene glycol)). The enzyme was purified about 1000-fold successively by chromatography on DEAE Sepharose CL 6B, CM 20 Poros, and Protein-Pak SW 300. The optimum pH and temperature activity were found to be similar to levels reported for other plant glycosidases. K_m and V_{max} values for pNP-β-rutinoside were 1.69 mM and 275 nkat/mg, respectively, with a molecular mass of 58 000. The enzyme was quite tolerant to glucose inhibition (K_i = 212 mM). Hydrolysis of monoterpenyl and 2-phenylethyl glycosides (arabinofuranosyl-, rhamnopyranosyl-, apiofuranosylglucosides) from grape and arabinopyranosyl- and xylopyranosylglucosides from other plants yielded the corresponding disaccharides and aglycons. The identity of the released sugars was confirmed by GC-EIMS/GC-NCIMS analysis of trifluoroacetylated derivatives.

Keywords: *Grape; endoglycosidase; disaccharide glycosides; aroma precursors; hydrolysis*

INTRODUCTION

Several attractive flavor compounds, monoterpenes, C13-norisoprenoids, and shikimate-derived compounds accumulate as odorless β-D-glucoside and diglycoside conjugates in some fruits and plant tissues (Williams et al., 1982; Günata et al., 1985; Krammer et al., 1991; Ogawa et al., 1997; Winterhalter and Skouroumounis, 1997). A common feature of these glycosidic flavor precursors is that the aglycon is linked to a β-D-glucose. Flavor enhancement through enzymatic hydrolysis of these glycosides has stimulated considerable research interest. The hydrolysis of monoglucosides requires the action of a β-glucosidase, while a sequential reaction is involved for the hydrolysis of disaccharide glycosides; first α-rhamnosidase, α-arabinofuranosidase, and β-apiosidase cleave the inter-sugar linkage and second β-glucosidase releases volatile compounds from the resultant β-D-glucosides (Günata et al., 1988, 1993). Besides these monosaccharide glycosidases that commonly occur in the plant kingdom and microorganisms (Günata et al., 1993), some plants such as *Rhamnus dahurica* (Suzuki, 1962), *Viburnum furcatum blume* (Imaseki and Yamamoto, 1961), and *Fagopyrum esculentum* (common buckwheat) (Bourbouze et al., 1975) contain a disaccharidase or endoglycosidase that is capable of hydrolyzing the heterosidic linkage of flavonoid diglycosides, thus liberating disaccharide and aglycon. Only a few genera have been found to possess an endoglycosidase out of 125 plant species studied (Plouvier, 1978). Production of such enzymes by filamentous fungi is inducible using adequate carbon sources (Hay et al., 1961; Shoseyov et al., 1988). Recently an endoenzyme (a primeverosidase) was isolated from tea leaves which releases flavor compounds from primeverosides (6-O-β-D-xylopyranosyl-β-D-glucopyranoside) (Guo et al., 1996; Ogawa et al., 1997). Previous work has shown the presence of monosaccharide glycosidases except β-apiosidase in grape berries (Aryan et al., 1987; Di Stefano, 1989; Günata et al., 1989; Lecas et al., 1991). The purpose of the present work was to study the occurrence of an endoglycosidase in grape berries and the hydrolytic activity of the enzyme toward several disaccharide glycosides. As plant material, grape berry skin was chosen because of its abundance of glycosidases (Biron et al., 1988).

MATERIALS AND METHODS

Enzyme Extraction. Muscat of Alexandria grapes picked at harvest maturity from the Salins du Midi vineyard near Montpellier, France, were kept frozen (−20 °C) until analysis. Grape skins (ca. 300 g) removed with a cutter from 3 kg of grape berries were ground in a Dangoumau ball grinder in liquid nitrogen. The skin powder was suspended in 1.5 L of phosphate−citrate buffer (100 mM, pH 7.2), containing 10 mM EDTA, 4 mM DTT, 1% PEG 4000 (w/v), and 3% PVP (w/v). After a gentle stirring (150 min at 4 °C), the slurry medium was centrifuged (30 min, 10000*g*, 4 °C). The pellet was discarded, and ammonium sulfate was added to the supernatant (80%). The precipitate was collected by centrifugation and suspended in 250 mL of the phosphate−citrate buffer (100 mM, pH 7.2). Chilled acetone (−20 °C) was added to the aqueous solution up to 60%. This ensemble was allowed to stand for 3 h and centrifuged. The pellet was recovered and dissolved in 40 mL of phosphate−citrate buffer to obtain the crude enzyme extract.

Purification. All the steps were carried out at 4 °C. The crude enzyme solution was equilibrated with imidazole buffer (25 mM, pH 7.4) by ultrafiltration on membrane (Amicon PM

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S0021-8561(98)00084-3 CCC: $15.00
Published on Web 07/01/1998

10, Grace, France) and loaded on a DEAE-Sepharose CL6B column (3.2 × 24 cm, Pharmacia, France) previously equilibrated with the above buffer. The proteins were eluted by NaCl gradient (0 → 1 M) in imidazole buffer. The flow rate was 0.25 mL/min. The fractions (2.5 mL) were assayed for glycosidase activities and protein (abs 280 nm). Endoglycosidase active fractions were combined, concentrated by ultrafiltration, and injected (100 μL) in several steps on a FPLC column (Poros CM 20, 4.6 × 100 mm, Perseptive Biosystems, Framingham, MA) previously equilibrated with acetate buffer (20 mM, pH 5.6). A NaCl gradient (0 → 1.5 M) was applied to elute the proteins. The flow rate was 5 mL/min. Fractions (1 mL) showing endoglycosidase activity were combined, dialyzed overnight against phosphate−citrate buffer (100 mM, pH 6.0), concentrated by Centricon 10 (Grace, France), and subjected to FPLC on a Protein Pak 300 SW column (7.5 × 300 mm, Waters, Milford, MA), equilibrated with the same buffer. The flow rate and fraction volumes were 1 mL/min and 1 mL, respectively.

Assay of Glycosidase Activities. The endoglycosidase and β-glucosidase activities were monitored through all purification steps with *p*-nitrophenyl-(*p*NP)-β-rutinoside and *p*NP-β-D-glucoside, respectively. The assay containing 75 μL of enzyme solution (diluted if necessary) and 75 μL of substrate (5 mM *p*NP-β-rutinoside or 20 mM *p*NP-β-D-glucoside in 100 mM acetate buffer, pH 5.0) was incubated at 40 °C for 30 min. The reaction was stopped by the addition of 450 μL of Na_2CO_3 (1 M), and the amount of *p*NP was determined by measuring the absorbance at 400 nm (Günata et al., 1988). Activity was expressed as nanokatals (nkat). Note that 1 nkat is 1 nmol of *p*NP liberated per s per mL of enzyme solution or mg enzyme. The protein concentration of samples was determined by the method of Pierce (bicinchoninic acid as protein reagent, Pierce, Rockford, IL) with bovine serum albumin as standard.

For the inhibition studies, various amounts of glucose were added to the assay solution and the inhibition constant for endoglycosidase was calculated from the Lineweaver−Burk plot.

Properties. *Molecular Mass.* MM was determined by gel filtration on a Protein-Pak 300 SW column as described above. The column was calibrated with the following molecular weight markers: ferritin (450 kDa), aldolase (158 kDa), bovine albumin (68 kDa), ovalbumin (45 kDa), chymotrypsin (25 kDa), cytochrome *c* (12.5 kDa).

Substrates. The K_m values for *p*NP-β-rutinoside (substrate concentration ranging from 0.62 to 3.5 mM) were calculated from the Lineweaver−Burk plot.

Several disaccharide substrates (1 mM each in acetate buffer, 20 mM, pH 5.0), rutinosides (6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside) of *p*NP, nerol, linalool, 2-phenylethanol (Voirin et al., 1990a), 6-O-α-L-arabinofuranosyl-β-D-glucopyranosides of nerol and linalool (Voirin et al., 1990a), 6-O-β-D-apiofuranosyl-β-D-glucopyranosides of geraniol and linalool (Voirin et al., 1990b), the vicianoside (6-O-α-L-arabinopyranosyl-β-D-glucopyranoside) of 4-methylumbelliferone, peltatoside (6-O-α-L-arabinopyranosyl-β-D-glucopyranoside of quercetine, Extrasynthèse, France), and the primeveroside (6-O-β-D-xylopyranosyl-β-D-glucopyranoside) of eugenol, were incubated with grape endoglycosidase (0.035 nkat) at 40 °C for 24 h. The reaction mixtures at different intervals were then subjected to TLC (15 μL of assay medium) and GC/MS analysis.

For TLC, 15 μL aliquots were loaded on silica gel, and ethyl acetate/2-propanol/water (65/30/10) was used as the solvent. Sugars were detected with 0.2% naphthoresorcinol in sulfuric acid−ethanol (5/95) after 10 min at 100 °C.

For the GC/MS analysis, trifluoroacetylated derivatives of sugars and glycosides were prepared. Phenyl-β-D-glucoside (10 μg of a 0.1% solution in ethanol) as standard was added to an aliquot (20 μL) of assay medium.The mixture was concentrated to dryness at 40 °C under nitrogen, and trifluoroacetylation was then performed (Voirin et al., 1992). EIMS and NCIMS spectra for TFA derivatives were recorded by coupling a Hewlett-Packard 5890 gas chromatograph equipped with a DB 5 MS fused-silica capillary column (60 m × 0.32 mm, i.d.; 0.2 μm film thickness, J&W Scientific, Folsom, CA) to a HP 5889 A mass spectrometer. The transfer line was heated to 290 °C. The carrier gas was helium (1.5 mL/min). The injector temperature was programmed at 180 °C/min from 110 to 250 °C and then held for 20 min. For EIMS, the quadrupole temperature was 120 °C, with a source temperature of 250 °C. Mass spectra were scanned at 70 eV in the *m*/*z* 29−350 range at 1 s intervals. For NCIMS, the conditions were as follows: energy of electrons 230 eV; emission current 300 μA; temperatures of the source and quadrupole 200 and 120 °C, respectively. Methane was used as reagent gas (1.3 Torr). The mass spectra were recorded in the *m*/*z* 100−1100 range at 1 s intervals.

pH and Temperature Effects. For optimum pH, endoglycosidase activity was measured in 100 mM phosphate−citrate buffer with pH ranging from 2.6 to 8.0. For the pH stability, the enzyme was incubated (22 °C, 21 h) in the phosphate−citrate buffer at various pHs and then the activity was measured at optimum pH. The optimum temperature activity for endoglycosidase was checked in the 10−80 °C temperature range in phosphate−citrate buffer (100 mM, pH 6.0).

RESULTS AND DISCUSSION

Study of the Endoglycosidase Extraction Procedure. *p*NP-β-rutinoside was used for the detection of activity during enzyme extraction and purification steps. Endoglycosidase activity was assayed by colorimetric measure of released *p*NP. The assay solutions at different steps were also subjected to TLC analysis to ensure that enzymatic cleavage of *p*NP-β-rutinoside was at an aglyconic linkage, thus yielding the disaccharide (rutinose). Further evidence indicating the presence of a dissaccharide was obtained by GC-MS analysis of corresponding TFA derivatives.

Endoglycosidase from grape skins was only extracted when a detergent was present in the extraction medium. Among the detergents, at the concentration used in the extraction medium (1%), poly(ethylene glycol) (PEG) was found to be the most efficient. This detergent is usually employed for protein precipitation (Ingham and Busby, 1980) and increased endoglycosidase extraction 7.5-fold in comparison to the other nonionic detergent (Triton X-100; Table 1). With Tween 80, the endoglycosidase was not extracted; however this detergent has been used in the extraction of β-glucosidase from grape berries (Aryan et al., 1987; Biron et al., 1988; Lecas et al., 1991). PEG-4000 was also interesting for the solubilization of β-glucosidase since the activity was 2-fold higher compared to the value obtained for Tween 80.

These results suggest that the structure of grape endoglycosidase differs from that obtained from tea and/or it could be a membrane-bound protein, considering that the extraction of the tea enzyme (primeverosidase) did not necessitate the use of detergent (Guo et al., 1996; Ogawa et al., 1997).

The yield of β-glucosidase and endoglycosidase extraction varied according to the PEG concentration. Enzymes were not extracted at 0.1% PEG, and the extraction yield was quite similar between 0.75 and 1%. Furthermore the use of a higher molecular weight detergent (PEG 20 000) at 1% concentration in the extraction medium did not improve enzyme recovery and hindered the acquisition of homogeneous extracts.

No noticeable differences were observed in endoglycosidase extraction, starting from an acetone powder of skins or directly from skin powder. In contrast, β-glucosidase extraction was 6-fold lower with acetone powder. Extraction of endoglycosidase from acetone powder also required the use of PEG 4000.

Table 1. Effect of Detergent on Glycosidase Extraction

detergent (1%)	β-glucosidase (nkat/g of skin)	endoglycosidase (nkat/g of skin)
none	0	0
PEG 4000	0.36	0.06
Triton X100	0.15	0.008
Tween 80	0.18	0

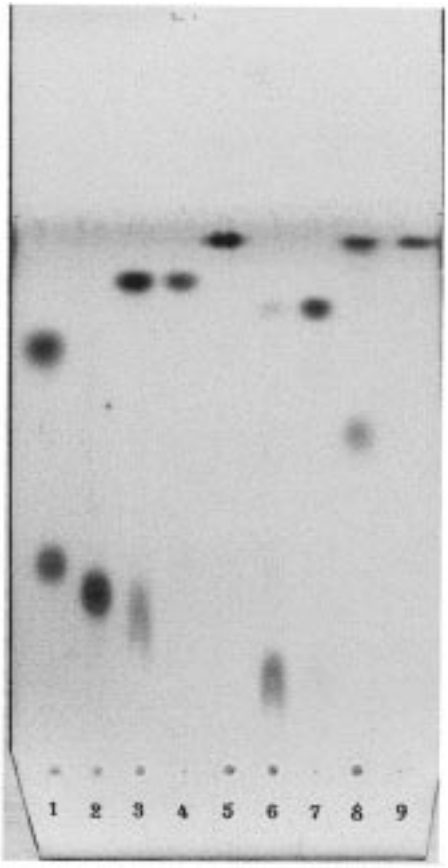

Figure 1. TLC of the hydrolysis products of glycosides by crude enzyme extract: (1) glucose and rhamnose; (2) rutinose; (3) enzyme (E) + *p*NP-rutinoside; (4) *p*NP-rutinoside; (5) E + *p*NP-rhamnoside; (6) E + eugenylprimeveroside; (7) eugenylprimeveroside; (8) E + *p*NP-xyloside; (9) *p*NP-xyloside.

Once the extraction procedure was established, before the chromatographic purification steps, hydrolysis of *p*NP-β-rutinoside, eugenyl-β-primeveroside, *p*NP-β-D-xylopyranoside, and *p*NP-α-L-rhamnoside by the crude grape enzyme extract was checked by TLC analysis of released sugars (Figure 1). Hydrolysis of *p*NP-β-rutinoside yielded only rutinose ($R_f = 0.29$), with no glucose or rhamnose. The enzyme extract had no action toward *p*NP-α-L-rhamnoside. This may be the reason of the absence of sequential hydrolysis of *p*NP-β-rutinoside (Günata et al., 1988). Hydrolysis of eugenyl-β-primeveroside also yielded a product at the R_f of a disaccharide (0.17), although the crude enzyme extract showed activity toward *p*NP-β-D-xylopyranoside (Figure 1). Consequently, we can assume that endoglycosidase activity dominated in the crude enzyme extract, in comparison to monosaccharide glycosidases. Confirmation of the disaccharide in assay conditions was further substantiated by GC/MS analysis of TFA derivatives.

Enzyme Purification. *Removal of PEG from the Crude Enzyme Extract.* Before chromatographic purification steps, the assays were carried out to remove more PEG 4000 from the crude enzyme extract. It is well-known that the PEG can affect the protein separation performance in chromatographic media (Ingham and Busby, 1980). Glycosidase activities were thus eluted with the void volume of a gel filtration column (Ultrogel AcA 44), when we used a grape crude enzyme extract containing 1% PEG 4000. In contrast, this phenomenon was not observed with grape crude enzyme extract containing Tween 80 as detergent (Lecas et al., 1991).

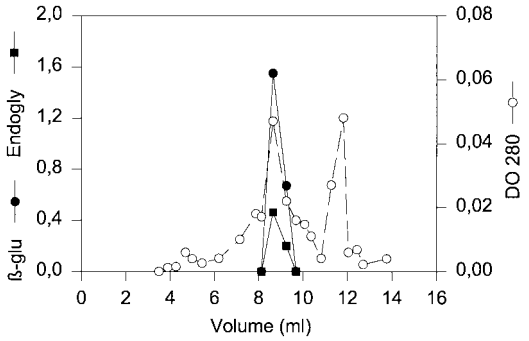

Figure 2. Ion-exchange chromatogram of the crude enzyme extract on a DEAE Sepharose CL-6B column.

Detergent removal from the crude enzyme extract was assayed by salt-induced phase separation, according to the described procedure (Busby and Ingham, 1980). Although this approach permitted quite good separation of PEG (in upper phase) and protein in biological liquids (Ingham and Busby, 1980), the results were not satisfactory with the crude grape enzyme extract. Glycosidase activities were almost equally distributed in the upper and lower phases, and the detergent was not sufficiently separated in both phases. Removal of Triton X-100 from aqueous protein solutions was found to be possible by adsorption on Amberlite XAD-2 resin (Cheetman, 1979). Although this procedure was successful for elimination of PEG from an aqueous solution (1% w/v), it failed when a crude grape enzyme extract containing 1% PEG was used. This suggests an interaction between endoglycosidase−PEG hinders PEG accesibility to the gel. Quite similar results were obtained when another hydrophobic adsorbent (Sep-Pak C 18, Waters) was used to remove PEG from the crude enzyme extract. As these assays failed to efficiently eliminate PEG from the medium, the crude enzyme extract was subjected to ammonium sulfate and acetone precipitations, respectively. Most of the detergent was eliminated in this way, particularly by the acetone treatment.

Purification. A crude enzyme extract obtained from 300 g of grape skins was first subjected to anion exchange chromatography on DEAE Sepharose CL-6B. Two peaks of β-glucosidase activity were obtained (Figure 2). The endoglycosidase was coeluted with the first peak of β-glucosidase in the void volume of the column. We had only observed the second peak of β-glucosidase activity in a previous study on grape β-glucosidase during chromatography with the same gel (Lecas et al., 1991). The main difference in both cases was due to the use of different detergents in the enzyme extraction assay. As endoglycosidase was not bound to the anion exchange gel, the pooled active fractions were subjected to cation exchange chromatography (CM 20) using the FPLC technique. Endoglycosidase was strongly bound to the gel and coeluted with a β-glucosidase at a high NaCl concentration (900 mM) (Figure 3). Further chromatography by FPLC gel filtration on a Protein-

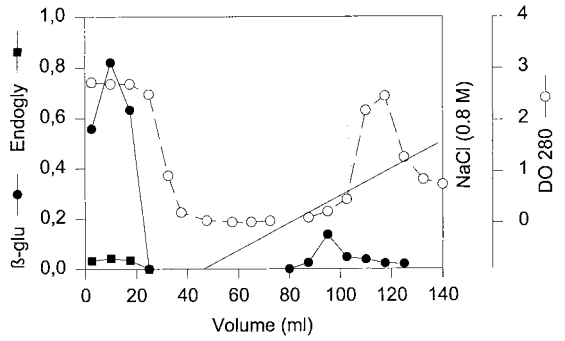

Figure 3. FPLC of grape glycosidases on a CM 20 column.

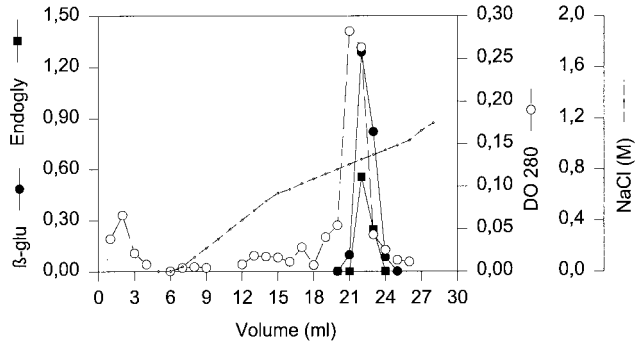

Figure 4. FPLC of grape glycosidases on a Protein-Pak SW 300 column.

Table 2. Purification of Endoglycosidase from 300 g of Grape Skins

purification step	activity (nkat)	protein (mg)	specific activity (nkat/mg)	purification factor
crude extract	27.7	378	0.07	1
$(NH_4)_2SO_4$ ppt	11.5	189	0.06	0.86
acetone ppt	2.6	9.12	0.29	4.1
DEAE CL-6B	2.1	0.42	5.0	71
CM 20	0.7	0.10	7.0	100
Protein-Pak	0.6	0.008	75	1071

Pak SW column allowed us to achieve a purification factor of 1071 (Table 2). Endoglycosidase and β-glucosidase activity were detected in the same peak (Figure 4).

Properties. The molecular mass (58 kDa) determined by FPLC gel filtration was close to that reported (61 kDa) for a primeverosidase from tea leaves (Ogawa et al., 1997). K_m and V_{max} values for *p*NP-β-rutinoside were 1.69 mM and 275 nkat/mg, respectively. The enzyme exhibited maximum activity between pH 4 and 5 and was stable between pH 4 and 8 (Figure 5). The optimum temperature was at 50 °C (Figure 6). Similar values were reported for plant glycosidases (Günata et al., 1993). Grape endoglycosidase was quite tolerant to glucose inhibition (K_i = 212 mM). This value is close to those reported for grape (Lecas et al., 1991) and almond β-glucosidase (Heyworth and Walker, 1962).

Action of the Enzyme against Various Disaccharide Glycosides. The hydrolysis products of geranyl, neryl, linalyl, 2-phenylethyl, eugenyl, 4-methylumbelliferyl, and quercetyl disaccharide glycosides (see Materials and Methods) by endoglycosidase after 24 h incubation at 40 °C were checked by TLC and GC/MS. Except for peltatoside, only disaccharides (R_f: rutinose, 0.35; apiosylglucose, 0.3; arabinofuranosylglucose, 0.33; xylosylglucose, 0.17; arabinopyranosylglucose, 0.08) were detected by TLC. The monosaccharides (R_f: arabinose, 0.38; glucose, 0.31) were observed in the assay with

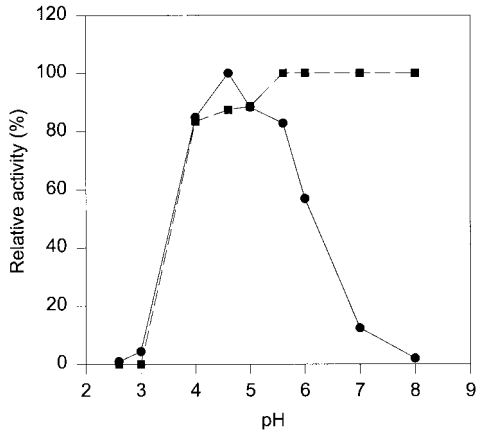

Figure 5. Effect of pH on activity (●) and stability (■) of endoglycosidase.

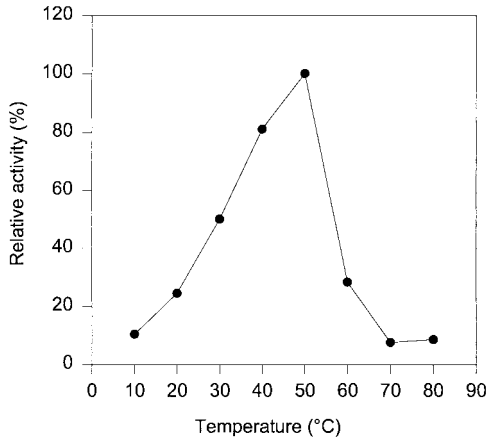

Figure 6. Effect of temperature on endoglycosidase activity.

peltatoside. For the sugar identification, trifluoroacetylated derivatives of the reaction mixtures were analyzed by GC-EIMS and GC-NCIMS. Information on the sugar composition of the disaccharides was obtained through the EI mass spectra (Voirin et al., 1992). The EI mass spectra of the rutinose detected during enzymatic hydrolysis of 2-phenylethyl-β-rutinoside, together with the total ion chromatogram, are given in Figure 7 as an example. Fragment ions at *m*/*z* 318 and 319 indicated the presence of glucose and at *m*/*z* 207 and 435 that of rhamnose. Similarly, ions at *m*/*z* 193 and 421 were fragment ions of pentosyl residues (apiose, arabinose, and xylose) detected in the enzymatic hydrolysis assays with related diglycosides. On the other hand, characteristic ions of trifluoroacetylated disaccharides were obtained by NCIMS (Table 3): A molecular ion M^-, an adduct ion $[M + TFAO]^-$, i.e., $[M + 113]$, and a fragment ion $[M - TFAO]^-$ were thus observed for rutinose and pentosylglucoses (Chassagne et al., 1995, 1996a). The NCI mass spectrum of the TFA derivative of arabinosylglucose is shown in Figure 8 as an example. The mass spectra of reference sugars (rutinose and arabinofuranosylglucose) as TFA derivatives in EI and NCI modes were closely correlated with those of the corresponding disaccharides generated upon the action of endoglycosidase on glycosides.

These results show that grape skin possesses an endoglycosidase capable of releasing aglycons from disaccharide glycosides in one step. In our assay conditions (24 h incubation at 40 °C), 2-phenylethyl and neryl rutinosides, geranyl apiosylglucoside, neryl arabinofuranosylglucoside, and eugenyl primeveroside were

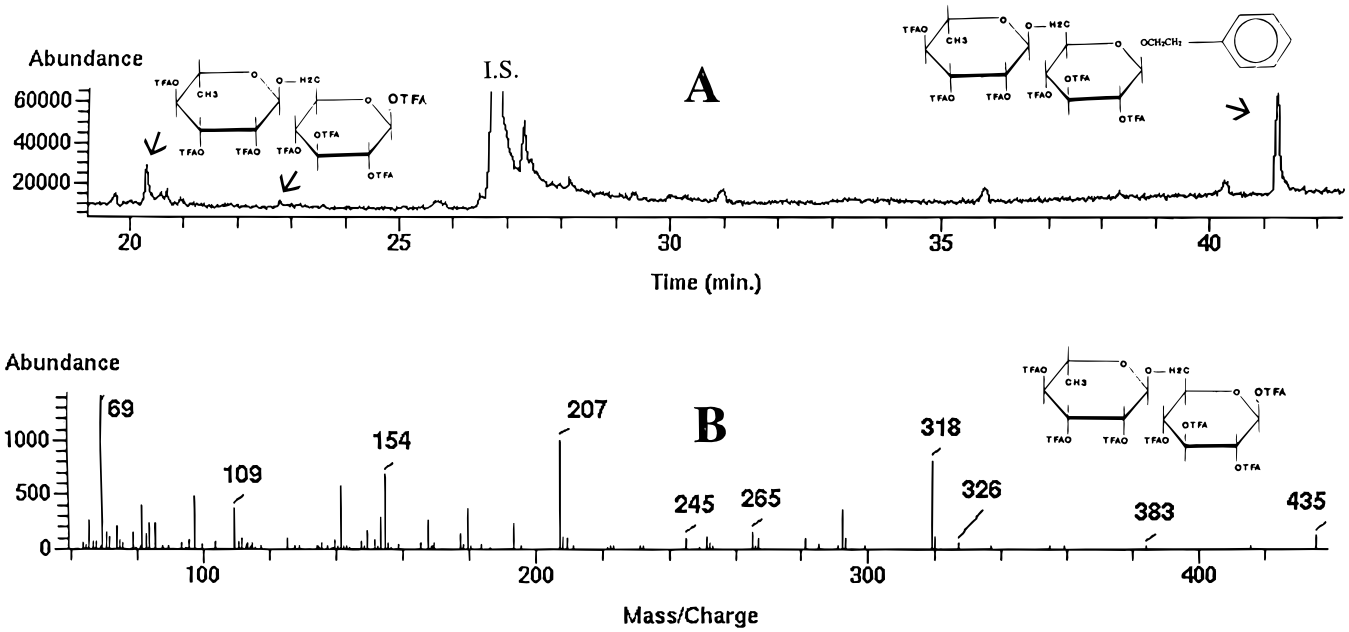

Figure 7. Hydrolysis of 2-phenylethyl-β-rutinoside by grape endoglycosidase: total ion chromatogram of TFA derivatives (A); EI mass spectrum of rutinose (B).

Table 3. Characteristic Fragment Ions in NCIMS of TFA Derivatives of Disaccharides Generated by Enzymatic Hydrolysis of Glycosides

rhamnosylglucose[a]		pentosylglucose[a]	
m/z	fragment ions	*m/z*	fragment ions
1111	$[M + TFAO]^-$	1097	$[M + TFAO]^-$
998	M^-	984	M^-
885	$[M - TFAO]^-$	871	$[M - TFAO]^-$

[a] The retention time (in GC) relative to phenyl-β-D-glucopyranoside was 0.758, 0.831, 0.834, and 0.964 for the rhamnosyl-, arabinosyl-, apiosyl-, and xylosylglucoses, respectively.

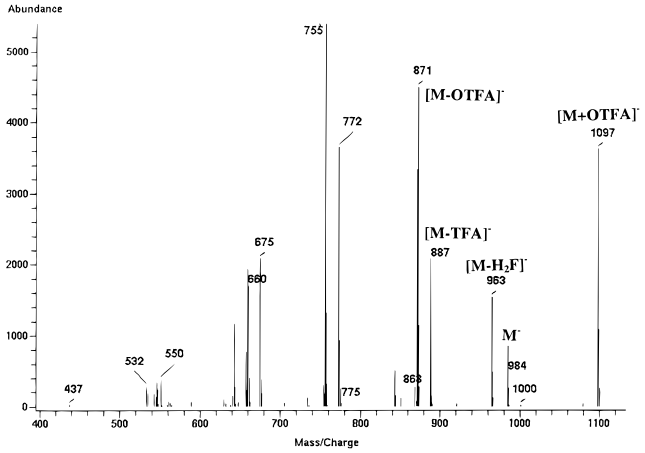

Figure 8. NCI mass spectrum of the TFA derivative of arabinosylglucoside generated during the hydrolysis of neryl-arabinofuranosylglucoside by grape endoglycosidase.

almost completely hydrolyzed. On the contrary, the activity was found to be very low toward tertiary alcohol (linalool) glycosides. The trend noted with aglycon was already observed for the hydrolysis of linalyl-β-D-glucoside by grape and almond β-glucosidases (Lecas et al., 1991; Günata et al., 1993). The mode of action of the enzyme was influenced by the aglycon moiety of arabinopyranosylglucosides tested. While the hydrolysis of 7-0-α-L-arabinopyranosyl-(1 → 6)-β-D-glucopyranoside of 4-methylumbelliferone yielded a disaccharide, the hydrolysis of peltatoside (3-0-α-L-arabinopyranosyl-(1 → 6)-β-D-glucopyranoside of quercetine) generated glucose and arabinose. The mode of action of some plant and fungal enzymes toward flavonoid glycosides was found to vary as a function of 3- or 7-substituted flavones (Suzuki, 1962).

Besides disaccharides from grape (arabinofuranosyl-, rhamnopyranosyl- and apiofuranosylglucosides), substrates from other plants, arabinopyranosylglucoside from passion fruit (Chassagne et al., 1996b), and xylopyranosylglucoside from tea (Guo et al., 1996; Ogawa et al., 1997) were found to be substrates for grape endoglycosidase. All of these substrates possessed a (1 → 6) intersugar linkage. Kinetic studies are envisaged using several substrates presenting different sugar and aglycon compositions to further characterize the catalytic action of the enzyme. Although plant glycosidases exhibit a marked specificity for different aglycons (Hösel and Conn, 1982; Lecas et al., 1991; Günata et al., 1993), there have been very few studies on the sugar specificity of endoglycosidases. An enzyme from *Viburnum furcatum* seems to require 6-O-β-D-apiofuranosyl-β-D-glucopyranose linkage for its action (Imaseki and Yamamoto, 1961), whereas that from *Rhamnus* (Suzuki, 1962) and from *Fagopyrum esculentum* (Bourbouze et al., 1975) showed a broad specificity toward flavonoid glycosides.

Many issues concerning grape endoglycosidase still require clarification, e.g. its involvement in flavor release during winemaking, its physiological function, and its tissue localization and changes with fruit development.

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Received for review January 30, 1998. Revised manuscript received April 29, 1998. Accepted April 29, 1998. This work was supported by an EU project (AIR 3 CT94 2193). We thank the Salins du Midi Co. for their generous donation of grape samples.

JF980084J