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SEQUENTIAL ENZYMIC HYDROLYSIS OF POTENTIALLY AROMATIC GLYCOSIDES FROM GRAPE

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ABSTRACT

The mechanism of action of α-L-arabinofuranosidase, α-L-rhamnopyranosidase, and β-D-glucopyranosidase on *p*-nitrophenyl and grape monoterpenyl disaccharide-glycosides has been studied. First, the (1→6) linkage is cleaved by either α-L-arabinofuranosidase or α-L-rhamnosidase, and arabinose, rhamnose, and the corresponding monoterpenyl β-D-glucosides are released. Subsequently, liberation of monoterpenol takes place after action of β-D-glucosidase. The possible use of these glycosidases for the enhancement of the aroma of grape juice and derived beverages is emphasised.

INTRODUCTION

Terpenols, which are important constituents of the flavor of Muscat grapes[1–3], are present partly free and partly as 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides (**1**), 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides (**2**, rutinosides), or β-D-glucopyranosides (**3**, traces) mainly of geraniol, nerol, and linalool[4,5]. These are the most aromatic terpenols and have the lowest olfactive thresholds among the Muscat monoterpenols[2,6]. Smaller quantities of bound forms of linalool oxides, 3,7-dimethylocta-1,5-diene-3,7-diol, α-terpineol, and citronellol are also found[5,7].

The ratio between bound and free monoterpenols ranges between 1 and 5 in the juice of mature grape cultivars of Muscat and Riesling[4,5] and up to 15 in the Gewurtztraminer variety[5]. Thus, these glycosidic precursors constitute an important potential aroma in juices[8] as well as in wines since they are hydrolysed very slightly during the fermentation process[9].

These precursors can be hydrolysed by acid[10,11] or enzymes[4,5,12]; under the former conditions, rearrangement of the monoterpenols may occur, whereas, under

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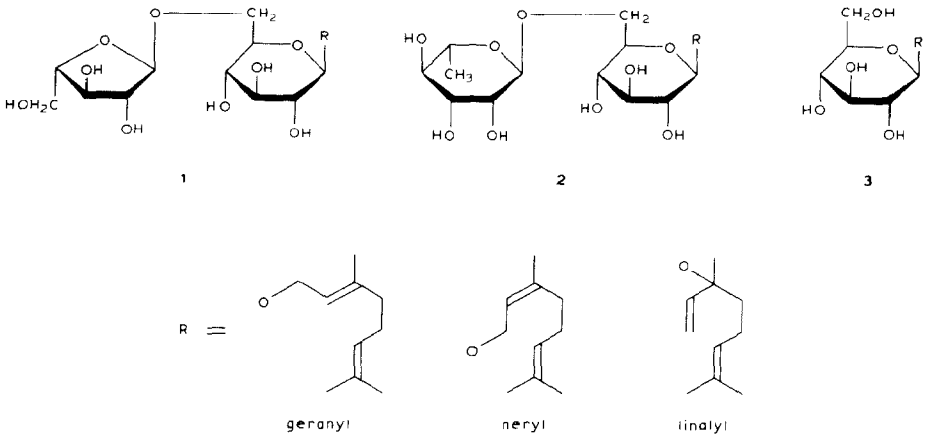

the latter conditions, the changes in the natural monoterpenol distribution are minimal. Enzymes from grape[5,12,13], buckweat[5], fungi[4,5,14], and yeast[15] have been used in the study of hydrolysis of grape monoterpenyl glycosides. Although the mechanism of hydrolysis of one of the grape monoterpenyl glycosides by an endo-β-D-glucosidase has been described[14], understanding of their enzymic breakdown is still limited.

We now report on the action of highly purified glycosidases on *p*-nitrophenyl and monoterpenyl glycosides[16], which could lead to a better utilisation of this aromatic potential in grapes.

EXPERIMENTAL

Chemicals. — *p*-Nitrophenyl β-D-glucopyranoside (*p*NP-Glc), α-L-arabinofuranoside (*p*NP-Ara), and α-L-rhamnopyranoside (*p*NP-Rha) were commercial products. *p*-Nitrophenyl 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside (*p*NP-AraGlc) was a gift from Dr. C. Tapiero. Geranyl, neryl, and linalyl β-D-glucopyranosides (Ger-Glc, Ner-Glc, and Lin-Glc, respectively), and *p*-nitrophenyl and geranyl 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides (*p*NP-RhaGlc and Ger-RhaGlc, respectively) were prepared by the Koenigs–Knorr method[17] as described[4,18].

Activated charcoal (CXV type) was purchased from Ceca S.A. (Velizy–Villacoublay), Amberlite XAD-2 resin from Rohm & Haas, and Tri-Sil reagent (49001) from Pierce.

General. — The contents of glycoside extracts (100 μL) were hydrolysed with 2M trifluoroacetic acid (120°, 1.25 h)[19], and the sugars were converted into their alditol acetates[19,20] and subjected to g.l.c. at 210° on a fused-silica column (30 m × 0.32 mm i.d.) bonded with OV-225 (DB-225, J&W Scientific) (0.25-μm film), with the injector and flame-ionisation detector at 250°, split ratio 1:10, and hydrogen as carrier gas at 65 kPa.

The contents of glycoside extracts (equivalent to 100 μL) were methylated by the Hakomori technique[21] as described by Jansson *et al.*[22], using a 2 h-contact with the methylsulfinylmethanide. The products were extracted with $CHCl_3$–CH_3OH (2:1), and the extract was washed thrice with water[23] and concentrated in a stream of air, before hydrolysis of the residue with aqueous 90% formic acid (1 h, 100°) and then 2M trifluoroacetic acid (1.25 h, 120°). The partially methylated sugars were converted into their alditol acetates and analysed by g.l.c. on two fused-silica capillary columns bonded with OV-1 (Delsi Instruments, France; 50 m × 0.32 mm i.d.; 0.20-μm film, on-column injection at 35°, then 5°/min to 165°, and, after 30 min, to 210° at 15°/min; flame-ionisation detector at 250°; hydrogen carrier gas at 120 kPa) and with OV-225 as above (170° for 15 min, then 5°/min to 210°). Peaks were identified on the basis of retention times, using partially methylated alditol acetates from standard polysaccharides[24], and confirmed by g.l.c.–m.s. with the OV-1 column coupled to a Nermag R 1010C mass spectrometer[25]. Peak areas were corrected by response factors as described by Sweet *et al.*[26].

T.l.c. of sugars and glycosides was performed on silica gel (Merck, 5552), using ethyl acetate–1-propanol–water (65:30:10) and detection with 0.2% naphthoresorcinol in sulfuric acid–ethanol (5:95) for 10 min at 105°. The fraction of grape glycosides resistant to enzymic hydrolysis was recovered from the t.l.c. plates by extraction with methanol.

Monosaccharides and synthetic or grape glycosides were separated by g.l.c. of their trimethylsilyl derivatives. To an aliquot (50 μL) of the glycoside extract was added *p*NP-Glc as internal standard (25 μL of a 0.005% solution in ethyl acetate), and the mixture was concentrated to dryness at 40° under nitrogen. After the addition of the Tri-Sil reagent (50 μL), the mixture was stored for 20 min at 40°, then cooled, and injected onto the above fused-silica column bonded with OV-1 (on-column injection at 90°, then 5°/min to 305°, and 305° for 15 min; flame-ionisation detector at 300°; hydrogen carrier gas at 120 kPa).

The monoterpenols released by enzymic hydrolysis were recovered by extraction with pentane (5 × 250 μL). The extracts were concentrated, and the residues were analysed by g.l.c. on a capillary column (25 m × 0.32 mm i.d.) coated with CP-Wax 52 CB (Chrompack; 1.28-μm film, on-column injection at 70° then, after 5 min, to 195° at 2°/min; flame-ionisation detector a 250°; hydrogen carrier gas at 60 kPa).

Plant material. — Mature, sound grapes (cultivar Muscat of Frontignan) were collected in the experimental vineyards of the INRA Pech Rouge Experimental Station (Gruissan). Must was obtained by standard winery procedures (crushing and pressing), with the addition of sulfur dioxide (50 p.p.m.), and clarified by centrifugation.

Isolation of grape glycosides. — Must (80 L) and activated charcoal (80 g) were stirred for 4 h before storage overnight. The charcoal was then collected on NCA3 type (40–50 μm) cellulose filters and washed with distilled water (4 × 150 mL) to remove sugars[27]. Glycosides were then extracted from the charcoal with acetone (5 × 250 mL), the combined extracts were concentrated to dryness at 35°,

and a solution of the residue in distilled water (40 mL) was injected onto a column (20 × 1 cm) of Amberlite XAD-2 resin previously equilibrated with water[5]. The column was washed with water and pentane in order to remove residual sugars and free terpenols, respectively. Glycosides were then recovered by elution with ethyl acetate. The eluate was concentrated to dryness and a solution of the residue in water (40 mL) was repurified as described above. Each step of the purification procedure was checked by t.l.c. for the fate of terpenyl glycosides. Finally, the sugar-free aqueous extract (18 mL) was washed with pentane (5 × 10 mL) and frozen (−20°).

Enzymes. — α-L-Arabinofuranosidase (EC 3.2.1.55) was purified from a commercial crude enzyme mixture (Hemicellulase REG 2) from *Aspergillus niger* (Gist Brocades, Seclin)[28]. α-L-Rhamnopyranosidase (EC 3.2.1.40) was obtained from naringinase from *Penicillium* sp. (Sigma, N 1385) and freed from contaminating β-D-glucosidase activity by chromatofocusing on PBE 94 (Pharmacia), using a pH gradient from 7.5 to 3.7. Rhamnosidase isoenzymes were eluted at pH 6.2 and 5.7, respectively, whereas β-D-glucosidase activity remained bound and was released by M sodium chloride in 0.1M acetate buffer (pH 3.7). Sweet-almond β-D-glucopyranosidase (EC 3.2.1.21) from Koch–Light (287201) was used without purification.

One nkat of enzyme liberated 1nmol of *p*-nitrophenol/s from the corresponding 4mM solution of *p*-nitrophenyl glycoside in acetate buffer (pH 4.5) at 40°. Each enzyme was devoid of the two other activities used in this study up to incubation times of 40 h.

Action of enzymes on synthetic glycosides. — Solutions (200 μL) of *p*NP-RhaGlc [4mM in 0.1M acetate buffer (pH 4.2)] and Ger-RhaGlc were incubated with α-rhamnosidase (50 μL, 0.15 nkat) for 90 min at 40°. Similarly, *p*NP-AraGlc [200 μL of a 4mM solution in 0.1M acetate buffer (pH 4.2)] was treated with α-L-arabinofuranosidase (50 μL, 0.15 nkat) under similar conditions. β-D-Glucosidase (50 μL, 0.15 nkat) was added to each digest and incubation was continued for 90 min. Each substrate was also treated under similar conditions with β-D-glucosidase alone (50 μL, 0.15 nkat). Controls were also run with heat-inactivated enzymes (30 min, 95°).

The media were analysed after each enzymic hydrolysis by t.l.c. (10 μL) and, for liberated *p*-nitrophenol, by the addition of an equal volume of M sodium carbonate (absorption at 400 nm). Geraniol liberated from Ger-RhaGlc was recovered by extraction with pentane and determined by g.l.c.

Action of enzymes on grape glycosides. — Aliquots (200 μL) of grape glycoside extract were treated with enzymes under conditions similar to those used for the synthetic glycosides, but for 16 h. The aglycons liberated were extracted with pentane and analysed by g.l.c. The residual material was analysed by t.l.c. and g.l.c. after trimethylsilylation.

Another aliquot (200 μL) of grape glycosides was submitted to the simultaneous action of the three enzymes (0.15 nkat of each activity; 16 h, 40°) and

the resistant fraction was recovered by t.l.c. as described above. This material was hydrolysed either at pH 3.0 (20 min, 100°)[29] or by 2M trifluoroacetic acid (1.25 h, 120°)[19]. After cooling, each hydrolysate was extracted with pentane and the released aglycons were analysed by g.l.c. Then pentane-extracted samples were checked for liberated monosaccharides by t.l.c. and g.l.c. of the alditol acetate derivatives.

RESULTS AND DISCUSSION

Action of enzymes on synthetic glycosides. — The individual or sequential action of α-arabinofuranosidase, α-rhamnosidase, and β-glucosidase on *p*NP-AraGlc, *p*NP-RhaGlc, and Ger-RhaGlc was studied. The sugar moieties of these compounds are identical to those of natural glycosides; the last substrate also has the same aglycon as that of some major grape glycosides.

T.l.c. of the products released by enzymes revealed that none of the substrates was cleaved by β-glucosidase, whereas *p*NP-AraGlc (R_F 0.69) was hydrolysed into *p*NP-Glc (R_F 0.80) and arabinose (R_F 0.31) by α-arabinofuranosidase. Similarly, *p*NP-RhaGlc (R_F 0.68) was hydrolysed by α-rhamnosidase, yielding *p*NP-Glc and rhamnose (R_F 0.59).

The action of β-glucosidase on the samples previously treated with α-arabinofuranosidase or α-rhamnosidase resulted in the cleavage of *p*NP-Glc into glucose (R_F 0.26) and *p*-nitrophenol. The same pattern was observed when Ger-RhaGlc was treated sequentially with α-rhamnosidase and β-glucosidase, rhamnose, then glucose and geraniol being released.

Thus, enzymic hydrolysis of the synthetic disaccharide-glycosides requires three glycosidases and involves cleavage first of the disaccharide (1→6) linkage to give *p*NP-Glc and rhamnose or arabinose by action of α-rhamnosidase or α-arabinofuranosidase. *p*-Nitrophenol is then released by action of β-glucosidase. A similar sequence was reported for the hydrolysis of naringin by α-L-rhamnosidase and β-D-glucosidase[30]. Furthermore, sweet-almond β-glucosidase is able to release glucose quantitatively from *p*-nitrophenyl and geranyl β-D-glucosides.

Action of enzymes on grape glycosides. — A preliminary study of the mixture of glycosides isolated from the juice of Muscat of Frontignan grapes revealed rhamnose, arabinose, glucose, and an unknown compound (Table I). Methylation analysis using methyl iodide[21] indicated that rhamnopyranose and arabinofuranose were present in non-reducing end positions and glucopyranose was in both terminal and 6-linked positions. The mass spectrum of the partially methylated alditol acetate of the unknown constituent did not correspond to that of any common sugar.

The presence of terminal rhamnopyranose, arabinofuranose (Ara/Rha ratio ~2), and 6-linked glucopyranose confirmed the assertion[4] that Muscat grapes contain rhamnopyranosyl- and arabinofuranosyl-glucosides. However, the large proportion of terminal and 6-linked glucose and of the unknown component indicated that other glycosides were present.

TABLE I

METHYLATION ANALYSIS OF GRAPE GLYCOSIDES

Methyl ether	T[a]		*Rel. mol. %*	
	A	*B*		
2,3,4-Tri-*O*-methylrhamnose	0.475	0.761	5.8[b]	(5.4)[c]
2,3,5-Tri-*O*-methylarabinose	0.469	0.70	12.6	(10.3)
Unknown	0.52	0.754	14.1	(13.0)
2,3,4,6-Tetra-*O*-methylglucose	1.00	1.00	30.6	(71.3)
2,3,4-Tri-*O*-methylglucose	1.71	1.35	36.9	

[a]Retention times relative to that of 2,3,4,6-tetra-*O*-methylglucose on OV-225 (A) and OV-1 (B). [b]Relative mol. % of the methyl ethers. [c]Relative mol. % of the monosaccharides from direct sugar analysis.

The extract of grape contained no free monosaccharides or terpenyl monoglucosides, as shown by t.l.c. but three components were detected (*1* minor, R_F 0.67; *2* major, R_F 0.71; *3* minor, R_F 0.76; *cf.* Ger-Glc R_F 0.79, Ger-RhaGlc R_F 0.67).

β-Glucosidase had little effect on components *1–3* except for the formation of minute amounts of glucose. α-Arabinofuranosidase converted much of component *2* into arabinose and a Ger-Glc which was hydrolysed by β-glucosidase. Similarly, α-rhamnosidase converted component *1* into rhamnose and a Ger-Glc which was hydrolysed by β-glucosidase.

Even when the glycoside extract was exhaustively hydrolysed by the combination of the three glycosidases, a major component (R_F 0.71) remained. After recovery by t.l.c., hydrolysis of this component either at pH 3.0 (100°) or by 2M trifluoroacetic acid (120°) did not release any monoterpenol, whereas glucose, rhamnose, and an unknown product (R_F 0.55) were liberated by the strong acid hydrolysis. This last compound was identical to that detected by compositional analysis (Table I).

The products of enzymic hydrolysis of the grape glycosides were also analysed by g.l.c. of their trimethylsilylated derivatives (Figs. 1 and 2). The glycoside extract gave four major peaks (d–g), but none for monosaccharides or monoterpenyl β-D-glucosides, which confirms that these latter compounds must be present in Muscat grapes only in trace amounts[4].

After the action of β-glucosidase, only small peaks eluted between the internal standard and peak d were affected; some glucose was produced without liberation of any monoterpenol. Treatment with α-arabinofuranosidase removed two peaks d and f (Fig. 1) which were identified, respectively, as neryl and geranyl arabinosyl-glucosides by enzymic hydrolysis[27]. Linalyl arabinosyl-glucoside could not be identified under our chromatographic conditions. Peaks e and g were not affected, but arabinose, and linalyl, neryl, and geranyl glucosides were liberated together with an unknown component x and traces of monoterpenols. The relative

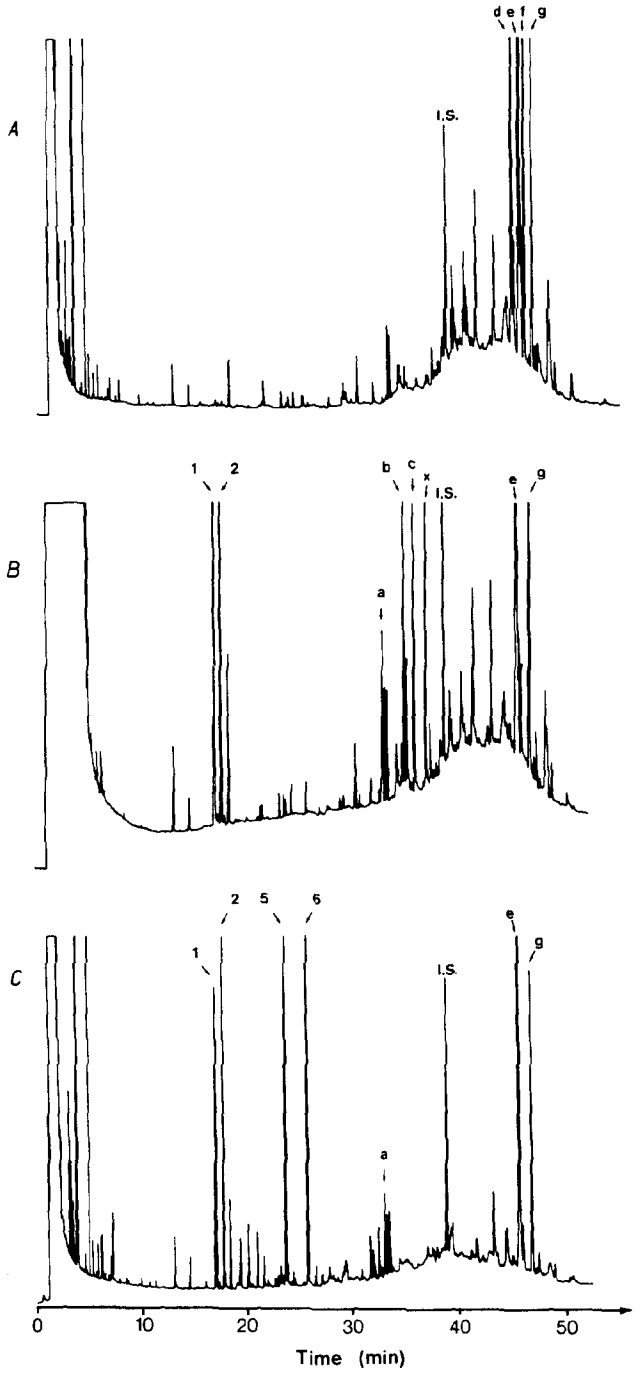

Fig. 1. G.l.c. of the trimethylsilylated products of the hydrolysis of grape glycosides (*A*) by α-L-arabinofuranosidase (*B*), and α-L-arabinofuranosidase + β-D-glucosidase (*C*). Key: 1 and 2, αβ-arabinose; 5 and 6, αβ-glucose; a, Lin-Glc; b, Ner-Glc; c, Ger-Glc; d, Ner-AraGlc; f, Ger-AraGlc; g, Ger-RhaGlc + unknown; e and x, unknowns; I.S., internal standard.

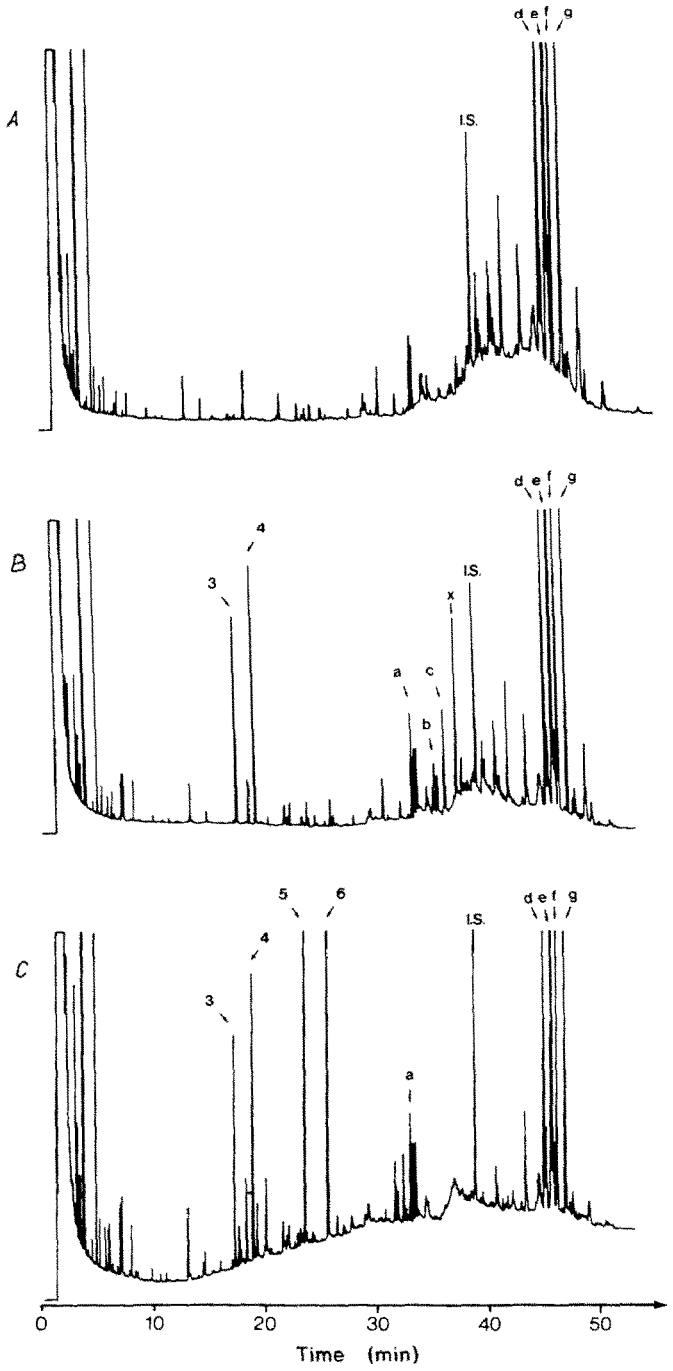

Fig. 2. G.l.c. of the trimethylsilylated products of hydrolysis of grape glycosides (*A*) by α-L-rhamnosidase (*B*), and α-L-rhamnosidase + β-D-glucosidase (*C*). Key: 3 and 4, αβ-rhamnose; for other peaks, see Fig. 1.

TABLE II

RELEASE OF MONOTERPENYL β-D-GLUCOSIDES FROM GRAPE GLYCOSIDES BY α-L-ARABINOFURANOSIDASE OR α-L-RHAMNOSIDASE

Monoterpenyl glucosides	*Enzymes*		
	α-L-Arabinofuranosidase	*α-L-Rhamnosidase*	*Total*
Linalyl glucoside	10[a]	9[a]	19[a]
Neryl glucoside	28	5	33
Geranyl glucoside	39	9	48
Total	77	23	100

[a]Percent of the total monoterpenyl glucosides liberated by both enzymes.

proportions of liberated monoterpenyl β-glucosides are shown in Table II, 77% of the total being produced by α-arabinofuranosidase. The relative abundance of terpenyl arabinosyl-glucosides in the grape juice of the Muscat of Frontignan cultivar, as deduced from Table II, was also observed in the Muscat of Alexandria[4]. Subsequent treatment with β-glucosidase eliminated the neryl and geranyl glucosides and component x, decreased the linalyl glucoside by only 37%, and released glucose and the corresponding monoterpenols. Minor quantities of 3,7-dimethylocta-1,5-diene-3,7-diol, *cis*- and *trans*-furan linalool oxides, α-terpineol, 2-phenylethanol, and benzyl alcohol were detected also.

The action of α-rhamnosidase (Fig. 2) reduced peak g, which had the same retention time as Ger-RhaGlc, by 36% and gave monoterpenyl β-D-glucosides, component x, and rhamnose. The relative proportions of the monoterpenyl β-D-glucosides (Table II) were different from those produced by the α-arabinofuranosidase, 23% of total being attributable to α-rhamnosidase. The low level of linalyl glucoside as compared to the neryl and geranyl glucosides confirms previous results[5]. Subsequent hydrolysis by β-glucosidase hydrolysed the linalyl glucoside incompletely.

The appearance of compound x by the action of α-arabinofuranosidase or α-rhamnosidase and its hydrolysis by β-glucosidase suggest that it might be present in the glycoside extract as both arabinosyl- and rhamnosyl-glucoside forms probably carrying one of the other aglycon moieties identified in the glycoside fraction of grape[5,7].

Furthermore, the incomplete hydrolysis of linalyl glucoside may be related to the lower activity of sweet-almond β-D-glucosidase on this glucoside than on neryl and geranyl glucosides, β-glucosidases from fungi being more efficient on this former compound[5,13].

Thus, the hydrolysis of grape monoterpenyl disaccharide-glycosides by α-L-arabinofuranosidase, α-L-rhamnopyranosidase, and β-D-glucopyranosidase involves removal first of the terminal non-reducing unit by cleavage of the (1→6)

linkage by either α-L-arabinofuranosidase or α-L-rhamnosidase with concomitant production of monoterpenyl glucosides. Monoterpenol is then liberated by action of β-D-glucosidase.

The use of purified glycosidases enabled the sequential hydrolysis of the grape glycosides to be elucidated and their tentative identification without the need for synthetic components. The enzyme-resistant grape glycosides are being studied further, as is the technological use of such glycosidase for the enhancement of the flavor of grape juice and wine, especially the tolerance to low pH (3.2–3.5), glucose (~0.5M in juice), ethanol (10–15% in wine), and also the aglycon specificity of β-glucosidases.

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