Production of β-Apiosidase by *Aspergillus niger*: Partial Purification, Properties, and Effect on Terpenyl Apiosylglucosides from Grape

Isabelle Dupin, Ziya Gunata,* Jean-Claude Sapis, and Claude Bayonove

Laboratoire des Arômes et des Substances Naturelles, Institut des Produits de la Vigne, Institut National de la Recherche Agronomique, 2 Place Viala, F-34060 Montpellier, France

Oubadjim M'Bairaroua and Claude Tapiero

Laboratoire de Chimie Bioorganique, Université des Sciences et Techniques du Languedoc, Place Eugène Bataillon, F-34060 Montpellier, France

Various aspects of fungus-originated β-apiosidase were studied. The production of the enzyme on various carbon sources by fungi appeared to be inducible as it was only produced when apiin, an apiosylglucoside, was present in the culture medium. The influence of apiin concentration, nitrogen source, and surfactants on enzyme production was studied. The enzyme was partially purified by filtration chromatography on Ultrogel AcA44 and ion-exchange chromatography on DEAE-Sepharose CL6B. The molecular weight of this enzyme was 38 000; K_m and V_{max} values for *p*-nitrophenyl β-D-apiofuranoside were, respectively, 16 mM and 0.192 nkat/mg of protein. With regard to activity, the optimum pH and temperature were, respectively, 5.6 and 50 °C. The optimum pH of stability was 7. Na^+, Mg^{2+}, Mn^{2+}, Cu^+, Cu^{2+} have an inhibitor effect on β-apiosidase activity. Conversely, the enzyme was inhibited neither by glucose nor by ethanol. The effect of β-apiosidase toward apiosylglucosides of terpenols, grape flavor precursors, was tested.

INTRODUCTION

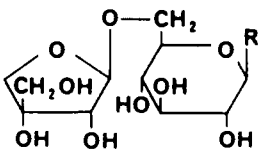
In the course of our previous studies on Muscat grape flavor precursors, novel monoterpene disaccharide glycosides were characterized as 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides (Figure 1) (Voirin et al., 1990). Together with other monoterpene glycosides identified in the past (Williams et al., 1982; Gunata et al., 1985), apiosylglucosides form a large part of flavor precursors (Voirin et al., 1992a). To hydrolyze these glycosides to enhance grape juice and wine flavor, recent studies gave evidence of the considerable interest of use of exogenous enzymes in wine making (Gunata et al., 1990). Among glycosidases involved in the release of free volatiles from grape glycosides, no data concerning β-apiosidase were available. This paper gives details of the production of fungus-originated β-apiosidase, properties of this enzyme, and its effect toward monoterpene apiosylglucosides from grape.

EXPERIMENTAL PROCEDURES

Chemicals. *Nitrogen and Carbon Sources.* Peptone was obtained from Biokar (La Source, France). Rutin (6-*O*-α-L-rhamnosyl-β-D-glucoside of quercetin) was purchased from Fluka (Buchs, Switzerland). Apiin (2-*O*-β-D-apiofuranosyl-β-D-glucoside of apigenin) was obtained from Extrasynthese (Genay, France). Arabinan of peas was donated by Polymers Laboratory, IPV (INRA, Montpellier, France). Parsley seeds were obtained from Abondance (La Verpillière, France).

Synthetic Substrates. For the measurement of glycosidase activities, *p*-nitrophenyl (pNP) glycosides (pNP α-L-arabinofuranoside, pNP α-L-rhamnopyranoside, pNP β-D-glucopyranoside) were commercially available (Sigma, St. Louis, MO).

With regard to *p*-nitrophenyl β-D-apiofuranoside (pNP Ap) [4-nitrophenyl-(3-*C*-hydroxymethyl)-β-D-erythrofuranose], its synthesis has been realized by the procedure of the fusion reaction (Gent et al., 1976) in vacuo of 1,2,3-tri-*O*-acetyl-(3-*C*-acetoxymethyl)-β-D-erythrofuranose [this latter compound being obtained according to the methods of Ho (1979) and Hettinger and Schildknecht (1984)] with 4-nitrophenol and APTS as catalyst. This reaction yielded a mixture of the two anomers, 4-nitrophenyl-2,3-di-*O*-acetyl-(3-*C*-acetoxymethyl)-α-D-erythrofuranose (1) and 4-nitrophenyl-2,3-di-*O*-acetyl-(3-*C*-acetoxymethyl)-β-D-erythrofuranose (2), separated by column chromatography on silica gel (respectively, 12% and 59%). Treatment of both compounds 1 and 2 with catalytic sodium methoxide in methanol gave quantitatively 4-nitrophenyl-α-D-apiofuranose (3) and 4-nitrophenyl-β-D-apiofuranose (4) (the enzymic substrate).



R = monoterpenyl

Figure 1. Structure of β-D-apiofuranosyl β-D-glucopyranoside from grape.

1: syrup, $[\alpha]^{25}_D$ +160° (*c* 1.0, $CHCl_3$), TLC R_f 0.32 (diethyl ether/petroleum ether 4:1); ^1H NMR ($CDCl_3$) δ 2.05–2.20 (2 s, 9 H, 3 OAc), 4.23 (d, 1 H, H-4a, J_{4a4b} = 10.6 Hz), 4.30 (d, 1 H, H-4b), 4.48 (d, 1 H, H-5a, J_{5a5b} = 12.0 Hz), 4.58 (d, 1 H, H-5b), 5.25 (d, 1 H, H-2, $J_{1,2}$ = 4.6 Hz), 5.90 (d, 1 H, H-1), 7.18 (m, 2 H, arom, $J_{H,H'}$ = 4.9 and 9.3 Hz), 8.18 (m, 2 H, arom); ^{13}C NMR ($CDCl_3$) δ 20.5, 20.6, 21.3 (CH_3CO), 63.1 (C-5), 71.9 (C-4), 72.6 (C-2), 81.1 (C-3), 98.8 (C-1), 116.9, 125.8, 161.4, 142.9 (C-arom), 169.7, 169.8, 170.3 (CH_3CO). Anal. Calcd for $C_{17}H_{19}NO_{10}$: C, 51.39; H, 4.82; N, 3.53. Found: C, 51.59; H, 4.94; N, 3.56.

2: mp 154–156 °C (diethyl ether/*n*-pentane); $[\alpha]^{25}_D$ −153° (*c* 1.0, $CHCl_3$); TLC R_f 0.35 (same conditions as for 1); ^1H NMR ($CDCl_3$) δ 2.08–2.14 (3 s, 9 H, 3 OAc), 4.32 (s, 2 H, H-4a,4b), 4.58 (d, 1 H, H-5a, J_{5a5b} = 12.4 Hz), 4.70 (d, 1 H, H-5b), 5.62 (s, 1 H, H-2), 5.75 (s, 1 H, H-1), 7.10 (m, 2 H, arom, $J_{H,H'}$ = 5.0 and 9.2 Hz), 8.2 (m, 2 H, arom, $J_{H,H'}$ = 5.0 and 9.2 Hz); ^{13}C NMR ($CDCl_3$) δ 20.5, 20.7, 21.1 (CH_3CO), 62.7 (C-5), 73.6 (C-4), 76.6 (C-2), 83.4 (C-3), 103.9 (C-1), 116.5, 125.8, 142.9, 160.7 (C-arom), 169.1, 169.6, 170.4 (CH_3CO). Anal. Calcd for $C_{17}H_{19}NO_{10}$: C, 51.39; H, 4.82; N, 3.53. Found: C, 51.60; H, 4.89; N, 3.54.

* Author to whom correspondence should be addressed.

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3: syrup, $[\alpha]^{25}_D$ +33° (c 1.0, methanol); TLC R_f 0.37 (dichloromethane/methanol 1:1); ^{1}H NMR [$(CD_3)_2SO$] δ 3.32 (d, 1 H, H-5a, J_{5a5b} = 11.1 Hz), 3.36 (d, 1 H, H-5b), 3.48 (d, 1 H, H-4a, J_{4a4b} = 9.4 Hz), 3.80 (d, 1 H, H-4b), 4.13 (d, 1 H, H-2, $J_{1,2}$ = 4.7 Hz), 5.79 (d, 1 H, H-1), 7.17 (m, 2 H, arom, $J_{H,H'}$ = 5.4 and 9.2 Hz), 8.20 (m, 2 H, arom); ^{13}C NMR (D_2O) δ 64.2 (C-5), 72.7 (C-2), 73.6 (C-4), 77.6 (C-3), 101.5 (C-1), 116.4, 126.8, 142.4, 163.6 (C-arom). Anal. Calcd for $C_{11}H_{13}NO_7$: C, 48.71; H, 4.83; N, 5.16. Found: C, 48.90; H, 4.97; N, 5.20.

4: mp 104–105 °C (diethyl ether/methanol/n-pentane); $[\alpha]^{25}_D$ −155° (c 1.0, $CHCl_3$); TLC R_f 0.37 (same conditions as for 3); ^{1}H NMR [$(CD_3)_2SO$] δ 3.37 (d, 1 H, H-5a, J_{5a5b} = 11.2 Hz), 3.42 (d, 1 H, H-5b), 3.77 (d, 1 H, H-4a, J_{4a4b} = 9.5 Hz), 4.06 (d, 1 H, H-4b), 4.22 (d, 1 H, H-2, $J_{1,2}$ = 3.6 Hz), 5.66 (d, 1 H, H-1), 7.16 (m, 2 H, arom, $J_{H,H'}$ = 5.6 and 9.3 Hz), 8.19 (m, 2 H, arom); ^{13}C NMR (D_2O) δ 64.1 (C-5), 75.4 (C-4), 77.7 (C-2), 80.1 (C-3), 107.1 (C-1), 117.2, 126.8, 142.8, 162.3 (C-arom). Anal. Calcd for $C_{11}H_{13}NO_7$: C, 48.71; H, 4.83; N, 5.16. Found: C, 48.89; H, 4.80; N, 5.22.

Natural Substrate. The bound fraction of aroma (glycosidic extract) was obtained by percolation of 30 mL of Muscat of Frontignan juice on an Amberlite XAD-2 resin column (Gunata et al., 1985). The glycosidic fraction was eluted with 30 mL of ethyl acetate.

Experimental Enzymic Preparations. Cellulase A, Hemicellulase, and Pectinase 263 were obtained from Gist Brocades (Seclin, France). Rohament CW was obtained from Rohm (Darmstadt, Germany), Pektolase 3PA 400 from Grinsted (Denmark), and Ultrazym 100 from Ciba-Geigy (Basel, Switzerland).

Organism and Cultural Conditions. The fungal cultures *Aspergillus niger* (AN) 55465 and 12648 and *Aspergillus oryzae* (*AO*) 12559 and 10207 were obtained from the CBS collection (Delft, The Netherlands). The organisms were maintained at ambient temperature by weekly subculturing on potato dextrose agar (PDA) (Difco, Detroit, MI) slants. The basal medium initially used for β-apiosidase production contained the following components (% w/v): $(NH_4)_2SO_4$, 0.8; $(NH_4)_2HPO_4$, 0.3; KH_2PO_4, 0.1; $MgCl_2 \cdot 6H_2O$, 0.1 (Westlake and Simpson, 1961). The carbon sources (glucose, rhamnose, apiin, arabinan, xylose, rutin), solubilized in water, were autoclaved (120 °C, 15 min) separately with water (see concentrations in Table I). The basal medium after sterilization (120 °C, 15 min) was added aseptically to the carbon source (50-mL Erlenmeyer flask). The final volume of culture was 20 mL. The inoculum was prepared by harvesting the spores from PDA cultures in sterile distilled water. An inoculum volume corresponding to 10^6 spores was added to the 20-mL cultivation medium under aseptic conditions. Incubation was carried out at 30 °C on a rotary shaker (Polytest 20, Bioblock Scientific, Illkirch, France) at 140 rpm. At various intervals of culture growth, samples were withdrawn and mycelium was removed by centrifugation (5000g, 10 min, 5 °C). The supernatant was used for the enzymic assays.

The change of biomass during fungi growth was followed by measuring the weight of mycelium. Seven basal media (20 mL) containing 2% (w/v) apiin as carbon source were inoculated and cultivated as described above. Samples (20 mL) at different intervals (24, 48, ..., 168 h) were centrifuged. The pellet was washed threefold with 0.1 N NaOH to remove the residual apiin and then rinsed with distilled water. The weight of mycelium was estimated after drying at 100 °C for 24 h.

Enzyme Assay. *Assay on Synthetic Substrates.* β-Apiosidase activity was assayed by incubating 1 volume of enzyme solution (diluted if necessary) in 1 volume of 2.2 mM pNP Ap in 100 mM acetate buffer (pH 4.4) for 20 min at 40 °C. The reaction was stopped by the addition of 6 volumes of 0.1 M Na_2CO_3, and absorbance was measured at 400 nm. β-Glucosidase, α-arabinosidase, and α-rhamnosidase activities were assayed according to the same protocol using the corresponding substrates. Enzyme activity was expressed as nanokatal (nkat), being nanomoles of pNP liberated per second per milliliter of enzymic solution or milligram of enzyme at the enzymic assay conditions.

Assay on Grape Glycosidic Extract. The 30-mL eluate of ethyl acetate was concentrated to dryness (Rotavapor, 40 °C) and dissolved in 2 mL of 100 mM citrate–phosphate buffer (pH 5.6); 1 mL of this extract was incubated with 400 μL of β-apiosidase solution (14 nkat, arising from second step of purification) during 12 h at 40 °C. The remaining 1 mL was incubated without enzymic solution in the same conditions. The samples were analyzed by gas chromatography after derivatization by trifluoroacetylation (Voirin et al., 1992b). The identification of peaks was carried out by gas chromatography–mass spectrometry (GC–MS) (Voirin et al., 1990, 1992b).

Protein Measurement. The Pierce method [bicinchoninic acid (BCA) as protein assay reagent; Pierce, Rockford, IL] was followed for the determination of protein during β-apiosidase purification using bovine serum albumin as standard.

Enzyme Purification. *Gel Filtration Chromatography.* The growth medium (4.7 mL) was dialyzed (4 °C, 5 h) against 2 L of 100 mM citrate–phosphate buffer, pH 6.9, and then applied at 4 °C to a column (0.8 × 95 cm) of Ultrogel AcA44 (IBF, Villeneuve-La-Garenne, France) equilibrated with the same buffer at a flow rate of 5.5 mL h^{-1}. Fractions were collected and assayed for protein at 280 nm and for glycosidase activities.

Ion-Exchange Chromatography. The whole fractions (21.3 mL) containing β-apiosidase activity were dialyzed against 2 L of 25 mM imidazole hydrochloride buffer, pH 7.4, and applied at 4 °C onto a DEAE-Sepharose CL6B (Pharmacia, Uppsala, Sweden) column (1.6 × 26 cm), equilibrated with the same buffer. Elution was developed using a linear gradient 0–0.5 M sodium chloride in the same buffer at a rate of 30 mL h^{-1} and with 3-mL fractions. The β-apiosidase active fractions were pooled and used for further determinations.

Properties of β-Apiosidase. *Molecular Weight.* MW determination was carried out with an Ultrogel AcA44 column using standard proteins (Boehringer, Mannheim, Germany): cytochrome c, 12 500; chymotrypsin, 25 000; ovalbumin, 45 000; bovine albumin, 68 000.

Effects of pH and Temperature. (a) *Effect of pH on Enzyme Activity.* The purified enzyme in imidazole buffer was dialyzed (4 °C) against water and then diluted in a citrate–phosphate buffer at various pHs from 2.6 to 8. The activity was measured under standard conditions.

(b) *Effect of pH on Enzyme Stability.* The enzyme solution was incubated in citrate–phosphate buffer of various pHs (2.6–8) and at 30 °C for 1 h. Residual activity was measured after dialysis (4 °C, 5 h) against 100 mM citrate–phosphate buffer, pH 5.6 (optimum pH for enzyme activity).

(c) *Effect of Temperature on Enzyme Activity and Stability.* The β-apiosidase activity was measured under standard conditions in the temperature range 10–80 °C. For stability, enzymic samples were maintained in 100 mM citrate–phosphate buffer, pH 7, for increasing times (from 5 to 30 min) at various temperatures, from 40 to 80 °C.

Inhibition. The effect of glucose and ethanol on β-apiosidase activity was studied in the incubation medium at 10% ethanol (v/v), 5 and 50 g/L glucose. Inhibitions by Na^+, Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^+, Cu^{2+}, and pCMB [p-(chloromercuri)benzoate] were carried out with a final concentration of 10 mM in the incubation medium.

RESULTS AND DISCUSSION

Production of β-Apiosidase by Fungi. *Effect of Carbon Sources.* The effect was tested using various carbon sources to determine the constitutive or inducible character of β-apiosidase production by fungi (Table I). The extracellular β-apiosidase appeared to be an inducible enzyme since it was only produced by fungi when growth occurred on apiin. This compound was the sole carbon source of apiose commercially available.

With regard to the production of other glycosidases, arabinan, rutin, and apiin served as good carbon sources.

As shown in Table II, fungus-originated commercial enzymic preparations contain higher levels of β-glucosidase and α-arabinosidase activities than do α-rhamnosidase and β-apiosidase. The latter was absent in Cellulase, Hemicellulase, and some pectinases, which indicates its nonconstitutive character.

For further studies, the *A. niger* 55465 strain was used as it yielded the highest level of β-apiosidase among fungi

Table I. Effect of Various Carbon Sources on Glycosidase Production by Various Strains of *Aspergillus*: β-Glucosidase (β-glu), β-Apiosidase (β-apio), α-Rhamnosidase (α-rha), α-Arabinosidase (α-ara)

carbon source[b] (% w/v)		glycosidase act.[a] with organism			
		AN 55465	AN 14628	AO 12559	AO 10207
rhamnose (1)	β-glu	+	+	−	−
	β-apio	−	−	−	−
	α-rha	+	−	+	+
	α-ara	−	+	−	−
glucose (0.5)	β-glu	+	−	−	−
	β-apio	−	−	−	−
	α-rha	−	−	−	−
	α-ara	+	+	−	−
xylose (1)	β-glu	+	+	−	−
	β-apio	−	−	−	−
	α-rha	−	−	−	−
	α-ara	+	+	−	−
apiin (0.1)	β-glu	+	+	+	−
	β-apio	+	+	+	+
	α-rha	+	−	−	+
	α-ara	+	−	+	−
rutin (1)	β-glu	+	+	+	−
	β-apio	−	−	−	−
	α-rha	+	+	+	−
	α-ara	+	+	−	−
arabinan (1)	β-glu	+	+	+	+
	β-apio	−	−	−	−
	α-rha	+	+	+	−
	α-ara	+	+	+	+

[a] Activities were measured from the first to the fifth day of culture: +, presence of activity; −, absence of activity. [b] Sugar concentrations were 1% w/v except for apiin (0.1%) and glucose (0.5%) to avoid catabolic repression (Dekker, 1981).

Table II. Glycosidase Activities (β-Apiosidase, β-Glucosidase, α-Arabinosidase, α-Rhamnosidase) in Some Experimental Enzymic Products

exptl enzymic products	glycosidase act., nkat (mg of product)$^{-1}$			
	β-apio	β-glu	α-ara	α-rha
Cellulase A	0	6.06	0.59	0.07
Hemicellulase	0	7.07	6.96	0.93
Pectinase 263	0.21	7.21	1.39	0.31
Rohament CW	0	3.27	0.71	0.38
Pektolase 3PA 400	0.33	1.49	3.80	0.04
Ultrazym 100	0.03	0.49	0.14	0
Pectinol VR	0	0.18	0.1	0.03

tested. As apiin was the carbon source inducer, it was of interest to know the effect of apiin concentration on enzyme synthesis. As shown in Figure 2 the highest production was obtained with 2% (w/v) apiin in medium. Production was highest after 120 h of growth and then declined steadily. When lower concentrations were used (0.5 and 1%), the maximum production occurred after 168 h of growth. On the other hand, β-apiosidase production increased 3-fold when apiin concentration in the medium passed from 1% to 2% (w/v).

Further studies were made with basal medium containing 2% (w/v) apiin as the carbon source. This medium was named M1 medium.

Effect of Nitrogen Sources. Different organic and inorganic nitrogen sources in M1 medium were tested (Table III). The presence of ammonium salts such as $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ did not allow high β-apiosidase production. These results are in agreement with those of Desai et al. (1982), who showed β-glucosidase production by *Scytalidium lignicola* was depressed by ammonium salts. Partial substitution of inorganic salts by peptone caused an increase of enzyme production with a maximum when peptone was used as the only nitrogen source. Under these conditions production was 10 times higher than with a medium containing both ammonium salts. This is in agreement with the results of Srivastava et al. (1987), who

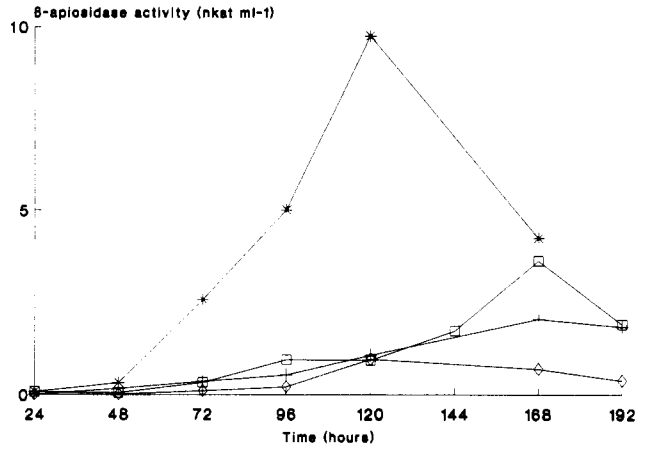

Figure 2. Effect of apiin concentration (% w/v) in the culture medium on β-apiosidase production by *A. niger*: 0.1% (◇); 0.5% (+); 1% (□); 2% (*).

Table III. Effect of Nitrogen Sources on β-Apiosidase Production by *A. niger* in M1 Medium[a]

nitrogen source (2.33 g of N L^{-1})	β-apiosidase act., nkat mL^{-1}	nitrogen source (2.33 g of N L^{-1})	β-apiosidase act., nkat mL^{-1}
control	5.50	peptone	65.01
$(NH_4)_2SO_4$	1.60	peptone + $(NH_4)_2SO_4$	25.90
$(NH_4)_2HPO_4$	3.71		
$(NH_4)_2SO_4$ + $(NH_4)_2HPO_4$	6.09	peptone + $(NH_4)_2HPO_4$	10.70

[a] Cells were carried out in M1 medium with the indicated nitrogen source(s). When more than one nitrogen substrate was used, the addition of the other was made in an equal proportion with respect to the nitrogen content. Activities were measured after 144 h of growth.

Table IV. Effect of Surfactants on Production of β-Apiosidase by *A. niger* in M2 Medium[a]

surfactants	addition, % v/v	β-apiosidase act., nkat mL^{-1}
none (control)	0	61
Tween 80	0.05	56.6
	0.15	217
Triton X-100	0.05	51
	0.15	84.2

[a] Activities were measrued after 144 h of growth.

found that peptone was the best nitrogen source for β-glucosidase production by *Aspergillus wentii*.

Accordingly, in further experiments, M1 medium was changed to M2 medium with addition of 2.04% (w/v) peptone as the nitrogen source.

Effect of Surfactants. The addition of certain surfactants to fungal cultures has been shown to increase significantly extracellular enzyme production (Reese and Maguire, 1969; Sternberg et al., 1977). The surfactants act by modifying cell membranes, in particular by increasing permeability (Reese and Maguire, 1969) that facilitates enzyme release. The effect of different surfactants such as Tween 80 and Triton X-100 at various concentrations in M2 medium is shown in Table IV. Tween 80 added at a level of 0.15% (v/v) increased 3.5-fold the enzyme production. Conversely, Triton X-100 at the same level was less effective and caused only a 1.3-fold increase in production. Similar results were reported by Srivastava et al. (1987) and Desai et al. (1982) about Tween 80 superiority as compared to Triton X-100 for β-glucosidase production by *A. wentii* and *S. lignicola*, respectively.

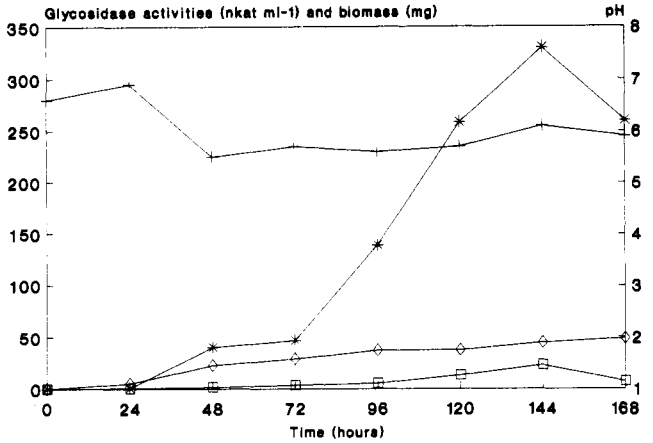

Figure 3. Formation of extracellular β-apiosidase (*) and β-glucosidase (□) during development of *A. niger*: pH (+); biomass (◇).

Table V. Effect of Parsley Variety on Glycosidase Production by *A. niger*[a]

parsley variety seeds	glycosidase act., nkat mL^{-1}			
	β-apio	α-ara	β-glu	α-rha
common parsley	3.24	7.64	0.38	0.01
curly parsley	8.6	12.4	0.33	0.01

[a] Maximum enzyme production occurred after 168 h of growth.

In consequence, for further experiments we changed M2 medium to M3 medium containing 0.15% (v/v) Tween 80.

Time Course of β-Apiosidase Production. According to the previous optimization of medium culture composition, this study was carried out on M3 medium containing $MgCl_2 \cdot 6H_2O$ 0.1% (w/v), KH_2PO_4 0.1% (w/v), apiin 2% (w/v), peptone 2.04% (w/v), and Tween 80 0.15% (v/v). As shown in Figure 3, the maximum rate of β-apiosidase production was at 144 h of growth culture and was 10-fold higher than β-glucosidase production. α-Arabinosidase and α-rhamnosidase were weak (not reported in Figure 3), with maximal activities, respectively, 173 and 1100 times lower than the highest β-apiosidase activity. When growth time increases from 144 to 168 h, β-apiosidase and β-glucosidase production decreases while biomass production estimated by mycelium weight increases slightly. Desai et al. (1982) demonstrated that a large variation of pH in the medium caused a fall of β-glucosidase production by *S. lignicola*. However, in our experiment, pH only changed slightly from the 6.9 initial value during growth.

Production of β-Apiosidase on Parsley Seeds. Apiose is found in a wide variety of plants, especially as flavonoid glycosides such as apiin in parsley seeds, stems, and leaves (Watson and Orenstein, 1975). Production of β-apiosidase was studied by replacing 2% (w/v) apiin with 5% (w/v) ground parsley seeds in the M3 medium. According to results of Table V, it was possible to produce β-apiosidase under these conditions. Production was higher with the curly parsley variety than with common parsley. The maximum enzyme production occurred for α-arabinosidase, and the production of β-glucosidase and α-rhamnosidase was negligible.

Partial Purification and Some Properties of β-Apiosidase. Two steps of β-apiosidase purification were carried out. The first one used a gel filtration on Ultrogel AcA44 of β-apiosidase crude solution, issued from M3 growth medium, which gave a single peak for the enzyme near the β-glucosidase peak (Figure 4). Of the β-apiosidase original activity, at this step, 132% was recovered in the pooled fractions (Table VI). This high value could be explained by the presence of activity inhibitors in the growth medium, eliminated by gel filtration. At this step, the residual contaminating α-arabinosidase and α-rhamnosidase activities were largely eliminated as were 67% of the proteins.

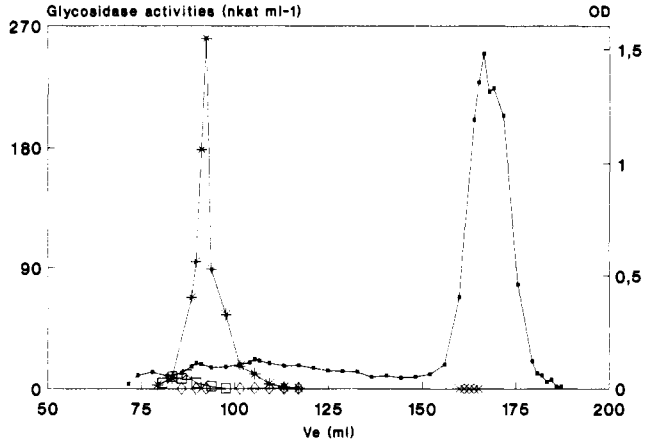

Figure 4. Gel chromatography (Ultrogel AcA44) of initial growth medium of *A. niger*: β-apiosidase (*); β-glucosidase (□); α-arabinosidase (◇); α-rhamnosidase (×); absorbance (OD) 280 nm (⊡).

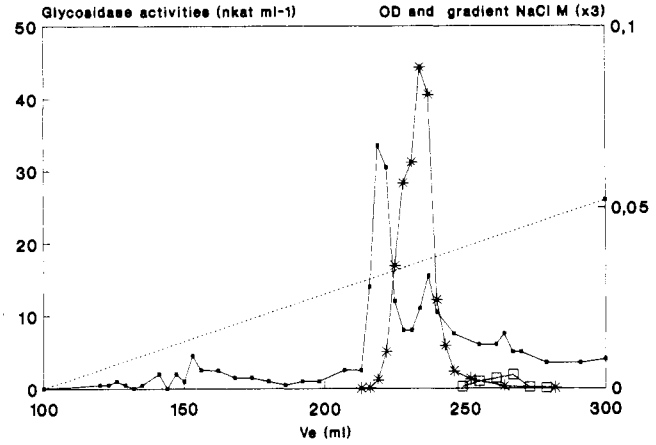

Figure 5. Ion-exchange chromatography (DEAE-Sepharose CL6B) of β-apiosidase: β-apiosidase (*); β-glucosidase (□); absorbance (OD) 280 nm (⊡); NaCl gradient (- - -).

Table VI. Purification of β-Apiosidase from *A. niger*

purifn step	vol, mL	total act., nkat	act. yield, %	total protein, mg	protein yield, %	sp act., nkat (mg of protein)$^{-1}$	purifn factor
initial extract	4.7	1473	100	2.25	100	655	1
Ultrogel AcA44	21.3	1946	132	0.74	32.8	2629	4
DEAE-Sepharose CL6B	20	492	25.3	0.08	11.6	6150	9.4

A second purification was made using ion-exchange chromatography on DEAE-Sepharose CL6B; this step yielded a β-apiosidase peak eluted by 0.112 M sodium chloride (Figure 5) containing 25% of the original activity and 11.6% of the original applied proteins (purification 9.4-fold, Table VI). β-Glucosidase was eluted by 0.125 M sodium chloride and efficiently eliminated at this step.

Properties. The β-apiosidase issued from DEAE-Sepharose chromatography was used to determine some of its properties.

The MW of the enzyme was found to be 38 000 as determined by gel filtration. This result belongs to the range of fungal β-glucosidases having a low M_r such as those of *Candida guilliermondii* (48 000) or *Aspergillus fumigatus* (41 000) (Woodward and Wiseman, 1982).

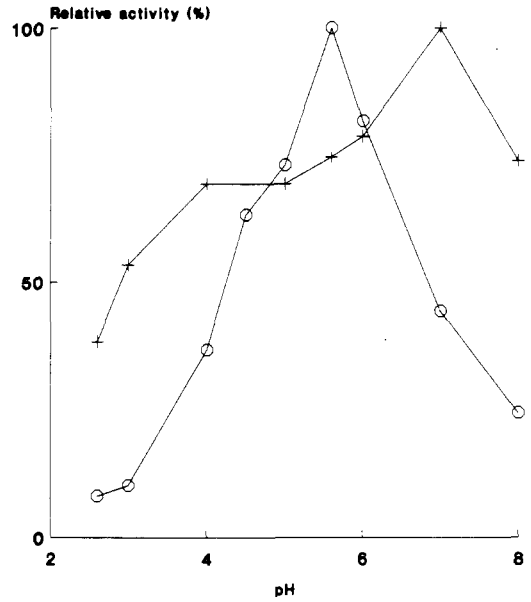

Figure 6. Effect of pH on activity (O) and stability (30 °C, 1 h) (+) of β-apiosidase.

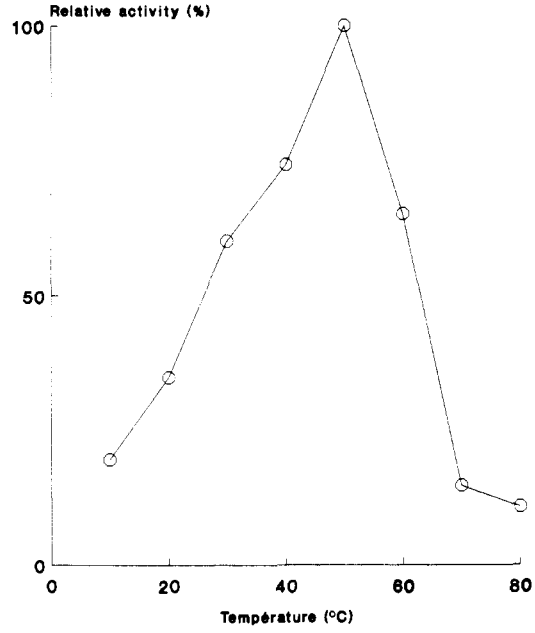

Figure 7. Effect of temperature on β-apiosidase activity.

K_m and V_{max}, determined according to the Lineweaver–Burk plot, were, respectively, found to be 16 mM and 0.192 nkat/mg of protein with pNP apiofuranoside as the substrate.

β-Apiosidase maximum activity was at pH 5.6, and it was most stable around pH 7 (Figure 6). At acidic pHs, as usually found in grape juices and wines (between 3 and 3.6), the β-apiosidase stability decreased to around 50% of the optimum value.

Optimum temperature for maximal enzyme activity was 50 °C with a rapid decrease for higher values (Figure 7). In the usual ranges of temperature encountered in wine making (20–30 °C), the β-apiosidase activity was reduced to 35–60%. At pH 7, the enzyme was quite stable from 40 to 50 °C. More than 90% inactivation was observed at 60 °C after 10 min (not shown).

Glucose, which is a strong inhibitor of fungal β-glucosidase (Aryan et al., 1987; Cordonnier et al., 1989), did not affect β-apiosidase activity at the levels studied (5 and 50 g/L). Ethanol at 10% (v/v) in the medium (average value in the wine) did not decrease β-apiosidase activity.

Table VII. Effect of Various Effectors toward *A. niger* β-Apiosidase (Values as a Percentage of the Untreated Enzyme)

effector[a]	β-apiosidase act., %	effector[a]	β-apiosidase act., %
Na^+	85.6	Cu^+	12.5
Mg^{2+}	12.6	Cu^{2+}	16.0
Mn^{2+}	64.2	pCMB	0
Ca^{2+}	22.5		

[a] The final concentration of the effectors in incubation medium is 10 mM.

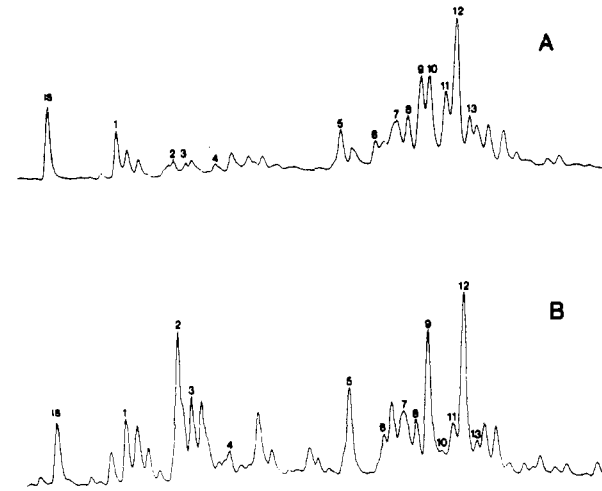

Figure 8. GC chromatograms after trifluoroacetylation of glycosidic extract from Frontignan grape juice enzymically nontreated (A) or treated (B) with β-apiosidase: IS, internal standard (phenyl β-D-glucoside); 1, furan linalool oxide β-D-glucoside; 2, neryl β-D-glucoside; 3, pyran linalool oxide β-D-glucoside; 4, geranyl β-D-glucoside; 5, 6, linalyl α-L-rhamnosyl- and α-L-arabinosyl-β-D-glucosides; 7, furan linalool oxide β-D-apiosyl-β-D-glucoside; 8, geranyl α-L-rhamnosyl-β-D-glucoside; 9, neryl α-L-arabinosyl-β-D-glucoside; 10, neryl β-D-apiosyl-β-D-glucoside; 11, pyran linalool oxide β-D-apiosyl-β-D-glucoside; 12, geranyl α-L-arabinosyl-β-D-glucoside; 13, geranyl β-D-apiosyl-β-D-glucoside.

The effects of several ions and pCMB are shown in Table VII. Na^+ and Mn^{2+} inhibited β-apiosidase activity less than Mg^{2+}, Ca^{2+}, Cu^{2+}, or Cu^+. pCMB totally inhibited enzyme activity, which is probably related to the presence of a mercapto group in the active site of *A. niger* β-apiosidase.

Action of β-Apiosidase on Grape Glycosidic Extract. Figure 8 chromatograms show that the purified β-apiosidase acted only on apiosylglucosides. The arabinosyl and rhamnosylglucosides were not affected. The sugar linkage (1→6) of apiosylglucosides of nerol, geraniol, and pyran linalool oxide was cleaved by the enzyme with a liberation of the corresponding monoglucosides. As the enzyme solution was devoid of β-glucosidase activity, the monoglucosides were not hydrolyzed. The same mechanism of hydrolysis of monoterpenyl arabinosyl- and rhamnosylglucosides by α-arabinosidase and α-rhamnosidase was reported in our previous studies (Gunata et al., 1988).

Furthermore, apiosylglucoside of furan linalool oxide did not appear to be a good substrate for β-apiosidase, unlike other apiosylglucosides. The aglycon specificity of β-apiosidase produced in this work and its ability to hydrolyze grape glycosides during wine making will be the subject of a future paper.

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