Purification and Some Properties of an *Aspergillus niger* β-Apiosidase from an Enzyme Preparation Hydrolyzing Aroma Precursors

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A β-apiosidase was isolated and purified to electrophoretic homogeneity from an enzyme preparation, Klerzyme 200, through ammonium sulfate precipitation, gel filtration chromatography, ion-exchange chromatography, and HPLC on ion-exchange and size exclusion columns. The purification of the enzyme was aided by the synthesis of 4-methylumbelliferyl β-D-apiofuranoside for the specific detection of activity on electrophoresis gels. The molecular mass estimated by SDS−PAGE was 120 kDa. The optimum activity of the β-apiosidase was found at pH 5 and 40 °C. The K_m and V_{max} for *p*-nitrophenyl β-D-apiofuranoside were 4.2 mM and 2460 nkat/mg of protein, respectively. The enzyme was not inhibited by glucose and ethanol. This enzyme hydrolyzed the intersugar linkages of apiofuranosylglucosides, aroma precursors from grape.

Keywords: *β-Apiosidase; Aspergillus niger; purification; aroma precursors; hydrolysis*

INTRODUCTION

Work since the 1980s has shown that several potent flavor compounds, such as monoterpenes and C13-norisoprenoids, accumulate as odorless nonvolatile glycoconjugates, β-D-glucoside and disaccharide glycosides, in fruits and plants tissues (Williams et al., 1982; Günata et al., 1985; Krammer et al., 1991; Winterhalter and Skouroumounis, 1997; Ogawa et al., 1997). Hence, the liberation of flavor compounds from the glycoside precursors through the enzymatic hydrolysis has been the subject of intensive research work (Aryan et al., 1987; Günata et al., 1993; Perez-Gonzalez et al., 1993; Rosi et al., 1994; Gueguen et al., 1996). The hydrolysis of monoglucosides requires the action of β-glucosidase, whereas disaccharide glycosides are hydrolyzed through two steps catalyzed by the relevant glycosidases (Günata et al., 1988). First, the terminal sugar is liberated by *exo*-glycosidases such as α-rhamnosidase, α-arabinofuranosidase, or β-apiosidase, and then the resultant monoglucoside is hydrolyzed by a β-glucosidase. Several studies have pointed out the interesting properties of glycosidases from *Aspergillus niger* and some yeast species for flavor release in fruit juice processing and wine-making (Shoseyov et al., 1990; Günata et al., 1993; Perez-Gonzalez et al., 1993; Rosi et al., 1994; Gueguen et al., 1996). However, our knowledge is quite limited with regard to β-apiosidase involved in the hydrolysis of apiofuranosylglucosides, which are among the most abundant glycosidic flavor precursors in grape (Dupin et al., 1992; Günata et al., 1993). This enzyme is often absent in enzyme preparations, occurs at low levels if present, contrary to other glycosidases (Dupin et al., 1992; Günata et al., 1993), and has been only partially purified (Dupin et al., 1992; Günata et al., 1997). We report here the purification and properties of a β-apiosidase from an enzyme preparation, Klerzyme 200, which could efficiently liberate volatile compounds from apiofuranosylglucosides in wine-making (Günata et al., 1990).

MATERIALS AND METHODS

Materials. Klerzyme 200 was obtained from Gist-Brocades Co. (Delft, The Netherlands). *p*-Nitrophenyl (*p*NP) β-D-glucopyranoside and 4-methylumbelliferyl β-D-glucopyranoside were purchased from Sigma Chemical Co. (St. Louis, MO). *p*-Nitrophenyl β-D-apiofuranoside was synthesized during previous work (Dupin et al., 1992).

Synthesis of 4-Methylumbelliferyl β-D-Apiofuranoside. The starting material, 3-*C*-(hydroxymethyl)-2,3-*O*-isopropyliden-β-D-erythrofuranose (**1**) (2.30 g, 12.1 mmol) prepared according to the method of Ho (1979) was acetylated to give 3-*C*-(acetoxymethyl)-1-*O*-acetyl-2,3-*O*-isopropylidene-β-D-erythrofuranose (**2**) (3.10 g, yield = 94%, R_f = 0.54 on TLC silica gel with diethyl ether/dichloromethane = 1:4).

The products, 3-*C*-(acetoxymethyl)-1,2,3-tri-*O*-acetyl-D-erythrofuranose (**3**) and 3-*C*-(acetoxymethyl)-1,2,3-tri-*O*-acetyl-L-threofuranose (**4**), were prepared from compound **2** (Tronchet and Tronchet, 1974). They were separated into two fractions containing **3**β,**4**α and **3**α,**4**β isomers, respectively, by flash chromatography on silica gel (petroleum ether/diethyl ether = 1:1). The differentiation of compounds **3**β and **4**α from compounds **3**α and **4**β was easily made by ^{1}H NMR (Tronchet and Tronchet, 1974). The mixture of **3**β and **4**α was further separated by flash chromatography on silica gel (dichloromethane/methanol = 99:1). Compound **3**β was obtained as an oil (0.728 g, yield = 29%, R_f = 0.58 on silica gel with dichloromethane/methanol = 99:1).

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A fusion reaction of **3**β (83.8 mg, 0.264 mmol) in 20 mL of 1,2-dichloroethane with an excess of 4-methylumbelliferone (**5**) (208.2 mg, 1.182 mmol) in conditions similar to those used for the synthesis of *p*-nitrophenyl β-D-apiofuranoside (Dupin et al., 1992) yielded 3-*C*-(acetoxymethyl)-2,3-di-*O*-acetyl-1-(4-methylumbelliferone)-β-D-erythrofuranose (**6**) as a light pink gummy solid: NMR δ_H ($CDCl_3$, ppm) 1.25 (*s*, 3H, CH_3), 2.18−2.19 (*s*, each, 9H, 3 × CH_3CO), 4.28 (*d*, 1H, $J_{4a,4b}$ = 10.6 Hz, H-4a), 4.39 (*d*, 1H, $J_{4a,4b}$ = 10.6 Hz, H-4b), 4.65 (*d*, 1H, $J_{5a,5b}$ = 12.4 Hz, H-5a), 4.84 (*d*, 1H, $J_{5a,5b}$ = 12.4 Hz, H-5b), 5.65 (*s*, 1H, H-2), 5.69 (*s*, 1H, H-1). Compound **6** was deacetylated in methanol catalyzed by sodium methoxide. The product, 3-*C*-(hydroxymethyl)-1-(4-methylumbelliferone)-β-D-erythrofuranose (4-methylumbelliferyl β-D-apiofuranoside, **7),** purified by flash chromatography on silica gel gave a yellowish oil (9.8 mg, yield = 12%, R_f = 0.61 on silica gel, with ethyl acetate/methanol = 2:1).

Enzyme Assay. Twenty microliters of properly diluted sample solution was mixed with 20 μL of 2.2 mM *p*NP β-apiofuranoside or 4 mM *p*NP β-glucopyranoside in 0.1 M citric acid−phosphate buffer (pH 5.6) and incubated at 40 °C for 10 min. The reaction was stopped by adding 120 μL of 1 M sodium carbonate, and the absorbance was measured at 400 nm (ϵ = 18300 M^{-1} cm^{-1}). The activity was expressed as nanokatals per milliliter (nkat/mL) or per milligram of protein, being nanomoles of *p*NP liberated per second.

Protein Measurement. The protein was precipitated and measured by the Pierce reagent (Smith et al., 1985). Fifty microliters of sample solution was mixed with 50 μL of 10% trichloroacetic acid and centrifuged (10000*g*, 5 min). The supernatant was removed, and 50 μL of 0.2 M NaOH was added to dissolve the protein. One milliliter of Pierce reagent was then added, and the mixture was heated at 60 °C for 30 min. The absorbance was observed at 562 nm. Albumin was used as standard.

SDS−PAGE. SDS−PAGE was carried out on an apparatus of Mini Protean-II (Bio-Rad) on 7.5% acrylamide gels. The standard proteins were high molecular weight−SDS calibration kit (Pharmacia Biotech, Uppsala, Sweden) consisting of myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa). For the denatured conditions, the sample was heated with Laemmli solution containing 2-mercaptoethanol at 100 °C for 5 min. For the detection of glycosidase activities, the sample was treated with Laemmli solution in the absence of 2-mercaptoethanol at 37 °C for 1 h. After electrophoresis, β-apiosidase and β-glucosidase activities were detected by washing the gel with 2.5% Triton X-100 for 5 min × 2 and with 0.1 M citric acid−phosphate buffer (pH 7.0) containing 0.1 mM EDTA for 5 min × 2 and then applying a solution of 1 mM 4-methylumbelliferyl β-D-apiofuranoside or 2 mM 4-methylumbelliferyl β-D-glucopyranoside (in 0.1 M citric acid−phosphate buffer, pH 5.6). The gel was heated at 40 °C for 20 min. Hydrolysis of these substrates releases methylumbelliferone, which is visualized under UV light at 365 nm. The gel was then stained by silver coloration (Bio-Rad, Hercules, CA).

Purification of β-Apiosidase. *Ammonium Sulfate Precipitation*. The Klerzyme 200 (300 mL) was mixed with 100 mL of 0.1 M citric acid−phosphate buffer (pH 7.0, 0.1 mM EDTA), 65.6 g of ammonium sulfate (30% saturation) was added, and the mixture was stirred for 2 h at 4 °C and then centrifuged (12000*g*, 20 min). The precipitate was discarded. To the supernatant was added with 135 g of ammonium sulfate (65% saturation), and the mixture was stirred for 2 h at 4 °C and then centrifuged (12000*g*, 20 min). The precipitate was dissolved in 50 mL of 0.1 M citric acid−phosphate buffer (pH 7) with 0.1 mM EDTA. This solution was dialyzed against the same buffer for 2 h at 4 °C and concentrated by ultrafiltration on PM-10 membranes (Amicon, Beverly, MA) to 20 mL.

Gel Filtration Chromatography. The concentrated brown solution was applied to a gel filtration column (2 × 90 cm) of Ultrogel AcA 44 (Biosepra, Marlborough, MA) equilibrated with 0.1 M citric acid−phosphate buffer (pH 7.0) and 0.1 mM EDTA. The elution was done at 0.2 mL/min flow rate, and the fraction volumes were 1.6 mL. The fractions 51−110 were then combined and concentrated by ultrafiltration to 50 mL. One gram of active charcoal was added into the solution. The mixture was stirred for 10 min to eliminate some pigments and centrifuged (7500*g*, 10 min). The charcoal precipitate was washed with 5 mL of buffer and centrifuged again. The supernatants were combined, equilibrated by dialysis (overnight at 4 °C) with 25 mM citric acid−phosphate buffer (pH 7.0) and 0.1 mM EDTA and concentrated to 20 mL.

Ion-Exchange Chromatography. The equilibrated sample was applied to an ion-exchange column (1.6 × 18 cm) of DEAE-Sepharose CL-6B (Pharmacia Biotech) and eluted with 25 mM citric acid−phosphate buffer (pH 7.0) and 0.1 mM EDTA. The flow rate was 0.5 mL/min, and the fraction volumes were 3 mL. A linear gradient of 0−0.5 M NaCl was made from 240 to 1080 min. The fractions 70−95 were combined, equilibrated by dialysis (overnight at 4 °C) with 25 mM citric acid−phosphate buffer (pH 7.0) and 0.1 mM EDTA, concentrated to 2.96 mL, and filtered through a 0.22 μm membrane.

Ion-Exchange HPLC. The sample was applied to ion-exchange HPLC (Beckman Gold System) on a TSK DEAE−5PW column (Waters, Milford, MA) (7.5 × 75 mm, injection = 100 μL) eluted with 25 mM citric acid−phosphate buffer (pH 7.0) and 0.1 mM EDTA with a flow rate of 2 mL/min and fraction volumes of 1 mL. An NaCl gradient was applied from 0 to 0.06 M (5−20 min) and from 0.06 to 0.15 M (30−35 min). The fractions 31−63 were combined, concentrated, and subjected to the same HPLC analysis but with an NaCl gradient of 0−0.06 M (5−20 min) and 0.06−1 M (30−35 min). The fractions 30−55 were combined for further purification on size exclusion HPLC.

Size Exclusion HPLC. The combined β-apiosidase fractions were concentrated to 600 μL and injected to size exclusion HPLC on an SEC 2000 column (Waters) (7.5 × 300 mm) (injection = 100 μL) eluted with 0.1 M citric acid−phosphate buffer (pH 7.0) and 0.1 mM EDTA, with flow rate of 1 mL/min. The volume of the collected fractions was 0.5 mL. β-Apiosidase active peak was subjected to the same HPLC analysis for further purification.

Properties of β-Apiosidase. *Effect of pH and Temperature. (a) Effect of pH on Enzyme Activity*. The purified enzyme was diluted 25 times with 0.15 M citric acid−phosphate buffer at various pH values from 2.6 to 8.0. The β-apiosidase activity was measured with 2.2 mM *p*NP apiofuranoside in water under the standard conditions.

(b) Effect of Temperature on Enzyme Activity. The β-apiosidase activity was measured under the standard conditions at temperatures ranging from 10 to 80 °C.

Inhibition. The effects of glucose and ethanol on the β-apiosidase activity were studied in the presence of 0.18, 18, and 90 g/L glucose and 10% (v/v) ethanol in the assay medium.

Hydrolysis of Apiofuranosyl Glucosides. Twenty microliters of 1.3 mM 6-*O*-β-D-apiofuranosyl β-D-glucopyranosides of geraniol and linalool (Voirin et al., 1990) in 0.1 M citric acid−phosphate buffer (pH 5.6) and 0.1 mM EDTA was separately mixed with 15 μL of β-apiosidase solution (1.5 nkat) and incubated at 40 °C for 15 h. The reaction mixtures were then subjected to TLC. Ten microliter aliquots were loaded on silica gel 60 F254 (0.25 mm thickness, Merck, Darmstadt, Germany), and ethyl acetate/2-propanol/water (60:30:10) was used as the solvent. Sugars were detected by spraying 0.19% (w/v) naphthoresorcinol in sulfuric acid/ethanol (5:95) and heating on flame (Günata et al., 1988).

RESULTS AND DISCUSSION

Purification of β-Apiosidase. The β-apiosidase was not stable during purification. Addition of 0.1 mM EDTA in buffers was found to be effective to keep enzyme activity. By ammonium sulfate precipitation, most β-apiosidase activity of the Klerzyme 200 was found in the precipitate of 30−65% saturation. This precipitate was dialyzed against 0.1 M citric acid−phosphate buffer (pH 7.0) and 0.1 mM EDTA, concentrated, and subjected to gel filtration chromatography, by which the β-apiosidase

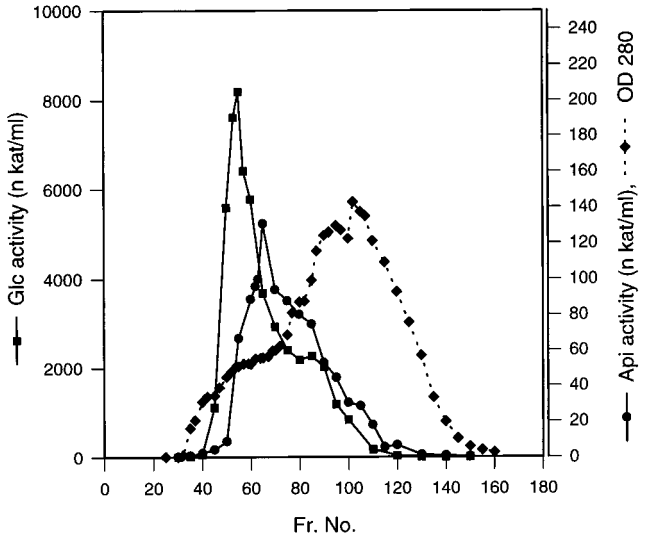

Figure 1. Gel filtration chromatography (Ultrogel AcA 44) of the enzyme from Klerzyme 200: OD 280, absorbance at 280 nm; Glc, glucosidase activity; Api, β-apiosidase activity.

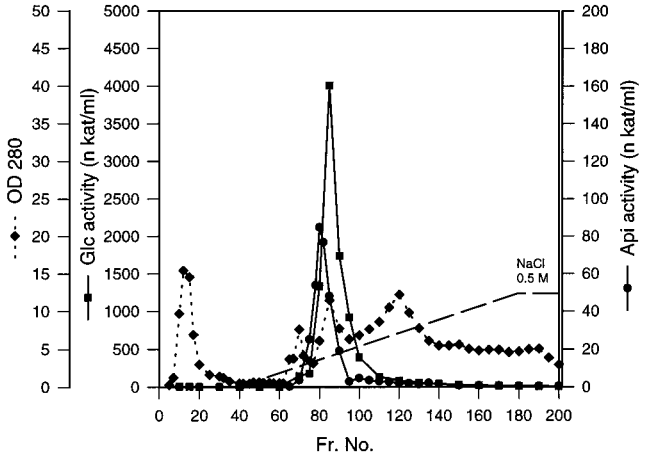

Figure 2. Ion-exchange chromatography (DEAE-Sepharose CL-6B) of the fractions from gel filtration chromatography: OD 280, absorbance at 280 nm; Glc, glucosidase activity; Api, β-apiosidase activity.

overlapped the β-glucosidase peak (Figure 1). During this purification step, most of the contaminating protein was eliminated. The fractions 51−110 showing β-apiosidase activity were pooled. A large part of the brown pigment was removed from the sample with active charcoal without any activity loss.

The sample was separated into several protein peaks by the ion-exchange (DEAE-Sepharose CL-6B) column chromatography (Figure 2). Again, the β-apiosidase was eluted with the β-glucosidase by ∼0.15 M NaCl. SDS−PAGE and the detection of activity on electrophoresis gels with relevant 4-methylumbelliferyl substrates showed that both enzymes had the same molecular masses (120 kDa). The fractions 70−95 containing β-apiosidase activity were pooled and subjected to ion-exchange HPLC. The β-apiosidase was separated into two peaks (Figure 3). The first peak was eluted by 0.04−0.06 M NaCl, whereas the second was eluted by 0.06−0.15 M NaCl together with a β-glucosidase activity. The combined fractions (31−63) of the first peak exhibited, however, a low level of β-glucosidase activity after concentration. This was due to the collected last fractions, which were eluted just before the β-glucosidase peak. The fractions 31−63 were eliminated from contaminating β-glucosidase by subjecting them to the same ion-exchange HPLC and by pooling the fractions (30−55) much before exclusion of β-glucosidase peak. When the fractions 30−55 were subjected to size exclusion HPLC, two incompletely separated peaks at $t_R = 8$ and 9.5 min were detected. Thus, the fractions of size exclusion HPLC were analyzed by SDS−PAGE both for the detection of the apiosidase activity and for the purity, besides the colorimetric assay for the activity measurement. The peak eluted at $t_R = 9.5$ min corresponded to an impurity protein, arising from the previous ion-exchange chromatography (Figure 4B, lane 6). The first peak contained 120 and 80 kDa bands. Only the 120 kDa band yielded a fluorescent spot after reaction with 4-methylumbelliferyl β-D-apiofuranoside (Figure 4). The β-apiosidase active fractions were pooled and subjected to the same size exclusion HPLC, which yielded a unique peak at 280 nm (Figure 5).

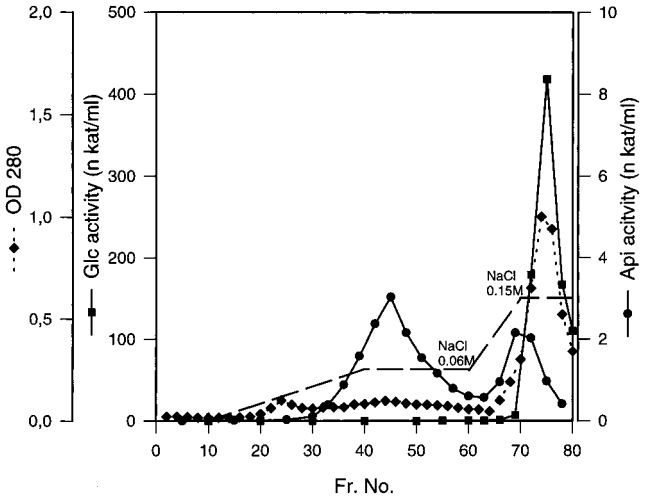

Figure 3. Ion-exchange HPLC (1) (TSK DEAE-5PW) of the fractions from ion-exchange chromatography on DEAE-Sepharose CL-6B: OD 280, absorbance at 280 nm; Glc, glucosidase activity; Api, β-apiosidase activity.

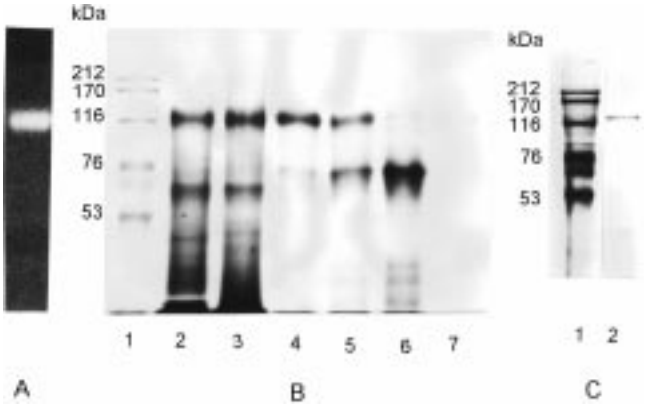

Figure 4. SDS−PAGE of enzymes during purification of β-apiosidase: (A) fluorescent spot of purified β-apiosidase indicated by 4-methylumbelliferyl β-D-apiofuranoside on 7.5% gel, nondenatured conditions; (B) samples at each purification step, on 7.5% gel, in electrophoresis conditions to detect enzyme activity [(lane 1) marker proteins; (lane 2) ammonium sulfate precipitation; (lane 3) gel filtration fractions 51−110; (lane 4) ion-exchange chromatography fractions 70−95; (lane 5) ion-exchange HPLC (1) fractions 31−63; (lane 6) ion-exchange HPLC (2) fractions 30−55; (lane 7) size exclusion HPLC (purified β-apiosidase); the fluorescent spots indicated by 4-methylumbelliferyl β-D-apiofuranoside were observed at 120 kDa in lanes 2−7]; (C) under denatured conditions [(lane 1) marker proteins; (lane 2) purified β-apiosidase].

Table 1. Purification of β-Apiosidase from Klerzyme 200

purification step	apiosidase activity (nkat)	apiosidase activity yield (%)	protein (mg)	protein yield (%)	specific activity (nkat/mg)	purification factor
Klerzyme 200	11600	100	2440	100	4.75	1
ammonium sulfate ppt	5800	50.0	2080	85.2	2.79	0.59
gel filtration CC	5720	49.3	1350	55.3	4.24	0.89
DEAE-Sepharose CC	1926	16.6	184	7.54	10.5	2.20
ion-exchange HPLC (1)	624	5.38	25.3	1.04	24.7	5.19
ion-exchange HPLC (2)	456	3.93	4.73	0.19	96.4	20.3
size exclusion HPLC	50.1	0.43	0.039	0.0016	1285	270

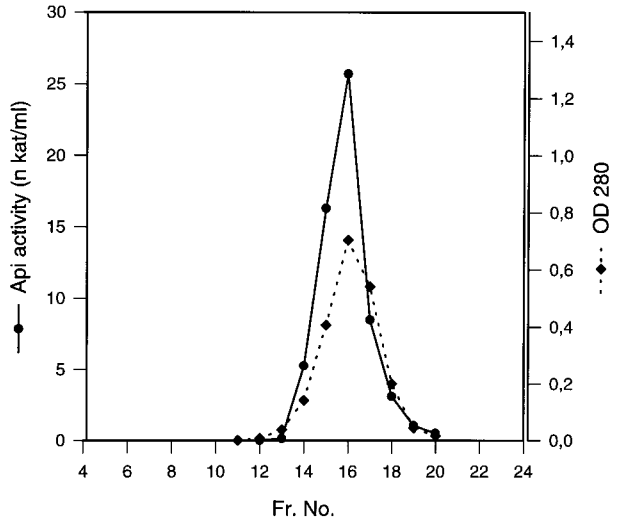

Figure 5. Size exclusion HPLC (SEC 2000) of the β-apiosidase fractions from the first size exclusion HPLC: OD 280, absorbance at 280 nm; Api, β-apiosidase activity.

Figure 4B shows the electrophoresis of the samples during each step of purification. β-Glucosidase was one of the major proteins in the sample and showed a behavior quite similar to that of β-apiosidase during several chromatographic purification steps. It has been coeluted with β-apiosidase until the second ion-exchange HPLC.

The purification procedure of β-apiosidase is summarized in Table 1. β-Apiosidase was a minor protein in Klerzyme 200, and the final purification factor was 270.

Properties of β-Apiosidase. The molecular mass of the β-apiosidase was estimated to be ~130 kDa according to the size exclusion HPLC. The SDS−PAGE analyses gave a value of 120 kDa, indicating that the β-apiosidase was a monomeric protein (Figure 4C).

The K_m and V_{max} were determined as 4.16 mM and 2460 nkat/mg of protein, respectively, by using *p*NP apiofuranoside as the substrate.

The maximum activity of β-apiosidase was at pH 5 (Figure 6A). The optimum temperature was ~40 °C (Figure 6B). The isoelectric point of the enzyme seemed to be ~3.5 according to the results obtained from the chromatofocusing technique (Günata et al., 1988) that was used in the preliminary work to purify the enzyme.

The enzyme acted on geranyl and linalyl β-D-apiofuranosyl β-D-glucopyranosides by cleaving the intersugar linkage. Hydrolysis products were apiose (R_f = 0.55) and the corresponding monoglucosides (geranyl β-D-glucopyranoside, R_f = 0.79, and linalyl β-D-glucopyranoside, R_f = 0.82). In the presence of a high concentration of glucose (90 g/L, average value in grape juice), which is an inhibitor of β-glucosidase (Günata et al., 1993), β-apiosidase activity was not inhibited at all.

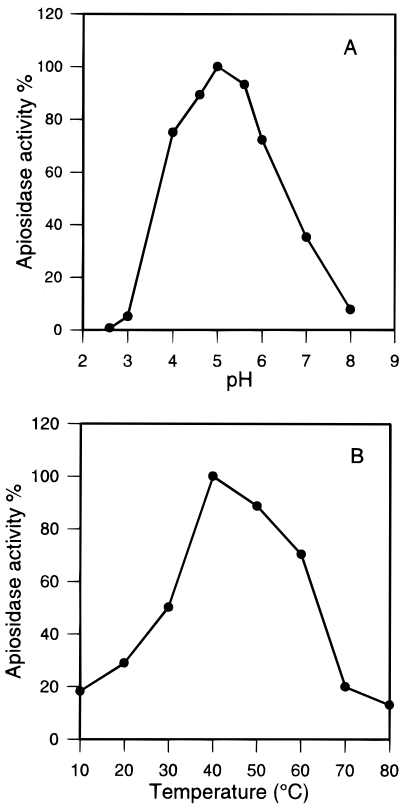

Figure 6. Effects of pH (A) and temperature (B) on β-apiosidase activity.

β-Apiosidase was resistant to 10% ethanol (average value in wine) as 84% of the activity remained.

The properties of β-apiosidase described here show the potential of this enzyme for flavor release in fruit juice processing and wine-making.

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