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ENDO-β-GLUCOSIDASE FROM *ASPERGILLUS NIGER* GROWN ON A MONOTERPENE GLYCOSIDE-CONTAINING MEDIUM*

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Key Word Index—*Aspergillus niger*, *Vitis vinifera*; wine; monoterpene glycosides; β-glucosidase.

Abstract—An endo-β-glucosidase was isolated from *Aspergillus niger* grown on a medium containing rutin as the sole carbon source, and was partially purified by affinity chromatography. The enzyme was found to be extracellular. Its optimum pH was 3.4, and its optimum temperature 65°. K_M values were 1 mM (PNPG) and 1.25 mM (geranyl-β-rutinoside), V_{max} values were 0.22 μmol/min/mg protein (PNPG) and 0.08 μmol/min/mg protein (geranyl-β-rutinoside), and K_I was 40 mM (glucose). The enzyme's activity was not inhibited by fructose, sucrose or SO_2, and was enhanced by ethanol. No loss of activity was found after seven days at 50°. M_r determined by gel filtration was 120 000. Using polyacrylamide isoelectric focusing gel, a pI of 3.9 was obtained.

INTRODUCTION

Monoterpenes play an important role in grape and wine flavour [1, 2]. Cordonnier and Bayonove [3] were the first to suggest that grapes of *Vitis vinifera* cv Muscat of Alexandria contain non-volatile forms of monoterpene glycosides. In grapes and wines of that variety, as well as of var. Rhine Riesling, Williams *et al.* [4] identified a series of glycosides such as β-rutinosides (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides) and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides of monoterpene alcohols. These glycosides are not hydrolysed during the fermentation process and remain as a non-utilized potential source of aroma [5]. The ratio between the potential (bound) and the free monoterpenes varies between 5:1 in Muscat of Alexandria and 1:1 in some non-muscat varieties [6]. A β-glucosidase obtained from almonds was found to be almost inactive towards the isolated glycosidic fractions [7].

Commercial enzymes such as rohapect C. and other available pectinases are inactive at vinification temperatures and pH levels, as well as in the presence of glucose or ethanol [2, 7]. Various authors have reported, hydrolysis of flavonoid glycosides containing β-rutinose by rhamnodiastase from *Rhamnus utilis* [8], *R. dahurica* [9], and buckwheat [10], as well as by enzymes from *Aspergillus flavus* and *A. niger* [11, 12].

We describe here the kinetics of an endo-β-glucosidase which was isolated and partially purified from *A. niger* grown on a medium containing rutin or monoterpene glycosides.

RESULTS

β-Glucosidase activity was found both in the supernatant and in the homogenates of the mycelium and the media, indicating that the enzyme is extracellular. The higher activity in the supernatant of the *Aspergillus* grown on rutin compared to that in the supernatant of the fungus grown on monoterpene glycosides (13.28 and 7.26 mg/l linalool equivalents respectively) may be attributable to the lower solubility of the rutin, which may have resulted in the secretion of more enzyme from the mycelium. However, the sum of the activities in the homogenate and in the supernatant was similar in both cases, thus confirming the suitability of rutin as a substrate.

The enzyme was found to be active over a relatively wide pH range with an optimum at 3.4, which accords well with the optimal pH values for vinification.

The activity of the enzyme declined with increasing ionic strength from 0.08 to 0.3 M. The 0.02 M concentration used for the standard assay was within the range consistent with high activity. the enzyme was active over a wide range of temperatures, with a sharp optimum at 65°; however, about 5% of the activity was still present at 12°, which is the optimal temperature for vinification and wine storage (not shown).

In another experiment, it was found that the enzyme activity was stable and did not decline after seven days at 50°. Activity was enhanced by increasing the concentration of ethanol up to a maximum of 9%, at which the activity was doubled; further increase in ethanol concentration (10–50%) resulted in a slight decline in activity. The K_M was found to be 1 mM for PNPG (*p*-nitropheynl-β-D-glucopyranoside) and 1.25 mM for geranyl-β-rutinoside, indicating relatively high affinities for the substrates. The V_{max} values were 0.22 and 0.08 μmol/min/mg protein for PNPG and geranyl-β-rutinoside, respectively. Glucose inhibited the enzyme competitively (PNPG substrate); the inhibition was fully competitive as evident from the linearity of the slopes obtained by the Lineweaver–Burk [13] plot versus glucose concentration. The K_I (glucose), calculated from the intercepts of

* Patent pending.

reciprocals of the glucose concentration on the Δ slope [13], was 40 mM.

Since we aim at hydrolysing monoterpene glycosides in musts and wines, we examined the inhibitory effects of grape juice, various sugars and sulphur dioxide on the enzyme activity. Fructose and sucrose were found to have very little effect, but 1 M glucose totally inhibited the activity and 0.25 M glucose inhibited it by 81%. Grape juice containing 21° Brix totally inhibited the activity, whereas 1% juice (diluted with buffer) inhibited it by 40%. It seems that the inhibition by grape juice can be entirely attributed to glucose, since 25% contains *ca* 0.14 M glucose and inhibits the activity accordingly (Fig. 1). Enzyme activity was not inhibited by sulphur dioxide (200 ppm).

Isoelectric focusing PAGE followed by X-glue visualization (see Methods) yielded a zymogram showing a single band at pH 3.9 (Fig. 2).

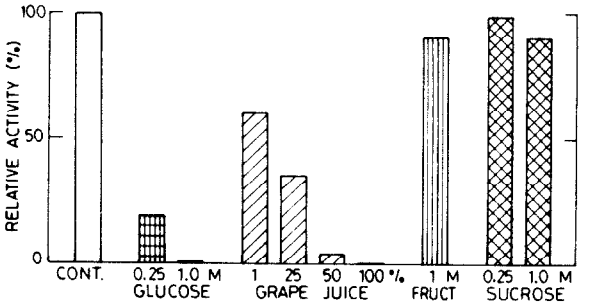

Fig. 1. Effect of various sugars and grape juice on β-Glucosidase activity. 100% = 0.21 μmol PNP/min/mg prot.

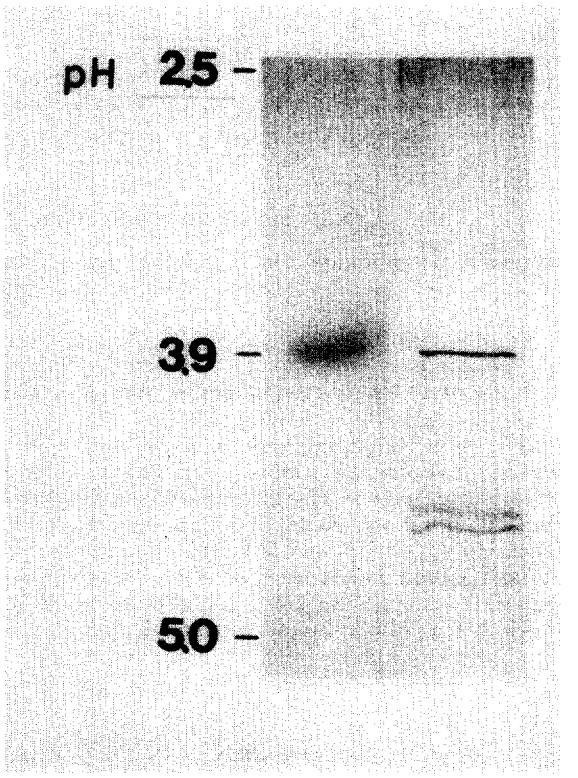

Fig. 2. Visualization of β-Glucosidase activity on isoelectric focusing polyacrylamide gel. Left, X-glue; Right, Coomassie Brilliant Blue.

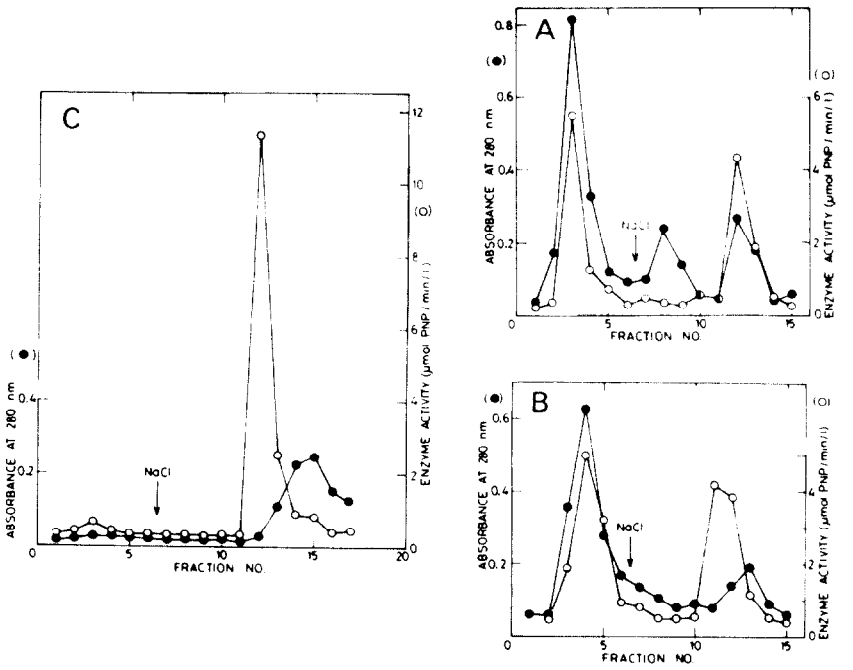

Fig. 3. Affinity chromatography separation of protein and β-Glucosidase activity. A. *p*-Aminophenyl-β-D-glucopyranoside sepharose (PAPGS). B. ξ-Aminocaproic *p*-aminophenyl-β-D-glucopyranoside sepharose (ξAPGS). C. Tyramine *p*-aminophenyl-β-D-glucopyranoside Upergit C (TPAPGU).

Affinity chromatography

Affinity chromatography was performed on three kinds of columns (see Methods and Fig. 3). The PAPGS and the ξAPGS columns split the protein into two portions, one prior to and one following the NaCl shock, whereas the third column, TPAPGU, adsorbed the whole protein and released the entire enzyme in a single fraction following the NaCl shock. The TPAPGU column yielded the highest specific activity of the enzyme. The M_r of the enzyme, as determined by gel filtration, was 120000.

DISCUSSION

Enzyme preparations from *A. niger* grown on flavonoid glycosides media have been shown to consist of β-glucosidase and α-rhamnosidase [12]. The disaccharide rutinose released in our preparation showed a single enzyme activity, i.e. endo-β-glucosidase. This β-glucosidase was found to have a lower pH optimum than that observed in other preparations [2, 14]; the enzyme may therefore be suitable for use in low-pH media, such as certain fruit juices, for aroma enrichment by hydrolysis of monoterpene glycosides and debittering by hydrolysis of flavonoid glcosides.

All known preparations are inhibited even at low glucose concentrations [2, 7]. Dekker [14] has described an enzyme from *A. niger* that was relatively resistant to inhibition by glucose; its K_I for glucose was 3.0 mM, as compared to 40 mM in our preparation. Enhancement by alcohols has been reported for the activities of other enzymes [15] but not for β-glucosidases. In the present case, however, the enzyme may be utilized during juice fermentation and also in finished wines. The partial inhibition by glucose may be overcome by subjecting the substrate to enzyme activity for long periods, since its thermal stability is far higher than that for other known preparations [14]. The high thermal stability is indicative of high stability under lower temperatures as well.

Recent reviews and articles [2, 7, 16, 17] have expressed an interest in utilizing β-glucosidases for enriching the aroma of musts and wines, as well as of other beverages, by the hydrolysis of monoterpene glycosides. It seems that the enzyme produced by this unique strain of *A. niger* may have a role to play in this process.

EXPERIMENTAL

Aspergillus niger *strain selection. A. niger* was grown from spores on a medium containing 0.4% monoterpene glycosides isolated and purified from Muscat of Alexandria wine (see below) as the sole carbon source; $(NH)_2SO_4$ 0.5 g/l, $KH_2PO_4 \cdot 3H_2O$ 0.2 g/l, $MgSO_4$ 0.2 g/l, $CaCl_2 \cdot 2H_2O$ 0.1 g/l, $FeSO_4 \cdot 6H_2O$ 0.001 g/l, $ZnSO_4 7H_2O$ 0.001 g/l, pH 3.4 adjusted by HCl. Following incubation for several weeks in an agitating incubator at 100 rpm and 30°, the slow-growing mycelium was crushed by ultratourax and incubated for a further 3 days. The resulting massive mycelium sporulated after several days and served as a source for the *A. niger* strain used.

Enzyme production by A. niger. The selected strain was grown in a 10 l fermentor with the same medium, rutin (quercetin-3-β-rutinoside) serving as an inexpensive sole carbon source. Temperature was kept at 35°, agitation 200 rpm and aeration at 10 l/min. After 48 hr the growing medium was filtered through cheesecloth, then through a 0.45 μ nitrocellulose membrane. The filtrate was ultrafiltrated and concd to 20 ml with a 20000 M_r cut-off membrane.

Isolation and purification of monoterpene glycosides. Vacuum-de-alcoholized wine (Muscat of Alexandria 30 l) was loaded on a C_{18} reversed-phase adsorbent (40–60 μ) packed in a 60 × 2 cm flush chromatography glass column. The column was first flushed with H_2O and glycosides were then eluted by MeOH. The MeOH was evapd and the remaining soln lyophilized.

Enzyme assay. β-Glucosidase activity was assayed by incubating 25 μl of enzyme preparation in 1 ml of 1 mM PNPG (4-nitro phenyl-β-D-glucopyranoside) for 2 min at 35°. The reaction was terminated by the addition of 200 μl 1 M Na_2CO_3 and the A was read at 399 nm.

Synthesis of geranyl-β-rutinoside. Geranyl hexa-*o*-acetyl-β-rutinoside was synthesized according to ref. [4], and 1 g was dissolved in 10 ml of dry MeOH. To this was added 1 ml of fresh 0.1 M NaOMe, and the soln was refluxed for 10 min for deacetylation. Cation exchange resin (Amberlite IR-120 H+]) was added till neutrality, and the soln filtered through a glass filter and lyophilized to dryness.

Quantitation of endo- and exocellular β-glucosidase activities. A. niger was grown on two kinds of liquid medium containing either monoterpene glycosides (isolated from a Muscat of Alexandria wine according to ref. [5] or rutin, 1 g/l. After incubation for 48 hr a 40 ml sample was filtered through a 0.45 μ nitrocellulose membrane to obtain 20 ml of filtrate. The non-filtered residue containing the mycelium was homogenized and centrifuged at 10000 rpm for 20 min.

Supernatants and filtered samples were then separately incubated with 20 ml of wine glycosides (1 mg/l) for 20 hr at 30°. Free monoterpenes were analysed according to ref. [18].

A. niger β-glucosidase activity was characterized using standard procedures for the determination of pH, ionic strength, temperature, EtOH response curves, K_M, V_{max} and K_I for glucose. Also examined were the effects of various concentrations of grape juice (Muscat of Alexandria), sucrose, fructose and SO_2 on the enzyme activity under standard conditions.

Isoelectric focusing on polyacrylamide gel. Analytical isoelectric focusing was performed in 0.5 mm polyacrylamide gel (T = 5%; C = 3%) containing 2.4% (w/v) ampholine (pH 2.5–5) using a multiphor unit LKB 2117 at 2000 V and 25 W for 1 hr. The slab was sliced longitudinally into three strips. One strip was cross-sectioned into 0.5 cm slices, which were immersed in 1 ml of H_2O for several hours and the pH gradient was then established. The second strip was immersed in 100 mg/l X-glue (5-bromo-4-chloro-3-indolyl β-D-glucopyranoside) for several hours and the β-glucosidase activity was visualized as previously described [19]. The third strip was stained with a general protein stainer, Coomassie Brilliant Blue 250-R.

Affinity chromatography. (a) Column preparation. Three kinds of columns were used: 1. *P*-aminophenyl-β-D-glucopyranoside sepharose (PAPGS). 2. ξ-Aminocaproic *p*-aminophenyl-β-D-glucopyranoside sepharose (EAPGS). 3. Tyramine *p*-aminophenyl-β-D-glucopyranoside Upergit C (TPAPGU).

The first two columns were prepared according to ref. [20]. The third column was prepared as follows: Tyramine 274 mg was dissolved in 20 ml of DMF and mixed with 20 ml of 1 M K_2HPO_4 containing 2 g preswollen Upergit C for 48 hr at room temp. The mixture was filtered through glass filter paper and the gel was washed first with DMF and then with 0.2 M Na_2CO_3 soln. Dialysed *p*-aminophenyl-β-D-glucopyranoside (100 μmol) was coupled through azo-linkage to the tyramine Upergit C gel according to Cuatrecasas [20] resulting in an orange-coloured product. Each column (standard Pasteur pipette) contained 2 ml of the gel. (b) Enzyme separation. One ml (0.5 mg/ml protein) was loaded onto each column and eluted

with 15 ml of 0.02 M citrate buffer, pH 3.4, folowed by 10 ml of 1 M NaCl in Na-citrate buffer. Fractions (1.5 ml) were collected at a rate of 60 ml/hr. A_{280} was determined and each fraction was then separately dialysed against deionized water. β-Glucosidase activity was assayed in all fractions.

Gel filtration chromatography. Gel filtration was performed using a Sepharose CL-6B column and a MWFG 1000 M_r marker kit (Sigma Tech. Bull. GF-3).

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