β-Glucosidases from Five Black *Aspergillus* Species: Study of Their Physico-Chemical and Biocatalytic Properties

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Five black *Aspergillus* strains (*A. aculeatus*, *A. foetidus*, *A. japonicus*, *A. niger*, and *A. tubingensis*) were cultivated on crude wheat arabinoxylan as the carbon source under defined pH, temperature, and oxygen conditions. Protein and *β*-glucosidase content differed remarkably within the obtained culture filtrates, of which eleven *β*-glucosidases were isolated. Seven *β*-glucosidases were purified to apparent electrophoretic homogeneity using anion-exchange and gel-permeation chromatography. They were found to be acidic proteins and most of them appeared to be glycoproteins with a molecular mass between 93 and 142 kDa. Classification of the *β*-glucosidases into four groups (I-A, I-B, II, and III) is suggested according to their physicochemical and biocatalytic properties. The major *β*-glucosidases were assigned to groups I-A and I-B, the minor *β*-glucosidases to groups II and III, comprising acid-tolerant and glucose-tolerant enzymes, respectively.

Keywords: *β-Glucosidase; Aspergillus aculeatus; Aspergillus foetidus; Aspergillus japonicus; Aspergillus niger; Aspergillus tubingensis; flavor release*

INTRODUCTION

Aspergillus species are one of the most common fungi in man's environment. Some are pathogenic to man and animals or produce harmful secondary metabolites such as aflatoxins or ochratoxins to cause food spoilage; others are of important industrial value. In Asian countries they have been used in food fermentation for hundreds of years. From the beginning of this century, *A. niger*, as a member of the black Aspergilli (*Aspergillus* sect. *Nigri*; Gams et al., 1985), was successfully applied for biotechnological production of organic acids. In addition, this fungus has been studied thoroughly with respect to its ability to produce extracellular enzymes such as cellulases or pectinases that can be applied easily in food processing, as they have achieved FDA recognition as GRAS (Generally Regarded as Safe) substances.

Aspergillus species are indeed efficient producers of cellulolytic enzymes (Berka et al., 1992) which comprise four types of enzymes acting synergistically. Endo-1,4-*β*-glucanase (1,4-(1,3;1,4)-*β*-D-glucan 4-glucanohydrolase, EC 3.2.1.4) and cellobiohydrolase (1,4-*β*-D-glucan cellobiohydrolase, EC 3.2.1.91) hydrolyze crystalline cellulose producing cellooligosaccharides and cellobiose. Subsequently, these products are converted to glucose by exo-1,4-*β*-glucosidase (1,4-*β*-D-glucan glucohydrolase, EC 3.2.1.74) and *β*-glucosidase (*β*-D-glucoside glucohydrolase, EC 3.2.1.21), thereby reducing cellulose inhibition of endo-1,4-*β*-glucanase and cellobiohydrolase (Wood, 1985). However, because most microbial *β*-glucosidases are themselves very sensitive to product inhibition (Woodward, 1982), glucose tolerant *β*-glucosidases are highly required for complete saccharification of cellulose.

In fruits and other plant tissues many secondary metabolites, including flavor compounds, are accumulated in their glycosylated form (Stahl-Biskup et al., 1993; Winterhalter and Skouroumounis, 1997). Because *β*-D-glucosides constitute the majority of the known glycoconjugated flavor compounds, *β*-glucosidases play an important role in flavor liberation from these precursors. In recent years, increasing interest has been focused on flavor enhancement in wine, fruit juices, and similar products (Gueguen et al., 1996; Schreier, 1997; Günata et al., 1998).

In the present work, representatives of five black *Aspergillus* species (*A. aculeatus* Iizuka, *A. foetidus* Thom & Raper, *A. japonicus* Saito, *A. niger* Tiegh, and *A. tubingensis* Mosseray) were selected and cultivated at defined pH, temperature, and oxygen conditions. Extracellular *β*-glucosidases were isolated, purified, and compared with respect to their physicochemical and biocatalytic properties. The presented data allow classification of the purified *β*-glucosidase into four groups (I-A, I-B, II, and III) in agreement with modern taxonomy of black Aspergilli.

MATERIALS AND METHODS

Materials. Phenyl *β*-D-glucopyranoside was from Aldrich. 4-Methylumbelliferyl α-D-galactopyranoside (4-MU α-D-gal*p*), 4-MU *β*-D-gal*p*, and 4-methylumbelliferyl *β*-D-xylopyranoside (4-MU *β*-D-xyl*p*) were from Koch Light Laboratories (Colnbrook Berks, UK). *p*-Nitrophenyl α-D-glucopyranoside (pNP α-D-GLC*p*), *p*-nitrophenyl α-D-mannopyranoside (pNP α-D-man*p*), and sophorose were from Serva. Benzyl *β*-D-GLC*p*, 2-phenylethyl *β*-D-GLC*p*, citronellyl *β*-D-GLC*p*, 1-*O*-*trans*-cinnamoyl *β*-D-GLC*p*, and strawberry glycosidic extract were available in our laboratory. All other substrates used were purchased from Sigma.

Culture Conditions. Conidiospores of *A. aculeatus* CBS 101.43, *A. foetidus* CBS 115.52, *A. japonicus* NW 215.pIM 803: 75, *A. niger* 5B NW 579.3, and *A. tubingensis* CBS 643.92 were

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Table 1. Production of *Aspergillus* β-glucosidases: Fermentation Conditions, Protein Concentrations, and β-glucosidase Activities

		A. japonicus[a]	*A. tubingensis*[b]	*A. aculeatus*[a]	*A. niger*[b]	*A. foetidus*[a]
fermentation time	h	168	168	168	163	163
biomass	g/L	4.25	4.62	5.24	5.98	6.90
volume	mL	1960	1930	2250	2396	2162
			protein			
concentration	μg/mL	709.9	551.3	436.0	864.2	500.0
total	mg	1391.4	1064.0	981.0	2070.6	1081.0
			β-glucosidase			
volume activity	mU/mL	81.1	168.5	122.4	131.6	192.1
total activity	U	159.0	325.2	275.4	315.3	415.3
specific activity	(mU/mg)	114.2	305.6	280.7	152.3	384.2
			after DEAE-Sephadex			
total activity	(U)	117.4 (74%)	172.5 (53%)	186.9 (68%)	288.0 (91%)	288.0 (75%)

[a] β-glucosidase activity determined at pH 5.0. [b] β-glucosidase activity determined at pH 4.5.

propagated on complete medium plates containing 1.2% (w/v) agar and 50 mM glucose. All media for *A. niger* cultures were supplemented with biotin, and plates for *A. japonicus* contained 0.6% xylose. Spores were harvested with a saline-Tween solution (0.9% NaCl + 0.005% Tween 80). The medium for β-glucosidase production contained 1.2 g/L $NaNO_3$, 0.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \times 7H_2O$, 2% crude wheat arabinoxylan (Latenstein, The Netherlands) as carbon source and 0.2 mL/L trace metal solution (Vishniac and Santer, 1957). Preculture and fermentation media for *A. japonicus* were supplemented with 10 and 5 mM uridine, respectively. The pH of the media was adjusted to 5.5, and the precultures (300 mL) were inoculated with 2.1×10^9 spores. After 6 h of incubation at 30 °C in a rotary shaker (New Brunswick, Edison, NJ) the precultures containing the germinated spores and 1 mL polypropylene glycol as antifoam were added to the fermentation media (2.1 L final volume). Fermentation was carried out for 163 (168) hours in a 3-L flat-bottom bioreactor (H/D ratio = 1.5) (Applikon, Schiedam, The Netherlands) at pH 5.5 ± 0.05, 28 °C, DOT > 30% and 800 rpm (cf. Table 1).

Isolation and Purification of β-Glucosidases. All purification steps were carried out at 6 °C. Bulk media, columns, peristaltic pumps, and FPLC (fast protein liquid chromatography) equipment used were from Pharmacia.

(i) Preparation of Crude Extract. Extracellular protein was separated from the mycelium by filtration. The culture filtrate was diluted 10-fold and the pH was adjusted to 6.0 to minimize protease activity. With 20 mM piperazine/HCl (at pH 6.0) preequilibrated DEAE-Sephadex A-50 (3 g dry weight) was added to the diluted culture filtrate. While the material was being stirred overnight, proteins with a pI below 6 were bound to the anion exchanger. After the protein was washed with 100 mL of buffer, the protein was pulse-eluted with 5 × 100 mL of 1 M NaCl in 20 mM piperazine/HCl (pH 6). Fractions containing activity against pNP β-D-GLC*p* were pooled and dialyzed against starting buffer for anion exchange chromatography.

(ii) Anion-Exchange Chromatography on DEAE-Sepharose Fast Flow (2.5 cm × 27 cm). Dialyzed sample was applied to the preequilibrated anion exchanger (20 mM piperazine/HCl (pH 6.0) or 25 mM imidazole/HCl (pH 7.0) for *A. aculeatus*) using a peristaltic pump. After the protein was washed with 200 mL of starting buffer, the protein was eluted with a 1.5-L linear gradient up to 0.4 M NaCl (0.9 M for *A. aculeatus*) in the corresponding buffer.

(iii) Anion-Exchange Chromatography on Q-Sepharose Fast Flow (1.6 cm × 13.8 cm). A. tubingensis β-glucosidase collected from DEAE-Sepharose was dialyzed against 20 mM *N*-methyl piperazine/HCl pH 4.8 and again submitted to anion-exchange chromatography on Q-Sepharose Fast Flow. After removal of unbound protein, β-glucosidase was eluted with a linear gradient up to 0.4 M NaCl (400 mL).

(iv) Gel-Permeation Chromatography on Sephacryl S-200 HR. Active fractions from DEAE-Sepharose Fast Flow or Q-Sepharose (*A. tubingensis*) were combined and concentrated by ultrafiltration (Amicon cell, 10 kDa cut-off). Samples of 1.2 mL were applied to the GPC column and eluted with 0.1 M potassium phosphate buffer (pH 6.2) containing 0.1 M NaCl. For determination of the Stokes radius (R_s), a calibration curve was constructed plotting $(-\log(k_{av}))^{1/2}$ versus R_s of the following marker proteins (Pharmacia): chymotrypsinogen (20.9 Å), ovalbumin (30.5 Å), bovine serum albumin (35.5 Å), aldolase (48.1 Å), and catalase (52.2 Å). Void volume and column volume were determined with dextran blue 2000 and 1% acetone, respectively.

(v) Anion-Exchange Chromatography on Mono P HR 5/20. β-Glucosidases dialyzed against the starting buffer (20 mM *N*-methyl piperazine/HCl (pH 4.6)) were applied to the ion exchanger and eluted with a linear gradient up to 0.5 M NaCl in 20 mM *N*-methyl piperazine/HCl (pH 4.6).

Protein Determination. Protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as the standard (Smith et al., 1985).

Enzyme Assays. Enzyme assays were generally performed at 30 °C in McIlvaine buffer (pH 5.0) preserved with 0.02% of sodium azide unless indicated otherwise. pNP β-D-GLC*p* (0.02%) was used as the substrate for determination of aryl β-D-glucosidase activity. After 15 min of incubation the reaction was terminated by adding an equal volume of 0.25 M Na_2CO_3. Liberated *p*-nitrophenolate was monitored spectrophotometrically (Shimadzu UV 2101 PC) at 400 nm using a calibration curve. Other glycosidase activities were measured in McIlvaine buffer at pH 5.0 using the following substrates at a concentration of 1 mM: pNP α-D-GLC*p*, pNP β-D-xyl*p*, *p*-nitrophenyl α-L-arabinofuranoside (pNP α-L-ara*f*), pNP α-D-gal*p*, pNP β-D-gal*p*, pNP α-D-man*p*, and pNP β-D-man*p*. The pH optimum of β-glucosidase activity toward pNP β-D-GLC*p* was determined in the pH range of 2.5−7.0 in McIlvaine buffer. Optimal temperature was determined by incubating at 30, 40, 50, 55, 60, 65, and 70 °C for 15 min at the optimal pH. Graphical evaluation of the Arrhenius equation plotting log *k* versus $1/T(K^{-1})$ was used for calculation of activation energy (E_a) from resulting straight line of slope $-E_a/2.303R$. Thermal stability at 60 °C with respect to the time was investigated at pH 3, 4, 5, 6, and 7 by measuring the remaining activity after several time intervals. Kinetic measurements were performed at 30 °C in McIlvaine buffer at the pH optimum of the investigated enzyme. pNP β-D-GLC*p* was used as the substrate at 8−10 different concentrations between 0.1 and 5 mM. For determination of the initial velocity (v_o) samples were incubated up to 15 min, and aliquots were taken at different time intervals. The Michaelis−Menten constants (K_m) and V_{max} values were calculated from Hanes plots by plotting S/v_0 versus *S* (substrate concentration). End-product inhibition by glucose ($K_{i(GLC)}$) was estimated from the intercept of abscissa ($-K_i$) of the secondary Hanes plots (K_m' and K_m'/V_{max}' plotted versus *I*).

Activity toward oligosaccharides and other nonchromogenic substrates was determined at a concentration of 1 mM (in 50 mM sodium acetate at pH 5.0 including 0.02% sodium azide) and incubation with the β-glucosidases for 30 min. After the sample was boiled for 10 min and cooled, 0.2 mM talose was

added as internal standard. High-performance liquid chromatography on a Lichrospher 100 Diol column (Knauer, Berlin, Germany) was used for analysis, and released glucose was monitored using an evaporative light-scattering detector (Sedex 55, Sedere, Alfortville, France). Elution was achieved with a linear gradient from A (12% H_2O and 88% CH_3CN) to B (12% H_2O and 25% MeOH and 63% CH_3CN) within 12 min at a flow rate of 1 mL min^{-1}.

β-Glucosidase activity toward flavor precursors was determined by incubating strawberry glycosidic extract (150 g of fresh strawberries) with 800 mU β-glucosidase in McIlvaine buffer (pH 5) at 37 °C for 22 h. 1-Dodecanol (60 μg) was added as internal standard; liberated aglycones were extracted with diethyl ether (3 × 8 mL) and dried over Na_2SO_4, and the solvent was removed by Vigreux distillation. The aglycones were analyzed by capillary gas chromatography−mass spectrometry (HRGC−MS).

Capillary Gas Chromatography−Mass Spectrometry (HRGC−MS). A Fisons gas chromatograph 8060 with split injector (1:30) equipped with a J&W fused silica DB−Wax capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was coupled to a Fisons MD 800 mass spectrometer with MassLab data systems. The temperature program was 3-min isothermal at 50 °C and then increased to 250 °C at 4 °C/min. The flow rate of the carrier gas was 2.0 mL/min He and the make up gas 30 mL/min N_2. Other conditions were injector temperature, 220 °C; temperature of ion source, 230 °C; temperature of all connecting parts, 200 °C; electron energy, 70 eV; cathodic current, 4 mA; mass range, 40−250.

Discontinuous Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Electrophoresis was performed using 7 or 10% polyacrylamide gels (Laemmli, 1970) in a Mini 2-D-Cell (Bio-Rad). Molecular mass was determined using a calibration curve derived from a mixture of marker proteins (Serva): myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumine (66 kDa), albumine (45 kDa), and carbonic anhydrase (29 kDa). Developed gels were stained with either Coomassie Blue R-250 or silver-staining (development file No. 210, Pharmacia, modified). Glycoproteins were specifically monitored by periodic acid-silver staining (Dubray and Bezard, 1982). Activity staining was used to identify active β-glucosidase in non-reducing SDS−PAGE gels. After the gel was washed with McIlvaine buffer (pH 5.0), β-glucosidase activity was monitored either directly by using the fluorogenic substrate 4-MU β-D-GLC*p* and detection of 4-methylumbelliferone at $\lambda = 366$ nm or by a coupled assay with β-naphthyl β-D-GLC*p* as the substrate. For the second method the gel was immersed in 1 mM β-naphthyl β-D-GLC*p* in McIlvaine buffer (pH 5.0) for a few minutes. By subsequent addition of the diazonium salt Fast Garnet GBC (0.02% in 0.5 M potassium phosphate (pH 6.0)) a highly insoluble, strongly red azo color was generated by its coupling to the liberated β-naphthol. Further staining with Coomassie Blue or silver staining was used to distinguish between β-glucosidase and protein being inactive toward β-naphthyl β-D-glucopyranoside.

Isoelectric Focusing. Analytical ultrathin-layer isoelectric focusing (UDIEF) was performed in the pH range 3−6 using Servalytes precotes (125 mm × 125 mm, 0.15 mm thickness) and the Pharmacia low pI calibration kit (pH 2.5−6.5). Gels were either stained with Coomassie Blue G-250 or treated as indicated for SDS−PAGE.

Chemical and Enzymatic Protein Deglycosylation. N-Glycans were specifically removed by enzymatic deglycosylation with peptide N-glycosidase F from *Flavobacterium meningosepticum* using the Glyco Shift kit (Oxford Glycosciences, Abington, UK). The kit was applied according to the suppliers instructions for complete chemical deglycosylation with anhydrous trifluoromethanesulfonic acid.

Amino Acid Sequencing. N-terminal amino acids were determined after SDS−PAGE and western blotting on poly(vinylidene difluoride) (PVDF) membranes using a gas-phase sequenator (Model 476A, Applied Biosystems).

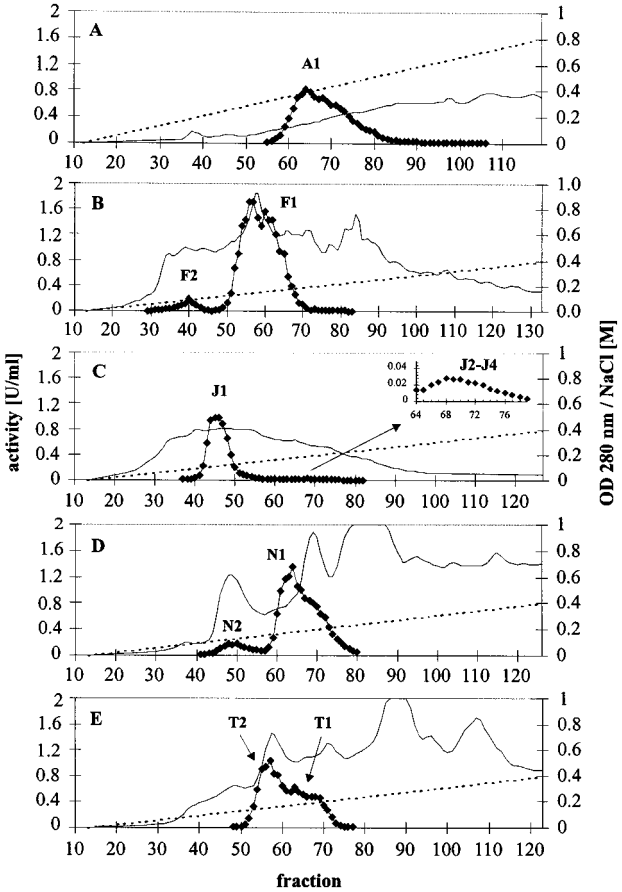

Figure 1. Chromatographic separation of *Aspergillus* β-glucosidases using DEAE-Sepharose Fast Flow. Pooled fractions containing β-glucosidase activity obtained from batch adsorption on DEAE-Sephadex-A50 were dialyzed against 20 mM piperazine/HCl (pH 6.0) (25 mM imidazole/HCl (pH 7.0) for *A. aculeatus*) and applied onto the column. After removal of unbound protein, β-glucosidases were eluted with a 1.5 L linear gradient of 0−0.4 M NaCl (0−0.9 M NaCl for *A. aculeatus*) at a flow rate of 2 mL/min. Fractions (10 mL) were assayed for β-glucosidase activity with pNP-β-D-GLC*p*; (-♦-) β-glucosidase, (−) $OD_{280\ nm}$, (- - -) NaCl. (A) *A. aculeatus*. (B) *A. foetidus*. (C). *A. japonicus*. (D) *A. niger*. (E) *A. tubingensis*.

RESULTS

Production of β-Glucosidases. The five *Aspergillus* species under study were cultivated for 163 to 168 h on crude wheat arabinoxylan until β-glucosidase activity reached its maximum. Table 1 summarizes the fermentation conditions, protein concentrations, and β-glucosidase activities. Prepurification by batch adsorption on DEAE-Sephadex recovered between 53 and 91% of the initial β-glucosidase activity of the Aspergillus culture filtrates.

Purification of β-Glucosidases. Anion-exchange chromatography on DEAE-Sepharose Fast Flow was used as a subsequent purification step (Figure 1). *A. aculeatus* was found to produce a single β-glucosidase (**A1**) (Figure 1A). β-Glucosidase activity of *A. foetidus*, *A. japonicus*, and *A. niger* was separated into a major (**F1**, **J1**, **N1**, respectively) and at least a minor β-glucosidase activity peak (**F2**, **J2**−**J4**, **N2**) (Figure 1B−D), the latter representing only between 4 and 16% of the initial activity. The shape of the activity peak of *A. tubingensis* in Figure 1E already indicates the presence of two β-glucosidases (**T1** and **T2**) which was confirmed by UDIEF. Attempts to separate these two enzymes

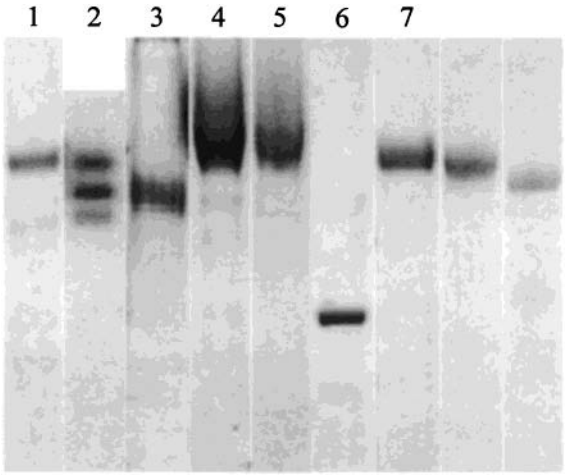

Figure 2. SDS−PAGE of isolated β-glucosidases. (1) A1, 93 kDa; (2) F1, 120−142 kDa; (3) F2, 45 kDa; (4) J1, 121 kDa; (5) J2−J4, 125, 110, and 98 kDa; (6) N1, 130 kDa; (7) N2 including xylosidase, 116−200 kDa; (8) T1, 111 kDa; (9) T2, 102 kDa. Electrophoresis was carried out on 7% and 10% (lane 3 and 5) polyacrylamide gels. They were stained with Coomassie Blue R-250, except lane 1 which was silver stained and lane 5 which was activity stained using β-naphthyl β-D-glucopyranoside.

with a pI of 4.2 and 3.9 by anion-exchange chromatography on Q-Sepharose Fast Flow at pH 4.8 failed.

β-Glucosidase active fractions from each *Aspergillus* strain were combined, concentrated by ultrafiltration, and subjected to gel-permeation chromatography on Sephacryl S-200 HR. With this purification procedure, the β-glucosidases **A1**, **F1**, **J1**, and **N1** were obtained in apparently homogeneous form as monitored by UDIEF (data not shown) and SDS−PAGE (Figure 2). At this stage, the relative activity of **N2** compared to that of contaminating xylosidase (similar pI and molecular mass) was too low (0.7 versus 29.5 U/ml) for further purification. Activity-stained SDS−PAGE gels using β-naphthyl β-D-glucopyranoside as substrate revealed that the second β-glucosidase activity peak from *A. japonicus* (fractions 65−78) contained three β-glucosidases (**J2**−**J4**) exhibiting differences in their molecular masses (Figures 1D and 2). The total activity of **J2**, **J3,** and **J4** however, amounted to approximately 0.5% of the initial activity, which made further purification impossible. To separate the two β-glucosidases of *A. tubingensis* (**T1** and **T2**) (Figure 1E), different chromatographic methods were applied, including perfusion chromatography on Poros HQ and QE as well as various chromatofocusing techniques. Finally, their purification, recovering 0.3 and 0.1% of the initial activity, was achieved using a Mono P HR 5/20 column at a pH of 4.6. **F2** was purified likewise with a yield of 0.4%. The recovery of activity for **A1**, **F1**, **J1,** and **N1** was 8.8, 34.3, 64.3, and 44.3%, with corresponding purification factors of 157, 30, 260, and 224, respectively.

Characterization of the β-Glucosidases. *(i) Molecular Mass, Stokes Radius, and Isoelectric Point.* Gel filtration and SDS−PAGE were used to determine the Stokes radii and molecular masses, respectively, of the isolated β-glucosidases. Most β-glucosidases were demonstrated to be glycoproteins with a Stokes radius between 51.2 and 57.0 Å and a molecular mass between 98 kDa and 140 kDa. As an exception, the Stokes radius and molecular mass of **F2** were found to be 24.9 Å and 45 kDa, respectively, and no reduction of molecular mass was observed after chemical or enzymatic deglycosylation. In contrast, the molecular masses of β-glucosidases **F1**, **N1**, **N2**, **T1,** and **T2** were equally reduced after treatment with peptide-N-glycosidase F and anhydrous trifluoromethanesulfonic acid, demonstrating that these enzymes are only N-glycosylated. Chemical deglycosylation of **J1** and **F1** caused greater reductions in molecular mass (97 and 99 kDa) than did enzymatic deglycosylation (102 and 109 kDa) indicating that these enzymes might be O- as well as N-glycosylated. UDIEF was applied for pI determination of the purified β-glucosidases. **A1** and **J1** were both isoelectric at pH 4.7. Lower pIs of 4.3, 4.2, and 4.1 were determined for **F1**, **T1** and **N1**, respectively. The minor β-glucosidases **T2** and **N2** turned out to be isoelectric at pH 3.9 and 4.0, whereas **F2** revealed an unusually low pI of 3.3. The physicochemical properties of the different *Aspergillus* β-glucosidases are summarized in Table 2.

(ii) pH and Temperature. The pH profiles of **T1** and **N1** were found to be more or less identical exhibiting maxima between pH 4.6 and 4.8. A similar pH profile with its maximum at pH 4.8 was found for **F1**. The pH optimum of **A1** and **J1** was equally shifted to pH 5.0, whereas **T2** and **N2** exhibited optima at pH 4.0 and 4.2, respectively. In contrast to the rather sharp pH optima of these enzymes, **F2** displayed its maximal activity in the range of pH 4 to 6 (Figure 3).

Apparent temperature optima were determined using an incubation time of 15 min, and were found to be 65 °C for **A1**, **F1**, **N1**, **N2**, **T1**, and **T2**. However, **F2** and **J1** displayed their apparent maximal activity at 60 °C (Table 2). Arrhenius plots between 30 and 55 °C provided corresponding activation energies for purified β-glucosidases in the range of 48 to 73 kJ/mol.

At 30 °C and pH 4 to 6 all purified β-glucosidases were remarkably stable; after 2 days of incubation no significant loss of activity was observed. For this reason, pH stability was investigated at 60 °C. At pH 3, **F1**, **F2**, **N1,** and **T1** were completely inactivated after 30 min, whereas **A1**, **J1**, **N2**, and **T2** maintained their activity up to 5 h. At pH 7 all enzymes retained some activity up to 3 to 5 h, except **F2** and **T2** which completely lost their activity after 1 and 2 h, respectively. All purified enzymes were most stable at pH 5. After 20 h of incubation at 60 °C most β-glucosidases retained 20 to 60% of their initial activity; only **F1** was completely inactivated. On the other hand, **J1** and **T2** were remarkably stable, maintaining between 85 and 90% of their initial activity.

(iii) N-terminal Amino Acid Sequencing. To reveal homology of the purified β-glucosidases to each other and to other β-glucosidases, a part of their primary structure was determined. At the time we had purified β-glucosidase from *A. aculeatus* (**A1**) its complete sequence was readily available in the SWISS−PROT database (Bairoch and Henrissat, 1999). The N-terminal amino acids of this enzyme, presented in Table 2, were found to be identical to those of **J1**. In addition, Edman degradation of **F1**, **N1**, and **T1** again revealed the same N-terminus for these three enzymes. Compared to the *A. aculeatus* sequence the first two tyrosine residues are replaced by phenylalanine. This 80% identity within the determined sequence strongly indicates the high homology of all major β-glucosidases isolated from the different *Aspergillus* strains, which are therefore all members of the glycosyl hydrolase family 3 (Henrissat, 1991; Henrissat and Bairoch, 1993, 1996; Bairoch and Henrissat, 1999).

Table 2. Physicochemical and Kinetic Properties of Purified β-glucosidases from *Aspergillus Species*

type of enzyme	species	M_r (10^3)	M_r (10^3)[a]	p*I*	K_m (mM)	$K_{i(GLC)}$ (mM)	optimum pH (°C)	optimum temp. (°C)	N-terminus
I-A	*A. aculeatus* (**A1**)	93	93	4.7	0.23 +/−0.02	3.7 +/−0.1	5.0	65	DELA**F**SPP**F**Y[b]
	A. japonicus (**J1**)	121	97	4.7	0.20 +/−0.02	9.2 +/−0.1	5.0	60	/ ELA**F**SPP**F**Y
I-B	*A. foetidus* (**F1**)	120−142	99	4.3	0.41 +/−0.05	8.1 +/−0.3	4.8	65	DELA**Y**SPP**Y**Y
	A. niger (**N1**)	130	91	4.1	0.64 +/−0.07	3.4 +/−0.3	4.8	65	DELA**Y**SPP**Y**Y
	A. tubingensis (**T1**)	111	96	4.2	0.59 +/−0.02	5.8 +/−0.1	4.6	65	DELA**Y**SPP**Y**Y
II	*A. niger* (**N2**)	116−200	91	4.0	-	-	4.2	65	-
	A. tubingensis (**T2**)	102	94	3.9	0.31 +/−0.06	1.3 +/−0.3	4.0	65	APSQ ? IST D T YFYGQSPAVY.P S ?E
III	*A. foetidus* (**F2**)	45	45	3.3	1.9 +/−0.2	520 +/−10	5.0	60	?NASQFDYK? QIVRGVNLG?

[a] After chemical deglycosylation. [b] Sequence obtained from SWISSPROT database.

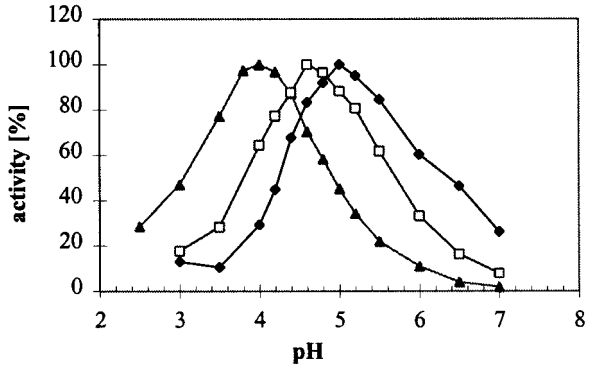

Figure 3. pH profiles of *Aspergillus* β-glucosidases. Activity toward pNP β-D-GLC*p* was determined in McIlvaine buffer from pH 2.5 to 7.0 at 30 °C. (-♦-) J1, type I-A. (-□-) **T1**, type I-B. (-▲-) **T2**, type II. pH profile of **F2** (type III) corresponds to the lower part of **T1** and the upper part of **J1**.

A completely different sequence was obtained for **T2**. Using FASTA3 at the European Bioinformatics Institute (EBI) for a SWISS−PROT database search, no homology to any other glycosyl hydrolase could be detected, implying that this enzyme, as well as the corresponding *A. niger* enzyme, represents a new type of β-glucosidase. The N-terminal amino acid sequence of **F2** was established as ?NASQFDYK?QIVRGVNLG?, thereby revealing homology to various glucan 1,3-β-glucosidases (1,3-β-D-glucan glucohydrolase, EC 3.2.1.58) that all contain GVNLG as conserved amino acids within a highly conserved region (Figure 4).

(iv) Kinetic Parameters and Substrate Specificity. All purified β-glucosidases obeyed typical Michaelis−Menten kinetics regarding pNP β-D-GLC*p* as substrate, except **A1** and **J1** which exhibited strong substrate inhibition at concentrations over 0.5 mM. Minimal substrate inhibition was also observed for **F1**, **N1,** and **T1** at concentrations above 1.5 mM, whereas **F2** and **T2** were not affected at all. With apparent Michaelis constants between 0.2 and 0.7 mM, as listed in Table 2, the various β-glucosidases mostly displayed a high affinity toward the artificial substrate pNP β-D-GLC*p*. As **F2** showed increased K_m value of 1.9 ± 0.2 mM, it was much less submitted to end product inhibition by glucose than all the other enzymes. Its corresponding apparent inhibition constant ($K_{i(GLC)}$) was determined to be 520 ± 10 mM, in contrast to 1.3 to 9.2 mM of the other β-glucosidases.

A1, **J1**, and **T1** were found to reveal high glycon specificity; no side activity toward the tested *p*-nitrophenyl derivatives was detected. **F1** and **T2** displayed also slight α-galactosidase or β-xylosidase activity, respectively. Both *A. niger* enzymes were demonstrated to act also effectively on pNP β-D-xyl*p*, pNP α-L-ara*f*, and pNP α-D-gal*p*. The β-glucosidases were further used for hydrolysis experiments of strawberry glycosidic extract. HRGC−MS analysis of the obtained hydrolysates demonstrated that all purified enzymes were able to liberate volatiles that contribute to the flavor of fresh strawberries from their glycosidically bound precursors, e.g. 2,5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol) or cinnamic acid as indicated in Table 3.

For further characterization of the purified β-glucosidases it was necessary to verify their ability to hydrolyze flavor precursors or disaccharides using pure substrates. Despite its high glycon specificity, **J1** appeared to be a broad-specificity β-glucosidase. Besides pNP β-D-GLC*p* (61.8%), it efficiently hydrolyzed other aryl β-D-glucosides such as 2-phenylethyl β-D-GLC*p* (50.6%) and, to a lesser extent, benzyl β-D-GLC*p* (17.0%), compared to its maximal activity of 100% toward phenyl β-D-GLC*p* (corresponding to 24.1 U/mg). Other well-accepted substrates were further important flavor precursors such as geranyl and citronellyl β-D-GLC*p* (62.7 and 61.4%). Alkyl β-D-glucosides (hexyl, octyl, and dodecyl), cellobiose, and sophorose were only hydrolyzed at 15 to 30%, whereas laminaribiose and gentiobiose, containing 1,3-β- and 1,6-β-glucosidic linkages, were more favorable substrates (67.2 and 90.9%). The Michaelis constant for cellobiose was determined as 1.16 mM which explains the low relative rate of hydrolysis at a substrate concentration of 1 mM. Maximal velocity was found to be in the same order of magnitude (28.7 U/mg) as for pNP β-D-GLC*p* (33.7 U/mg).

Owing to low yields, limited information with respect to substrate specificity of the *A. tubingensis* β-glucosidases is available. **T1** was demonstrated to hydrolyze efficiently cellobiose which was only a poor substrate for **T2**.

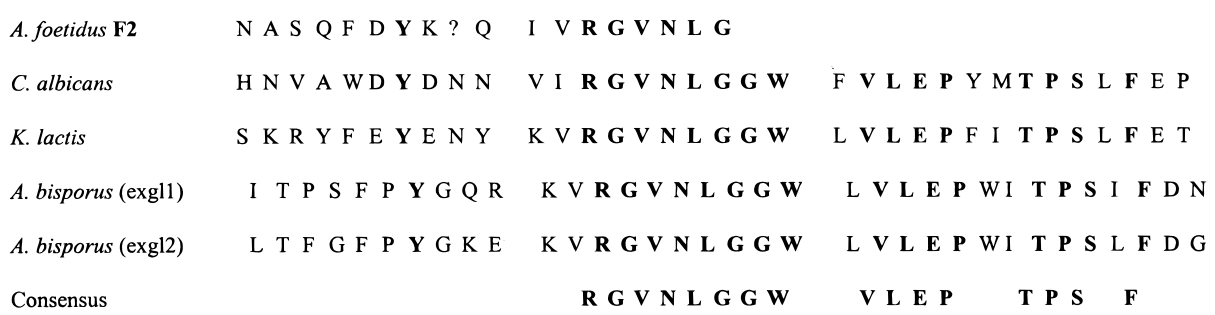

Figure 4. Partial sequence alignment of *A. foetidus* F2 with glucan 1,3-β-glucosidases. *C. albicans*, Swiss−Prot Entry P29717. *K. lactis*, Q12628. *A. bisporus* (exgl1), Q12539. *A. bisporus* (exgl2), Q12540. SWISS−PROT searches were performed with Fasta3.

Table 3. Selected Aglycones (μg/kg)[a] Liberated from Strawberry Glycosidic Extract[b] by the Purified *Aspergillus* β-Glucosidases

compound	A1	F1	F2[c]	J1	N1	N2	T1	T2
furaneol	116	300	97	295	277	338	338	256
1-hexanol	7	32	<1	27	33	48	19	32
2/3-methyl-1-butanol	5	12	<1	11	13	19	8	15
methyl 3-hydroxybutanoate	307	104	<1	715	190	190	145	68
cinnamic acid	805	2170	6440	6025	2230	2720	9610	7020
hexanoic acid	38	180	83	<1	153	254	<1	436
2-methylbutanoic acid	50	86	12	115	58	194	54	233

[a] Mean values of double HRGC−MS analyses using external standard. [b] Incubation at pH 5 with 800 mU β-glucosidase for 22 h at 37 °C. [c] Incubation with 80 mU β-glucosidase for 46 h.

DISCUSSION

The selected *Aspergillus* strains, i.e., *A. japonicus*, *A. aculeatus*, *A. niger*, *A. tubingensis*, and *A. foetidus*, were found to differ strongly in their production of β-glucosidases on crude wheat arabinoxylan. All Aspergilli produced at least two different β-glucosidases, except *A. aculeatus*. From the 2-liter culture filtrate we were able to isolate only one single β-glucosidase produced by this strain under the applied fermentation conditions. As Sakamoto et al. (1985) used a 50-times larger scale they were able to purify three different β-glucosidases from *A. aculeatus* no. F-50 with low yields of 3.2, 0.9, and 0.002%. The major β-glucosidase was reported to have the same pI of 4.7 as the enzyme we purified, but an increased molecular mass of 133 kDa versus 93 kDa of our enzyme. The higher molecular mass can be attributed to differences in the carbohydrate content, because our enzyme became somehow deglycosylated during the purification process. Further characterization of the three reported β-glucosidases is lacking, which makes proof of identity to **A1** impossible. On the other hand, *A. japonicus*, whose major β-glucosidase **J1** has properties similar to those of the *A. aculeatus* enzyme (identical pI, similar K_m, identical pH optimum, strong substrate inhibition, and identical N-terminus; cf.Table 2), was also able to produce multiple β-glucosidases. Three of them were found at such low levels that purification and characterization are still required.

Besides **A1** and **J1** that strongly resemble each other, also the major β-glucosidases from *A. niger*, *A. tubingensis*, and *A. foetidus* revealed similar pIs, K_m values, pH optima and, in particular, an identical N-terminal amino acid sequence (Table 2), which is closely related to the sequence from the corresponding *A. japonicus* and *A. aculeatus* enzymes. For this reason, the major β-glucosidases assigned to group I were further divided into subgroups I-A and I-B, the former comprising the *A. aculeatus* and *A. japonicus* enzyme and the latter comprising the *A. niger*, *A. tubingensis*, and *A. foetidus* enzymes. Because *A. niger* has been widely studied, many different publications on β-glucosidases from this fungus are available, and the properties attributed to this enzyme strongly differ from one publication to another. High molecular weight β-glucosidase (described between 96 kDa (Witte and Wartenberg, 1989) and 325 kDa (Yeoh et al., 1988) was reported to be isoelectric at pH 3.8 (Unno et al., 1993) to 4.3 (Ashadi et al., 1996) and to exhibit a K_m value toward pNP β-D-GLC*p* between 0.22 mM (Adikane and Patil, 1985) and 63 mM (Witte and Wartenberg, 1989). Within this study, we determined the pI of the 120 to 142 kDa protein as 4.1 and the K_m at the lower end of the range, at 0.64 mM.

As well-characterized as *A. niger* might be, as little is known about *A. tubingensis* and *A. foetidus*. Mar'yanovskaya et al. (1986) have reported the purification of a β-glucosidase from *A. foetidus* with a molecular mass of 130 kDa and a pI of 4.3, which correlates well with our results, in contrast to K_m toward pNP β-D-GLC*p* of 0.86 mM compared to 0.41 mM of our enzyme.

A. niger and *A. tubingensis* were both found to produce a second β-glucosidase (**N2** and **T2**) with slightly lower molecular mass than the major β-glucosidases **N1** and **T1**. Although we were not able to separate the *A. niger* enzyme from its contaminating xylosidase, we assume, due to similar pI, pH optimum, temperature optimum, and relationship between *A. niger* and *A. tubingensis*, that this enzyme might be classified into the same group of β-glucosidases (II) as the corresponding *A. tubingensis* β-glucosidase. This enzyme was demonstrated to represent a new class of β-glucosidase which is acid tolerant; it is still highly active at pH 2.5 and remarkably stable at 60 °C. In addition, no sequence homology of its established N-terminal amino acids to other published sequences could be detected. Because of such unique properties and, in particular, its ability to hydrolyze efficiently a wide variety of flavor precursors (Decker et al., 2000), β-glucosidases of type II have a great potential with respect to their application in biotechnological aroma production or flavor enhancement.

The second *A. foetidus* β-glucosidase (**F2**), representing the type III, differed considerably from all other

purified enzymes. The molecular mass was determined as 45 kDa and its pI of 3.3 is also much lower compared to those of the other β-glucosidases. But the most striking property is its high glucose tolerance expressed by the inhibition constant of 0.52 M. Although clearly active toward the artificial pNP β-D-GLC*p*, its sequence homology to glucan 1,3-β-glucosidases indicates that this enzyme might not be a true β-glucosidase, though purification of other glucose-tolerant β-glucosidases of such low molecular mass has already been reported (Saha et al., 1996; Riou et al., 1998).

The resulting classification of β-glucosidases correlates well with recent findings concerning the classification of black Aspergilli. Taxonomy based on morphological characteristics has been re-interpreted on the basis of more reliable restriction fragment length polymorphism (RFLP) analysis of mitochondrial or ribosomal DNA (Kusters-van Someren et al., 1990, 1991; Varga et al., 1993; Parenicová et al., 1997), and interrelationship between the *Aspergillus* species of the section *Nigri* was established. Differentiation of *A. aculeatus* and *A. japonicus* by their SmaI-digested rDNA pattern was not possible and *A. niger*, *A. tubingensis*, and *A. foetidus* were described to form the so-called *A. niger* aggregate. In accordance with their taxonomy, the major β-glucosidases from *A. japonicus* and *A. aculeatus* resemble each other as do the major β-glucosidases from species belonging to the *A. niger* aggregate.

β-Glucosidases of the type I-A were found to be highly specific toward the glycon portion of the substrate, but less specific with respect to the aglycon as reported for *A. japonicus*. These properties designate this type of enzyme for its application for cellulose degradation as well as aroma liberation from glucosidically bound precursors. The enzymes of type I-B differ with respect to their glycon specificity. The *A. foetidus* enzyme exhibited some α-galactosidase activity and the *A. tubingensis* enzyme exhibited some β-xylosidase activity, but only the *A. niger* enzyme revealed β-xylosidase, α-L-arabinofuranosidase, and α-galactosidase activity, of which the latter could be separated from the β-glucosidase by UDIEF as monitored by staining with the 4-methylumbelliferyl derivatives. It remains questionable whether the *A. niger* β-glucosidase has some intrinsic arabinofuranosidase and xylosidase activity. The latter could be attributed to sequence similarity between β-glucosidases and xylosidases of glycosyl hydrolase family 3 and the conserved Asp in their active site (Van Peij et al., 1997, 1998). If this were the case, we should expect the same side activities among the other closely related β-glucosidases from *A. tubingensis* and *A. foetidus*, which is not the case. Presumably, the remaining activities toward substrates with other glycones than glucose arise from insufficient purification.

In conclusion, the presented data clearly support the classification of purified β-glucosidases into four groups with different potential uses. The first group, I-A, comprises β-glucosidases with little specificity; they equally hydrolyze oligosaccharides and glucosidically bound flavor precursors. β-Glucosidases designed to group I-B act preferably toward cellooligosaccharides, which offers their application in cellulose degradation. β-Glucosidases of group II seem to be most promising due to their unique physicochemical and biocatalytic properties, e.g. their ability to hydrolyze efficiently flavor precursors at low pH and in the presence of ethanol (Decker et al., 2000). Finally, glucose-tolerant β-glucosidases are represented by group III; suitable substrate specificity provided, they are very attractive for application in cellulose degradation and aroma enhancement in wine or fruit juices.

ABBREVIATIONS USED

pNP, *p*-nitrophenyl; GLC*p*, glucopyranoside; man*p*, mannopyranoside; xyl*p*; xylopyranoside; gal*p*, galactopyranoside; 4-MU, 4-methylumbelliferyl; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IEF, isoelectric focusing; **A1**, β-glucosidase of *A. aculeatus*; **F1**, major β-glucosidase of *A. foetidus*; **F2**, minor β-glucosidase of *A. foetidus*; **J1**, major β-glucosidase of *A. japonicus*; **J2**, **J3**, and **J4**, minor β-glucosidases of *A. japonicus*; **N1**, major β-glucosidase of *A. niger*; **N2**, minor β-glucosidase of *A. niger*; **T1**, major β-glucosidase of *A. tubingensis*; **T2**, minor β-glucosidase of *A. tubingensis*.

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