β-Glucosidase from *Chalara paradoxa* CH32: Purification and Properties

Rosario Lucas, Ana Robles, Gerardo Alvarez de Cienfuegos, and Antonio Gálvez*

Area of Microbiology, Faculty of Experimental Sciences, University of Jaén, 23071-Jaen, Spain

The hyphomycete *Chalara paradoxa* CH32 produced an extracellular β-glucosidase during the trophophase. The enzyme was purified to homogeneity by ion-exchange and size-exclusion chromatography. The purified enzyme had an estimated molecular mass of 170 kDa by size-exclusion chromatography and 167 kDa by SDS−PAGE. The enzyme had maximum activity at pH 4.0−5.0 and 45 °C. The enzyme was inactivated at 60 °C. At room temperature, it was unstable at acidic pH, but it was stable to alkaline pH. The purified enzyme was inhibited markedly by Hg^{2+} and Ag^{2+} and also to some extent by the detergents SDS, Tween 80, and Triton X-100 at 0.1%. Enzyme activity increased by 3-fold in the presence of 20% ethanol and to a lesser extent by other organic solvents. Purified β-glucosidase was active against cellobiose and *p*-nitrophenyl-β-D-glucopyranoside but did not hydrolyze lactose, maltose, sucrose, cellulosic substrates, or galactopyranoside, mannopyranoside, or xyloside derivatives of *p*-nitrophenol. The V_{max} of the enzyme for *p*-NPG (K_m = 0.52 mM) and cellobiose (K_m = 0.58 mM) were 294 and 288.7 units/mg, respectively. Hydrolysis of *p*NPG was inhibited competitively by glucose (K_i = 11.02 mM). Release of reducing sugars from carboxymethylcellulose by a purified endoglucanase produced by the same organism increased markedly in the presence of β-glucosidase.

Keywords: *β-Glucosidase; Chalara paradoxa; purification, kinetics*

INTRODUCTION

Cellulose is the most abundant polymer on earth. Its annual productivity is about 4.0×10^{10} tons (Coughlan, 1992). The cellulose-degrading enzyme system includes endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). The breakdown of native cellulose is only effected by the synergistic action of multienzyme systems (Filho et al., 1989; Coughlan, 1992). β-Glucosidase catalyzes the hydrolysis of β-*O*-glucosidic linkages of alkyl- and aryl-β-glucosides as well as diglucosides and oligosaccharides. It also plays an important role in the process of saccharification of cellulose by removing cellobiose, which is known to be a strong inhibitor of cellobiohydrolase and endoglucanase activities (Shewale, 1982; Filho et al., 1989; Coughlan, 1992).

β-Glucosidases have been isolated from members of all three domains of life, i.e., eukarya, bacteria, and archaea (Woodward and Wiseman, 1982). The physiological roles postulated for β-glucosidases are extremely diverse: glucoside ceramide catabolism in human tissue, cell wall, pigment and cyanoglucoside metabolism, defense against pathogens in plants, and utilization of oligosaccharide substrates by many fungi and bacteria (Leclerc et al., 1987). These enzymes are widely used in various biotechnological processes, including the production of fuel ethanol from cellulosic agricultural residues (Bothast and Saha, 1997; Pemberton et al., 1980; Xin et al., 1993) and the synthesis of useful β-glucosides (Shinoyama et al., 1991). In the flavor industry, β-glucosidases are also key enzymes in the enzymatic release of aromatic compounds from glucosidic precursors present in fruits and fermentating products (Gueguen et al., 1996).

The hyphomycete *Chalara paradoxa* CH32 isolated from olive mill wastewater (a byproduct of the olive oil extraction industry) disposal ponds produces extracellular cellulolytic enzymes, including a low molecular mass endoglucanase (Lucas et al., manuscript in preparation) and a β-glucosidase. Olive mill wastewaters contain a high load of organic matter composed mainly of polysaccharidic substances, reducing sugars, and phenolic compounds derived from the olive fruit after maceration (Borja et al., 1990). Microbial strains having cellulolytic activity may contribute to the biodegradation of this waste. Production of extracellular β-glucosidase may stimulate cellulase activity and therefore play an important role in solubilization of cellulosic materials. The purification and characterization of the β-glucosidase produced by this strain was undertaken to assess the main properties of this enzyme and its capacity to enhance endoglucanase activity.

MATERIALS AND METHODS

Strain and Cultivation Conditions. The strain *Chalara paradoxa* CH32 was used for production of extracellular β-glucosidase activity. The organism was propagated on yeast-malt agar (YMA; Scharlau, Barcelona). For β-glucosidase production, *C. paradoxa* CH32 was cultivated on Roux flasks containing 500 mL of modified yeast-malt broth (MYM), consisting (per liter) of 0.8 g of yeast malt, 0.4 g of yeast extract, and 1.6 g of glucose, and incubated at 28 °C. At regular intervals of incubation, the mycellium was separated by filtration. The biomass dry weight and the amount of enzyme released into the cultured broth were determined.

Enzyme Assays. The enzyme activity toward *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) was measured by the amount of *p*-nitrophenol released from *p*NPG using a calibration curve

* To whom correspondence should be addressed. Telephone: 34-953-212160. Fax: 34-953-212141. E-mail: agalvez@ujaen.es.

10.1021/jf0002591 CCC: $19.00
Published on Web 07/20/2000

at 410 nm (McCleary and Harrington, 1988). The reaction mixture (1 mL) containing 4 mM *p*NPG, 50 mM sodium acetate buffer (pH 5.0), and appropriately diluted enzyme solution was incubated at 40 °C for 30 min. Then, the reaction was stopped by adding 2 mL of 1 M Na_2CO_3 and the color that developed as a result of *p*-nitrophenol liberation was measured at 410 nm. The activity against other *para*-nitrophenyl glycosides was assayed by the same procedure. One unit of β-glucosidase activity was defined as the amount of enzyme required to catalyze the formation of 1.0 μmol of *p*-nitrophenol/min. Endoglucanase activity was assayed in 50 mM sodium acetate buffer, pH 5.0, at 37 °C, using carboxymethylcellulose as substrate. The release of reducing sugars resulting from hydrolysis of substrates was determined by monitoring A_{540} by the method of Miller (Miller, 1959) using glucose as the standard.

The protein content was determined at A_{595} by using the Bio-Rad protein assay based on the Bradford procedure (Bradford, 1976) using bovine serum albumin as a standard.

Enzyme Purification. Standard chromatographic procedures were carried out at 4 °C. High-performance liquid chromatography experiments were done at ambient temperature. The UV light absorbance of the column effluents was monitored at 280 nm. Fractions collected from the different chromatographic experiments were tested for β-glucosidase activity and protein content as described above.

Step 1. Sephadex DEAE-A50 Column Chromatography. Cultured broths (4 L; pH 8.0) were mixed with 300 mL of Sephadex DEAE-A50 gel slurry previously equilibrated in 20 mM Tris-HCl buffer, pH 8.0, and kept under stirring for 30 min. The gel slurry was then packed into a 10 by 30 cm glass column and washed with 500 mL of the same equilibration buffer. The material retained in the gel was eluted by adding 500 mL of 0.25 M NaCl followed by 500 mL of 0.5 M NaCl dissolved in the same Tris-HCl buffer. Fractions (50 mL each) of the column effluent were collected.

Step 2. Sephacryl S-200 HR Gel Filtration. Active fractions from the previous step were concentrated 10-fold by lyophilization. After this procedure, samples were tested for activity to ensure that no inactivation occurred during lyophilization. Concentrated samples were loaded on a Sephacryl S-200 HR column (2 by 100 cm). The column was eluted at 3 mL/min using 20 mM Tris-HCl buffer, pH 7.2, as eluent, and the effluent was collected in 10 mL fractions.

Step 3. MonoQ Chromatography. Active fractions obtained after size-exclusion chromatography were loaded on a MonoQ HR 5/5 column (0. 5 by 5.0 cm; Pharmacia) previously equilibrated in 20 mM Tris-HCl buffer, pH 7.2. This buffer was passed through the column following sample application until the UV absorbance of the column effluent decreased to baseline. The material retained in the column was eluted with a linear gradient of 0−0.3 M NaCl in the same Tris-HCl buffer over 20 min followed by a 0.3−0.5 M salt gradient over 10 min, at a flow rate of 1.5 mL/min.

Step 4. TSK DEAE-5PW Anion Exchange. Active samples from previous step were dialyzed overnight against 20 mM Tris-HCl buffer, pH 8.0, before they were loaded on a 0.75 by 7.5 cm Progel-TSK DEAE-5PW column (Supelco, Inc., Bellefonte, PA) equilibrated in the same Tris-HCl buffer. The material retained in the column was eluted with a linear NaCl gradient of 0−0.2 M over 20 min, followed by 0.2−0.4 M over 10 min, at 1.5 mL/min.

Step 5. Superose 12 Gel Filtration. Active fractions previously concentrated to 200 μL by lyophilization were loaded on a Superose 12 HR 10/30 (1.0 by 30.0 cm; Pharmacia) column equilibrated in 20 mM Tris-HCl, pH 7.2, plus 0.2 M NaCl. Elution was carried out at 0.5 mL/min. Standard markers (kit MW-GF-200, Sigma) were run through this column to estimate the molecular mass of the purified β-glucosidase.

Polyacrylamide Gel Electrophoresis. Samples containing purified enzyme were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) (Laemmli, 1970) on 10% gels using a Miniprotean II unit (BioRad). Proteins separated in the gel were stained with Coomassie Brilliant Blue, and their molecular mass was estimated with standard markers (Sigma). For β-glucosidase activity staining, a nonacid fixed gel was washed twice with 20% 2-propanol for 20 min to remove SDS (Blank et al., 1982) and then incubated at 40 °C in 100 mM sodium acetate buffer (pH 5.0) containing 0.1% esculin and 0.03% ferric chloride until a black band appeared (Kwon et al., 1994).

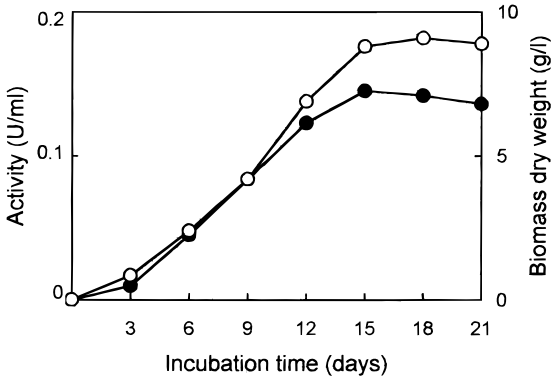

Figure 1. Fungal growth (closed symbols) and β-glucosidase production (open symbols) by cultures of *C. paradoxa* CH32.

Effect of Temperature and pH on Enzyme Activity and Stability. The optimum temperature for the hydrolysis of *p*NPG was measured between 4 and 60 °C by incubating each reaction mixture in 50 mM sodium acetate buffer, pH 5.0, for 30 min. For determination of the pH dependence of the purified β-glucosidase, reaction mixtures in Britton and Robinson buffers (pH 3.0−9.0) were incubated for 30 min at 40 °C.

To study pH and temperature stabilities, samples containing purified β-glucosidase in Britton and Robinson buffers (pH 3.0−9.0) were incubated at different temperatures (28−70 °C) for 30 min. Following the specified treatments, samples were diluted 10-fold in 100 mM sodium acetate buffer, pH 5.0, and incubated at 40 °C for 10 min. Then *p*NPG was added, and the amount of substrate transformed during a further 30 min incubation period was determined.

Effect of Chemical Reagents, Metal Ions, and Organic Solvents on Enzyme Activity. The effects of different chemical reagents, metal ions, and organic solvents on the enzyme activity were measured by addition of the tested compounds to the reaction mixtures, which were incubated at 40 °C for 10 min, separately. Then the remaining activity was determined by adding the substrate *p*NPG and incubating samples at 40 °C for 30 min. The percentage of remaining activity was calculated by comparing the amount of substrate transformed with that of a control reaction mixture lacking the test compounds.

Kinetic Study. The kinetic parameters of the purified β-glucosidase were carried out by adding *p*NPG (0.03−20 mM) as substrate into the reaction mixture and assaying hydrolysis activity as described previously. The K_m and V_{max} values were calculated by the double-reciprocal plot method of Lineweaver and Burk. The inhibition of glucose on enzyme activity was determined by adding various concentrations of glucose into reaction mixtures with *p*NPG as substrate. The inhibition constant (K_i) was obtained at the line intersection of a Dixon plot.

Chemicals. Aryl glucosides, cellobiose, and other natural substrates, as well as molecular mass markers, were purchased from Sigma Chemical Co. (Madrid). Unless specified otherwise, all other chemicals used were of analytical grade.

RESULTS

Enzyme Production. The time course of β-glucosidase production by *C. paradoxa* CH32 is shown in Figure 1. The enzyme was detected in cultured broths at day 3 of cultivation, and it reached highest concentration at day 15. Some decrease of enzyme activity was detected after prolonged incubation of cultures.

Table 1. Purification of β-Glucosidase from *C. paradoxa* CH32

step	tot. protein (mg)	tot. activity (U)	specific activity (U/mg)	yield (%)	purification (-fold)
cultured broth	388	580	1.5	100	1
DEAE A-50	70	438	6.3	75.5	4.2
Sephacryl S-200	7.3	359	49.2	61.9	32.8
MonoQ	0.83	188	226.5	32.4	151
TSK DEAE-5PW	0.15	80	533.4	13.8	355.6
Superose 12	0.07	41.3	590.0	7.1	393.4

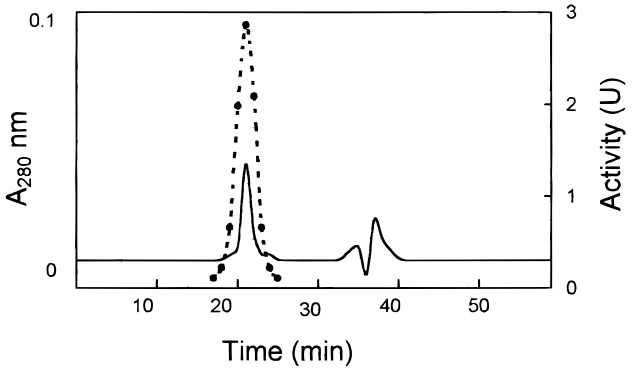

Figure 2. Purification of *C. paradoxa* CH32 β-glucosidase by size-exclusion chromatography on a Superose 12 HR 10/30 column. The A_{280} nm UV absorbance spectrum (solid line) and the β-glucosidase activity (●) are shown.

Enzyme Purification. An extracellular β-glucosidase was purified from cultured broths of *C. paradoxa* CH32. A summary of the purification procedure is presented in Table 1. Activity in cultured broths was concentrated by anion-exchange chromatography on DEAE-Sephadex A-50. The active samples were further concentrated by lyophilization. Enzyme activity was not modified notably by this procedure, and concentrated samples contained over 95% of their initial activity. A second step, consisting of Sephacryl S200 size-exclusion chromatography, served to eliminate pigmented material present in cultured broths and carried over in the anion-exchange concentrate. The partially purified fractions obtained after this step yielded a single peak of β-glucosidase activity during elution from a MonoQ column. The specific activity of samples increased after a second chromatographic step on a TSK DEAE-5PW column at pH 8.5, resulting in separation from an accompanying contaminating protein. The specific activity of the single β-glucosidase activity peak obtained after the last purification step (Figure 2) was 590 U/mg of protein. The purified enzyme showed a single protein band on SDS−PAGE (Figure 3, lane B) that coincided with the band obtained after activity staining (Figure 3, lane C).

General Properties. The molecular mass of the purified β-glucosidase was determined to be 170 kDa by Superose 12 gel filtration (Figure 2) and 167 kDa by SDS−PAGE (Figure 3).

The temperature and pH dependences of purified β-glucosidase are shown in Figure 4. The temperature optimum was 45 °C (Figure 4A). Under optimal temperature conditions the purified enzyme showed maximum activity in the pH range of 4.0−5.0 (Figure 4B). β-Glucosidase activity decreased markedly at pH below 3.0 as well as above 7.0.

The thermostability of the enzyme was investigated by measuring the residual activity after 30 min of incubation at temperatures ranging from 4 to 60 °C. Under the conditions used (50 mM Britton and Robinson buffer, pH 7.0), the purified β-glucosidase was highly stable at temperatures up to 50 °C but was almost completely inactivated at 60 °C (Figure 4A).

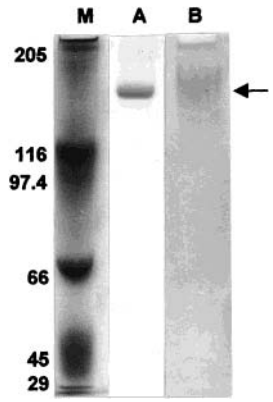

Figure 3. SDS−PAGE of purified *C. paradoxa* CH32 β-glucosidase (lane A) and activity staining with esculin after removal of SDS from the gel (lane B). The arrow indicates the position of the β-glucosidase protein. Molecular mass markers are shown (lane M).

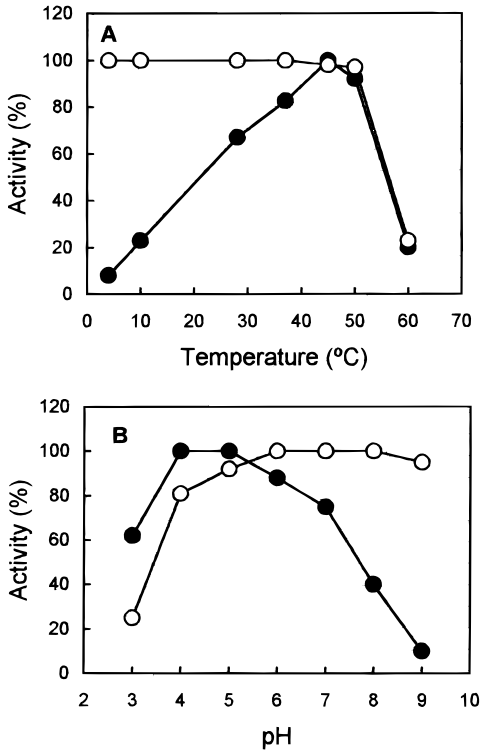

Figure 4. Effects of temperature (A) and pH (B) on the activity (closed symbols) and stability (open symbols) of purified β-glucosidase from *C. paradoxa*.

The pH stability was also investigated by measuring the residual activity after 1 h of incubation at 28 °C at pH values ranging from 3.0 to 9.0. The enzyme was almost inactivated at pH 3.0, but it retained over 75% of its activity at pH 4.0 and it was totally stable at pH 6.0 or above (Figure 4B).

The purified enzyme was almost totally inhibited by Hg^{2+} at 10 mM (Table 2), although the enzyme retained a high percentage of its activity at lower concentrations (e.g., 76% at 1 mM; Table 2). Similarly, the enzyme activity was reduced to 16% by 10 mM Ag^{+} but only to 87% at 1 mM (Table 2). Other cations (Zn^{2+}, Fe^{2+}, Mg^{2+},

Table 2. Effect of Various Reagents on β-Glucosidase Activity

	relative activity (%) at final concn of	
reagent[a]	1 mM	10 mM
none	100	100
$AgNO_3$	87	16
$CaCl_2$	100	100
$FeCl_2$	100	90
$HgCl_2$	76	1
$MgCl_2$	100	99
$MnCl_2$	100	100
$ZnSO_4$	97	82
SDS[a]	78	
Tween 80[a]	60	
Triton X-100[a]	85	
DTT		99
2-mercaptoethanol		100
cysteine		100
EDTA		100
δ-gluconolactone		0

[a] Detergents were tested at a final concentration of 0.1%.

Table 3. Effect of Organic Solvents on *C. paradoxa* β-Glucosidase Activity[a]

	relative activity (%) at solvent concn of	
	20% v/v	40% v/v
methanol	268	181
ethanol	297	158
acetonitrile	276	39
ethyl acetate	271	291
dimethyl sulfoxide	192	55

[a] The activity measured without any organic solvent addition was considered 100%

Table 4. Substrate Specificity of *C. paradoxa* CH32 β-Glucosidase

substrate	rel activity (%)
celobiose	89
esculin	87
p-nitrophenyl-β-D-cellobioside	8
p-nitrophenyl-β-D-glucopyranoside	100
p-nitrophenyl-β-D-glucosamine	27

and Ca^{2+}) caused some inhibition at 10 mM (Table 2). The enzyme activity was partially inhibited by the detergents SDS, Tween 80, and Triton X-100, but it was not affected noticeably by reducing agents or by the chelating agent EDTA. The specific β-glucosidase inhibitor δ-gluconolactone caused total inhibition.

The enzyme activity on *p*NPG could be activated by organic solvents (Table 3). Ethanol was the most effective one and could activate the enzyme by about 3-fold at the final concentration of 20%. At a final solvent concentration of 40% the enzyme was still greatly activated by ethyl acetate, methanol, and ethanol, but it was inhibited by acetonitrile and dimethyl sulfoxide.

Substrate Specificity and Kinetic Parameters. The substrate specifity of the purified β-glucosidase is shown in Table 4. None of the cellulosic substrates tested (including Avicel, carboxymethylcellulose, cotton fiber, and filter paper) were hydrolyzed, indicating the lack of endoglucanase activity in the purified preparation. The disaccharide cellobiose was hydrolyzed efficiently, while lactose, maltose, and sucrose were not. The enzyme had no activity on *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-xyloside, or *p*-nitrophenyl-α-D-mannopyranoside. The V_{max} of the enzyme for *p*-NPG (K_m = 0.52 mM) and cellobiose (K_m = 0.58 mM) were 294 and 288.7 units/mg, respectively (Figure 5).

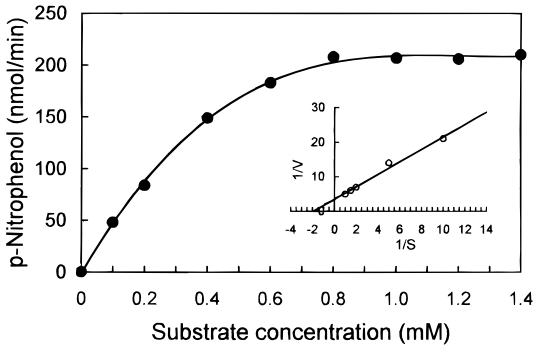

Figure 5. Effect of various concentrations of *p*NPG on the activity of purified β-glucosidase from *C. paradoxa* CH32. Inset: Lineweaver−Burk plot.

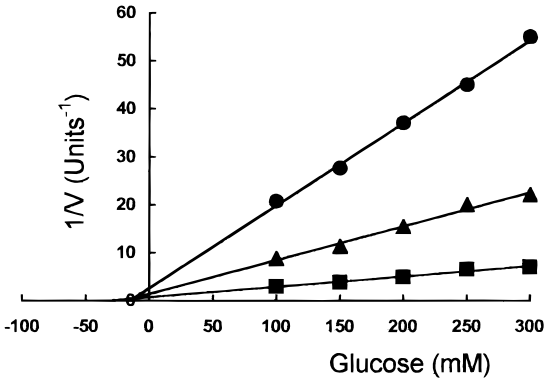

Figure 6. Dixon plot of inhibitory effects of glucose on *p*NPG hydrolysis by β-glucosidase from *C. paradoxa* CH32. The reaction time was 15 min at pH 4.5 and 45 °C. The pNPG concentrations used were 2.5 mM (●), 5 mM (▲), and 10 mM (■).

Hydrolysis of *p*NPG was inhibited competitively by glucose. The K_i value was calculated to be 11.02 mM (Figure 6). Fructose, galactose, mannose, and xylose did not inhibit the enzyme activity at 35 mM.

Synergistic Activity. To study the synergistic action of the purified β-glucosidase, a purified endoglucanase from *C. paradoxa* CH32 (Lucas et al., manuscript in preparation) was used in an assay in the presence of increasing concentrations of β-glucosidase, using carboxymethylcellulose as the endoglucanase substrate. The amounts of reducing sugars accumulated in the reaction medium increased noticeably as the amount of added β-glucosidase increased (Figure 7).

DISCUSSION

Production of hydrolytic enzymes by the hyphomycete *Chalara paradoxa* has been the subject of a limited number of studies. Kainuma et al. (1985) reported amylase production by *C. paradoxa* isolated from the pith of the sago palm. The glucoamylase component was purified and characterized (Ishigami et al., 1985a, 1985b; Monma and Kainuma, 1992) and tested for semicontinuous hydrolysis of sweet potato raw starch (Noda et al., 1992). More recently, several strains of *C. paradoxa* isolated from olive mill wastewater disposal ponds were found to produce laccase activity (Robles et al., 2000). The strain *C. paradoxa* CH32 isolated from olive mill wastewater disposal ponds was also found to produce laccase activity as well as two cellulolytic enzymes: a low molecular mass endoglucanase (Lucas et al., in preparation) and a β-glucosidase. The latter

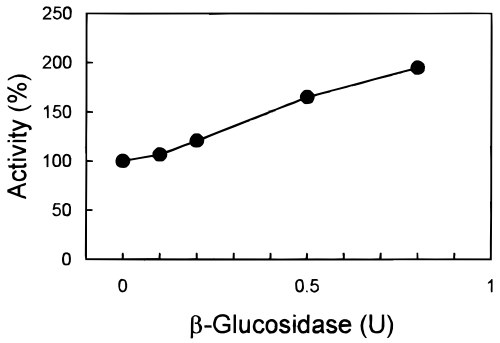

Figure 7. Potentiation of endoglucanase activity by purified β-glucosidase from *C. paradoxa* CH32. Activity of a purified endoglucanase from *C. paradoxa* CH32 (Lucas et al., manuscript in preparation) against carboxymethylcellulose (●) was measured in the presence of increasing concentrations of purified β-glucosidase.

was released into the culture medium during exponential growth, from where it was purified to homogeneity by a combination of ion-exchange and size-exclusion chromatographic steps with an average yield of 7%. The single protein band detected on SDS−PAGE gels coincided with the band of β-glucosidase activity and had a molecular mass very close to that estimated by size-exclusion chromatography, indicating that the enzyme was monomeric.

The fungal β-glucosidase from this study had a relatively broad range of pH and temperature dependence, with optimum at pH 4.0−5.0 and 45 °C. Nevertheless, activity in the pH range of 4.0−7.0 and 28−50 °C was always above 60% of maximum. Although the enzyme was not active at alkaline pH, it was remarkably stable up to pH 9.0.

The β-glucosidase produced by *C. paradoxa* CH32 was inhibited by sulfhydryl oxidant metals (Ag^{+}, Hg^{2+}) at relatively high concentration (10 mM), but it was not inhibited by thiol-reducing agents as DTT, 2-mercaptoethanol, or cysteine. These results suggest that thiol groups are not essential for catalytic activity. Inhibition of β-glucosidase activity by sulfydryl oxidant metals may be due to nonspecific salt formation rather than to complex formation with and (or) catalysis of oxidation of specific residues (thiol groups) (Ruttersmith and Daniel, 1993).

Interestingly, alcohols increased the hydrolytic activity of the *C. paradoxa* CH32 β-glucosidase toward *p*NPG. Activation by organic solvents has been observed for β-glucosidases from *Aspergillus niger* (Yan and Lin, 1997; Watanabe et al., 1992), *B. cinerea* (Gueguen et al., 1995), *C. peltata* (Saha and Bothast, 1996), *Thermotoga* sp. (Ruttersmith and Daniel, 1993), and *F. oxysporum* (Christakopoulos et al., 1994). Some β-glucosidases preferentially utilize alcohols rather than water as acceptors for the glycosyl moiety during catalysis, yielding ethyl-β-D-glucoside in the reaction. The effect of ethanol has been attributed to β-glucosyl transferase activity, the ethanol acting as a suitable acceptor for this reaction (Ruttersmith and Daniel, 1993; Pemberton et al., 1980). Nevertheless, little is known on the effects of organic solvents other than alcohols. Activity enhancement of β-glucosidase form *C. paradoxa* CH32 by acetonitrile and DMSO could also be attributed to the interaction between the organic solvent and the relatively hydrophobic aglycone moiety of the substrate. Nevertheless, activation by water-immiscible solvents such as ethyl acetate could also be attributed to some property of this enzyme. β-Glucosidases having glucosyltransferase activity are of value in the biotechnological industry for production of oligosaccharides with special functionality as healthy food additives.

The purified β-glucosidase from *C. paradoxa* has a relatively high substrate specificity. It can tolerate a variety of aglycons, provided that the glycosyl residue of the substrate has the β-D-glucosyl configuration. A number of β-glucosidases from various biological sources are accompanied by β-galactosidase and/or β-xylosidase activity (Kengen et al., 1993; McCleary and Harrington, 1988; Sanyal et al., 1988; Woodward and Wiseman, 1982; Yan and Lin, 1997). In contrast, *C. paradoxa* β-glucosidase showed no activity toward lactose, maltose, or sucrose, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-mannoside, or *p*-nitrophenyl-β-D-xyloside, suggesting that the enzyme recognizes precisely the C-4 configuration of the terminal, nonreducing β-D-glucose residue in the substrate. In this respect, it resembles the β-glucosidases from *A. niger* (Saha et al., 1994; Unno et al., 1993; Watanabe et al., 1992; Yan et al., 1998).

Most microbial β-glucosidases reported are completely inhibited by glucose (Saha et al., 1994; Woodward and Wiseman, 1982; Yeoh et al., 1986). The calculated inhibition constant (K_i) of β-glucosidase from *C. paradoxa* CH32 for glucose (11 mM) was slightly higher than inhibition constants reported for other enzymes (0.6−8 mM; Yan et al., 1998). Utilization of the released sugars by fermentating microorganisms is a suitable way to paliate end-product inhibition. In this respect, release of reducing sugars from carboxymethylcellulose by a purified endoglucanase obtained from *C. paradoxa* CH32 increased noticeably in the presence of β-glucosidase, suggesting that this enzyme relieved endoglucanase from end-product inhibition due to cellobiose. One of the most important attributes is that its pH and temperature optima match those of *C. paradoxa* endoglucanase (Lucas et al., manuscript in preparation), as well as those of many other endoglucanases having acidic pH optima, indicating a potential for supplementing such endoglucanases with *C. paradoxa* β-glucosidase.

Wastewaters generated during the olive oil extraction process contain a high load of organic matter, which is composed mainly of the polysaccharidic substances and phenolic compounds present in the pulp and skin of the olive fruit (Borja et al., 1990). Therefore strains such as *C. paradoxa* CH32 endowed with cellulolytic and phenol-oxidase activities could be helpful to achieve biodegradation of this toxic waste.

LITERATURE CITED

Blank, A.; Sugiyama, R. H.; Dekker: C. A. Activity staining of nucleotide enzymes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis: use of aqueous 2-propanol to remove detergent from gels. *Anal. Biochem.* **1982**, *120*, 267−275.

Bothast, R. J.; Saha, B. C. Ethanol production from agricultural biomass substrates. *Adv. Appl. Microbiol.* **1997**, *44*, 261−286.

Borja, R.; Martín, A.; Fiestas, J. A.; Maestro, R. Efecto de inhibición en el proceso de biometanización del alpechín en biorreactores con microorganismos inmovilizados en diversos tipos de soportes. (Inhibition of alpechin biomethanization in bioreactors with microorganisms immobilized on different types of supports). *Grasas Aceites (Sevilla)* **1990**, *41*, 397−403.

Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−252.

Coughlan, M. P. Enzymatic hydrolysis of cellulose: an overview. *Biores. Technol.* **1992**, *39*, 107−115.

Christakopoulos, P.; Goodenough, P. W.; Kekos, D.; Macris, B. J.; Claeyssen, M.; Bhat, M. K. Purification and characterization of an extracellular β-glucosidase with transglycosylation and exo-glucosidase activities from *Fusarium oxysporum*. *Eur. J. Biochem*. **1994**, *224*, 379−385.

Filho, E. X. F.; Caldas, R. A.; Félix, C. R. Kinetics of substrate hydrolysis by partially purified β-glucosidase from *Humicola* sp. *Arch. Biol. Technol.* **1989**, *32*, 687−701.

Gueguen, Y.; Chemardin, P.; Arnaud, A.; Galzy, P. Purification and characterization of an intracellular β-glucosidase from *Botrytis cinerea*. *Enzyme Microb. Technol.* **1995**, *15*, 127−132.

Gueguen, Y.; Chemardin, P.; Janbon, G.; Arnaud, A.; Galzy, P. A very efficient β-glucosidase catalyst for the hydrolysis of flavor precursors of wines and fruit juices. *J. Agric. Food Chem*. **1996**, *44*, 2336−2340.

Ishigami, H.; Hashimoto, H.; Kainuma, K. Determination of optimum culture conditions for the *Chalara* enzyme production. *J. Jpn. Soc. Starch Sci.* **1985a**, *32*, 189−196.

Ishigami, H.; Hashimoto, H.; Kaiuma, K. Purification and some properties of the *Chalara paradoxa* enzyme. *J. Jpn. Soc. Starch Sci.* **1985b**, *32*, 197−204.

Kainuma, K.; Ishigami, V; Hashimoto, H. Isolation of a novel raw starch-digesting amylase from a strain of black mold-*Chalara paradoxa*. *J. Jpn. Soc. Starch Sci.* **1985**, *32*, 136−141.

Kengen, S. W. M.; Luesink, E. J.; Stams, A. J. M.; Zehnder, A. J. B. Purification and characterization of an extremely thermostable β-glucosidase from the thermophilic archaeon *Pyrococcus furiosus*. *Eur. J. Biochem*. **1993**, *213*, 305−312.

Kwon, K. S.; Lee, J.; Kang, H. G.; Han, Y. C. Detection of β-glucosidase activity in polyacrilamide gels with esculin as substrate. *Appl. Environ. Microbiol.* **1994**, *60*, 4584−4586.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage *T4*. *Nature (London)* **1970**, *227*, 680−685.

Leclerc, M.; Arnaud, A.; Ratomahenina, R.; Galzy, P. Yeast β-glucosidases. *Biotechnol. Genet. Eng. Rev*. **1987**, *5*, 269−295.

Lucas, R.; Robles, A.; Alvarez de Cienfuegos, G: Gálvez, A. Production, purification and properties of an endoglucanase produced by the hyphomycete *Chalara paradoxa* CH32. Manuscript in preparation.

McCleary, B. V.; Harrington, J. Purification of β-D-glucosidase from *Aspergillus niger*. *Methods Enzymol.* **1988**, *160*, 575−583.

Miller, G. L. Use of the dinitrosalicilic acid reagent for the determination of reducing sugars. *Anal. Chem*. **1959**, *31*, 426−428.

Monma, M.; Kainuma, K. Heterogeneity of the glucoamylase components of the raw-starch-digesting amylase from *Chalara paradoxa*. *Carbohydr. Res.* **1992**, *217*, 385−388.

Noda, T.; Ohtani, T.; Shiina, T.; Nawa, Y. Semi-continuous hydrolysis of sweet potato raw starch by *Chalara paradoxa* glucoamylase. *J. Food Sci*. **1992**, *57*, 1348−1352.

Pemberton, M. S.; Brown, R. D.; Emert, G. H. The role of β-glucosidase in the bioconversion of cellulose to ethanol. *Can. J. Chem. Eng.* **1980**, *58*, 8, 723−729.

Robles, A.; Lucas, R.; Alvarez de Cienfuegos, G.; Gálvez, A. Phenol-oxidase (laccase) activity in strains of the Hyphomycete *Chalara paradoxa* isolated from olive mill wastewater disposal ponds. *Enzyme Microb. Technol.* **2000**, *26*, 484−490.

Ruttersmith, L. D.; Daniel, R. M. Thermostable β-glucosidase and β-gxylosidase from *Thermotoga* sp. strain FjSS3-B.1. *Biochim. Biophys. Acta* **1993**, *1156*, 167−172.

Saha, B. C.; Freer, S. N.; Bothast, R. J. Production, purification, and properties of a thermostable β-glucosidase from color variant strain of *Aureobasidium pullulans*. *Appl. Environ. Microbiol.* **1994**, *60*, 3774−3780.

Saha, B. C.; Bothast, R. J. Production, purification, and characterization of a highly glucose tolerant novel β-glucosidase from *Candida peltata*. *Appl. Environ. Microbiol.* **1996**, *2*, 3165−3170.

Sanyal, A.; Kundu, R. K.; Dube, S.; Dube, P. K. Extracellular cellulolytic enzyme system of *Aspergillus japonicus*: 2. Purification and characterization of an inducible extracellular β-glucosidase. *Enzyme Microb. Technol.* **1988**, *10*, 91−99.

Shewale, J. G. β-Glucosidase: its role in cellulase synthesis and hydrolysis of cellulose. *Int. J. Biochem*. **1982**, *14*, 435−443.

Shinoyama, H.; Takei, V.; Ando, A.; Fujii, T.; Sasaki, M.; Doi, Y.; Yasui, T. Enzymatic synthesis of useful alkyl-β-glucosides. *Agric. Biol. Chem*. **1991**, *55*, 1679−1681.

Unno, T.; Ide, K.; Yazaki, T.; Tanaka, Y.; Nakakuki, T.; Okada, G. High recovery purification and some properties of a β-glucosidase from *Aspergillus niger*. *Biosci. Biotechnol. Biochem*. **1993**, *57*, 2172−2173.

Watanabe, T.; Sato, S.; Yoshioka, T.; Koshijima, T.; Kuwahara, M. Purification and properties of *Aspergillus niger* β-glucosidase. *Eur. J. Biochem*. **1992**, *209*, 651−659.

Woodward, J.; Wiseman, A. Fungal and other β-glucosidases-their properties and applications. *Enzyme Microb. Technol.* **1982**, *4*, 73−79.

Xin, Z.; Yinbo, Q.; Peiji, G. Acceleration of ethanol production from paper mill waste fiber by supplementation with β-glucosidase. *Enzyme Microb. Technol.* **1993**, *15*, 62−65.

Yan. T. R.; Lin, Y. H. Purification and characterization of a glucose-tolerant β-glucosidase from *Aspergillus niger* CCRC 31494. *Biosci. Biotechnol. Biochem*. **1997**, *61*, 965−970.

Yan, T. R.; Lin, Y. H.; Lin, Ch. L. Purification and characterization of an extracellular β-glucosidase II with high hydrolysis and transglucosylation activities from *Aspergillus niger*. *J. Agric. Food Chem*. **1998**, *46*, 431−437.

Yeoh, H. H.; Tan, T. K.; Koh, S. K. Kinetic properties of β-glucosidase from *Aspergillus ornatus*. *Appl. Microbiol. Biotechnol.* **1986**, *25*, 25−28.

Received for review March 1, 2000. Revised manuscript received May 16, 2000. Accepted May 16, 2000.

JF0002591