Production, Purification, and Properties of an Endoglucanase Produced by the Hyphomycete *Chalara* (Syn. *Thielaviopsis*) *paradoxa* CH32

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The hyphomycete *Chalara* (syn. *Thielaviopsis*) *paradoxa* produces endoglucanase activity during the late trophophase. The low molecular mass (35 kDa) endoglucanase purified from cultured broths works optimally at 37 °C and pH 5.0. The enzyme inactivates at pH below 3.0 and also at temperatures of 50 °C or higher, but it is stable at lower temperatures, including refrigeration temperature and freezing. The enzyme is inhibited by detergents, by EDTA, and by the divalent cations Hg^{2+} and Ag^{2+}. It is also inhibited to some extent by 10 mM Zn^{2+}, Fe^{2+}, and Mg^{2+}, but it is stimulated by Mn^{2+}. Enzyme activity is not affected by reducing agents. In the presence of low concentrations of water miscible organic solvents (20%) endoglucanase activity is inhibited by 7% (for methanol) to 50% (for acetonitrile), and it is totally inhibited at higher solvent concentrations (50%). Enzyme activity is not affected by the water immiscible solvent ethyl acetate. Carboxymethylcellulose is the preferred substrate ($K_{m(app)} = 8.3$ g/L; $V_{max(app)} = 1.1$ μM/min). Hydrolysis of crystalline cellulosic substrates is very limited, but it is greatly enhanced by phosphoric acid swelling. The purified enzyme shows no activity toward disaccharides or aryl-glucosides. Its activity is inhibited by cellobiose.

Keywords: *Endoglucanase; Chalara (Thielaviopsis) paradoxa; purification*

INTRODUCTION

Cellulose accounts for an average 40 wt % of the biomass on earth. Some 7.2×10^{11} tonnes are stored in higher plants, whereas annual productivity is $\sim 4.0 \times 10^{10}$ (*1*, *2*). The molecular structure of cellulose is a linear polymer of up to 12000 β-linked glucosyl units (*1*, *3*). Each glucose residue is rotated 180° with respect to its neighbors along the main axis chain (*1*). Therefore, cellobiose is the repeating unit.

Cellulose degradation requires the action of three major classes of enzymes: endoglucanases (EG; EC 3.2.1.4), which randomly cleave β-glycosidic bonds of soluble derivatives as well as amorphous cellulose; cellobiohydrolases (CBH; EC 3.2.1.91), which catalyze the cleavage of the second β(1,4)-glycosidic bond from the end of the cellulose chain, producing cellobiose; and β-glucosidases, which hydrolyze cellobiose and other low molecular weight oligosaccharides into glucose (*4*). A large number of cellulolytic enzymes have been described, both in bacteria and in fungi (*5*−*8*).

Treatment of cellulose by cellulolytic enzymes for practical purposes has attracted continuing attention from biotechnologists. There has been a great interest in the commercial use of cellulases in the agricultural and chemical industries to produce glucose for feed, food, and the manufacture of other chemicals (*9*−*12*). Deinking and fiber modification in the paper industry, as well as biopolishing in the textile industry, are less known but not less important applications (*13*−*17*).

In this paper we describe a low molecular mass cellulolytic enzyme from the black mold *Chalara* (syn. *Thielaviopsis*) *paradoxa*. The cellulolytic system of this mold is composed of at least one endoglucanase and one β-glucosidase (*18*). This is the first report on endoglucanase production by *C. paradoxa*. This mold was isolated from ponds where wastewaters generated during the olive oil extraction process (olive mill wastewaters, OMW) are disposed off. OMW is a highly toxic recalcitrant effluent (*19*, *20*). Production of laccase activity by strains of *C. paradoxa* isolated from this toxic environment has also been reported (*21*). The potential of strains from this hyphomycete with cellulolytic and ligninolytic activity for biodegradation of OMW is discussed.

MATERIALS AND METHODS

Microbial Strain and Cultivation Conditions. The strain *Chalara* (syn. *Thielaviopsis*) *paradoxa* CH32 isolated from an olive mill wastewater disposal pond was propagated on yeast−malt agar (YMA; Scharlau, Barcelona, Spain). For endoglucanase production, this strain was cultivated in Roux flasks containing 500 mL of modified yeast−malt broth (MYM), consisting (per liter) of 0.8 g of malt extract, 0.4 g of yeast extract, and 1.6 g of glucose, and incubated at 28 °C. At regular intervals of incubation, the mycelium was separated by filtration on preweighed Whatman GF/C glass fiber filters and dried to constant weight. Enzyme activity released into the cultured broths was measured.

Enzyme Assays. Endo-β(1→4)-glucanase activity was determined in 50 mM sodium citrate buffer, pH 5, at 37 °C, using 1% sodium carboxymethylcellulose (CMC; low viscosity; Sigma) as substrate. The release of reducing sugars was determined by the 3,5-dinitrosalicylic (DNS) method (*22*), with one unit defined as the amount of enzyme liberating 1 μmol/min of reducing sugars. Phosphoric acid swollen cellulose (PASC) was prepared from Avicel (Merck) as described elsewhere (*23*).

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Whatman No. 1 filter paper was cut into 3 mm squares before use. Dewaxed cotton cellulose (Procter and Gamble) and cellulose powder CF11 (Whatman) were also tested as substrates. Activity toward *p*-nitrophenyl derivatives (Sigma) was determined by measuring their light absorbance at 410 nm (*24*).

Enzyme Purification. Endoglucanase activity present in cultured broths (4 L; pH 8.0) was recovered by anion exchange chromatography on a 10 × 50 cm Sephadex DEAE-A50 glass column. The material retained in the gel matrix was washed with 500 mL of 20 mM Tris-HCl buffer, pH 8.0, and eluted by passing 500 mL of 0.25 M NaCl M through the column followed by 500 mL of 0.5 M NaCl, dissolved in the same buffer as above. The column effluent was collected in fractions (50 mL each), which were analyzed for endoglucanase activity and protein content (*25*). Active fractions were dialyzed against 20 mM Tris-HCl buffer, pH 7.2, using 12000 molecular weight cutoff dialysis tubing (Sigma) and concentrated 10-fold by lyophilization before they were loaded on a Sephacryl S-200 HR column (2 × 100 cm; Pharmacia). The column was eluted at 3 mL/min using 20 mM Tris-HCl buffer, pH 7.2, as eluent, and the effluent was collected into 10-mL fractions, which were tested for endoglucanase activity and protein content as above.

Active fractions obtained after size exclusion chromatography were loaded on a MonoQ HR 5/5 column (0.5 × 5.0 cm; Pharmacia) previously equilibrated in 20 mM Tris-HCl buffer, pH 7.2. The material retained in the column after extensive washing with buffer was eluted with a linear gradient of 0−0.3 M NaCl 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. Active fractions obtained after this step were dialyzed overnight against 20 mM Tris-HCl buffer, pH 8.0, before they were loaded on a 0.75 × 7.5 cm Progel-TSK DEAE-5PW column (Supelco, Inc., Bellefonte, PA) equilibrated in 20 mM Tris-HCl buffer, pH 8.0. 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.

Fractions containing endoglucanase activity were concentrated to 200 μL by lyophilization before they were loaded on a Superose 12 HR 10/30 column (1.0 × 30.0 cm; Pharmacia) 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 (Sigma) were chromatographed on this column to estimate the molecular mass of the purified endoglucanase.

Polyacrylamide Gel Electrophoresis. Samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels (*26*) with a Miniprotean II unit (Bio-Rad). Proteins separated in the gel were stained with Coomassie Brilliant Blue, and their molecular masses were estimated with standard markers (Sigma). For endoglucanase activity stain, gels were washed immediately after electrophoresis with 20% 2-propanol (two washes of 20 min each) to remove SDS (*27*) and then incubated for 4 h at 37 °C in 100 mM sodium acetate buffer (pH 5.0) plus 1% CMC (*28*). After a 5 min wash with distilled water, the CMC adsorbed to the polyacrylamide gel was stained for 15 min with 0.1% Congo red. Bands of hydrolyzed substrate became visible as clear zones on a red background after a washing with 1 M NaCl.

Effect of Temperature and pH on Enzyme Activity and Stability. The optimum temperature for the hydrolysis of CMC 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, 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 endoglucanase 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 30 min with CMC to determine remaining enzyme activity. The activity of enzyme solutions preincubated at neutral pH at different temperatures for increasing periods of time was also tested.

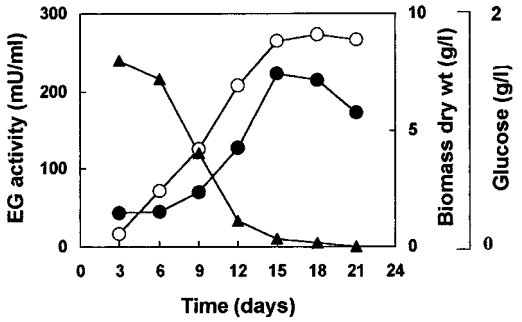

Figure 1. Biomass formation (○), endoglucanase production (●), and glucose consumption (▲) in liquid cultures of *C. paradoxa* CH32.

Effect of Chemical Reagents, Metal Ions, and Organic Solvents on Enzyme Activity. The different compounds were incorporated in the reaction mixtures at the specified concentrations. The amount of reducing sugars released from CMC after 30 min of incubation at optimum temperature was then calculated and compared with that of a control reaction mixture.

Kinetic Constants. The kinetic constants of the purified endoglucanase were carried out by adding CMC (0.03−2.0%) as substrate into the reaction mixture and assaying hydrolytic activity as described previously. The apparent K_m and V_{max} values were calculated by the double-reciprocal plot method of Lineweaver and Burk. The inhibition of cellobiose on enzyme activity was determined by adding various concentrations of this saccharide into reaction mixtures with CMC as substrate.

RESULTS

Enzyme Production in Liquid Cultures. Endoglucanase activity was detected in cultured broths after prolonged incubation but not at earlier stages (Figure 1). The levels of activity increased noticeably from day 9 to day 15 of cultivation. At this point, production of endoglucanase ceased, and the levels of activity decreased after day 21.

The relationship between formation of fungal biomass, glucose consumption, and endoglucanase production was studied. Fungal biomass increased markedly from day 3 to day 15 of cultivation (Figure 1). During early stages of cultivation the levels of glucose decreased rapidly, and most of the glucose from the growth medium was exhausted by days 12−15 (Figure 1). Glucose exhaustion coincided with highest production of endoglucanase activity.

Enzyme Purification. Endoglucanase activity was concentrated and partially purified by anion exchange chromatography. The activity was found in fractions eluted from the colum at 0.25 M NaCl. A high percentage of the initial activity (~84%) was recovered by this procedure (Table 1). Fractions from this step were dialyzed and concentrated by lyophilization, after which they retained >95% of their previous activity. The concentrated samples were separated by size exclusion chromatography on a Sephacryl S-200 column. The recovery of activity at this step was low (~60%), but removal of pigmented material as well as other contaminating proteins allowed a high degree of purification. When the colorless enzyme solutions obtained by Sephacryl S-200 chromatography were loaded on a MonoQ column, endoglucanase activity eluted at low salt concentration (~0.15 M) under a single UV-absorbance peak. The fractions from this column were dialyzed against 20 mM Tris-HCl buffer, pH 8, and loaded on a DEAE-5PW column. Elution at a higher pH

Table 1. Purification of Endoglucanase Activity

step	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)	purification (-fold)
cultured broth	388	892	2.3	100	1
Sephadex DEAE A-50	70	747	10.7	83.8	4.6
dialysis and lyophilization	65	720	11.1	80.7	4.8
Sephacryl S-200	8.8	530	60.2	59.4	26.2
MonoQ	2.2	360	163.6	40.3	71.1
TSK DEAE- 5PW	0.3	111	370.0	12.4	160.8
Superose 12	0.16	74	462.3	8.3	201.0

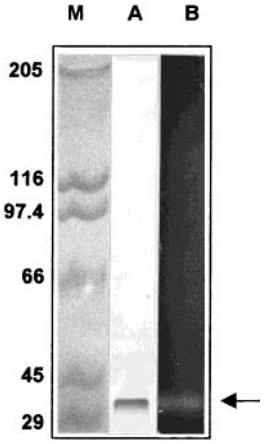

Figure 2. SDS-PAGE of purified endoglucanase after Coomassie Blue staining (lane A) and Congo red stained zymogram after removal of SDS and incubation with carboxymethylcellulose (lane B). Lane M: Molecular weight markers (in kDa).

on a different anion exchange column with a less steep salt gradient allowed the separation of other contaminating proteins, to yield a single endoglucanase peak with a high specific activity (Table 1). The size and purity of this fraction were determined both by size exclusion chromatography on an analytical Superose 12 column and by polyacrylamide gel electrophoresis. Endoglucanase activity eluted from the Superose 12 column coincidently with a single UV-absorbance peak corresponding to a molecular mass of 35 kDa according to the elution volumes of standard protein markers. The specific activity of the purified fractions was 450 units/mg of protein.

The purified fractions yielded a main protein band after SDS-PAGE, corresponding to a molecular mass of 35 kDa (Figure 2). When non-denatured samples were analyzed, a single band of endoglucanase activity was detected with apparently the same gel mobility as the main denatured protein band (Figure 2).

General Properties. The pH and temperature dependences of endoglucanase activity and stability are shown in Figure 3. Highest activity was detected in the pH interval of 4.0−6.0, the pH optimum being 5.0. Enzyme activity decreased markedly at pH <3.0 or >7.0 (Figure 3A). The lack of activity at alkaline pH was not due to enzyme inactivation, because enzyme preparations held at pH 8.0 and 9.0 retained >90% of their activity. However, the enzyme was inactivated under acidic pH conditions (Figure 3A).

Endoglucanase activity was higher in the temperature interval of 30−45 °C, and the optimum temperature was 37 °C under the described assay conditions (Figure 3B). Enzyme activity decreased above 45 °C as also did its thermal stability. Enzyme preparations were very stable at 40 °C and retained at least 95% of their activity after being incubated for 24 h at this temperature (Figure 3C). However, all of the activity was lost after 5 h of incubation at 50 °C or after 2 h of incubation at 60 °C. The purifed enzyme was stable at 4 °C for at least 24 h. No loss of activity was detected during freezing at −20 or −70 °C or during freeze-drying.

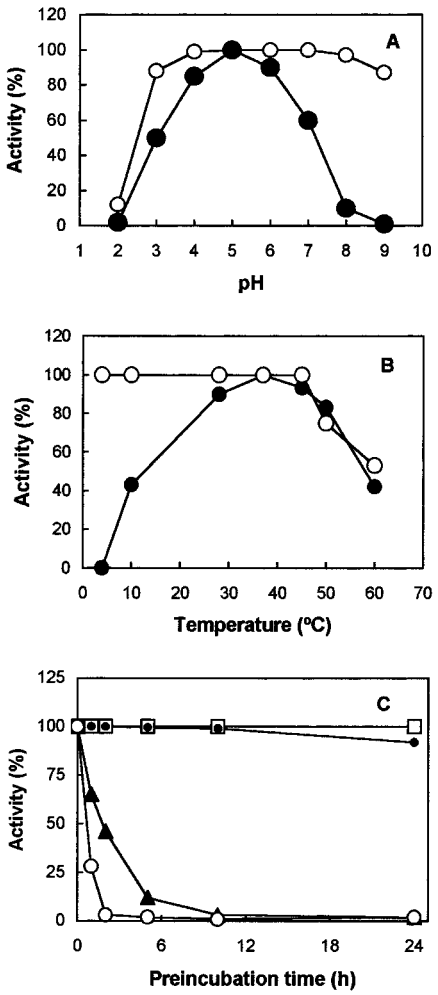

Figure 3. Effect of pH (A) and temperature (B) on endoglucanase activity (solid symbols) and stability (open symbols). (C) Heat stability over incubation time of samples incubated at 4 (□), 40 (●), 50 (▲), or 60 °C (○).

Endoglucanase activity was strongly or completely inhibited by Hg^{2+}, depending on the concentration tested (Table 2). Other divalent cations (Ag^{2+}, Zn^{2+}, Fe^{2+}, and Mg^{2+}) caused variable degrees of inhibition. The enzyme activity was stimulated 2-fold by 10 mM $MnCl_2$ (Table 2). Endoglucanase activity was reduced by almost 50% in the presence of 10 mM EDTA (Table 2).

Activity of the purified enzyme was also inhibited by the detergents Triton X-100 and Tween 80 as well as by SDS, the most potent inhibitor (Table 2). No loss of activity was detected when reducing agents such as

Table 2. Effect of Various Reagents on Endoglucanase Activity

reagent[a]	relative activity (%) at final concentration of 1 mM	10 mM
none	100	100
$AgNO_3$	73	34
$CaCl_2$	100	100
$FeCl_2$	100	60
$HgCl_2$	42	0
$MgCl_2$	100	78
$MnCl_2$	100	201
$ZnSO_4$	97	71
SDS[a]	13	
Tween 80[a]	46	
Triton X-100[a]	43	
DTT		100
2-mercaptoethanol		100
cysteine		100
EDTA		54

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

Table 3. Effect of Organic Solvents on *C. paradoxa* Endoglucanase Activity[a]

solvent	relative activity (%) at solvent concentration of 20% v/v	50% v/v
methanol	93	5
ethanol	60	2
acetonitrile	50	0
ethyl acetate	100	100
dimethyl sulfoxide	73	0

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

Table 4. Substrate Specificity of *C. paradoxa* CH32 Endoglucanase

substrate	relative activity (%)
carboxymethylcellulose	100
Avicel	22
swollen Avicel	72
cellulose powder	33
cotton fiber	38
filter paper	18

DTT, 2-mercaptoethanol, or cysteine were incorporated into the reaction mixtures (Table 2).

Hydrolysis of carboxymethylcellulose by the purified endoglucanase was not inhibited to a great extent by 20% methanol or by the water immiscible solvent ethyl acetate (Table 3). Other organic solvents such as dimethyl sulfoxide, ethanol, and acetonitrile caused variable degrees of inhibition when tested at a final concentration of 20% (by volume). Enzyme activity was severely inhibited by higher concentrations of all of the organic solvents tested, except for ethyl acetate (Table 3).

Substrate Specificity and Kinetic Parameters. The purified endoglucanase showed highest activity against carboxymethylcellulose (Table 4). No activity was detected toward crystalline substrates Avicel, cotton fiber, filter paper, or cellulose powder when we used a standard assay procedure for soluble reducing sugars consisting of removal of insoluble substrate material before treatment with dinitrosalicylic acid reagent. However, boiling of endoglucanase-treated samples with the dinitrosalicylic acid reagent before removal of the insoluble substrate resulted in the development of an intense color reaction as a consequence of reducing ends formed during enzyme attack (Table 4). Such a reaction was not observed when reaction mixtures lacking endoglucanase were treated in the same way. The activity against Avicel (measured by the standard dinitrosalicylic acid method) also increased markedly if this substrate had been swollen previously with orthophosphoric acid (Table 4).

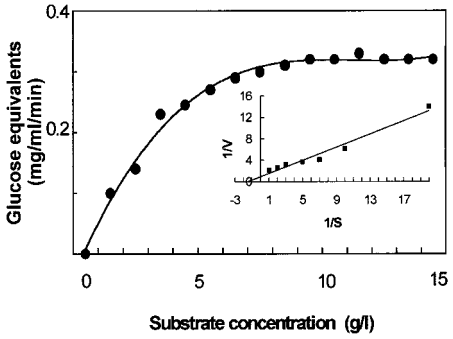

Figure 4. Effect of carboxymethylcellulose concentration on endoglucanase activity and Lineweaver−Burk plot (inset).

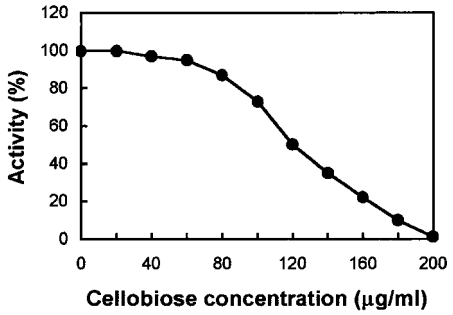

Figure 5. Effect of cellobiose concentration on endoglucanase activity toward carboxymethylcellulose.

Purified preparations of this enzyme did not show any hydrolytic activity against disaccharides (cellobiose, lactose, maltose, or sucrose) or the *p*-nitrophenyl derivatives of β-D-cellobioside, β-D-glucopyranoside, β-D-glucosamine, β-D-galactopyranoside, β-D-xyloside, or α-D-mannopyranoside. The values of apparent K_m and V_{max} for carboxymethylcellulose were 8.3 g/L and 1.11 μM/min (expressed as glucose equivalents; Figure 4), respectively. The rate of the reaction increased when the carboxymethylcellulose concentration was increased up to 8.5 g/L (Figure 4).

Endoglucanase activity was sensitive to end-product inhibition by cellobiose and decreased markedly as the concentration of added cellobiose in the reaction mixture increased above 80 μg/mL (Figure 5). The K_i values for glucose and cellobiose were 350 and 0.2 mM, respectively. A recent study indicates that end-product inhibition by cellobiose could be relieved by the addition of a purified β-glucosidase produced by the same strain (*18*).

DISCUSSION

Plant cell walls are a major reservoir of fixed carbon in nature. In recent years there has been considerable interest in the utilization of plant material as a renewable source of fermentable sugars that could be subsequently converted into useful products such as liquid fuels, solvents, chemicals, food, or feed (*29*, *30*). Such bioconversion processes are particularly attractive for the elimination of residues and wastes produced by agriculture and forestry. As a result of this interest, a wealth of knowledge on cellulolytic enzymes has accumulated (*5*−*8*).

This is the first report on endoglucanase production by *Chalara* (syn. *Thielaviopsis*) *paradoxa*. This dematiaceous hyphomycete (*31*) has been associated with a variety of diseases in coconuts, such as stem bleeding (*32*), bitten leaf (*33*), leaf spot (*34*), and leaf rot disease (*35*, *36*). Production of cellulolytic enzymes has been reported in other plant pathogenic fungi, such as *Macrophomina phaseolina* (*37*), *Phytophthora infestans* (*38*), or *Sclerotium rolfsii* (*39*). Cellulolytic enzymes should play a role in the penetration of plant cell walls (*40*, *41*). The strain used in this study, however, was isolated from olive mill wastewater disposal ponds in southern Spain, and its role as a plant pathogen in this region has not been established yet.

The cellulolytic system of *C. paradoxa* CH32 consists of at least one endoglucanase and one β-glucosidase, which has been characterized recently (*18*). Production of endoglucanase activity in liquid cultures takes place during the late trophophase, coincidently with glucose exhaustion. This behavior seems to be in agreement with the general observation that cellulase systems are repressed in the presence of more easily metabolizable carbon sources, for example, glucose (*42*). Under these conditions, endoglucanase formation in various fungi starts only when the repressing carbohydrate glucose is completely metabolized (*43*, *44*).

Endoglucanase activity produced by strain *C. paradoxa* CH32 seemed to be associated with a single polypeptide of low molecular mass. In this respect, it resembles endoglucanases produced by other fungi such as *Aspergillus niger* (*45*) or *Aspergillus niveous* (*46*). Several endoglucanases of low molecular mass have also been described in cellulolytic fungi producing multiple endoglucanases such as *Trichoderma viridae* (*47*) or *Trichoderma reesei* (*48*).

The endoglucanase enzyme from *C. paradoxa* CH32 showed optimum activity under acidic pH values and under moderate temperatures of incubation. In this respect it resembles other endoglucanases produced by mesophilic fungi, although endoglucanases differ markedly in their pH and temperature optima for activity (*49*). The activity of this enzyme was not modified by reducing agents, but it was inhibited by detergents and by heavy metals, especially by Hg^{2+}. The concentrations required for inhibition by divalent cations and heavy metals were relatively high. These data suggest that thiol groups are not essential for enzyme activity, because thiol groups are one of the main targets of heavy metals. Inhibition by EDTA and stimulation by Mn^{2+} suggest that this cation plays an important role in the activity of the purified endoglucanase. The different endoglucanases described to date vary considerably in their inhibition by reducing agents, chelators, or heavy metals as well as in their activation by divalent cations (*50*).

Purified preparations containing endoglucanase from *C. paradoxa* CH32 did not show any hydrolytic activity on aryl-glucosides, indicating that the β-glucosidase activity that was also present in the crude extracts was separated efficiently during the purification process. By using the standard assay procedure for determination of free reducing ends with the dinitrosalicylic acid reagent, we found a high activity against carboxymethylcellulose but no activity on crystalline cellulosic substrates. This procedure involves separation of solids before boiling with Miller's reagent. Nevertheless, if enzyme-treated samples were boiled with Miller's reagent before separation of solids, then we were able to detect a large increase in the concentration of reducing equivalents formed for all of the crystalline substrates tested. We suspect that reducing ends formed by endoglucanase action remained bound to the solid substrate. Boiling with Miller's reagent would facilitate release of endoglucanase-cleaved fragments into the medium. Furthermore, the increased activity found on acid-swollen Avicel strongly suggested that the purified endoglucanase cleaved within the amorphous regions of cellulosic materials. Endoglucanases have been reported to act preferentially on amorphous substrates (*51*). However, degradation of crystalline cellulose by the low molecular mass endoglucanase S from *Streptomyces* sp. LX has also been reported (*52*).

Strains of *C. paradoxa* also produce other extracellular enzymes. Amylase production by *C. paradoxa* isolated from the pith of the sago palm was reported (*53*), and the glucoamylase component was purified and characterized (*54*−*56*) and tested for semicontinuous hydrolysis of sweet potato raw starch (*57*). More recently, several strains of *C. paradoxa* isolated from olive mill wastewater disposal ponds were found to produce laccase activity (*21*). Among them, the strain *C. paradoxa* CH32 also produced β-glucosidase activity (*18*).

Olive mill wastewaters contain a high load of organic matter derived mainly from the pulp of the olive fruits (*19*). This effluent contains a high percentage of polysaccharidic substances, but it is also heavily loaded with phenolic substances, which make its biodegradation very difficult because many microbial consortia are inhibited (*19*, *20*, *58*). Therefore, the use of strains that produce enzymes that can degrade both the phenolic constituents and the polysaccharidic materials of OMW should be helpful to achieve an efficient biodegradation of this waste.

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