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Isolation and Properties of a Thermostable Endoglucanase from a Thermophilic Mutant Strain of *Thielavia terrestris*

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ABSTRACT

A heat-stable enzyme was isolated from the cellulase complex of a thermophilic strain of the micromycete *Thielavia terrestris*. The purified enzyme exhibited both endoglucanase and xylanase activities and had a mol mass of 69,000 Daltons and an isoelectric point of 6.4. When the cells were grown at 48°C, the initial activity of the purified enzyme using carboxymethylcellulose as a substrate was 150 nkat/mg and the Michaelis constant was 6.6 g/L. The heat stability of the enzyme was high, losing only 20% of the initial activity after a 6-h incubation at 65°C. When cultures were grown on microcrystalline cellulose and xylose was added after 48 h of growth, endoglucanase and xylanase activities were more than doubled. Similar increases in these activities were observed by growing the cultures on straw.

Index Entries: *Thielavia terrestris* (*Allesheria terrestris*); cellulase complex; endoglucanase; xylanase; thermostability.

Abbreviations: CMC, carboxymethyl cellulose; MCC, microcrystalline cellulose.

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INTRODUCTION

Rational usage of cellulose waste is a potentially attractive source of food and energy (*1,2*). In this context, efficient enzymatic hydrolysis of cellulose is an important biotechnological challenge.

For practical purposes, industrial processing of cellulosic wastes would be facilitated if performed at elevated temperatures (*3,4*), and, for this reason, scientists have searched extensively for thermophilic cellulases. Such enzymes are well known in thermophilic bacteria (*5–7*), but little is known about thermophilic fungal cellulases (*8–10*). Fungal systems are known for their production of large amounts of extracellular cellulases (*11*), and the description of heat stable thermophilic cellulases is thus of great interest. In this article, we describe the isolation and characteristics of a heat-stable endoglucanase from *Thielavia terrestris*.

MATERIALS AND METHODS

Organism and Culture Conditions

The strain of was obtained from the culture collection of the Institute of Plant Biochemistry, Academy of Sciences of the Georgian Republic. Cultures were grown in shake flasks (200 rpm) for 4 d at 48°C. The 500-mL vessels contained 100 mL of synthetic medium, consisting of the carbon source (20 g/L), peptone (15 g/L), maize extract (25 g/L), K_2HPO_4 (6.8 g/L), $(NH_4)_2SO_4$ (13 g/L), $MgSO_4 \times 7H_2O$ (0.5 g/L), and $CaCl_2$ (0.2 g/L). The medium was brought to a pH of 5.5. Carbon sources used in the different experiments were microcrystalline cellulose (MCC), xylan, and straw, as designated later in the text. The straw was collected, cut into 1.5–2-cm strips, washed with distilled water, and sterilized with the medium before inoculation. Following cultivation, the cells were removed by centrifugation. Protein content, enzyme activities, and end products were determined in the culture filtrates.

Isolation Procedure

The thermostable endoglucanase was isolated from the cell-free medium by affinity chromatography on MCC followed by ion exchange chromatography.

The culture was first subjected to ethanol precipitation. Four volumes of 96% ethanol were added per fraction volume, and the proteins were redissolved to a final concentration of 0.74 mg/mL in 0.5*M* acetate buffer (pH 4.5). The solution was heated at 65°C for 1.5 h. The resultant precipitant was removed by centrifugation, and 4 mL of the supernatant fluids were adsorbed onto 0.5 g of MCC for 4 h at 4°C. The resin was washed successively by centrifugation in batchwise fashion with 100 mL of 0.1*M*

sodium-potassium phosphate buffer (pH 6.0), and then 0.01*M* of the same buffer. The adsorbed proteins were removed from the resin using 100 mL of water.

The enzyme was purified further on a DEAE Toyoperl 650*M* column (1.5 × 12 cm; TSK Toyo Soda Mfg. Co. Ltd., Tokyo, Japan). The water eluent was brought to 0.66*M* sodium-potassium phosphate buffer, pH 7.6, and added to the column. Protein was eluted using a stepwise gradient consisting of 0.1, 0.2, and 1.0*M* buffered solutions of NaCl. The active endoglucanase was desorbed from the carrier at a concentration of 0.2*M* NaCl. This fraction was then desalted by ultrafiltration and concentrated on a rotary evaporator.

Analytical Methods

Endoglucanase and xylanase activities were determined using carboxymethyl cellulose and larch xylan as substrates (*12,13*). Reducing sugars were measured by Somogyi (*14*) and Nelson (*15*). Final hydrolysis products were determined by thin layer chromatography [butanol:ethanol:water (5:3:2)] using glucose, xylose, and cellobiose as markers (*16*). Thermostability of the enzyme was determined according to Bailey and Poutenen (*13*). Protein concentration was determined according to Bradford (*17*) using bovine serum albumin as a standard. Molecular weight of the enzyme was estimated by 12% SDS-PAGE (*18*) and isoelectric focusing was performed in an Ampholine gradient (3.5–10, Sigma, St. Louis, MO) (*19*).

RESULTS AND DISCUSSION

Isolation of a thermostable endoglucanase from a thermophilic strain of *Thielavia terrestris* was carried out by affinity chromatography on microcrystalline cellulose followed by ion-exchange chromatography. The isolated fraction showed a single band on SDS-PAGE, having a relative mol mass of 69,000 Daltons (Fig. 1). The isoelectric point of the purified protein corresponded to 6.4. The initial endoglucanase activity of the enzyme was 150 nkat/mg and a relatively high Michaelis constant (6.6 g/L) was obtained. A similar level of xylanase activity (150 nkat/mg) was also observed.

One of the distinctive characteristics of the purified protein is its heat stability, or the half-time of inactivation ($\tau_{1/2}$) upon incubation at high temperatures. The heat stability of the purified enzyme was thus studied at 65°C in the absence of substrate. As seen from Fig. 2, only about 20% of the initial endoglucanase and xylanase activities were lost upon 6 h of incubation at that temperature. It should be mentioned that the heat stability of cellulases of fungal origin is not high, and representative values for $\tau_{1/2}$ are generally between 5 min and 1 h at such temperatures (*20*). It is

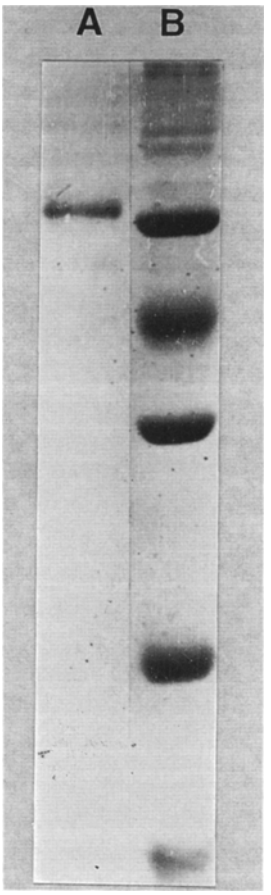

Fig. 1. SDS-PAGE of the purified endoglucanase in 12% polyacrylamide gels. Lane A, the purified endoglucanase; Lane B, molecular weight standards (bovine serum albumin [68,000], horseradish peroxidase [44,000], carbonic anhydrase [29,000], soybean trypsin inhibitor [20,100], and lysozyme [14,300]).

also notable that mesophilic fungi are also known to synthesize heat stable cellulases, although they tend to comprise only a very minor portion of the enzymes in the cellulase complex (*9*). It is thus remarkable that the portion of the heat-stable enzyme seems to have been increased in the thermophilic strain of *T. terrestris*.

Since the enzyme expressed both endoglucanase and xylanase activities, it was of interest to further examine whether xylose had a regulatory effect on the biosynthesis of these activities. We thus grew cultures of *T. terrestris* on MCC or xylose as the sole carbon sources. We also grew cells on a mixture of the two carbon sources. The effect of adding xylose to cells growing on MCC after 48 h of growth was also examined. The results are presented in Table 1. When xylose was added to cultures grown

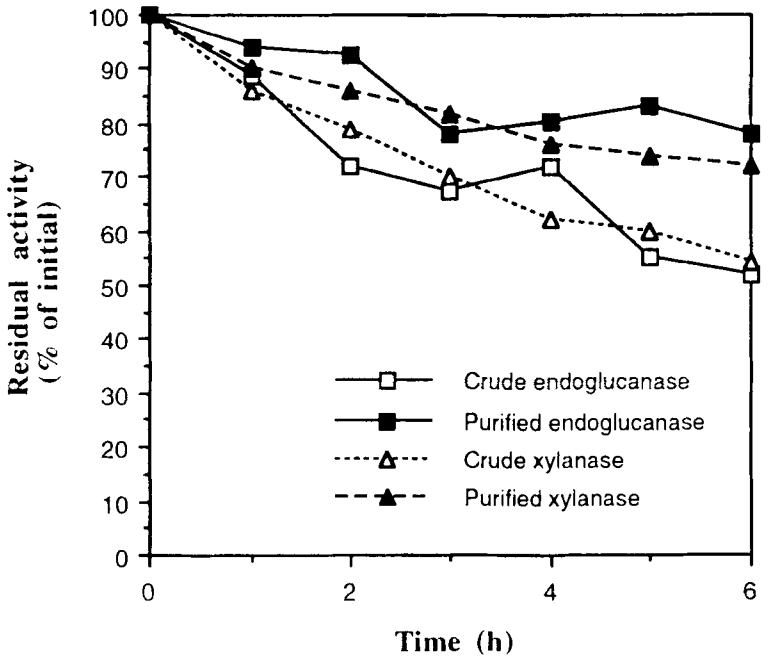

Fig. 2. Heat inactivation (in the absence of substrate) at 65°C of the purified enzyme and the crude enzyme preparation.

Table 1
Influence of Xylose as a Carbon Source
on Endoglucanase and Xylanase Activities of the Purified Enzyme[a]

Carbon source	Protein content, mg/mL	Endoglucanse activity		Xylanase activity	
		nkat/mL	nkat/mg	nkat/mL	nkat/mg
MCC	0.075	1.8	24.0	2.7	36
Xylose	0.064	1.9	29.7	2.7	42
MCC+xylose	0.100	2.0	20.0	4.2	42
MCC+xylose[b]	0.064	3.0	46.9	7.1	111

[a]Cultures were harvested after 96 h growth.
[b]Xylose was added to the designated culture after 48 h of growth.

on MCC after 48 h of growth, a significant increase in both endoglucanase and xylanase was observed, both in terms of total activity as well as specific activity. Future studies will show whether or not this increase is owing solely to the increase in the relative portion of the heat stable cellulase. Nevertheless, these data may bear relevance to natural substrates, since growth of *T. terrestris* on straw led to similar increases in both activities (Table 2).

Table 2
Comparative Endoglucanase and Xylanase Activities of Purified Enzymes, Derived from Cells Grown on MCC or Straw as Carbon Sources[a]

Carbon source	Protein content, mg/mL	Endoglucanse activity		Xylanase activity	
		nkat/mL	nkat/mg	nkat/mL	nkat/mg
MCC	0.023	1.25	54.3	0.83	36.1
Straw	0.045	4.80	106.7	2.40	53.3

[a]Cultures were harvested after 96 h growth.

It is interesting to note that growth of *T. terrestris* on MCC as a substrate led to the production of a single sugar, glucose, as the end product (data not shown). This phenomenon may reflect the high levels of β-glucosidase synthesized by this organism (*21*). Growth on straw, however, was accompanied by the presence of a variety of sugars (including glucose) with various degrees of polymerization. Hydrolysis of other natural substrates, which contain mainly cellulose and hemicellulose (e.g., canning industry wastes from apple, pear, and citrus pressing), generally continued to monosaccharides as the final end products. *T. terrestris* and its heat-stable enzymes are thus expected to be attractive vehicles for degradation of cellulosics in future industrial processes.

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