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Identification of Multiple Active Forms in Cellulase-xylanase of *Aspergillus* sp. 8-17 by Active Staining

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Abstract A fungal strain able to produce filter paper activity (FPase) was isolated from soil by testing the ability to hydrolyze using filter paper. The isolated strain was identified as an *Aspergilus* sp. judging from its morphological and microscopical characteristics. The cellulase-xylanase system of *Aspergillus* sp. 8-17 was detected *in situ* after gel electrophoresis in the presence of SDS and showed that each protein pattern had a distinct polypeptide composition. β-1,4-Glucanase, cellobiohydrolase, and xylanase activity profiles differ from protein patterns. The *Aspergillus* sp. 8-17 hydrolytic enzymes responsible for the hydrolysis of β-glucan, MUC, and xylan have multiple active forms.

Key words: *Aspergillus*, cellulase, xylanase, multiple active form

Cellulose is the most abundant renewable organic resource in nature. It comprises approximately 45% of dry wood weight. Microbial degradation of cellulose has been intensively studied for decades. In recently years, fungal enzymes have attracted much attention in pulp and paper manufacturing. Mechanisms of cellulose degradation have been hampered by numerous factors including substrate complexity and the multiplicity of enzymes involved. During the past few years, these investigations have made significant progress toward elucidating the enzymology of cellulose degradation [1, 19].

Cellulolytic fungi degrade biopolymers (e.g., cellulose, hemicellulose, and lignin) into monosaccharide with cellulolytic enzymes. This saccharification process is catalyzed by a complex enzyme system which has endoglucanase (EC 3.2.1.91), or xylanase (EC 3.2.1.8) activity [3, 8].

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Cellulase enzyme systems derived from different microorganisms differ markedly in their ratio of constituent enzymes and, consequently, in their ability to degrade native cellulose. Kollar [10] has characterized 12 isozymes from the cellulase system of *Venturia inaequalis*. For these multiple applications microbial xylanolytic systems have been extensively investigated [6, 12, 15, 20]. Recently, Pohlschroder *et al.* [14] demonstrated a multicomplex cellulase-xylanase system by chromatography of *Clostridium papyrosolvens*.

For the characterization of these enzymatic activities standing after SDS-PAGE appears to be the most suitable analyical procedure. This technique combines the advantages of enzyme identification with the resolution and molecular weight dependence of gel electrophoresis in the presence of SDS [4, 6, 16].

Here we report that the *Aspergillus* sp. 8-17 cellulolytic enzyme responsible for the hydrolysis of cellulose and xylan exists in multiple active forms.

MATERIALS AND METHODS

Isolation of Fungal Strains Producing Cellulolytic Enzymes

Soil samples obtained from an Rural Development Administration (RDA) area of Suwon in Korea were incubated in Rose-Bengal medium (RB medium: 1 g KH_2PO_4, 0.5 g $MgSO_4$, 20 g glucose, 0.035 g Rose-Bengal, and 20 g agar in 1 liter H_2O) containing 2% CMC (carboxy methyl cellulose) as a sole carbon source for enrichment. The microorganisms were tested filter paper hydrolytic activity (FPase) by hydrolysis of filter paper (Whatman No.1, 15×100 mm). The fungal strains revealed FPase activity were observed for morphological characteristics by a light microscope and agar plates.

Preparation of Crude Enzyme

The isolated strains were grown in PDA (Potato dextrose agar) medium supplemented as substrate with CMC, filter paper, cellulose powder, avicel and rice straw. After incubation for 7 days, cells were removed by centrifugation at 10,000 g for 10 min at 4°C and the supernatant was precipitated with 2 volumes of ethanol. The precipitate was dissolved with 10 mM sodium phosphate buffer (pH 6.8) in minimum volume. The precipitate was used as crude enzyme for the assay of enzyme activity.

Assay of Cellulolytic Enzyme Activity

Enzyme assay mixtures contained 500 μl of each 0.5% (wt/vol) CMC (medium viscosity, Sigma, St. Louis, MO, U.S.A.), 0.5% cellulose (fibrous cellulose power), 0.2% filter paper or 0.2% rice straw in 100 mM sodium acetate buffer (pH 5.0) and 500 μl of enzyme solution. Enzyme reactions were incubated at 37°C for 1 h. All cellulolytic activities were determined by measuring the amount of glucose released from each substrate by the Somogyi methood [13]. One unit of cellulolytic activity was defined as the amount of enzyme that released 1 mmole of glucose, respectively, per min. The protein patterns were measured by the method of Bradford [5] with a protein assay kit from Bio-Rad Laboratories using bovine serum albumin as the protein standard.

Detection of Cellulase and Xylanase Activities after SDS-PAGE

For the identification of existing isozymes and molecular weights in the gel, the methods of Schwarz *et al.* [16] and Chen and Buller [6] were used. SDS-PAGE was performed in 12% polyacrylamide slab gels in the presence of SDS as described by Laemmli [11]. Depending on the enzyme assay, barley β-glucan or xylan as a substrate was incorporated into the separating gel prior to the addition of ammonium persulfate and polymerization. Protein samples were applied to the gel after heating for 10 min at 60°C or without heating. Electrophoresis was conducted at a constant current of 30 mA until the dye reached the bottom of the slab gels. Upon completion of electrophoresis the gels were washed twice for at least 10 min with cold 0.1 M succinate buffer, pH 5.8, containing 10 mM dithiothreitol. The gels were then submerged in above buffer for 2 h at 60°C. For active staining of cellobiohydrolase, incubations were carried out in the presence of 1 mM MUC (4-Methylumbeliferyl-β-D-cellobioside) and positive bands were detected by fluorescence under UV illumination at 346 nm. Protein bands were detected by staining with Coomassie Blue R-250. β-Glucanase and xylanase bands were then visualized by staining the gel with Congo red (1 mg/ml) for 10 min at room temperature and destaining in 1 M NaCl for another 10 min. The band patterns of light yellowish activity were visible as a deep red background. The bands were photographed within the first hour after staining.

RESULTS

Selection of Cellulolytic Fungal Strains

Strains of seven fungi which produce cellulolytic enzymes, judging by the halo formation around their colonies, were isolated from soil samples. The FPase activities of isolated strains were tested by hydrolysis of filter paper. As shown in Fig. 1, strain 8-17 was chosen for showing the best FPase activity. The morphological properties of the isolated fungal strain 8-17 which produced FPase activity is shown in Fig. 2. Their colonies thus appear to be brown with hyphae showing multinucleate condition. The strain commonly occurs in conidiophore with one raw of sterigmata. According to "A manual of the *Aspergillus*" [17], the isolated strain 8-17 was identified as a strain of *Aspergillus* of an unnamed species. Thus, the isolated fungal strain was named as *Aspergillus* sp. 8-17.

Activity of Cellulolytic Enzymes on Various Substrates

To test for cellulolytic activity with a substrate as the carbon source, *Aspergillus* sp. 8-17 was grown at 30°C for 7 days in substrate-containing medium and enzyme activity was measured. As shown in Table 1, maximum enzyme activity appeared in β-1,4-glucanase with a native substrate of 2% rice straw. The *Aspergillus* sp. 8-17 of cellulolytic activity showed a high enzyme activity with avicel and rice straw and cellobiase activity was mimicked. This result indicated that rice straw and avicel

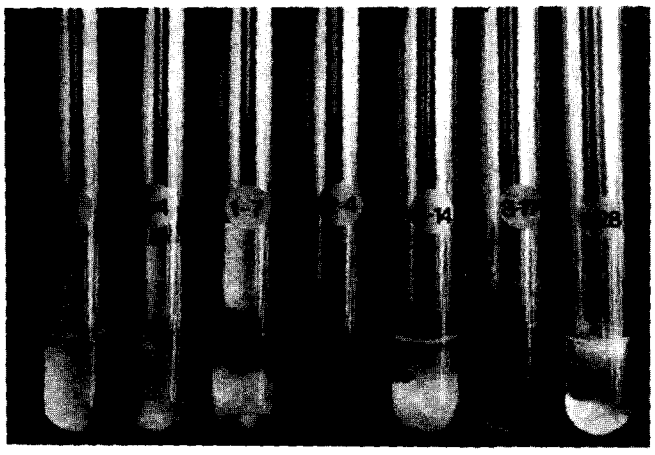

Fig. 1. The selected strain 8-17 shows hydrolysis of filer paper in medium at 30°C for 30 days.

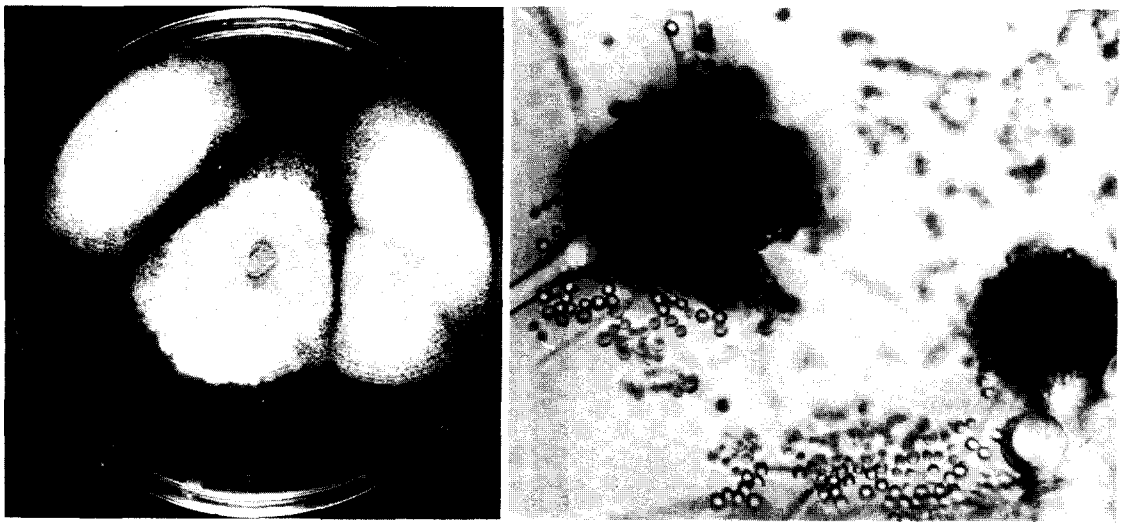

Fig. 2. Morphlogical appearance of strain 8-17.
A, Plate; B, Growth on slide.

Table 1. Comparison of enzyme activities of β-1,4-glucanase, cellobiohydrolase and cellobiase on five cellulosic substrates.

Enzyme	Activity[a]				
	CMC	Cellulose	Filter paper	Avicel	Rice straw
β-1,4-Glucanase	471.3	446.9	487.7	478.7	607.6
Cellobiohydrolase	47.1	116.6	216.0	305.4	300.1
Cellobiase	24.0	50.7	66.8	1.0	5.7

[a]Activity as moles of reducing sugar (as glucose) per mole of enzyme per min.

were revealed as effective substrates for cellulolytic production in *Aspergillus* sp. 8-17.

Cellulase-xylanase Complex Activity after SDS-PAGE

To determine the number of isozymes and the molecular weight of cellulase and xylanase in *Aspergillus* sp. 8-17, a cultural supernatant was analysed using SDS-PAGE and the active staining method. As shown in Fig. 3B, *Aspergillus* sp. 8-17 cellulases exhibit major acitivity bands with molecular masses of about 52 kDa. These activity bands could correspond to the products of β-1,4-glucanases, which are highly conserved among the cellulolytic fungi of *Aspergillus*. The complexity of the patterns demonstrated that isozymes with slight differences in molecular mass presumably arising from proteolytic processing can be readily differentiated. The gel shown in Fig. 3C was assayed for hydrolysis of MUC, a substrate for exocellulase (1,4-β-D-glucan cellobiohydrolases), prior to staining with Congo red. The cellobiohydrolase exhibited major activity bands with a molecular mass aroud 120 kDa and minor activity bands with molecular mass around 52 kDa. Thus, both endoglucanase and exoglucanase activities can be readily

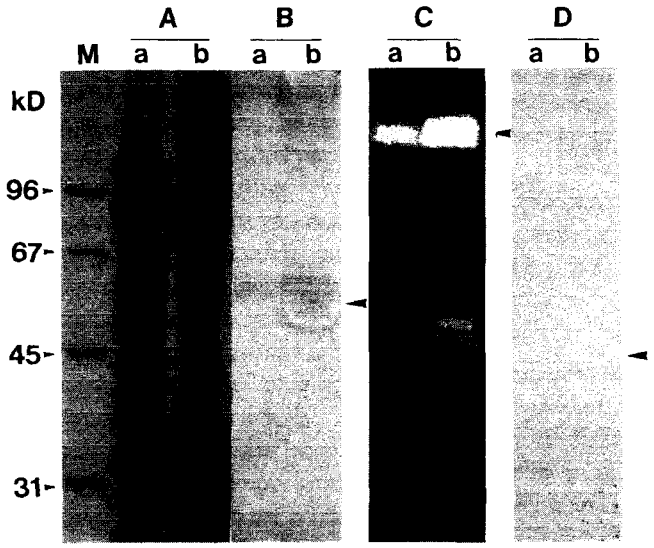

Fig. 3. Zymograms of cellulolytic enzyme from *Aspergillus* sp. 8-17 after SDS-PAGE.
(A) Protein patterns, (B) β-1,4-Glucanase, (C) Cellobiohydrolase and (D) Xylanase. Arrow indicates active band of cellulolytic enzymes. Gels contained 0.1% β-glucan and xylan as substrates were stained for protein and cellulolytic activities with Coomassie Blue R-250, Congo-red and MUC as described in Materials and Methods. Samples was denatured at 60°C for 10 min (lane a) or not denatured (lane b).

detected in the same gel. Fig. 3D shows the results of xylanase activity on xylan, being uniformly suspended in the separating gel, from *Aspergillus* sp. 8-17.

DISCUSSION

Despite the recent knowledge obtained concerning the synthesis, processing, and gene expression of cellulases

and xylanases, there is no available information regarding number, electrophoretic separation, and active forms of cellulases. The heterogeneity of cellulase protein is based mainly on charge properties. It is possibly caused by one or more of IEF (protein ampholyte interactions), extraction procedure, posttranslational modification, and cellulases and xylaneses small family and/or message heterogeneity [7].

Recently, SDS-PAGE has an extraordinary power for sensitive and specific *in situ* detection of separated cellulases and xylanases such as multiple active forms with slight difference in molecular weight and heterogeneity. All xylanases described appear to exhibit differing properties, suggesting that either strain diversity, susceptibility to proteolysis, gene multiplicity or combinations of these influence the type of enzymes produced [14, 18]. Kanellis and Kalaitzis [9] suggested the existence of multiple forms of cellulase protein in ripe avocado based on native IEF gels and visualed by enzyme activity staining and immunodetection. This is responsible for all the cellulase transcript in ripe fruit, then the message multiplicity would be explained by post-transcriptional processing, i.e. exon shulffling [2].

Cellulases and xylanases of *Aspergillus* sp. 8-17 exist into multiple active forms based and visualized by enzyme activity staining. It presumes that the multiplicity of cellulases and xylanases active forms were the result of post-translational modifcation by glycosylation and message heterogeneity.

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