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A method of purification, identification and characterization of β-glucosidase from *Trichoderma koningii* AS3.2774

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ABSTRACT

In this study, we used native gradient-polyacrylamide gel electrophoresis and electroelution (NGGEE) to purify enzymatic proteins from *Trichoderma koningii* AS3.2774. With this method, we purified eight enzymatic proteins and classified them to the cellulase system by comparing secretions of *T. koningii* in inductive medium and in repressive medium. It resulted in 24-fold β-glucosidase (BG) purification with a recovery rate of 5.5%, and a specific activity of 994.6 IU mg^{-1} protein. The final yield of BG reached 8 μg under purifying procedure of NGGEE. We also identified BG using the enzyme assay with thin-layer chromatography and MALDI-TOFMS. This BG had one subunit with a molecular mass of 69.1 kDa as determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis. The hydrolytic activity of the BG had an optimal pH of 5.0, an optimal temperature of 50 °C, an isoelectric point of 5.68 and a K_m for p-nitrophenyl-β-D-glucopyranoside of 2.67 mM. Taken together, we show that NGGEE is a reliable method through which μg grade of active proteins can be purified.

1. Introduction

Cellulose is a major polysaccharide constituent of plant cell walls, which is β-1, 4 linked linear polymer of 8000–12,000 glucose units. Three major enzymes are involved in the degradation from cellulose to glucose: endoglucanase (endo-1, 4-β-D-glucanase, EG, EC 3.2.1.4), cellobiohydrolase(exo-1, 4-β-D-glucanase, CBH, EC 3.2.1.91), and β-glucosidase (1, 4-β-D-glucosidase, BG, EC 3.2.1.21). EG acts in a random fashion, cleaving β-linked bonds within the cellulose molecule; CBH removes cellobiose units from the nonreducing ends of the cellulose chain; and BG degrades cellobiose and cellooligosaccharides to glucose. The *Trichoderma reesei* cellulase system has been the most widely studied among the cellulolytic fungi (Saha Badal and Bothast Rodney, 2009).

BG splits the cellobiose into two glucose monomers, removing the inhibition exerted by cellobiose on certain cellulase, such as CBH I of *T. reesei* (Gong et al., 1977). BGs are also important in the regulation of cellulase genes because they are the key enzymes in the synthesis of sophorose, an efficient inducer of the cellulolytic system of *T. reesei* (Mandels et al., 1962). Cellulase can be induced by Molasses alcohol stillage (Ling et al., 2009). Recently, BGs have become the focus of many applied studies because they are essential not only in the cellulose breakdown but also in the synthesis of oligomers and other complex molecules (e.g., alkyl-glucosides) by transglycosylation (Hanssona and Adlercreutza, 2002). The structural organization of this enzyme is a considerably diversified. The native BG from *Pyrococcus furiosus* has a molecular mass of 230 kDa and is composed of four subunits, each with a molecular mass of 58 kDa (Kengen et al., 1993). In contrast, the BG from *Clostridium stercorarium* is a monomer with a molecular mass of 85 kDa (Bronnenmeier and Staudenbauer, 1988). Some studies indicate that some BGs of white-rot fungi also have a high molecular mass. For example, two BGs from *Sporotrichum pulverulentum* have high molecular masses of 165 kDa and 182 kDa, respectively (Deshpande et al., 1978). Moreover, BGs with similar molecular masses have been purified: the BG from *Pisolithus tinctorius*, 450 kDa composed of three homo subunits; and the BGs from *Volvariella volvacea*, 158 kDa and 256 kDa (Cai et al., 1998; Cao and Crawford, 1993). *Stachybotrys microbispora* has a rich BG system consisting of more than four enzymes. Two of them were purified as dimeric with each monomer of 85 kDa (Amouri and Gargouri, 2006).

Due to their wide biological activities, the preparation of BGs with high purity has been of great interest to biologists. However, it is very difficult to obtain pure active proteins by conventional separation methods, such as column chromatography, two-dimensional electrophoresis and thin-layer chromatography (TLC). Combining with transfer membrane blotting, blue native electrophoresis (BN-PAGE) has been successfully used in the preparative separation of active proteins (Offord et al., 1991; Schägger, 1994). So far, no studies have been reported about two-cycle native gradient-polyacrylamide gel electrophoresis and electroelution (NGGEE) without transfer membrane blotting for isolating and purifying active proteins. Here we

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reported an efficient method for the preparative isolation and purification of BG.

2. Materials and methods

2.1. Microorganisms, culture conditions and preparation of cellulase

Trichoderma koningii AS3.2774 was used for cellulase production, which was obtained from the Institute of Microbiology, Chinese Academy of Sciences. Spore suspension of *T. koningii* (approximately 10^8 conidia ml^{-1}) was prepared from one potato dextrose agar slant after being maintained at 28 °C for 4 days. The enzyme production was carried out in 250-ml Erlenmeyer flasks in inductive medium or repressive medium, respectively. Inductive medium contained 40-mesh straw powder (inductor) 8.0 g, wheat bran 4.0 g, and tap water 20 ml, with natural pH. Repressive medium contained 40-mesh straw powder 8.0 g, wheat bran 4.0 g, glucose (repressor) 2.0 g and tap water 20 ml, with natural pH. Both of them were sterilized for 25 min at 121 °C. Each medium was inoculated with 2 ml spores, equably suspended and statically incubated at 28 °C for 4.5 days. The mould koji of medium was turned over axenic once a day for ventilation. Cultures of mould koji were harvested at the late exponential phase (4.5 days), and each was mixed with 100 ml citric acid-sodium citrate buffer (50 mM, pH 4.8) and slowly stirred in the flask shaker at room temperature for 1 h. Then, the extract was filtered through two-layer gauze. Filtrates were followed by centrifugation at 12,000 × *g* at 4 °C for 20 min. The supernatant, crude cellulase was used to purify cellulolytic enzymes and for the activity assay.

2.2. Zymogram analysis

The cellulase of *T. koningii* AS3.2774 was revealed by the zymogram analysis. For this purpose, ultra-condensates of cellulase excreted from inductive medium and repressive medium were loaded on the same native gradient acrylamide gel (4.0%–8.0% separating gel and 4% stacking gel without SDS and β-mercaptoethanol). When the run finished, the gel was stained with Coomassie brilliant blue R-250 and destained in a solution composed of 10% acetic acid and 20% absolute methanol to identify whether a protein band belonged to the cellulase system or not. The cellulase-related protein strips induced from inductive medium were named P_1 to P_{10}, respectively (Fig. 2). The cellulase excreted from inductive medium was also loaded on each gel under different purifying conditions.

2.3. Purification of cellulase

Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as the standard. Unless stated otherwise, all the purification procedures were performed at 4 °C. Isolation of cellulase excreted from inductive medium by *T. koningii* AS3.2774 was performed as follows.

Step 1 Ultrafiltration
The crude cellulase was ultra centrifugal filtrated 8 fold with Amcion Ultra-4 (Millipore, MW 10000, 4 ml, USA). The condensate was used for both NGGEE and zymogram analysis.

Step 2 Native gradient-polyacrylamide gel electrophoresis
To purify native BG, NGGEE was performed with a vertical electrophoresis system, which contained 4.0%–8.0% native gradient acrylamide separating gel and 4.0% stacking gel without SDS and β-mercaptoethanol. The glass plate of gel electrophoresis system apparatus (Model DYCZ-24A, 200 × 200 × 1.0 mm, 20 pockets) was purchased from Beijing Liuyi Instrument Factory, China. The electrophoresis conditions described by Schagger were modified as follows (Schagger et al., 1994): the voltage and current of 4% stacking gel were limited to 80–100 V and 20–30 mA, respectively. For the separation gel, the voltage was limited to 180–200 V, and the current was no more than 50 mA. The electrophoresis buffer was 12.5 mM Tris-glycin 96 mM at pH 8.8. The protein sample was 0.5–5 mg ml^{-1}, and the sample load was 10 μl $pocket^{-1}$. The electrophoresis was run at 4 °C for 10–12 h. When the run finished, the gel was subjected to the next step as quickly as possible.

Step 3 Location of cellulase in gel
Location of cellulase bands in the gel is the key of this method. Gel (20 lanes) after electrophoresis was cut into five parts vertically. Part 1 (lanes 1 and 2), part 3 (lanes 10 and 11) and part 5 (lanes 19 and 20) were cut out and subjected to staining with Coomassie brilliant blue R-250 and background destaining at 50 °C for 10–20 min, respectively. Part 2 (lanes 3 to 9) and part 4 (lanes 12 to 18) were stored on the glass plate at 4 °C. To locate protein bands, each part of a gel between stained and unstained was also marked by oblique cut along the vertical dotted line beforehand, because the gels became longer after staining and destaining. Then, all the gels (stained or unstained) were pieced together again, and according to the results of the zymogram analysis, native protein bands unstained in gels were cut out, respectively.

Step 4 Recovering native cellulase bands from gels
In this case, all gels (stained or unstained) were put together according to the declining dotted line. Native protein bands in gels (part 2 and part 4) were cut horizontally according to the stained gels (part 1, part 3 and part 5) by comparing with the declining dotted lines as described in the section "Location of cellulase in gel". Protein strips in part 2 and part 4 named P_1 to P_{10} were cut out and collected, respectively.

Step 5 Electroelution and recovery of native proteins
Each native cellulase strip collected from gels of part 2 and part 4 named P_1 to P_{10} was transferred and sealed in a dialysis bag (DM-21, cutoff value of 12–14 kDa, USA) with electrophoresis buffer, respectively. Dialysis bags were dipped into the cathodal sides of the electrophoresis tank and applied to electroelution during native electrophoresis as described in the section "Native gradient-polyacrylamide gel electrophoresis". In this case, any air bubble in the dialysis bag was removed. Electroelution should be run for 2–3 h during NGGEE till native proteins migrated out from gels to solution in the dialysis bag. After electroelution, native proteins sealed in the dialysis bag were transferred together to a tank with a magnetic stirrer and dialyzed against citric acid-sodium citrate buffer (20 mM, pH 4.8). During the dialysis, the buffer was changed with fresh ones at least third times within 4 h. After the dialysis, the gel strips were discarded, the liquid part was collected, and then the material was condensed by lyophilization. By then, the first purification cycle of cellulase was finished.

Step 6 Second time electrophoresis on the uniform gel
After the components of native proteins named P_1 to P_{10} were purified for the first time, the materials of native proteins were applied to electrophoretic isolation for a second time on the uniform gel. For this purpose, the separating gel was appropriately adjusted according to migrations in the first electrophoresis. Gels for P_1, P_2, P_3 and P_4 with low migrations were changed to 4%–5.0%, whereas gels for P_5, P_6, P_7, P_8 and P_{10} with high migration were changed to 5%–8.0%. Each native protein isolated in the first electrophoresis was isolated solely in the second electrophoresis. When the procedures were applied to the step "Electroelution and recovery of native proteins" for the second time, the isolation was finished. The purified components of cellulytic enzymes were stored at 4 °C for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE), identification and characterization studies.