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Purification and characterization of a cellulolytic multienzyme complex produced by *Neocallimastix patriciarum* J11

Hui-Chang Wang [a], Yo-Chia Chen [b,1], Ruey-Shyang Hseu [a,*]

[a] Department of Biochemical Science and Technology, National Taiwan University, Taipei, Taiwan
[b] Department of Biological Science and Technology, National Pingtung University of Science and Technology, Pingtung, Taiwan

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ABSTRACT

Understanding the roles of the components of the multienzyme complex of the anaerobial cellulase system, acting on complex substrates, is crucial to the development of efficient cellulase systems for industrial applications such as converting lignocellulose to sugars for bioethanol production. In this study, we purified the multienzyme complex of *Neocallimastix patriciarum* J11 from a broth through cellulose affinity purification. The multienzyme complex is composed of at least 12 comprised proteins, based on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Eight of these constituents have demonstrated β-glucanase activity on zymogram analysis. The multienzyme complex contained scaffoldings that respond to the gathering of the cellulolytic components. The levels and subunit ratio of the multienzyme complex from *N. patriciarum* J11 might have been affected by their utilized carbon sources, whereas the components of the complexes were consistent. The trypsin-digested peptides of six proteins were matched to the sequences of cellulases originating from rumen fungi, based on identification through liquid chromatography/mass spectrometry, revealing that at least three types of cellulase, including one endoglucanase and two exoglucanases, could be found in the multienzyme complex of *N. patriciarum* J11. The cellulolytic subunits could hydrolyze synergistically on both the internal bonds and the reducing and nonreducing ends of cellulose. Based on our research, our findings are the first to depict the composition of the multienzyme complex produced by *N. patriciarum* J11, and this complex is composed of scaffoldin and three types of cellulase.

1. Introduction

Creating a renewable source of energy by breaking down polysaccharides and subsequently converting them into bioethanol is a relevant topic. The current method of bioethanol production mainly relies on converting starches into sugars, which are in turn converted into ethanol through fermentation processes. Although this method is technically feasible and economically viable, assuming the cost of petroleum continues to increase, this large-scale starch-based bioethanol production might lead to eventual food shortages. Recent efforts have focused on converting starches into fermentable sugars through the enzymatic process involving bioethanol production from lignocellulosic feedstocks [1].

Cellulose, which is composed of insoluble fibers of D-glucose linked by β-1, 4-glucosidic bonds, is the most abundant renewable resource in nature. It will request various cellulolytic enzymes to completely degrade these materials. Studies have shown that anaerobic fungi distributed extensively in the digestive tracts of herbivores can utilize various carbohydrates and possess an efficient glycosyl hydrolase system that hydrolyzes plant carbohydrates [2]. Research has also demonstrated that cellulase secretion depends the carbon source [3–8]. The degradation of plant cell walls by anaerobic microorganisms involves the formation of a large extracellular enzyme complex known as cellulosome, which consists of a scaffolding protein and many bound cellulases and hemicellulases [9–11]. Wilson and Wood found that the extracellular cellulase of *Neocallimastix frontalis*, a common anaerobic fungus, contains a multienzyme complex that is responsible for the activity toward crystalline cellulose [12]. However, the influence of multienzyme complexes in their production and subunit composition is rarely discussed, and the member involving the hydrolysis of lignocellulose must also be confirmed. In the current study, multienzyme complexes were induced and purified from the anaerobic

* Corresponding author. Address: No.1, Sec. 4, Roosevelt Road, Taipei, Taiwan, ROC.
E-mail addresses: ox@mail.npust.edu.tw (Y.-C. Chen), rshseu@ntu.edu.tw (R.-S. Hseu).
[1] Co-corresponding author. Address: No.1 Shuehfu Road, NeiPu, Pingtung 912, Taiwan, ROC. Fax: +886 8 7740550.

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fungus *N. patriciarum* J11, which was grown on different carbon substrates. The components of the multienzyme complex were also analyzed and determined to depict the possible action mode of the complex. The results showed that *N. patriciarum* J11 produced a range of enzymes required for the degradation of a wide range of carbohydrates.

2. Materials and methods

2.1. Microorganism and growth condition

The anaerobic rumen fungus *N. patriciarum* J11 was isolated from the rumen of a water buffalo and cultured in a rumen fluid-containing basal medium supplemented with 0.5% (w/v) glucose, Avicel, cellobiose, carboxymethyl cellulose (CMC), and xylan, as well as rice straw and glucose as carbon sources for maintenance [13] in a multienzyme complex production experiment. The cultures were incubated at 39 °C for 5 d.

2.2. Purification of multienzyme complexes

The culture supernatant of *N. patriciarum* J11, grown for 5 d, was harvested by centrifugation (15,000*g* for 30 min at 4 °C). The purification process was modified based on a method described by Steenbakkers et al. [14]. The supernatant was concentrated approximately 15-fold by using ultrafiltration with a Vivaflow 200 cassette (10 kDa cut-off) (Sartorius Stedim Biotech, Goettingen, Germany) and dialyzed against a 100 mM potassium phosphate buffer (pH 6.5). Subsequently, 4% crystalline cellulose Avicel was added to the concentrated supernatant to absorb the multienzyme complex at 4 °C for 1 h. After centrifugation, the pellet was washed three times by resuspending the pellet in a 100 mM phosphate buffer and subsequently subjecting it to centrifugation (10,000*g* for 15 min at 4 °C). Finally, the absorbed proteins were eluted three times from the Avicel with 20 mL of deionized water. The eluted fractions were pooled and concentrated using an Amicon-stirred cell Model 8050 (5 kDa cut-off membrane) (Millipore, MA, USA) to 3 mL and dialyzed against a 50 mM citrate buffer containing 200 mM NaCl.

2.3. Gel electrophoresis and zymograms

Native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) were performed on 6% and 10% polyacrylamide gels with and without SDS, as described by Laemmli [15]. After electrophoresis, the proteins were stained using either Coomassie brilliant blue R-250 or a silver staining kit (Thermo Fisher Scientific, MA, USA). Zymogram analysis was conducted using an 8% SDS–PAGE gel containing 0.2% (w/v) barley β-glucan to reveal enzyme activity. Protein concentration was determined by implementing a bicinchoninc acid (BCA) assay kit (Thermo Fisher Scientific) and using bovine serum albumin as a standard.

2.4. Recombinant protein expression and purification

The DNA fragment encoding the docking domain was amplified from the genomic DNA of *N. patriciarum* J11 through a polymerase chain reaction, using forward primer DD-F (5′-GGGATCCGGTGAA CGTTTTGGTC-3′) and reverse primer DD-R (5′-GCGGCCGCTCAAAT ACCACACCAT-3′). The primer set was designed according to the DNA fragment of *N. frontalis cel*A (GenBank NO. U38843) by encoding the fungal docking domain sequence. The underlines and double underlines indicate the restriction sites of *Bam*HI and *Not*I, respectively. The expression vector, pGEX 4T-1 (GE Healthcare Life Sciences, NJ, USA), was used for introducing the N-terminus glutathione S-transferase (GST) tag. The resultant plasmids were transformed into *Escherichia coli* Rosetta-gami B (DE3) (Novagen, WI, USA) to express and purify the recombinant proteins.

2.5. Western blotting of multienzyme complexes

The multienzyme complexes were separated using SDS–PAGE (10% polyacrylamide) and then renatured, as described by Ye et al. [16]. After being electroblotted onto a polyvinylidene transfer membrane, the membranes were incubated at room temperature for 1 h with gelatin-NET (0.25% gelatin, 0.15 M NaCl, 5 M EDTA, 0.05% Tween-20, 50 mM Tris, pH 8.0). The membranes were then probed using either DD-GST or GST control fusion proteins in gelatin-NET at room temperature for 1 h. After being washed three times for 10 min with PBST (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween-20, pH 7.3), the membranes were incubated at room temperature for 1 h in gelatin-NET containing goat anti-GST antibodies (GE Healthcare) diluted 1:10,000. Washing was repeated as described and the membranes were incubated at room temperature for 1 h in gelatin-NET containing a rabbit anti-goat IgG-alkaline phosphatase conjugate (Sigma–Aldrich, MO, USA) diluted 1:5000. After being washed three times for 10 min with PBST, the membranes were incubated in an alkaline phosphatase buffer (100 mM NaCl, 10 mM $MgCl_2$, 100 mM Tris, pH 9.5) at room temperature for 15 min and then transferred to NBT/BCIP (Nitroblue tetrazolium and bromo-4-chloro-3-indolyl phosphate) (PerkinElmer, MA, USA) to develop the signals.

2.6. Gel filtration chromatography

The chromatographic process was modified based on the method described by Dijkerman et al. [17]. The concentrated adsorbed enzyme preparation was applied to a Sephacryl S-300 HR column (16 × 900 mm) (GE Healthcare) that was equilibrated and eluted with a 50 mM citrate buffer containing 200 mM NaCl at a flow rate of 1 mL/min. Every 5 mL of fraction was collected. The column was calibrated using a set of molecular weight markers (thyroglobulin 669 kDa; ferritin 440 kDa; aldolase 158 kDa, GE Healthcare) under identical conditions.

2.7. Protein identification by liquid chromatography/mass/mass

After the silver staining process, the overexpressed protein that showed constitutive or unusual bands were excised from gels, trypsin digested, and then subjected to liquid chromatography/mass/mass spectrometry (LC/MS/MS) analyses (Mass Solutions Technology, Taipei, Taiwan). To identify the protein, a peptide mass from Q-TOF MS was searched against the reference peptides in the NCBInr database by using MASCOT software [18].

3. Results

3.1. Purification of multienzyme complexes

The multienzyme complexes of *N. patriciarum* J11induced by Avicel were purified in the cellulose-affinity interaction. The binding fraction was eluted and examined using native-PAGE and zymogram. As shown in Fig. 1, the purified product resulted in distinct bands with an approximate molecular weight of 670 kDa, which could be found in the native-PAGE. A clear zone resulting from β-glucan hydrolysis could also be observed on the correspondent site of the zymogram gel. The result indicated that the high molecular-weight multienzyme complex exhibiting β-glucanase