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

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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

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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

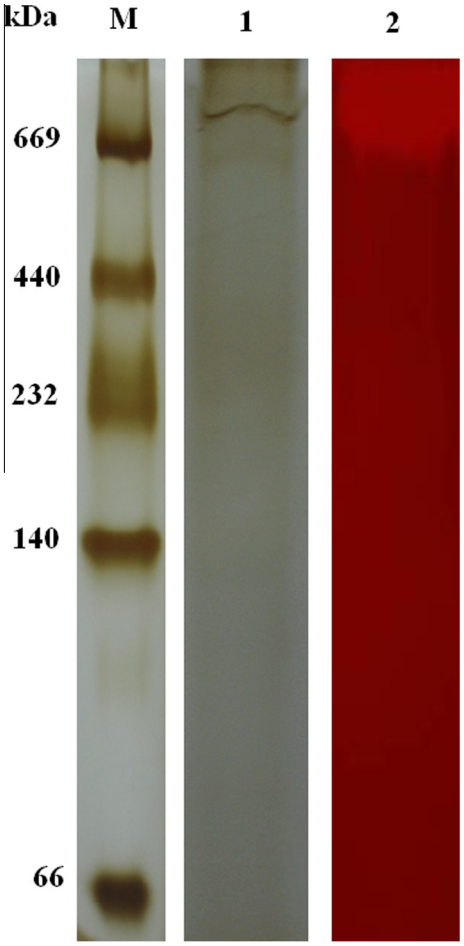

Fig. 1. Examination of the multienzyme complex purified from the broth of *N. patriciarum* J11 by using native PAGE (Lane 1) and zymogram (Lane 2) analyses. The protein was electrophoresed on a 6% polyacrylamide gel and visualized using silver staining in native-PAGE analysis. A 6% native-PAGE gel containing 0.2% barley β-glucan was used in the zymogram analysis. The electrophoresed gel was stained with Congo-red solution.

activity was produced and isolated from the broth of *N. patriciarum* J11.

3.2. Effect of carbon sources on the production of the N. patriciarum J11 multienzyme complex

The Avicel binding fractions of cultured liquids from *N. patriciarum* J11 grown on various carbon sources were applied to Sephacryl S-300 HR columns for purifying multienzyme complexes. The results of gel filtration chromatography revealed that the multienzyme complex, shown in 75 min in Fig. 2, is the predominant product in the broth supplemented by Avicel, cellulobiose, CMC, glucose, and rice straw as substrates for the growth of fungi. The results also showed that the multienzyme complexes could reach their highest levels when the insoluble substrates were used. The multienzyme complex could also be found in the xylan induced broth, but its ratio in the Avicel affinity fraction was significantly lower than in other substrate-induced broths. These results demonstrated that the multienzyme complex of *N. patriciarum* J11 could be expressed at the basal level, and that it could be induced when cellulosic substrates existed in the media.

3.3. Detection of the scaffolding protein in multienzyme complexes

The sequences of docking domain fused GST (DD-GST) were recombinantly expressed by *E. coli*, and the purified DD-GST was

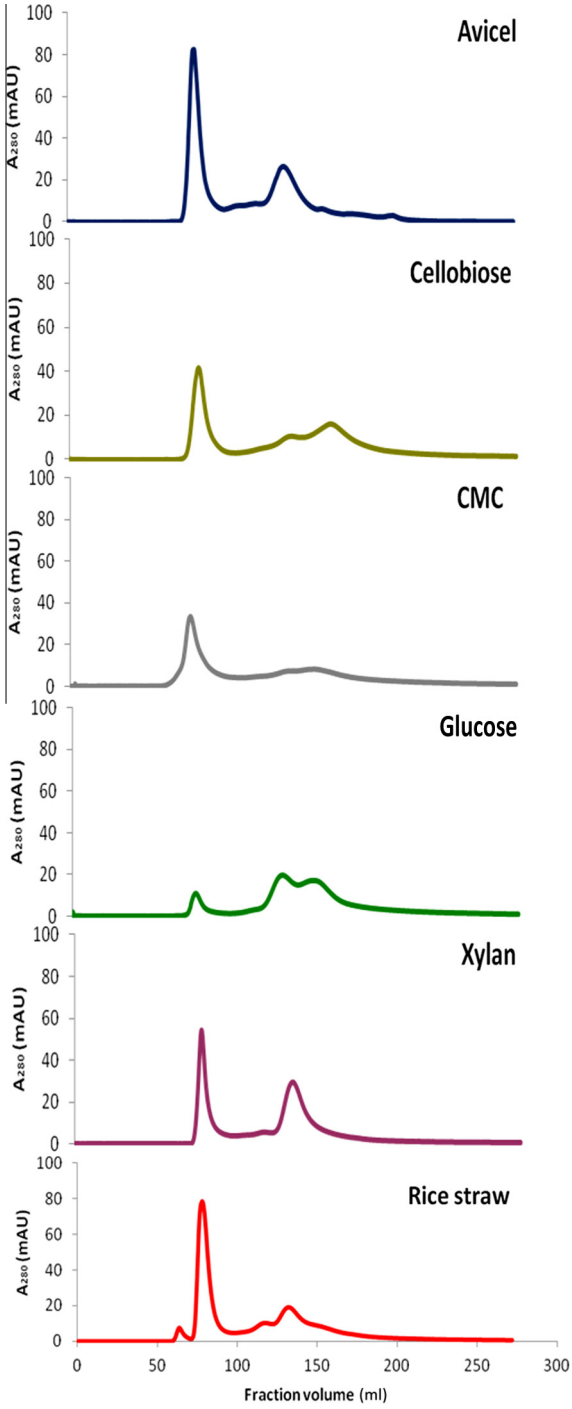

Fig. 2. Gel filtration chromatography of the multienzyme complex from *N. patriciarum* J11 grown on different carbon sources. Samples were applied to a Sephacryl S-300 column which was equilibrated and eluted with 50 mM Na-citrate buffer (pH 6.2) containing 0.2 M NaCl at a flow rate of 0.5 mL/min. The volume of the collected fraction was 5 ml.

used to detect the scaffolding protein in the multienzyme complex of *N. patriciarum* J11. As shown in Fig. 3, the DD-GST could bind the protein that was observed in the site of approximate 79-kDa, whereas the GST protein could not have any significant affinity to the same proteins. This revealed that scaffolding protein, a strut, exists in the multienzyme complex, suggesting that the complex may be assembled through the linkage of docking domains in comprised proteins and scaffolding proteins.

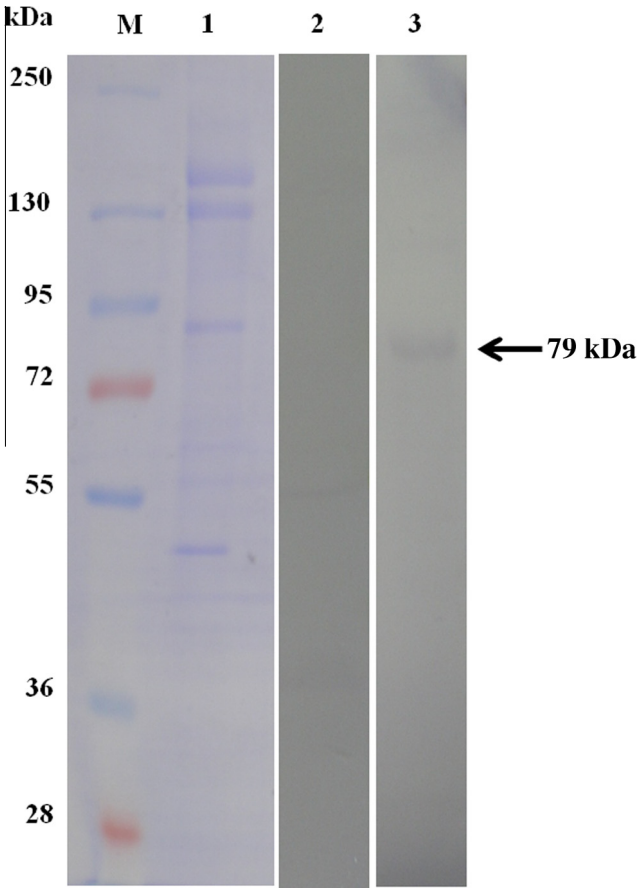

Fig. 3. Detection of the scaffolding protein in the multienzyme complex western blot analysis. The comprised proteins were resolved on 10% SDS–PAGE gels and stained using Coomassie blue (Lane 1). The gels were also mixed with either GST protein (Lane 2) or DD-GST fusion protein (Lane 3) at 37 °C for 1 h, and the reacted gels were detected using an anti-GST antibody.

3.4. Analysis of the comprised subunits in multienzyme complexes

The multienzyme complex induced from *N. patriciarum* J11 was collected, and the SDS-containing solution-treated proteins were synchronously separated using SDS–PAGE and zymogram analysis. As shown in Fig. 4 and 12 main bands in the range of 28 to 250 kDa could be clearly observed on the SDS–PAGE gel (Fig. 4A, Lane 1). This result indicated that the multienzyme complex is assembled by more than 12 proteins through noncovalent interaction. Among the predominant proteins of the multienzyme complex, eight proteins with the molecular weights in the range 72–250 kDa exhibited their β-glucanase activities on the zymogram gel (Fig. 4A, Lane 2), revealing that the multienzyme complex comprised both the fibrolytic enzyme and nonfibrolytic proteins. The purified multienzyme complexes of *N. patriciarum* J11 were induced by different carbon sources, and their subunits were separated and visualized on sliver-stained gels. The subunits of the multienzyme complexes followed a similar pattern, as shown in Fig. 4B; however, the levels of each subunit in the multienzyme complex were evidently varied according to the inductions of carbon substrates. The protein bands near the site of 130 kDa were the most significant among the comprised proteins in the multienzyme complexes induced by Avicel and rice straw. The bands in the range 36–72 kDa are more obvious than other components in the multienzyme complexes induced by cellulobiose and methyl carboxylcellulose, revealing that the ratio in the components of multienzyme complexes are based on the changes in utilized substrates. These bands also revealed that the protein composition of multienzyme complexes induced by different substrates is similar, but the levels of comprised subunits change according to the utilized substrates.

3.5. Identification of comprised proteins in multienzyme complexes

The band labeled in P1 to P11 in Fig. 4 was excised from the polyacrylamide gel for protein identification through typsin digestion, LC/MC/MS analyses, and database searching to identify the comprised subunits of the multienzyme complexes. The proteins were identified as having significant protein scores ($p < 0.05$) based on Mascot searches of peptide mass fingerprints (Table 1). The peptide fragments of P4, P5, P8, P10, and P11 subunits could match the amino acid sequences of GH5, GH6, and GH48 cellulolytic enzymes originating from rumen fungi. The protein bands of P4 and P5 were the homologies of *N. patriciarum* CelD and *N. frontalis* cellulase, respectively. P7 and P8 protein bands were homologous with the *Piromyces equi* Cel48A and *Piromyces* sp. E2 Cel48A, respectively, whereas the P10 and P11 protein bands were similar to the same protein, *Orpinomyces* sp. PC-2 CelH. These matched cellulases possess docking domains in their N- or C-terminus and offer emphatic evidence that they are also the subunits of multienzyme complexes. According to the proposed hydrolysis mechanism, GH6 and GH48 cellulases are exotype enzymes acting with glycosidic bonds from the nonreducing end and the reducing end, respectively. By contrast, GH5 cellulases generally demonstrate an endo-acting hydrolysis of β-1,4-glucan [19–21]. As shown in Fig. 4B, the levels of P10 and P11 subunits were significantly increased when the multienzyme complexes were produced by the induction of CMC and cellobiose, whereas the P4 and P5 subunits were obviously accumulated when the broth supplemented Avicel and rice straw as a carbon source. These results indicated that the endotype and exotype cellulolytic subunits of the multienzyme complexes from *N. patriciarum* J11 might be stimulated by soluble and insoluble substrates, respectively.

4. Discussion

Studies have shown that the cellulase and xylanase from rumen fungi possessed docking domains, and that these domains could bind to the secreted cellulases in the culture supernatants of anaerobic fungi [14,22]. However, preparing various antibodies for identifying different docking domain-contained proteins is laborious. In this study, the docking domain sequence of cellulases from *N. patriciarum* J11 and GST coding sequences were fused and expressed by *E. coli*. The fused protein could be easily purified by performing affinity chromatography and used in evaluating the interaction between the scaffolding and docking domains. The GST domain in the bound could be reorganized by a commercially available antibody in the standard western blot protocol.

Two rumen fungi, *N. frontalis* and *Piromyces* sp. strain E2, could produce cellulosome-like multienzyme complexes. The multienzyme complex of anaerobic rumen fungi was assumed to possess the cellulose-binding domain (CBD) in the individual enzyme subunit or the fungal scaffolding to assist the fungi in attaching themselves onto the surface of plant tissues [12,17]. The CBD of the multienzyme complex also provided a moiety for the purification of cellulosome-like complexes. Thus, we speculated that the 670 kDa protein was a multienzyme complex produced by *N. patriciarum* J11. This result was similar to the cases of *N. frontalis* and *Piromyces* sp. strain E2. However, the size of the multienzyme complex derived from Avicel-grown *N. patriciarum* J11 is smaller than these anaerobic fungi, with an apparent molecular weight of approximately 750–1400 kDa [17]. This may be due to the differences in the size and composition between species.

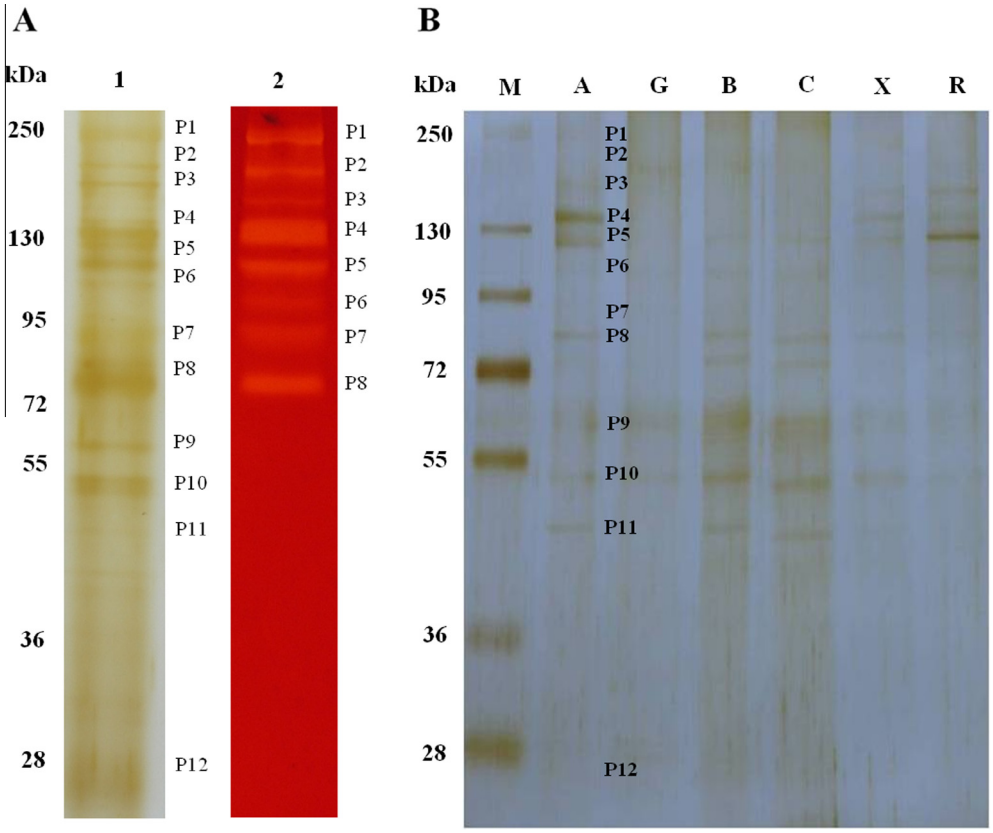

Fig. 4. Protein subunit patterns of multienzyme complexes from *N. patriciarum* J11. Lane 1, SDS–PAGE analysis of the multienzyme complex; Lane 2, zymogram analysis using β-glucan as substrates. The fungus was inoculated in the broth with Avicel (Lane A), glucose (Lane G), cellobiose (Lane B), carboxymethyl cellulose (Lane C), xylan (Lane X), and rice straw (Lane R) as carbon sources. The protein mass standard was loaded in Lane M. The protein samples were electrophoresed on a 10% SDS–PAGE gel and stained using silver nitrate.

Table 1
Identified sequences corresponding to the GenBank data by LC/MS/MS.

Protein name	Significant protein score	Sequence coverage	Significant protein name	Modular architecture[a]	Mode of action	Reference
P1	48	0%	gi|145602830 hypothetical protein MGG_13754 *Magnaporthe oryzae* 70–15	–	–	
P2	N/A	–	–	–	–	
P3	48	0%	gi|145602830 hypothetical protein MGG_13754 *Magnaporthe oryzae* 70–15	–	–	
P4	428	13%	gi|2981484 cellulase CelD, *Neocallimastix patriciarum*	GH5-GH5-GH5-Doc-Doc	Endo-type	[23]
P5	77	3%	gi|3712668 cellulase, *Neocallimastix frontalis*	GH5-Doc-Doc	Endo- type	[24]
P6	N/A	–	–	–	–	
P7	136	3%	gi|25990957 cellulase Cel48A precursor, *Piromyces equi*	GH48-Doc-Doc	Exo-type (reducing end)	[25]
P8	108	5%	gi|25990955 cellulase Cel48A precursor, *Piromyces* sp. E2	GH48-Doc-Doc	Exo-type (reducing end)	[25]
P9	N/A	–	–	–	–	
P10	155	7%	gi|15529294 cellobiohydrolase II-like cellulase CelH,*Orpinomyces* sp. PC-2	Doc-Doc-GH6	Exo-type (non reducing end)	[14]
P11	93	4%	gi|15529294 cellobiohydrolase II-like cellulase CelH,*Orpinomyces* sp. PC-2	Doc-Doc-GH6	Exo-type (non reducing end)	[14]

[a] The modular architectures were predicted by the Conserved Domain Database (CDD) of the National Center for Biotechnology Information (NCBI). GH, glycosyl hydrolase family; Doc, fungal docking domain.

According to the hydrolysis mechanism, GH5 is the endo-acting enzyme, and GH6 and GH48 are exotype enzymes acting as glycosidic bonds from the nonreducing end and reducing end, respectively. These three types of glycosyl hydrolase (Table 1) enabled the multienzyme complex to be an efficient composition for the hydrolysis of crystal cellulose because this complex can degrade both the internal linkages and the two ends of the cellulose fibril simultaneously. Based on our research, this is the first time that the architecture of anaerobic fungal multienzyme complexes has been depicted. These results reveal that the multienzyme complexes may have great potential for industrial applications such as bioethanol production.

References

[1] Y. Lin, S. Tanaka, Ethanol fermentation from biomass resources: current state and prospects, Appl. Microbiol. Biotechnol. 69 (2006) 627–642.
[2] A. Singh, K. Hayashi, Microbial cellulases: protein architecture, molecular properties, and biosynthesis, Adv. Appl. Microbiol. 40 (1995) 1–44.
[3] P.J.T. Anthony, D.R. Davies, K. Guii, M.I. Lawrence, B.B. Nielsen, A. Rickers, M.K. Theodorou, Anaerobic fungi in herbivorous animals, Mycol. Res. 98 (1994) 129–152.
[4] S.E. Lowe, M.K. Theodorou, A.P. Trinci, Cellulases and xylanase of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose, and xylan, Appl. Environ. Microbiol. 53 (1987) 1216–1223.
[5] M.J. Teunissen, G.V. de Kort, H.J. Op den Camp, J.H. Huis in 't Veld, Production of cellulolytic and xylanolytic enzymes during growth of the anaerobic fungus *Piromyces* sp. on different substrates, J. Gen. Microbiol. 138 (Pt 8) (1992) 1657–1664.
[6] A.G. Williams, C.G. Orpin, Polysaccharide-degrading enzymes formed by three species of anaerobic rumen fungi grown on a range of carbohydrate substrates, Can. J. Microbiol. 33 (1987) 418–426.
[7] P.D. Pearce, T. Bauchop, Glycosidases of the rumen anaerobic fungus *Neocallimastix frontalis* grown on cellulosic substrates, Appl. Environ. Microbiol. 49 (1985) 1265–1269.
[8] C.G. Orpin, A.J. Letcher, Utillisation of cellulose, starch, xylan, and other hemicelluloses for growth by the rumen phycomycete, *Neocallimastix frontalis*, Curr. Microbiol. 3 (1979) 121–124.
[9] L.G. Ljungdahl, The cellulase/hemicellulase system of the anaerobic fungus *Orpinomyces* PC-2 and aspects of its applied use, Ann. N. Y. Acad. Sci. 1125 (2008) 308–321.
[10] H.J. Flint, E.A. Bayer, Plant cell wall breakdown by anaerobic microorganisms from the Mammalian digestive tract, Ann. N. Y. Acad. Sci. 1125 (2008) 280–288.
[11] R.H. Doi, A. Kosugi, Cellulosomes: plant-cell-wall-degrading enzyme complexes, Nat. Rev. Microbiol. 2 (2004) 541–551.
[12] C.A. Wilson, T.M. Wood, The anaerobic fungus *Neocallimastix frontalis*: isolation and protperties of a cellulosome-type enzyme fraction with the capacity to solubilize hydrogen-bond-ordered cellulose, Appl. Microbiol. Biotechnol. 37 (1992) 125–129.
[13] Y.C. Chen, S.D. Tsai, H.L. Cheng, C.Y. Chien, C.Y. Hu, T.Y. Cheng, *Caecomyces sympodialis* sp. nov., a new rumen fungus isolated from *Bos indicus*, Mycologia 99 (2007) 125–130.
[14] P.J. Steenbakkers, X.L. Li, E.A. Ximenes, J.G. Arts, H. Chen, L.G. Ljungdahl, H.J. Op Den Camp, Noncatalytic docking domains of cellulosomes of anaerobic fungi, J. Bacteriol. 183 (2001) 5325–5333.
[15] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[16] X.Y. Ye, Purification and characterization of a cellulase from the ruminal fungus *Orpinomyces joyonii* cloned in *Escherichia coli*, Int. J. Biochem. Cell Biol. 33 (2001) 87.
[17] R. Dijkerman, M.B. Vervuren, H.J. Op Den Camp, C. van der Drift, Adsorption characteristics of cellulolytic enzymes from the anaerobic fungus *Piromyces* sp. strain E2 on microcrystalline cellulose, Appl. Environ. Microbiol. 62 (1996) 20–25.
[18] H.L. Cheng, C.Y. Tsai, H.J. Chen, S.S. Yang, Y.C. Chen, The identification, purification, and characterization of STXF10 expressed in *Streptomyces thermonitrificans* NTU-88, Appl. Microbiol. Biotechnol. 82 (2009) 681–689.
[19] H.R. Harhangi, A.C. Freelove, W. Ubhayasekera, M. van Dinther, P.J. Steenbakkers, A. Akhmanova, C. van der Drift, M.S. Jetten, S.L. Mowbray, H.J. Gilbert, H.J. Op den Camp, Cel6A, a major exoglucanase from the cellulosome of the anaerobic fungi *Piromyces* sp. E2 and *Piromyces equi*, Biochim. Biophys. Acta 1628 (2003) 30–39.
[20] H.R. Harhangi, A. Akhmanova, P.J. Steenbakkers, M.S. Jetten, C. van der Drift, H.J. Op den Camp, Genomic DNA analysis of genes encoding (hemi-)cellulolytic enzymes of the anaerobic fungus *Piromyces* sp. E2, Gene 314 (2003) 73–80.
[21] X.L. Li, H. Chen, L.G. Ljungdahl, Monocentric and polycentric anaerobic fungi produce structurally related cellulases and xylanases, Appl. Environ. Microbiol. 63 (1997) 628–635.
[22] C. Fanutti, T. Ponyi, G.W. Black, G.P. Hazlewood, H.J. Gilbert, The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain, J. Biol. Chem. 270 (1995) 29314–29322.
[23] G.P. Xue, K.S. Gobius, C.G. Orpin, A novel polysaccharide hydrolase cDNA (celD) from *Neocallimastix patriciarum* encoding three multi-functional catalytic domains with high endoglucanase, cellobiohydrolase and xylanase activities, J. Gen. Microbiol. 138 (1992) 2397–2403.
[24] Y. Fujino, K. Ogata, T. Nagamine, K. Ushida, Cloning, sequencing, and expression of an endoglucanase gene from the rumen anaerobic fungus *Neocallimastix frontalis* MCH3, Biosci. Biotechnol. Biochem. 62 (1998) 1795–1798.
[25] P.J. Steenbakkers, A. Freelove, B. Van Cranenbroek, B.M. Sweegers, H.R. Harhangi, G.D. Vogels, G.P. Hazlewood, H.J. Gilbert, H.J. Op den Camp, The major component of the cellulosomes of anaerobic fungi from the genus *Piromyces* is a family 48 glycoside hydrolase, DNA Seq.: J. DNA Seq. Mapp. 13 (2002) 313–320.