



Original Article

Thermal stability of *Cryptococcus* sp. S-2 carboxymethyl cellulase (CSCMCCase) having a cellulose binding domain from a fungal exoglucanase: Comparison to recombinant CSCMCCase

Jantaporn Thongekkaew^{1*} and Haruyuki Iefuji²

¹ Department of Biological Science, Faculty of Science,
Ubon-Rajathanee University, Warin Chamrap, Ubon Ratchathani, 34190 Thailand.

Graduate School of Biosphere Science,
Hiroshima University, 3-7-1 Kagamiyama, Higashi-hiroshima 739-0046 Japan.

Received 7 May 2008; Accepted 11 February 2009

Abstract

The *Cryptococcus* sp. S-2 carboxymethyl cellulase gene (CSCMCCase) containing 1023 nucleotides and fused to a cellulose-binding domain (CBD) which originated from cellobiohydrolase I of *Trichoderma reesei* (CSCMCCase-CBD) was cloned and expressed in the methylotrophic yeast, *Pichia pastoris*. The fusion of CBD to CSCMCCase resulted in a greatly enhanced binding toward cellulose for CSCMCCase-CBD compared with that for CSCMCCase. This was useful for enzyme purification and enzyme immobilization. Furthermore, the recombinant CSCMCCase-CBD showed high thermal stability after incubating at 90°C for 3 h, whereas the recombinant CSCMCCase rapidly lost its activity after incubating at 90°C for 1 h.

Keywords: *Cryptococcus* sp. S-2, Thermal stability, Carboxymethyl cellulase, Cellulose binding domain, *Pichia pastoris*

1. Introduction

Cellulases catalyze the hydrolysis of cellulose and comprise mainly three types: (1) endoglucanases (EC. 3.2.1.4), (2) cellobiohydrolases (EC. 3.2.1.91) and (3) β -glucosidases (EC.3.2.1.21) (Enari, 1983). Most cellulases have two separate domains, a catalytic and a cellulose-binding domain (CBD), usually connected by a flexible linker (Linder and Teeri, 1997). The main function of the binding domain is to anchor the catalytic domain close to its soluble substrate, thereby increasing the local enzyme concentration (Lemos *et al.*, 2003). Moreover, it also increases the thermal stability (Iefuji *et al.*, 1996). Several enzymes have been fused to CBDs for simplified and efficient immobilization onto cellulose (Ong *et al.*, 1991; Shpigel *et al.*, 1999; Rotticci-Mulder

et al., 2001). Some CBDs bind irreversibly to cellulose and can be used for applications involving immobilization, others bind reversibly and are more useful for separations and purifications. CBDs with affinity for crystalline cellulose are useful tags for classical column affinity chromatography (Ito *et al.*, 2004).

Cellulases have been widely studied and utilized for industrial applications in food, animal feed, textile, fuel, chemical industries, waste management and paper and pulp industry (Merja *et al.*, 1987). In industrial applications, thermostable enzymes are ideal because they can withstand high temperature; thus, enzyme engineering of *Cryptococcus* sp. S-2 carboxymethyl cellulase was studied with the aim of achieving its thermostability.

The carboxymethyl cellulase from *Cryptococcus* sp. S-2 (CSCMCCase) has previously been purified and characterized and could degrade carboxymethyl-cellulose to cello-oligosaccharide. It is a 34-kDa carboxymethyl cellulase with an optimum pH and temperature for activity of pH 3.5 and

*Corresponding author.

Email address: jantaporn_25@yahoo.com

40-50°C, respectively. Furthermore, it was stable at temperatures up to 90°C and retained about 50% of its activity at 90°C for 1 h. However, this cellulase lacks the cellulose binding domain (Thongekkaew *et al.*, 2008).

In the present study, domain engineering was performed to link the non-cellulose binding domain from the *Cryptococcus* sp. S-2 carboxymethyl cellulase to a CBD comprising 36 amino acid residues of the cellobiohydrolase I (CBHI) from *Trichoderma reesei*. Recombinant enzymes were constructed with none and single copies of CBD fused at the N-terminus of CSCMCase. The recombinant enzymes were expressed in *P. pastoris* and characterized for their binding functions. We also report on the thermal stability of the fusion protein in comparison with recombinant CSCMCase.

2. Materials and Methods

2.1 Strains and plasmids

Cryptococcus sp. S-2 (CS) was obtained from the National Research Institute of Brewing culture collection, Japan, and used as the carboxymethyl cellulase cDNA source. *Escherichia coli* strain JM109 (TAKARA BIO INC, Japan) was employed as the host of plasmid vector pGEM-T easy. *Pichia pastoris* strain GS115 (Invitrogen®, USA) served as the host strain for the transformation and vector pPIC3 served as the yeast expression vector.

2.2 Vector construction of mutant CSCMCase fused to cellulose binding domain of *Trichoderma reesei* cellobiohydrolase I and Transformation

The pGEM-T easy/CSCMCase plasmid and pGEM-T easy/CBDCBHI plasmid were transformed to *E. coli* JM109. These plasmids were digested with *KpnI* and *SphI*. The pGEM-T easy/CSCMCase/*KpnI/SphI* fragment was ligated to the pGEM-T easy/ CBDCBHI/*KpnI/SphI* fragment. The constructed mutant enzyme plasmid, pGEM-T easy/CSCMCase-CBD, was transformed into *E. coli* JM109. Sequencing was performed with a Genetic Analyzer ABI 310. *BamHI*-digested cDNA of CSCMCase and CSCMCase-CBD were ligated to the yeast expression vector pPIC3. Constructed plasmids, pPIC3/CSCMCase and pPIC3/CSCMCase-CBD, were transformed into *E. coli* and transformants were selected on LB-Amp agar plates. The CSCMCase and CSCMCase-CBD gene were sequenced on both strands.

Pichia pastoris GS115 (*his4*; Invitrogen®) was transformed with pPIC3/CSCMCase and pPIC3/CSCMCase-CBD by electroporation with a Bio-Rad Gene Pulser (Bio-Rad®, U.S.A) following the instruction of the manufacturer. Before transformation, pPIC3/CSCMCase and pPIC3/CSCMCase-CBD were linearized by digestion with *StuI*. As a reference, yeast cells were also transformed with the empty vector (pPIC3) using the same method. His⁺ transformants

were recovered on minimum dextrose (MD) plates (13.4 gL⁻¹ YNB without amino acids, 4x10⁻⁴ gL⁻¹ biotin and 20 gL⁻¹ glucose, supplemented with 20 gL⁻¹ Bacto agar) (Cregg *et al.*, 2000). To screen for carboxymethyl cellulase-producing transformants, the His⁺ transformants were spotted on the minimal methanol (MM)-CMC-Na salt agar (13.4 gL⁻¹ YNB without amino acids, 5 gL⁻¹ methanol, 4x10⁻⁴gL⁻¹ biotin and 5 gL⁻¹ carboxymethyl cellulose sodium salt supplemented with 20 gL⁻¹ Bacto agar) (Thongekkaew *et al.*, 2008) and incubated at 30°C for 3 days. After incubation, the hydrolysis halos were made visible by Congo red staining. *Pichia pastoris* transformants were randomly selected and checked for integration of the *CSCMCase* and *CSCMCase-CBD* gene into the genome by PCR with 5' and 3' *AOXI* primers. Positive clones yielded an approximately 1100 and 1300-bp DNA product which was the predicted size of *CSCMCase* and *CSCMCase-CBD*, respectively.

2.3 Cellulase expression in shake-flask cultures of *P. pastoris*

A single colony of recombinant *P. pastoris* GS115 carrying pPIC3/CSCMCase or pPIC3/CSCMCase-CBD or pPIC3 from an MD plate was precultured in 20mL buffered minimal medium (BMG) (13.4 gL⁻¹ YNB without amino acids, 4x10⁻⁴ gL⁻¹ biotin, 10 gL⁻¹ glycerol and 100mM potassium phosphate, pH 6.0) (Cregg *et al.*, 2000) at 30°C for 24 h in a shaking incubator (OD₆₀₀ ~ 6-8). Cells were harvested, pellets resuspended in 100 mL BMM medium (100mM potassium phosphate, pH 6.0, 13.4 gL⁻¹ YNB without amino acids, 4x10⁻⁴ gL⁻¹ biotin and 5 gL⁻¹ methanol) (Cregg *et al.*, 2000) and grown at 30°C for 4 days in a shaking incubator. Absolute methanol was added at intervals of 24 h to a final concentration of 0.5% to maintain gene expression. After 4 days, the supernatant was collected and assayed for carboxymethyl cellulase activity.

2.4 Protein and enzyme activity assay

The protein concentrations were determined using the Bio-Rad Protein Assay Kit according to the manufacturer's instructions with bovine serum albumin (Sigma) as the standard. For the assay of carboxymethyl cellulase (CSCMCase), 0.5 ml of each recombinant enzyme solution and 0.45 ml of 5 gL⁻¹ CMC-Na salt in 50 mM citrate buffer, pH 3.5 were mixed and incubated for 15 min 50°C (Thongekkaew *et al.*, 2008). The reducing sugar formed was quantified as glucose by the method of Somogyi-Nelson (Somogyi, 1952). One unit of enzyme activity was defined as the amount of protein that produced 1 mmol of product per min under the standard assay conditions.

2.5 Absorption assay of recombinant enzymes to Avicel

Adsorption assays were performed in a similar manner as described by Gal *et al.* (1997). Crystalline cellu-

lose (Avicel) was washed with water and 50 mM citrate buffer, pH 3.5, respectively. 50 μ l of each recombinant enzyme and 0.45 ml of 2 gL⁻¹ Avicel in 50 mM citrate buffer, pH 3.5 were mixed in an end-over-end rotation at 40 rpm at room temperature for 6 h. Reference samples containing buffer instead of cellulose suspension were made and treated under the same conditions. The supernatants containing the unbound protein were separated from the cellulose pellet (Avicel). In addition, the cellulose pellets was washed and centrifuged 3 times. The amounts of free carboxymethyl cellulase present in samples were determined by comparing the carboxymethyl cellulase activity in the supernatant with the activity of the corresponding reference sample.

2.6 Determination of thermal resistance of recombinant enzyme

The thermal resistance of recombinant enzyme was also determined by incubating an aliquot of the enzyme in 50 mM citrate buffer, pH 3.5, at different temperatures (70-90°C). The enzyme was then assayed for the remaining activity at 30 min intervals until 3 hour of incubation.

3. Results and Discussion

3.1 Expression of CSCMCase and CSCMCase-CBD in *P. pastoris*

The genes encoding a 341-amino acid for CSCMCase or 378-amino acid for CSCMCase-CBD expressed in the yeast *P. pastoris* strain GS115 and the carboxymethyl cellulase activity were detected by Congo red staining on MM-CMC-Na salt, where a clear halo could be seen around the colonies of yeast containing pPIC3/CSCMCase or pPIC3/CSCMCase-CBD, as shown in Figure 1. The enzyme activities were also detected in the supernatant of BMM liquid cultures. The production of the recombinant CSCMCase was

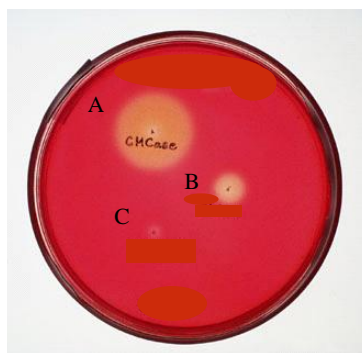


Figure 1. Detection of carbobymethyl cellulase (CSCMCase) activity in *Pichia pastoris* transformants. Yeast cells harboring pPIC3/CSCMCase plasmid (A), pPIC3/CSCMCase-CBD plasmid (B) and pPIC3 plasmid; control (C) were spotted on MM-CMC Na salt plate, and incubated at 30°C for 3 days. The plate was then stained with Congo Red as described in Materials and Methods.

8-fold than that of recombinant CSCMCase-CBD (1450.62 and 185.38 U/L, respectively) after 4 days of cultivation, indicating that the leader sequence of CSCMCase had been recognized and processed as efficiently by *P. pastoris*.

3.2 Characterization of cellulose-binding ability

About 80% of the total carboxymethyl cellulase coupling with CBD expressed by *P. pastoris* was adsorbed to Avicel (crystalline cellulose) due to the presence of carbohydrate binding module, indicating that the cellulose-binding domain was functional (Figure 2). Bound CSCMCase, but not CSCMCase-CBD, could be removed from the crystalline cellulose (Avicel) by washing with water. This result suggests that the binding ability to Avicel of the CSCMCase-CBD could be used for purification and immobilization of the enzymes. It is well documented that cellulases/enzymes with CBD show Avicel adsorbable activity as previously reported by Ogel *et al.* (2001). Moreover, it had earlier been reported for the interaction of CBD for insoluble/soluble cellulosic substrates makes its potential affinity tags for enzyme purification (Sa-Pereira *et al.*, 2003). These potential affinity tags had also previously been reported for the purification of cellulases from *Humicola insolens* by Schulein (1997).

3.3 Characterization of thermal stability of recombinant cellulases

An investigation of the thermal stability of the recom-

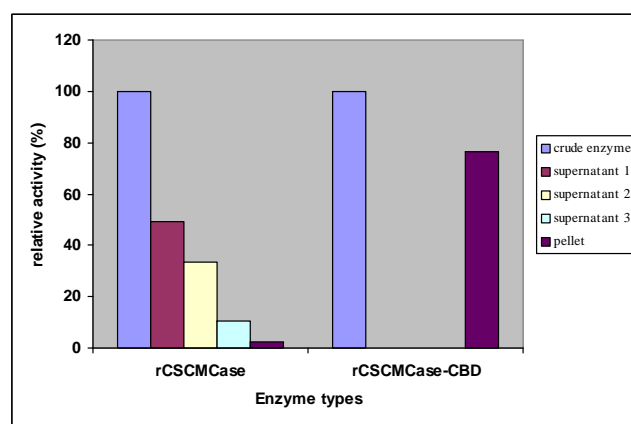
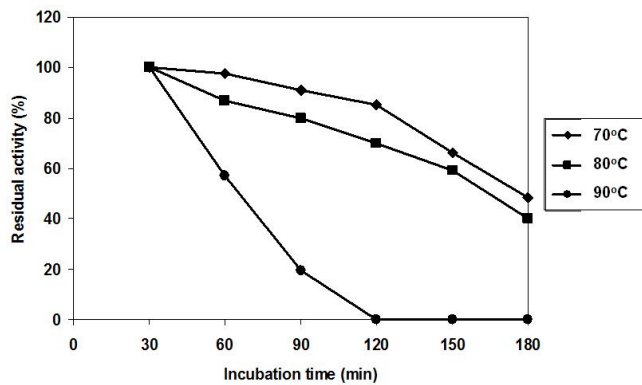


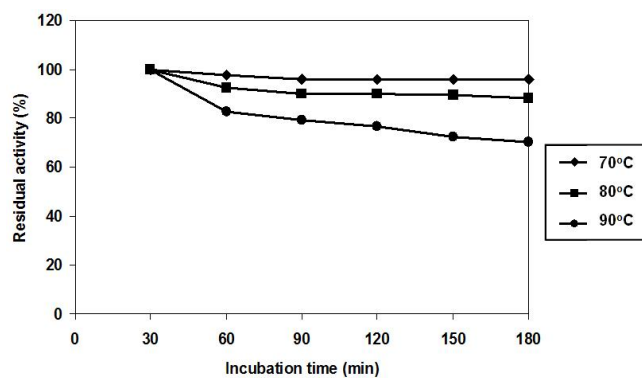
Figure 2. Adsorption properties of recombinant enzymes toward crystalline cellulose (Avicel)

Adsorption assays were performed by incubating the recombinant enzymes in 50 mM citrate buffer, pH 3.5 containing 2 gL⁻¹ Avicel in an end-over-end rotation at 40 rpm at room temperature for 6 h before the free enzyme activity was assayed (at 50°C in 50 mM citrate buffer, pH 3.5 for 15 min) in the supernatant and the cellulose pellet. 100% relative activity corresponds to free enzyme activity before incubating in 50 mM citrate buffer, pH 3.5 containing Avicel under the standard assay conditions.

binant enzymes showed that the recombinant CSCMCase-CBD had higher thermal stability than the recombinant CSCMCase (Figure 3). It retained more than 60% of its activity after keeping the enzyme at 90°C for 3 h whereas the activity of recombinant CSCMCase was rapidly lost after 1 h of incubation at 90°C. Our data suggest that the CBD endowed the recombinant enzymes with this thermostability property, and this reiterates the designation of CBDs as “thermostabilising domains” which had earlier been exploited by Lymar (1995) and Kataeva (2001).



(A)



(B)

Figure 3. Thermal stability of recombinant CSCMCase (A) and CSCMCase-CBD (B) Thermal stability was determined by incubating the enzyme in 50 mM citrate buffer, pH 3.5 at 70°C, 80°C and 90°C for 30, 60, 90, 120, 150 and 180 min before the remaining activity was assayed (at 50°C in 50 mM citrate buffer, pH 3.5 for 15 min).

4. Conclusion

The recombinant CSCMCase and CSCMCase-CBD were secreted into culture medium reaching levels of 1450.62 and 185.38 U/L, respectively. The fusion of CBD to cellulase resulted in a greatly enhanced binding toward cellulose, which might be useful for enzyme purification and enzyme immobilization. Furthermore, the recombinant CSCMCase-CBD showed higher thermal stability than the recombinant CSCMCase. Due to its high temperature stability (up to

90°C), our CSCMCase-CBD might be useful for some industrial applications which will be investigated in further study.

Acknowledgements

This research was supported financially by scientific cooperative research under Core University Program between Japan Society for the Promotion of Science and National Research Council of Thailand (JSPS-NRCT). We gratefully acknowledge their assistance.

References

- Cregg, J.M., Cereghino, J.L., Shi, J. and Higgins, D.R. 2000. Recombinant protein expression in *Pichia pastoris*, *Molecular Biotechnology* 16, 23-52.
- Enari, T.M. 1983. “Microbial cellulase: Microbial Enzymes and Biotechnology,” ed. By W. M. Foogarty, Applied Science Publishers, Laondin., pp. 183-223.
- Gal, L., Pages, S., Gaudin, C., Belaich, A., Reverbel-Leroy, C., Tardif, C. and Belaich, J. P. 1997. Characterization of the cellulolytic complex (cellulosome) produced by *Clostridium cellulolyticum*. *Applied and Environmental Microbiology* 63, 903-909.
- Iefuji, H., Chino, M., Kato, M. and Imura, Y. 1996. Raw-starch-digesting and thermostable α -amylase from the yeast *Cryptococcus* sp. S-2: purification, characterization, cloning and sequencing. *Biochemical Journal* 318, 989-996.
- Ito, S., Kuno, A., Suzuki, R., Kaneko, S., Kawabata, Y., Kusakabe, I. and Hasegawa, T. 2004. Rational affinity purification of native *Streptomyces* family 10 xylanase. *Journal of Biotechnology* 110, 137-142.
- Kataeva, I. A., Blum, D. L., Li, X. L. and Ljungdahl, L. G. 2001. Do domain interactions of glycosyl hydrolases from *Clostridium thermocellum* contribute to protein thermostability? *Protein Engineering* 14, 167-172.
- Lemos, M.A., Teixeira, J. A., Domingues, M. R. M., Mota, M. and Gama, F. M. 2003. The enhancement of the cellulolytic activity of cellobiohydrolase I and endoglucanase by the addition of cellulose binding domains derived from *Trichoderma reesei*. *Enzyme and Microbial Technology* 32, 35-40.
- Linder, M. and Teeri, T. T. 1997. The roles and function of cellulose-binding domains. *Journal of Biotechnology* 57, 15-28.
- Lyman, E. S., Li, B. and Renganathan, V. 1995. Purification and characterization of a cellulose-binding β -glucosidase from cellulose-degrading cultures of *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* 61, 2976-2980.
- Merja, E., Lars, A., Markku, S., Paivi, L. and Jonathan, K. C. K. 1987. Expression of two *Trichoderma reesei* endoglucanases in the yeast *Saccharomyces cerevisiae*. *Yeast* 3, 175-185.

- Ogel, Z. B., Yarangumeli, K., Dundar, H. and Ifrij, I. 2001. Submerged cultivation of *Scytalidium thermophilum* on complex lignocellulosic biomass for endoglucanase production. *Enzyme and Microbial Technology* 28, 689-695.
- Ong, E., Gilkes, N. R., Miller, R. C. Jr., Warren, A.J., Kilburn, D.G. 1991. Enzyme immobilization using a cellulose-binding domain: properties of a beta-glucosidase fusion protein. *Enzyme and Microbial Technology* 13, 59-65.
- Rotticci-Mulder, J. C., Gustavsson, M., Holmquist, M., Hult, K., Martinelle, M. 2001. Expression in *Pichia pastoris* of *Candida antarctica* Lipase B and Lipase B fused to a cellulose-binding domain. *Protein Expression and Purification* 21, 386-392.
- Sa- Pereira, P., Costa-Ferreira, H., Aires-Barros, M. 2003. A new look at xylanases: an overview of purification strategies. *Molecular Biotechnology* 24, 257-281.
- Schulein, M. 1997. Enzymatic properties of cellulases from *Humicola insolens*. *Journal of Biotechnology* 57, 71-81.
- Shpigel, E., Goldlust, A., Efroni, G., Avraham, A., Eshel, A., Dekel, M., Shoseyov, O. 1999. Immobilization of recombinant heparinase I fused to cellulose-binding domain. *Biotechnology and Bioengineering* 65(1), 17-23.
- Somoygi, M. 1952. Notes on sugar determination. *The Journal of Biological Chemistry* 195, 19-23.
- Thongekkaew, J., Ikeda, H., Masaki, K., Iefuji, H. 2008. An acidic and thermostable carboxymethyl cellulase from the yeast *Cryptococcus* sp. S-2: Purification, characterization and improvement of its recombinant enzyme production by high cell-density fermentation of *Pichia pastoris*. *Protein Expression and Purification* 60, 140-146.