CLONING AND EXPRESSION OF PUTATIVE BETA-XYLOSIDASE B FROM PHANEROCHAETE CHRYSOSPORIUM

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SUMMARY

White-rot fungus P. chrysosporium has been reported to produce complex hemicelluloses degradation enzymes including β-xylosidases. Genes encode for these enzymes have been predicted, namely β-xylosidase A and β-xylosidase B, however, the functional of these genes have not been characterized. This study aimed to clone a putative cDNA encoding for β-xylosidase B (PcXylB) from P. chrysosporium BKM-F-1767. Sequence analysis indicated the gene consisted of 981 nucleotides, coding for 326 amino acids. N-terminal containing 18 amino acids was identified as secretion signal peptide. The predicted secondary structure of PcXylB was composed of 27 beta strands, while three catalytic size residues Asp37, Asp152, and Glu221 were found to be general base, pKa module, and general acid, respectively. PcXylB was constructed with expression vectors pPICZαA and pPICZαA and integrated into P. pastoris genome using electroporation method. The rPcXylB secretion of recombinant pPICZαA-PcXylB P. pastoris was driven by intrinsic secretion signal while pPICZαA-PcXylBα P. pastoris was promoted of α-secretion signal from Saccharomyces cerevisiae. The rPcXylB secretion was carried out by adding methanol to final concentration of 1 % for every 12 h. The recombinant pPICZαA-PcXylB constructed P. pastoris did not secret rPcXylB. Western dot blot analysis showed the difference on recombinant protein released among of twenty pPICZαA-PcXylBα transformants. Free-cell medium culture of recombinant P. pastoris on the fourth day cultivation was harvested to purify enzyme using anti his-tag column. Purified rPcXylB exhibited a single band of approximately 33 kDa on SDS-PAGE. The present study was first report on the cloning and expression of putative P. chrysosporium β-xylosidase B.

Keyword: xylosidase, P. chrysosporium, P. pastoris, expression, rPcXylB.

INTRODUCTION

Hemicellulose, the second abundant plant natural component after cellulose, generally accounts for 15-35 percent of plant dry weight. Hemicellulose is composed of xylans, manans, xylogluccans, glucomannans, and β-(1→ 3, 1→ 4)-glucans. Among them, xylans are major hemicelluloses which constitute up to 50 percent dry weight in some tissues of grasses and cereals (Girio et al., 2010; Scheller, Ulvskov, 2010). Xylans are mainly consisted of β-D-xylopyranosyl units linked by β-1,4-glycosidic bonds with various side chains such as 4-O-methyl-β-D-glucuronic acid and acetic acid in hardwood, l-arabinofuranose residues in non-acetylated softwood, or p-cumaric acid and ferulic acid in grasses. The degradation of xylans are usually required a synergistic action of different enzyme classes including endo-xylanase which randomly hydrolyzes the internal β-1,4-glycosidic backbone, yielding short xylooligomers, β-xylosidase acts as exo-type to cleave xylooligomers into single xylose units, while α-L-arabinofuranosidase and esterase remove the side groups (Shallom, Shoham, 2003).

β-xylosidase is classified into seven groups of glycoside hydrolases family, named 3, 30, 39, 43, 52, 54, and 116. β-xylosidase is widely produced by hemicelluloses utilization microorganisms such as fungi, bacteria, and yeast (Sunna A, Antranikian G, 1997). β-xylosidase has been reported to enhanced xylanase activity in the synergistic action because β-xylosidase prevents xylanase, cellulase inhibition by hydrolysis end product (Poutanen, Puls, 1988; Qng, Wyman, 2011). This enzyme is cell-associated in most bacteria and yeast, but it is freely found in the culture media of some fungi (Guerfali et al., 2008; Sunna A, Antranikian G, 1997).

The white-rot fungus Phanerochaete chrysosporium is known to have capacity to completely hydrolyze biomass by producing an enzyme complex including lignin peroxidases, cellulases and hemicellulases. The genome sequence
and computational analysis indicated its putative hemicellulases system involves in endo-xylanase, β-
xylosidase, endo-arabinanase, α-1,5-L-
arabinofuranosidase, β-mannanase, β-mannosidase and
acetyl xylan esterase (Martinez et al., 2004; Wymelenberg et al., 2006), which are found on the
secreted protein matrix in the culture medium containing lignocellulosic biomass (Adav et al.,
2012).

MATERIAL AND METHODS

Strains, plasmids and medium cultures

The white rot fungus P. chrysosporium BKM-F-1767 was obtained from the Korean Collection for
Type Culture and maintained in medium as described by Tien (Tien, Kirk, 1988). Escherichia coli Top10 was used for general cloning. Recombinant vectors were transferred into P. pasteuris GS115 (Invitrogen, Carlsbad, CA, USA). Gene expression was carried out by recombinant P. pasteuris cultured in YP medium (1% yeast extract, and 2% peptone) containing 1 % methanol.

Polymerase chain reaction (PCR) product was ligated with pGEM-T Easy vector (Promega,
Madison, WI, USA) and sequenced. The P. pasteuris expression vectors pPICZc and pPICZaC
(Invitrogen, Carlsbad, CA, USA) were constructed with PcXylB. The standard gene and protein
manipulation were carried out as described by Sambrook and Russell (Sambrook, Russell, 2003).

Total cDNA synthesis

Total P. chrysosporium mRNA was isolated using the Oligotex mRNA Mini kit (Qiagen,
Valencia, CA, USA). cDNA library was synthesized using the SMARTer PCR cDNA Synthesis kit
(Clontech, Mountain View, CA, USA).

Construction and transformation of the recombinant plasmid

The nucleotide sequence of the putative PcXylB was obtained from the P. chrysosporium genome database of the DOE Joint Genome Institute website at http://genome.jgi-psf.org/Pchrl/Pchrl.home.html as predicted by Wymelenberg (Wymelenberg et al.,
2006). The cDNA of PcXylB including the signal peptide was amplified by PCR using the forward
PcXylB-F 5’-CTCGAGATGTTTCCACGGTGATC
TCAC-3’ and reverse PcXylB-R 5’-
TACGTACGTGAAGTGCACTCCTTG-3’

Primers. The PCR products as respected size was excised and purified from 0.8% agarose gel, then
ligated to pGEM-T Easy Vector. Three recombinant plasmids were randomly isolated and sequenced. The PcXylB gene was excised using XhoI and SnaBI, then inserted into the pPICZA vector, resulting in
pPICZA-PcXylB.

To cloning the gene into pPICZαA, the forward primer
PcXylBaF 5’-GAATTCCGGCGGATACAGAAGCGC
and the reverse primer PcXylBaR 5’-
TCATAGTGGAAGTGCACTCCTTG-3’ were used for the gene amplification. Then, PCR product, PcXylBa, was cloned into the pGEM-T Easy Vector, excised with EcoRI and XbaI. The gene restricted by EcoRI-XbaI was purified from agarose gel, and inserted into pPICZαA to generate pPICZαA-
PcXylBaA. The presence of the inserts in recombinant vectors was confirmed both by PCR
and restriction enzyme digestion. A sequence analysis was performed on pPICZA-PcXylB and
pPICZαA-PcXylBa using AOX1 primer as manufacture’s recommendation.

The pPICZA-PcXylB and pPICZαA-PcXylBa were linearized by PmeI and transferred into P. pasteuris GS115. The transformants were screened on YPD agar plates containing 100 μg ml-1 zeocin and 1 M sorbitol at 30°C. The presence of PcXylB in recombinant P. pasteuris genomes was confirmed by
PCR using AOX1 primers.

Expression and production of recombinant PcXylB (rPcXylB)

The rPcXylB production of twenty positive P. pasteuris transformants were screened by dot blot
analysis using his-tag antibody as described by Vasu et al (Vasu et al., 2012). The P. pasteuris
transformant secreting the highest recombinant protein was seeded in 5 ml YPD medium at 30°C
and 200 rpm for 24 hrs, then, transferred to 50 ml of fresh YP medium containing 1% glycerol.
The cultivation was continued at 30°C and 180 rpm for overnight. The cell pellets were harvested by
centrifugation at 2000 rpm for 5 min, and resuspended in 100 ml YP medium supplemented of
1 % methanol for induction at 25°C, 180 rpm.

Enzyme purification

To purify, cell-free supernatants on the fourth
day of culture were collected by centrifugation at
2,000 rpm for 5 min and filtered through 0.45 μm
filters. The supernatant was mixed with 10x binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole; pH 7.4) at ratio 9:1. Ni²⁺ charged his-tag column (Histrap-GE Healthcare, Picataway, NJ, USA) was used to separate rPcXylIB using the ÄKTA fast protein liquid chromatography purification system. rPcXylIB was eluted with an elution buffer (20 mM sodium phosphate, 0.5 M NaCl and 500 mM imidazole; pH 7.4). The purified rPcXylIB was dialyzed with cellulose dialysis tubing membrane (Sigma, St. Louis, MO, USA) in distilled water at 4°C overnight. Protein concentration was measured by the Bradford method, using a Thermo Scientific Protein Assay kit (Rockford, IL, USA) with serum albumin as the standard. Molecular mass of purified rPcXylIB was determined by SDS-PAGE as standard procedure.

RESULTS AND DISCUSSION

The sequence of putative β-xyalosidase was searched on P. chrysosporium RP78 genome sequence database. We found two predicted sequences related with β-xyalosidase, hence named PcXylA and PcXylB. These putative enzymes were classified to glycoside hydrolase family 43, which PcXylA, and PcXylB contain 598 and 326 amino acids, respectively. PcXylA is composed of 10 exons and 9 introns, whereas PcXylB contains 5 exons and 4 introns. There was no similarity between these two putative enzymes. Base on the predicted cDNA PcXylB sequence, we designed the primers to amplify this gene from cDNA library. Figure 1 shows a stronger DNA band of approximately of 950 bp. The full length cDNA nucleotide sequence of PcXylB was deposited in GenBank (accession no. JX625153).

The function of PcXylB was predicted by homology modeling with crystal structure protein database using Phyre server (Kelley, Sternberg, 2009). PcxylB highly matched with β-xyalosidase of Bacillus subtilis (PDB 1YIF), Geobacillus stearothermophilus (PDB 2EX1), B. halodurans (PDB 1YRZ), and Clostridium acetobutylicum (PDB 1Y17) structures, suggesting PcXylB might be a β-xyalosidase. A secretion signal peptide of 18 amino acids at N-terminus was found using SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). While there is no predicted glycosylation site or fungus carbohydrate binding module was identified on PcXylB. The predicted secondary structure of PcXylB was composed of 27 beta strands (Fig. 2). Three catalytic size residues Asp37, Asp152, and Glu221 were found to be general base, pKa module, and general acid, respectively which similar to member of glycoside hydrolase family 43 (Pons et al., 2004).

The constructions of pPICZA-PcXylB and pPICZaA-PcXylBa were transferred into P. pastoris GS115 using electroporation method. The transformants were screened on YPDS supplementation with Zeocin. Ten colonies from each plasmid were randomly selected to check the presence of PcXylB gene. As the results, the PCR products of transformants were observed of two bands, which correspond to AOX1 gene of P. pastoris and pPICZaA-PcXylBa genes (Fig. 3) indicated pPICZaA-PcXylBa was successfully homologous integrated into P. pastoris genome. Similarly, we also obtained the transformants of pPICZA-PcXylB in P. pastoris genome.

In view of expression of recombinant P. pastoris, twenty positive colonies of each transformant were selected. Colonies were cultured for 24 h in YPD medium for biomass production, then the pellets were harvested and replaced by YP medium, induction by supplementation with 1 % methanol for 2 days.
Western dot-blot analysis was carried out for expression determination using 5 µl free-cell supernatants. The recombinant xylanase C was used as positive control (Huy et al., 2011), while negative control was P. pastoris GS115. The results indicated nineteen recombinant pPICZA-PcXylBa colonies were efficiently secreting recombinant putative β-xylanosidase. Among them, number fourth colony showed the highest hybridization significant (Fig. 4). However, the recombinant construction pPICZA-PcXylB did not displayed hybridization significant, resulting in an unsuccessful secretion by intrinsic signal peptide. There have been reported the expression of P. chyrososporium genes using intrinsic secretion signal (Gu et al., 2003; Huy et al., 2011; Wang et al., 2004). The signal peptide is responsible for the translocation of the pro-protein into the endoplasmic reticulum and is subsequently cleaved by signal peptidase during the translocation process, and then the pro-protein is transported to the Golgi where the pro-region is cleaved by Kex2 protease to release the mature protein (Daly, Hearn, 2005). An incorrect process at this step may result in a cell that does not release rPcxylB. Thus, the intrinsic PcXylB signal peptide does not have an efficient protein translocation processing in P. pastoris.

Figure 2. Deduced amino acid sequence and predicted secondary structure of PcXylB. The arrow indicated the signal peptide cleaving site. The closed inverted triangles show the three conserved catalytic residues. The secondary structure containing 27 beta strands was generated flowing three dimension structure of B. subtilis (PDB 1YIF).

Figure 3. The colony PCR for confirmation of pPICZaA-PcXylBa construct. M: 1 kb DNA standard molecular marker; 1: P. pastoris genome; 2: pPICZaA-PcXylBa recombinant vector; 3-12: the recombinant P. pastoris with pPICZaA-PcXylBa.
Figure 4. Western dot-blot analysis of P. pastoris expressed protein using his-tag antibody. PC: free-cell supernatant of recombinant xylanase C from recombinant P. pastoris (Huy et al., 2011); NC: free-cell supernatant from P. pastoris culture; 1-20: twenty transformants of pPICZαA-PcXylBα P. pastoris.

Figure 5. SDS-PAGE analysis of purified recombinant β-xylosidase (rPcXylB). M: molecular mass markers; 1: purified rPcXylB; 2: free-cell supernatant of recombinant rPcXylB from P. pastoris culture. The molecular weight of rPcXylB was estimated to be 33 kDa.

The rPcxylIB was further purified by anti-his-tag column. The molecular mass of purified rPcxylIB was determined by SDS-PAGE, showing a single band around 33 kDa (Fig. 5). rPcXylB molecular mass was very closed to theoretical molecular mass of the fusion mature PcXylB, c-myc- and his-tag tail of 34.7 kDa. The methylotrophic P. pastoris yeast is a useful system to express milligram-to-gram quantities of proteins for both basic laboratory research and industrial purposes. Moreover, the glycosylation system of P. pastoris is less efficient than that of other heterologous expression systems such as S. cerevisiae; therefore, it secretes a native recombinant protein or a less hyperglycosylated form (Daly, Hearn, 2005). Similarly, recombinant P. pastoris released rPcXylB as its native form which might have the same function as P. chrysosporium produced.

CONCLUSION

In the present study, we reported the cloning and expression of a putative β-xylosidase from P. chrysosporium, which play an important role in the biomass degradation processing. rPcXylB were efficiently produced by recombinant P. pastoris without glycosylation and further purified. Although, the biochemical properties of this enzyme are still unknown, however, the characterization may be easy carried out using purified rPcXylB on specific substrate such as p-nitrophenyl-β-D-xylopyranoside. Moreover, the characterization and application on biomass degradation in the synergistic action with other hemicellulases such as xylanaseC should be further topic of research.

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TẠO DÒNG VÀ BIỂU HIỆN BETA-XYLOSIDASE B T PHANEROCHAETE CHRYSSORPIUM

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TÓM TẮT

N m s i tr ng P. chrysosporium t lâu dâ d c bi t đ n có kh nang ti t ra t h p enzyme th y phan hemicelluloses nh β-xylosidase. Hai gene mã hóa cho enzyme này đội d c bảo v i tên g i β-xylosidase A và B. Tuy nhiên, ch c nang c a các gene này đ n nay v n ch a đ c xác đ nh. M c dịch c a nghiên c u này là t o đong do n cDNA gi d nh mà hóa cho enzyme β-xylosidase B (PcXylB) t P. chrysosporium BKM-F-1767 dâ c t o đong. Phân tích trình t nuclide cho th y gene có 981 nucleotide, mà hóa cho chu i polypeptide v i 326 amino acid. Chu i polypeptide g m 18 amino acid d c xác đ nh đong và trò tín hi u t t d u t n cùng N. D oan c u truc b c hai c a protein d c mà hóa b i gene PcXylB cho th y nó đ c c u thành bày b i 27 chu i beta, trong đó ba v trị xúc tác Asp37, Asp152 và Glu221 đong và trò tín base, pKa và acid module. Gene PcXylB dâ c g n vào vector bi i hi n pPICZA and pPICZαA và d c đung h p v i genome c a P. pastoris b ng ph ng phap đ n bi n n p. S t i t enzyme tài t h p d c c m ng b ng cách b sung methanol đ n n ng d c u i 1 % trong m i 24 h. Th tài t h p P. pastoris và pPICZA-PcXylB không tài t ra protein tài t h p PcXylB. Phân tích bi i hi n b ng k thu t Western dot-blot cho th y s khác nhau v m c d bi u hi n c a hai m i th tài t h p pPICZAa-PcXylBα. D ch nuôi c y d a lo i b t bão n m ngay th t d c s d ng d tinh s ch enzyme tài t h p s d ng c t anti his-tag. M t band protein có kh i l ng phân t kho ng 33 KDa x u t hi n trên đ n đ c a rPcXylB tinh s ch. Dây là nghiên c u đ u tiên v t o đong và bi u hi n gene gi đ nh mà hóa cho β-xylosidase B t P. chrysosporium.

T khóa: xylosidase, P. chrysosporium, P. pastoris, bi i hi n, rPcXylB.

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