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Characterization of a novel thermostable GH45 endoglucanase from *Chaetomium thermophilum* and its biodegradation of pectin

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A novel thermostable endoglucanase (CTendo45) encoding gene was cloned from *Chaetomium thermophilum* and heterologously expressed in *Pichia pastoris*. Sequence alignment indicated that the CTendo45 enzyme belonged to glycoside hydrolase family 45. The recombinant enzyme was purified by Ni^{2+} affinity chromatography, and its apparent molecular mass was estimated to be 32 kDa by SDS-PAGE. The purified enzyme displayed maximum activity at 70°C and pH 4. CTendo45 was stable at 60°C for 1 h, and residual activities of 78.9% and 65.6% were estimated after 1 h at 70°C and 80°C, respectively. Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+} and Mn^{2+} were found to have beneficial effects on the enzyme activity to different degrees. The specific activity of purified CTendo45 was 1.52 IU mg^{-1} and the K_m value was 59.6 μg ml^{-1} with a sodium carboxymethyl cellulose substrate. Moreover, CTendo45 exhibited high hydrolysis activity towards pectin, and the hydrolysis products were mainly galacturonic acid oligosaccharides. CTendo45 is the first reported bifunctional enzyme in glycoside hydrolase family 45 from *C. thermophilum* that is able to hydrolyze both cellulose and pectin. The biochemical properties of this recombinant CTendo45 make it a potentially effective glycoside hydrolase for industrial applications.

[**Key words:** Glycoside hydrolase family 45; Endoglucanase; Heterologous expression; Thermostability; Bifunctional enzyme]

Cellulose is regarded as the most abundant renewable carbon resource on the planet. Endoglucanases (EC 3.2.1.4) are responsible for the endohydrolysis of β-1,4-glucosidic linkages in cellulose. For this reason, endoglucanases have attracted extensive attention in recent years, particularly in biotechnological and industrial applications, such as in the ethanol biorefinery and the feed industries (1). According to the classification of the carbohydrate-active enzymes database (CAZy), endoglucanases are assigned to 14 glycoside hydrolase (GH) families: GH5-GH10, GH12, GH26, GH44, GH45, GH48, GH51, GH74, and GH124 (2). GH45 endoglucanases, in particular, have attracted attention due to their low molecular weights and high activities. These properties make GH45 family enzymes important in many specialized bioindustrial applications. All of the members of the GH45 family are reported to be endoglucanases in CAZy, with no other hydrolytic activities. Most of the GH45 endoglucanases identified to date originate from fungal sources. At present, only five crystal structures of GH45 endoglucanases are available from different organisms: *Humicola grisea* var. *thermoidea* (PDB: 1HD5), *Humicola insolens* DSM 1800 (PDB: 3ENG) (3,4), *Melanocarpus albomyces* (PDB: 1OA7) (5), *Mytilus edulis* (PDB: 1WC2) and *Phanerochaete chrysosporium* K-3 (PDB: 3X2G) (6).

Chaetomium thermophilum, a thermophilic fungus of the phylum Ascomycota, has been used to identify novel glycoside hydrolases. In the past few decades, many enzymes from *C. thermophilum* have been characterized and commercialized, in particular, thermostable glycoside hydrolases. Generally, significant thermal stability at high temperatures is an important requirement for a commercial cellulase because thermostable enzymes can effectively improve the hydrolysis efficiency and reduce possible contamination in industrial processes (7). To satisfy the demand for thermostable enzyme production, heterologous expression has become the principal method in the enzyme preparation industry (8). Many enzymes from *C. thermophilum* have been heterologously expressed, such as a GH55 β-1,3-glucanase (9), a manganese superoxide dismutase (MnSOD) (10) and a GH6 cellobiohydrolase (11). However, although there are significant glycoside hydrolases in thermophilic *C. thermophilum*, GH45 endoglucanases have not been reported to date. In this study, a novel GH45 endoglucanase, CTendo45, was isolated and characterized. This is the first report that shows that a GH45 endoglucanase from *C. thermophilum* has the ability to hydrolyze pectin.

MATERIALS AND METHODS

Strain, vector, and materials The *C. thermophilum* strain was isolated from bovine feces at Tengchong (Yun'nan, China). The strain was deposited in the publicly accessible culture collection CGMCC (no. 3.17990), Beijing, China. *Escherichia coli* T1 (TransGen Bio, Beijing, China) was used for gene cloning. *Pichia pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) was used for recombinant protein production. The *Pichia* secretory protein expression vector pPIC9K (Invitrogen) was used in the expression system. Primers were synthesized by Sangon Biotech (Shanghai, China).

Gene cloning and construction of the yeast expression system Total RNA was isolated from the mycelia of *C. thermophilum* using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed according to the RNA PCR Kit 3.0 instructions (Takara Bio, Shiga, Japan). The *CTendo45* gene (Genbank accession number KC441877), based on the genomic DNA of *C. thermophilum* (http://ct.bork.embl.de/), was amplified using the primers 5′-

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CCTACGTACAAGGTGCCCAAGGCA-3′ and 5′- TTGCGGCCGCTTAGTGGTGGTGGTGGT GGTGGGGAGTCCAAGTCG-3′ (the primers included a C-terminal 6× histidine tag sequence for the purification of the expressed product and also compatible restriction sites, *Sna*BI and *Not*I, respectively). PCR was performed using the following protocol: 94°C for 5 min; 30 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 60 s; and 72°C for 10 min. The amplified product was digested with *Sna*BI and *Not*I. The pPIC9K vector, which contained an α-factor secretion signal peptide sequence for secreted expression, was also digested with *Sna*BI and *Not*I. The digested amplified product was then ligated into the pPIC9K vector to produce the *P. pastoris* secretory expression plasmid pPIC9K/*CTendo45*. The recombinant plasmid was subsequently confirmed by restriction analysis and DNA sequencing using self-primers and *AOX1* gene primers 5′-GACTGGTTCCAATTGACAAGC-3′ and 5′-GCAAATGGCATTCT GACATCC-3′.

Transformation and heterologous expression of the recombinant enzyme in *P. pastoris* *P. pastoris* GS115 was transformed with 10 μg of *Sac*I-linearized recombinant plasmid. Transformants that grew normally on MD and MM plates were seeded onto YPD medium plates containing G418 at a final concentration of 1–4 mg ml^{-1} and cultured at 28°C for 3 days to select multi-copy integrants. Although the colony morphology of these multi-copy colonies should be the same, the biggest one, which might have contained the most copies of pPIC9K/*CTendo45*, was selected for the next step. This multi-copy colony was grown in 5 ml of minimal medium overnight for genomic DNA extraction. PCR amplifications were carried out with 1 μg of genomic DNA and *AOX1* primers to confirm the presence of pPIC9K/*CTendo45*. Enzyme induction in *P. pastoris* was performed according to the *Pichia* Yeast Expression System Kit (Invitrogen) (12).

Purification and SDS-PAGE analysis After induction by methanol for seven days, the crude culture was harvested by centrifuging at 8000 rpm for 15 min to prepare the cell-free extract of fermentation liquor. Then, it was adjusted to 80% saturation in $(NH_4)_2SO_4$ overnight at 4°C. The suspension was centrifuged for 15 min at 8000 rpm, and the precipitate was dissolved in phosphate buffer solution at pH 7.4. Histidine-tagged recombinant CTendo45 was purified using Ni^{2+} affinity chromatography (HisTrap FF crude; GE Healthcare, Buckinghamshire, UK). The purity and molecular weight were confirmed by 12% (w/v) SDS-PAGE. The enzyme bands were stained with Coomassie brilliant blue R-250 or a PierceR Glycoprotein Staining Kit (Thermo Scientific, Waltham, MA, USA).

Enzyme assay CTendo45 activity was assayed by measuring the amount of reducing sugars using 3,5-dinitrosalicylic acid (13). Sodium carboxymethyl cellulose (CMC-Na), with a viscosity of 400–800 centipoise (cps) in water at room temperature, was purchased from Sigma–Aldrich (St. Louis, MO, USA). The reaction mixture was composed of 150-μl of 1% (w/v) CMC-Na and 15 μg of purified enzyme in a 300-μl total reaction volume. After incubation for 30 min at 50°C, the reaction was terminated by adding 300 μl of a 3,5-dinitrosalicylic acid reagent. The absorbance was measured at 540 nm. One unit (IU) of enzyme activity was defined as the amount of enzyme that catalyzed the liberation of reducing sugar equivalent to 1 μmol of glucose per minute under the assay conditions.

Characterization of the purified CTendo45 The optimal pH for enzyme activity was determined in various buffer solutions in the pH range 3–9. The buffer solutions used were 50 mM acetate buffer (pH 3–6), 50 mM sodium phosphate buffer (pH 6–8) and 50 mM Tris-HCl buffer (pH 8–9). The optimal temperature was evaluated by measuring activity at 30°C–70°C at the optimal pH. Thermal stability was determined by assaying the residual activities after the enzyme was incubated at 30°C–90°C for 1 h. The effect of different metal cations on the CTendo45 activity was measured at a final metal ion concentration of 1 mM.

Substrate specificity and kinetic analysis β-1,4-D-Glucan from barley, pectin from citrus peel, chitin from shrimp shells, amylose from potato, β-1,4-D-xylan from birch wood, D-galacto-D-mannan from *Ceratonia silique* and soluble starch were purchased from Sigma–Aldrich. (+)-Arabinogalactan from larch wood was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Sucrose was obtained from Solarbio Science & Technology Co., Ltd. (Beijing, China). Ashless quantitative filter paper (no. 1) was purchased from Whatman (Chiltern, UK). Untreated wheat straw was from Tai'an (Shandong, China). The concentration of β-1,4-D-glucan, pectin and (+)-arabinogalactan were 0.2% (w/v). One percent (w/v) CMC-Na, amylose, sucrose, β-1,4-D-Xylan, D-galacto-D-mannan and soluble starch were used. Twenty milligrams of chitin, filter paper and wheat straw were used in each reaction. The commercial endo-1,4-β-D-glucanase from *Trichoderma longibrachiatum* (Megazyme, Bray, Co., Wicklow, Ireland) was used as a control for substrate specificity. The reaction was performed as described above at the optimal reaction conditions of 40°C and pH 4.5. K_m and V_{max} were determined using 1–10 mg ml^{-1} CMC as a substrate under optimum conditions and calculated using the Michaelis–Menten equation.

Hydrolysis of pectin and analysis of the hydrolysis products The 200-μl reaction mixture contained 15 μg of purified CTendo45 protein and 150 μg of pectin. Samples were incubated at 50°C for various times: 10 min, 30 min, 60 min, 90 min and 120 min. The hydrolytic activity was measured using 3,5-dinitrosalicylic acid. The reaction products and oligosaccharides mixture standard (Gentaur, Kampenhout, Belgium) solution were applied onto a silica plate (type 60 F254; Merck, Germany), which was subsequently developed with ethyl acetate:methanol:water:acetic acid (4:2:1:0.5, v/v/v/v) as the developing solvent. The developed silica plate was completely dried and then visualized by dipping in a solution containing 2% (w/v) *N*-phenylaniline, 2% (v/v) phenylamine and 85% (v/v) phosphoric acid in acetone, followed by heating at 85°C for 15 min.

RESULTS AND DISCUSSION

Isolation and characterization of CTendo45 The *CTendo45* gene contains a 744-bp ORF, encoding a protein of 247 amino acids with a deduced molecular mass of 24.5 kDa and calculated pI of 5.85, according to the online analytical tool ProtParam. The SignalP 4.1 server analysis identified the sequence of the first 18 N-terminal amino acids (MHLSQLALPLLLAAGAHA) as a signal peptide, suggesting that CTendo45 is secreted extracellularly. Blastp analysis comparing the sequence with published endoglycosidases indicated that CTendo45 should be classified as a member of the glycoside hydrolase family 45. Two N-linked glycosylation sites (N81 and N88) and two O-linked glycosylation sites (T27 and T237) were identified in the amino acid sequence using the NetNGlyc 1.0 Server and NetOGlyc 4.0 Server, respectively. These data suggested that the CTendo45 protein could be glycosylated.

The NCBI-blast results revealed a high degree of identity with other glycoside hydrolase family 45 members. CTendo45 shared 77% identity with the *Phaeoacremonium minimum* GH45 family protein (XP_007916894) (14), 78% identity with the *Thielavia terrestris* GH45 family protein (XP_003652266) (15) and 72% identity with the *Colletotrichum gloeosporioides* GH45 family protein (EQB52129) (unpublished). According to the structure determination of *H. insolens* endoglucanase V (EGV), which was the first representative of the glycoside hydrolase family 45, Aspl21 acted as a catalytic acid in the glycosyl group hydrolysis and Aspl0 acted as the base, enhancing the nucleophilicity of the catalytic water (3,4). Both residues are structurally invariant in *M. albomyces* GH45 endoglucanase maEG, based on crystal structure analysis (5). Compared to EGV and maEG, two aspartates (Asp32 and Asp144) in CTendo45 could be considered to be the proton donor and nucleophile in the substrate catalytic domain. The conserved amino acid Tyr30 in CTendo45 seemed to be situated below the two aspartate residues, at the floor of the active site. In addition, highly conserved aromatic residues Ser37, Ser68 and Trp40 were found in CTendo45, which were expected to be cellulose substrate-binding sites (3–5,16,17) (Fig. 1).

Heterologous expression and purification To investigate its biological function, the CTendo45 encoding sequence, which contains 708 bp with a 6× histidine tag sequence, was expressed in *P. pastoris*. After methanol induction, a cell-free extract of the fermentation liquor was prepared, and the activity was determined to be 0.57 IU ml^{-1} when CTendo45 was expressed as a secretory form in *P. pastoris*. A similar result of 0.56 IU ml^{-1} was obtained for the GH45 endoglucanase EGL3 from *H. grisea* (AB003107) under the same heterologous expression conditions. The recombinant CTendo45 protein was purified using Ni^{2+} affinity chromatography. The molecular weight of the purified CTendo45 protein was approximately 32 kDa based on SDS-PAGE (Fig. 2). Glycoprotein staining showed that the CTendo45 protein was a glycoprotein (Fig. 2). These data were consistent with our predictions, and glycosylation could explain why the molecular mass was slightly higher than we previously calculated. Sugar residues are commonly linked to Ser, Thr, Hyl and Asn by O- or N-linked glycosylation. The glycosylation of CTendo45 may be involved in protein stability (18). However, more research is necessary to elucidate the potential roles of glycosylation.

Enzyme activity assay The optimum activity temperature was 70°C (Fig. 3A). The thermostability of CTendo45 was also investigated. After incubation for 1 h at different temperatures from 30°C to 60°C, there was little impact on the hydrolysis

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