Extremophiles (2006) 10:79–82
DOI 10.1007/s00792-005-0475-y

NOTE

Runying Zeng · Pengjun Xiong · Jianjun Wen

Characterization and gene cloning of a cold-active cellulase from a deep-sea psychrotrophic bacterium *Pseudoalteromonas* sp. DY3

Received: 31 March 2005 / Accepted: 25 July 2005 / Published online: 24 August 2005

Abstract The *celX* gene encoding an extracellular cold-active cellulase was isolated from a psychrotrophic bacterium, which was isolated from deep-sea sediment and identified as a *Pseudoalteromonas* species. It encoded a protein consisting of 492 amino acids with a calculated molecular mass of 52.7 kDa. The CelX consisted of an N-terminal catalytic domain belonging to glycoside hydrolase family 5 and a C-terminal cellulose-binding domain belonging to carbohydrate-binding module family 5. The long linker sequence connecting both domains was composed of 105 residues. The optimal temperature for cellulase activity of CelX was 40°C. The enzyme was most active at pH 6–7 and showed better resistance to alkaline condition. The zymogram activity analysis indicated that the CelX consisted of single enzyme component. The cellobiose was main hydrolysate of CelX.

Keywords Deep sea · *Pseudoalteromonas* · Cold-active cellulase

Cellulose is the major polysaccharidic compound in plants and the most abundant renewable energy on Earth. Its turnover plays an important role in global carbon cycle for all living organism. In natural environment, the degradation of cellulose is mainly carried out by the multiple enzyme components produced by microorganism. Microbial cellulases have been widely studied in various bacteria, as well as in fungi (Garsoux et al. 2004; Grant et al. 2004; Gaudin et al. 2000; Singh and Hayashi 1995). In contrast to their mesophilic counterparts, cold-active enzymes are more attractive not only because of their high activity in low temperature which is more valuable in application, but also because they represent the lower natural limit of protein stability and is a useful tool for studies in the field of protein folding (Feller et al. 1996). Many cold-active enzymes have been isolated from permanent environment such as the deep sea and the Antarctic, but the cold-active cellulase is still short of study up to now. Among the limited studies, one cellulase CelG was isolated from an Antarctic bacterium *Pseudoalteromonas haloplanktis* (Violot et al. 2003), another endoglucanase was characterized from a mesophilic ruminal anaerobe *Fibrobacter succinogenes* S85 (Iyo and Forsberg 1999), the rest of the endoglucanase was isolated from a soil psychrotrophic yeast *Rhodotorula glutinis* KUJ 2731 (Oikawa et al. 1998). Deep sea is the largest region on the Earth, which is permanently below 5°C. But to our knowledge, the isolation and characterization of cellulase gene from deep sea has not been reported. Here we report the isolation, characterization, and gene clone of a cold-active endoglucanase CelX from a deep-sea psychrotrophic bacterium.

The strain DY3, which showed strong cellulolytic activity, was isolated from deep-sea sediment collected from a depth of 5000 m at 8°21′N, 145°19′W in the east Pacific with screening medium containing carboxymethyl cellulose (CMC) and yeast extract. It could grow at temperature ranging from 5 to 30°C and had an optimum growth temperature of 20°C. As determined by 16S rDNA sequence, strain DY3 was most closely related to the genus *Pseudoalteromonas* (Fig. 1), exhibiting the highest affiliation with *P. citrea* and *P. elyahovii*. Thus we named this strain as *Pseudoalteromonas* sp. DY3. Most of the bacteria exhibiting high levels of 16S rDNA sequence similarity (upwards of 98%) to strain DY3 were undefined species of *Pseudoalteromonas*, indicating that the strain DY3 may be a new species.

The endoglucanase activity of the enzyme was determined by the method described previously (Iyo and Forsberg 1996). According to the cell growth, extracellular cellulolytic activity was detected at 5–25°C, and

Communicated by K. Horikoshi

R. Zeng (✉) · P. Xiong · J. Wen
Third Institute of Oceanography, Key Laboratory of Marine Biogenetic Resources, State Oceanic Administration, Daxue road 178#, 361005 Xiamen, Fujian, China
E-mail: runyingzeng@yahoo.com.cn
Tel.: +86-592-2195323
Fax: +86-592-2085376

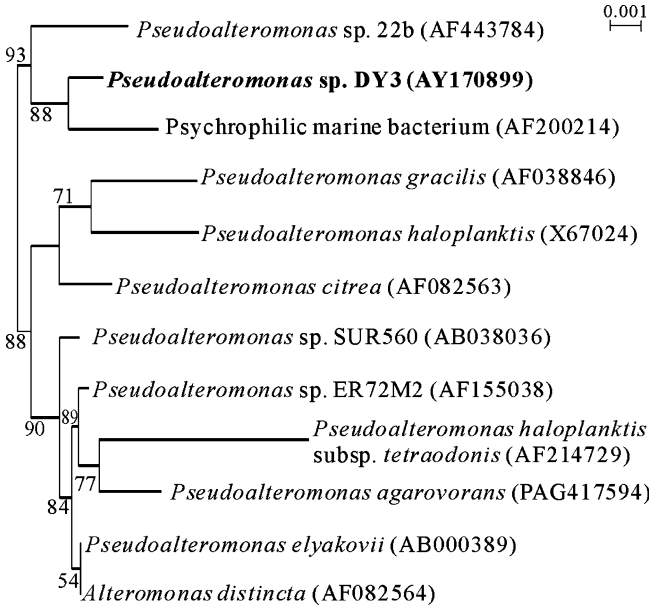

Fig. 1 Phylogenetic analysis of *Pseudoalteromonas* sp. DY3. The numbers in brackets are the EMBL accession numbers of the 16S rDNA sequences. The tree was constructed by neighbor-joining method. The *scale bar* shows 0.001 substitutions per base position. *Numbers* refer to bootstrap values for each node out of a total of 100 replicate resamplings

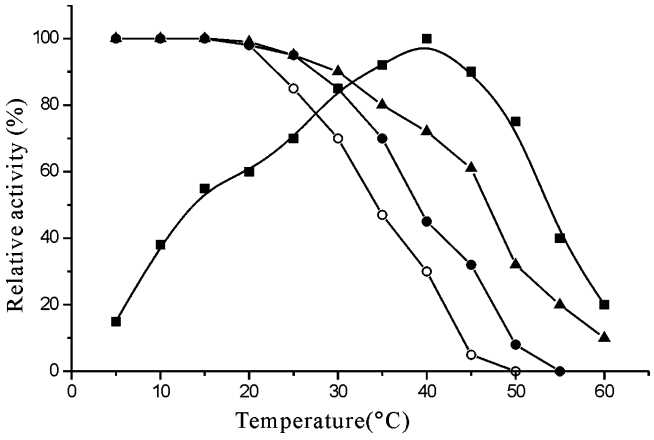

Fig. 2 The effect of temperature on activity (■) and stability of CelX. The thermostability of CelX was determined by detecting the relative activity after incubating the enzyme at temperatures from 5 to 60°C with increment of 5°C for 30 min (▲), 60 min (●), and 120 min (○)

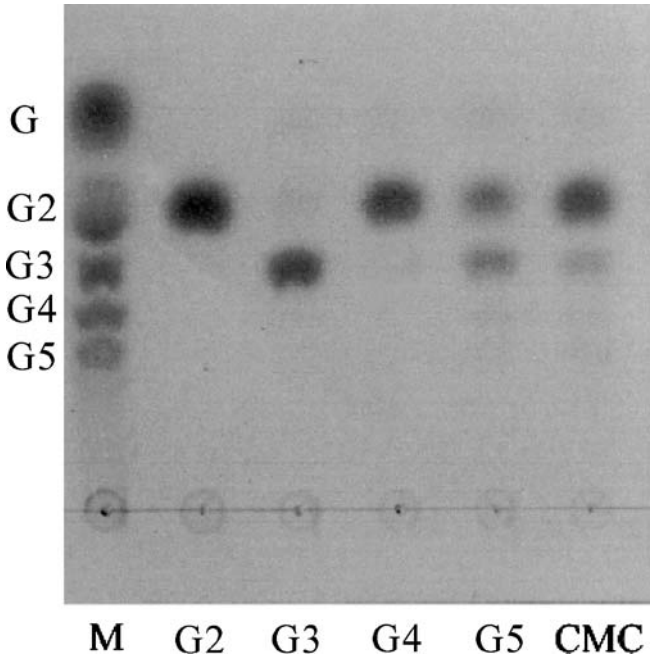

Fig. 3 Analysis of CelX digestion products by TLC. *M* marker, *G* glucose, *G2* cellobiose, *G3* cellotriose, *G4* cellotetrose, *G5* cellopentose

reaching the highest value at 20°C. The purification CelX was achieved at 4°C as follows. The cell-free supernatant was brought to 90% saturation in $(NH_4)_2SO_4$, and the precipitate was dissolved and dialyzed in 50 mM phosphate buffer (pH 8.0). The preparation was loaded onto a DEAE Sepharose CL-6B column that was pre-equilibrated with 10 mM Tris–HCl buffer (pH 8.0) and elution was carried out with a linear gradient from 0 to 500 mM NaCl in the same buffer. The active fractions showed a single clear band that equal to a molecular mass of about 52 kDa on SDS-PAGE by zymogram activity staining.

The maximal cellulolytic activity of CelX was obtained at 40°C, 55 and 15% residual activity was detected at 15 and 5°C, respectively (Fig. 2). But the activity decreased quickly at temperature higher than 45°C. The CelX was stable at temperature below 20°C, and no loss of activity was observed up to 12 h at 20°C. At temperature above 40°C, the enzyme was rapidly inactivated; only 30 and 10% residual activity was detected after 30 min and 1 h incubation at 50°C, respectively (Fig. 2). The CelX was most active at pH 6–7 and retained 73 and 63% residual activity at pH 9 and 10, respectively. It showed better stability in the buffers with high pH values, keeping 64.3 and 37.6% residual activity after incubation at pH 9 and 10 for 1 h separately.

The CelX digestion products of various substrates were submitted to thin-layer chromatography (TLC) on silica gel plate according to the method described previously (Jung et al. 1993). The CelX digested G4 to G2, and did not show activity toward G2 and G3. The digestion product of the G5 and CMC were all mixture of G2 and G3, but the concentration of G2 produced from CMC was obviously higher than that from G5. The results showed that the G2 was the main hydrolysate of CelX (Fig. 3).

Based on known endoglucanase sequences, oligonucleotide primers were designed and used for *celX* gene cloning as follows: 5′-GGATCCATGAATAACAGTTCAAATAATCACA-3′ (celXF) and 5′-GCTAGCTTAATTACAAGTATAAAGAAGCGTCC-3′ (celXR). The PCR amplification was performed with an initial

denaturation step of 2 min at 95°C and then 30 cycles of 30 s denaturation at 94°C, 30 s at 52°C for primer annealing, and 2 min at 72°C for primer extension. The PCR product was cloned into pGEM-T vector (Promega, WI, USA) and sequenced. Sequence analysis of the amplified DNA revealed an open reading frame (ORF) of 1479 nucleotides, encoding a protein of 492 amino acids (AAs), with a calculated molecular mass of 52,700 Da and an estimated pI of 4.05. It shared 95% identity in AAs with *P. haloplanktis* endoglucanase CelG (Violot et al. 2003).

The CelX contained three domains with distinct structure and function, including catalytic domain in N-terminal, cellulose-binding domain in C-terminal, and a linker between them. The AA sequence of CelX catalytic domain, between 33 and 327 from N-terminal, displayed 97 and 64% of similarity to *P. haloplanktis* endoglucanase CelG and *Erwinia chrysanthemi* endoglucanase Cel5, respectively (Fig. 4). They all belonged to glycoside hydrolase family 5 (Davies and Henris1995), characterized by five strictly conserved residues in the active site namely Arg57, His100, His194, Glu135, and Glu222 (Bortoli-German et al. 1995; Wang et al. 1993). The primary structure alignment of the catalytic domain revealed that the cellulase Cel5 from *E. chrysanthemi* (Py et al. 1991) was the mesophilic homologue of the CelX. The C-terminal domain was a cellulose-binding domain, belonging to family 5 of carbohydrate-binding modules (Tomme et al. 1995). The CelX had a long linker sequence, which was much longer than that in Cel5. The type of these additional residues was mainly provided by an increase of Asp, Gly, Asn, Ser, and Val residues.

Cold-active enzymes from psychrophilic microorganisms are generally characterized by the following specialties (Feller et al. 1996). At first, they have an activity curve displaced toward low temperatures compared with mesophilic counterparts. Second, they have a higher catalytic activity and catalytic efficiency than their mesophilic counterparts in the temperature range of 0–30°C. Third, they have a limited thermostability owing to denaturation at moderate and high temperatures. The optimal temperature for cellulase activity of CelX was 40°C, which was 15°C lower than that of Cel5. The CelX kept 55% remnant activity at 15°C, whereas its mesophilic counterpart Cel5 was totally inactive at this temperature. On the other hand, the CelX was more sensitive to the raising of temperature than Cel5. In view of the characteristics described above, the CelX from *Pseudoalteromonas* sp. DY3 was a typical psychrophilic cellulase.

Similar to the psychrophilic cellulase CelG, the CelX had 20 AA residues that interacted with the substrate (Garsoux et al. 2004), as indicated in Fig. 4 with asterisk. These conserved residues were consistent with the structure of Cel5 from *E. chrysanthemi*, with the excep-

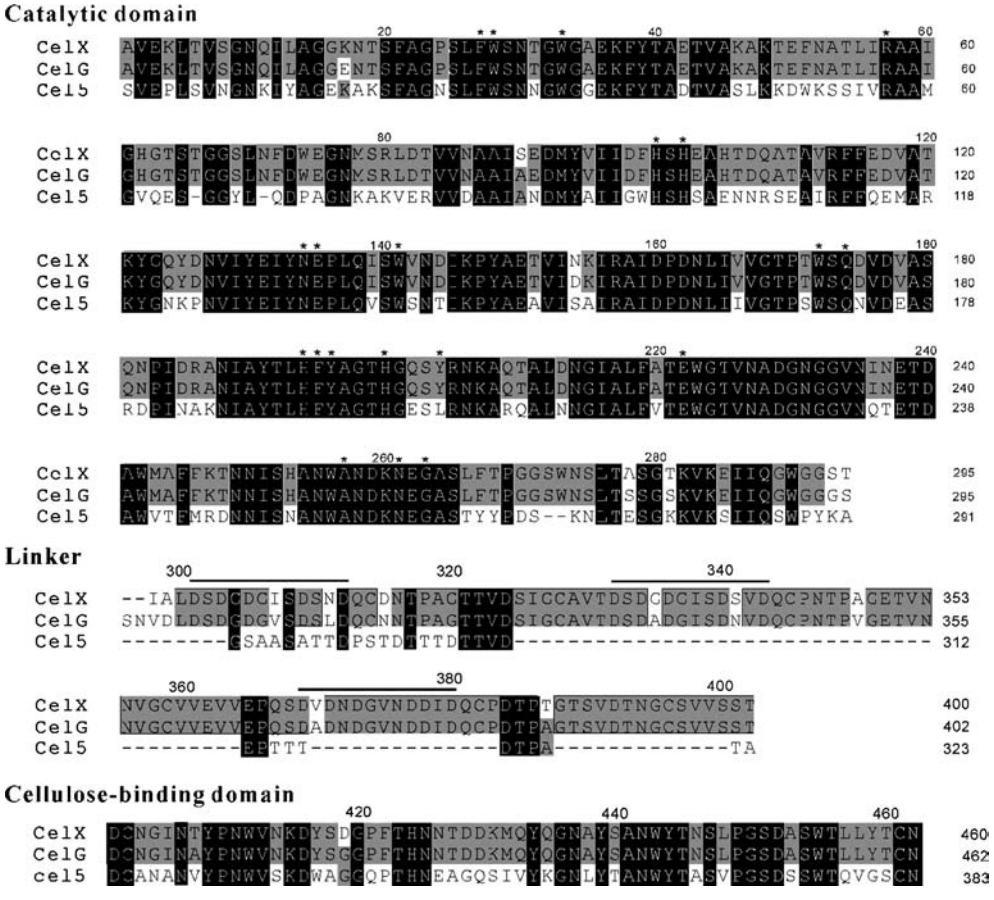

Fig. 4 Amino acid sequence alignment of the psychrophilic (CelX, CelG) and mesophilic (Cel5) cellulases. The accession numbers of *celX* gene and CelX in GenBank were AY250997 and AAP04424, respectively

tion of Tyr 204 in CelX, which is a Leu in Cel5 (Fig. 4). No specific function has been assigned to this residue in family 5 cellulases. Therefore, no obvious structural change within the active site that can be related to the activity of CelX at low temperature, implying that the protein structures outside the active site were responsible for cold-activity. The apparent difference between psychrophilic cellulase and its mesophilic counterpart was the linker sequence connecting catalytic domain and cellulose-binding domain. In comparison with the structure of Cel5, the psychrophilic cellulase CelX had a longer linker sequence containing 73 additional AA residues. The long linker endued the enzyme with better extending and folding ability, which increased the surface of substrate available to the catalytic domain. Three DxDxDGxxDxxD motifs (Fig. 4) in linker sequence of CelG were considered to be a possible structural basis for the conformational organization of the enzyme chain (Garsoux et al. 2004). In this study, the same motifs were found in the linker sequence of CelX also, indicating that they were special structure of psychrophilic cellulase. Moreover, most of the difference in AAs between CelX and CelG were found in these DxDxDGxxDxxD motifs (Fig. 4), impling that these motifs might be responsible for the enzymatic characteristics at low temperature.

Acknowledgments This work was supported by COMRA DY105-4-2-4 Project.

References

Bortoli-German I, Haiech J, Chippaux M, Barras F (1995) Informational suppression to investigate structural functional and evolutionary aspects of the *Erwinia chrysanthemi* cellulase EGZ. J Mol Biol 246:82–94

Davies G, Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases. Structure 3:853–859

Feller G, Narinx E, Arpigny J, Aittaleb M, Baise E, Genicot S, Gerday C (1996) Enzymes from psychrophilic organisms. FEMS Microbiol Rev 18:189–202

Garsoux G, Lamotte J, Gerday C, Feller G (2004) Kinetic and structural optimization to catalysis at low temperatures in a psychrophilic cellulase from the Antarctic bacterium *Pseudoalteromonas haloplanktis*. Biochem J 384(Pt 2):247–253

Gaudin C, Belaich A, Champ S, Belaich JP (2000) CelE, a multidomain cellulase from *Clostridium cellulolyticum*: a key enzyme in the cellulosome? J Bacteriol 182:1910–1915

Grant S, Sorokin DY, Grant WD, Jones BE, Heaphy S (2004) A phylogenetic analysis of Wadiel Natrun soda lake cellulase enrichment cultures and identification of cellulase genes from these cultures. Extremophiles 8:421–429

Iyo AH, Forsberg CW (1996) Endoglucanase G from *Fibrobacter succinogenes* S85 belongs to a class of enzymes characterized by a basic C-terminal domain. Can J Microbiol 42:934–943

Iyo AH, Forsberg CW (1999) A cold-active glucanase from the ruminal bacterium *Fibrobacter succinogenes* S85. Appl Environ Microbiol 65:995–998

Jung ED, Lao G, Irwin D, Barr BK, Benjamin A, Wilson DB (1993) DNA sequences and expression in *Streptomyces lividans* of an exoglucanase gene and an endoglucanase gene from *Thermomonospora fusca*. Appl Environ Microbiol 59:3032–3043

Oikawa T, Tsukagawa Y, Soda K (1998) Endo-beta-glucanase secreted by a psychrotrophic yeast: purification and characterization. Biosci Biotechnol Biochem 62:1751–1756

Py B, Bortoli-German I, Haiech J, Chippaux M, Barras F (1991) Cellulase EGZ of *Erwinia chrysanthemi*: structural organization and importance of His98 and Glu133 residues for catalysis. Protein Eng 4:325–333

Singh A, Hayashi K (1995) Microbial cellulase: protein architecture, molecular properties and biosynthesis. Adv Appl Microbiol 40:1–44

Tomme P, Warren RA, Gilkes NR (1995) Cellulose hydrolysis by bacteria and fungi. Adv Microb Physiol 37:1–81

Violot S, Haser R, Sonan G, Georlette D, Feller G, Aghajari N (2003) Expression, purification, crystallization and preliminary X-ray crystallographic studies of a psychrophilic cellulase from *Pseudoalteromonas haloplanktis*. Acta Cryst D59:1256–1258

Wang Q, Tull D, Meinke A, Gilkes NR, Warren RA, Aebersold R, Withers SG (1993) Glu280 is the nucleophile in the active site of *Clostridium thermocellum* CelC, a family A endo-beta-1,4-glucanase. J Biol Chem 268:14096–14102