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Cloning, Sequencing and Expression Analysis of the First Cellulase Gene Encoding Cellobiohydrolase 1 from a Cold-adaptive *Penicillium chrysogenum* FS010

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Abstract A cellobiohydrolase 1 gene (*cbh1*) was cloned from *Penicillium chrysogenum* FS010 by a modified thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). DNA sequencing shows that *cbh1* has an open reading frame of 1590 bp, encoding a putative protein of 529 amino acid residues. The deduced amino acid sequence revealed that CBHI has a modular structure with a predicted molecular mass of 56 kDa and consists of a fungal type carbohydrate binding module separated from a catalytic domain by a threonine rich linker region. The putative gene product is homologous to fungal cellobiohydrolases in Family 7 of the glycosyl hydrolases. A novel *cbh1* promoter (1.3 kb) was also cloned and sequenced, which contains seven putative binding sites (5′-SYGGRG-3′) for the carbon catabolite repressor CRE1. Effect of various carbon sources to the *cbh1* transcription of *P. chrysogenum* was examined by Northern analysis, suggesting that the expression of *cbh1* is regulated at transcriptional level. The *cbh1* gene in cold-adaptive fungus *P. chysogenum* was expressed as an active enzyme in *Saccharomyces cerevisiae* H158. The recombinant CBHI accumulated intracellularly and could not be secreted into the medium.

Key words *Penicillium chrysogenum*; cellobiohydrolase; TAIL-PCR; promoter of *cbh1*

Cellulose is the world's most abundant biopolymer, and as such, its degradation is of considerable ecological, agricultural and commercial importance. Cellobiohydrolase 1 (CBHI EC 3.2.1.91) is a retaining exo-cellulase that hydrolyzes the β-1,4-linkages of a cellulose chain from its reducing end liberating β-cellobiose as the main product. It belongs to Family 7 of the glycosyl hydrolases [1]. The GH Family 7 comprises enzymes responsible for hydrolysis of β-1,4-*D*-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains [2]. Fungal cellobiohydrolase 1 enzymes share a modular structure consisting of a fungal type cellulose-binding module and a catalytic binding domain separated by a proline/serine/threonine rich linker peptide [3]. CBHs play a key role in degradation of crystalline cellulose. Cellobiohydrolases genes were cloned and characterized from a number of fungal sources including *Penicillium janthinellum* [4], *Trichoderma reesei* [3,5], *Phanerochaete chrysosporium* [6,7], *Aspergillus aculeatus* [8], *Aspergillus niger* [9], *Fusarium oxysporum* [10], *Irpex lacteus* [11–13], and the thermophilic fungus *Talaromyces emersonii* [14,15]. Although cellulose utilizations by terricolous fungi have been widely investigated [16], studies on CBHs from marine fungi have rarely been reported [17]. Identification and characterization of these genes from marine fungi are of great importance in the ocean carbon cycle.

Penicillium chrysogenum is an important industrial organism due to its capacity to produce penicillin, which is still one of the main commercial antibiotics, and the saprobic ascomycete fungus is also known for its ability of secreting a variety of cellulolytic enzymes [18]. In

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addition, a wide spectrum of lytic enzymes is secreted by *P. chrysogenum*, including hemicellulases, xylanases, and amylolytic enzymes [18–20]. *P. chrysogenum* FS010 isolated from Huanghai Sea was identified as *P. chrysogenum* by the analysis of 18S rDNA sequence (AY593254) and small subunit ribosomal RNA sequence (AY553613) reported previously [21]. The optimal temperature of the crude CBHI from *P. chrysogenum* FS010 was 35 ºC, whereas the optimal temperatures of other CBHs from moderate thermophilic fungi were usually 50–60 ºC, suggesting that the CBHI from marine *P. chrysogenum* FS010 had great advantages in hydrolyzing the crystaline cellulase at room temperature. However, cellulase genes have not been isolated and characterized from this fungus.

In this paper, we cloned the *cbh1* from the cold-adaptive *P. chrysogenum* FS010 by a modified TAIL-PCR approach and examined the transcription of this *cbh1* gene. The heterologous expression of CBHI in yeast was also studied.

Materials and Methods

Strains, plasmids and media

P. chrysogenum strain FS010 [21] was used as a DNA donor in this study. Stock cultures were kept on potato glucose agar and subcultured monthly. *P. chrysogenum* FS010 conidia were inoculated in minimal medium as described by Mandels and Andreotti [22] at a final concentration of 10^8 conidia/L. Flasks were incubated in an orbital shaker (220 rpm) at 15 ºC for 48 h. The mycelium was recovered by filtration on a nylon filter (30 μm spore) washed with 0.9% (*W*/*V*) NaCl and dried by pressing between two filter papers. To examine the effects of various carbon sources (1%, *W*/*V*) on cellulase expression, the replacement technique described by Sternberg and Mandels [23] was used. The induction time of various carbon sources was 18 h. Avicel cellulose, sophorose, gentibiose, cellobiose and sorbitol were purchased from Sigma-Aldrich (St. Louis, USA).

Escherichia coli DH5α and the plasmid pGEM-T (Promega, Madison, USA) were used for general DNA manipulations and for DNA sequencing. *Saccharomyces cerevisiae* H158 (*his*$^-$ *leu*$^-$ *ura*$^-$) and the expression vector pAJ401 (*ura3*, 2 μ plasmid replicate origin, PGK promoter, and PGK terminator) derived from plasmid pFL60 [24] were used for the heterologous expression of CBHI in *S. cerevisiae* H158.

General recombinant DNA techniques

The genomic DNA was isolated from *P. chrysogenum* FS010 using the method developed by Raeder and Broda [25]. DNA fragments were recovered from agarose gels by using the E.Z.N.A gel extraction kit (Omega Bio-Tek, Jinan, China) and PCR clean-up system (Promega). The purification of plasmid and other general DNA manipulation procedures were carried out as described by Sambrook and Russell [26].

Cloning and sequencing of the full-length *cbh1* gene by TAIL-polymerase chain reaction

The multiple alignment (DNAMAN) using the primary structure of known fungal CBHIs including *P. janthinellum cbh1*, *T. reesei cbh1*, *P. chrysosporium cbh1*, and *A. aculeatus cbh1* shows high conservation of the protein sequences V-L-D-A-N-W-R-X-V-H and N-M-L-W-L-D-S-D-Y-P (data not shown). Based on the conserved sequences, two degenerate oligonucleotide primers were designed and synthesized to amplify a fragment of the *cbh1* gene: forward, 5′-NTCATTMACGCCAYCTGG-3′; reverse, 5'-MCTMTCGAGCCACAACAT-3' (N, M, and Y represent A/G/C/T, C/G, and A/T, respectively). Genomic DNA of *P. chrysogenum* FS010 was used as the template. Polymerase chain reaction (PCR) was performed under the following conditions: an initial denaturation at 94 ºC for 5 min followed by 35 cycles of amplification (94 ºC for 30 s, 54 ºC for 30 s, and 72 ºC for 1 min), and an additional extension step at 72 ºC for 10 min. The amplified fragment (1062-bp PCR product) was analyzed by gel electrophoresis and purified, then cloned into the pGEM-T vector (Promega), and its nucleotide sequence was determined.

To isolate the 5′-end of the *cbh1* gene fragment, TAIL-PCR was performed according to the protocol developed by Liu *et al.* [27] with a modification. The modification is shown on the use of an asymmetric thermocycling pattern of the tertiary PCR. The PCR pattern was: 94 ºC for 4 min (1 cycle); 94 ºC for 30 s, 61 ºC for 1 min, and 72 ºC for 2 min, 94 ºC for 30 s, 61 ºC for 1 min, and 72 ºC for 2 min, 94 ºC for 30 s, 40 ºC for 1 min, and 72 ºC for 2 min (12 cycles); and 72 ºC for 10 min (1 cycle). Five arbitrary degenerate primes (AD) such as AD1 (5′-NTGCANTNTGCNGTT-3′), AD2 (5′-NGTCAGNNNGANANGAA-3′), AD3 (5′-NGTGNGANANCANCAG-3′), AD4 (5′-TGNGNGANANCANAG-3′) and AD5 (5′-AGNGNAGNANCANAGC-3′), in which N represents A/G/C/T, were designed. Three interlaced specific reverse primers complementary to the known nucleotide sequence

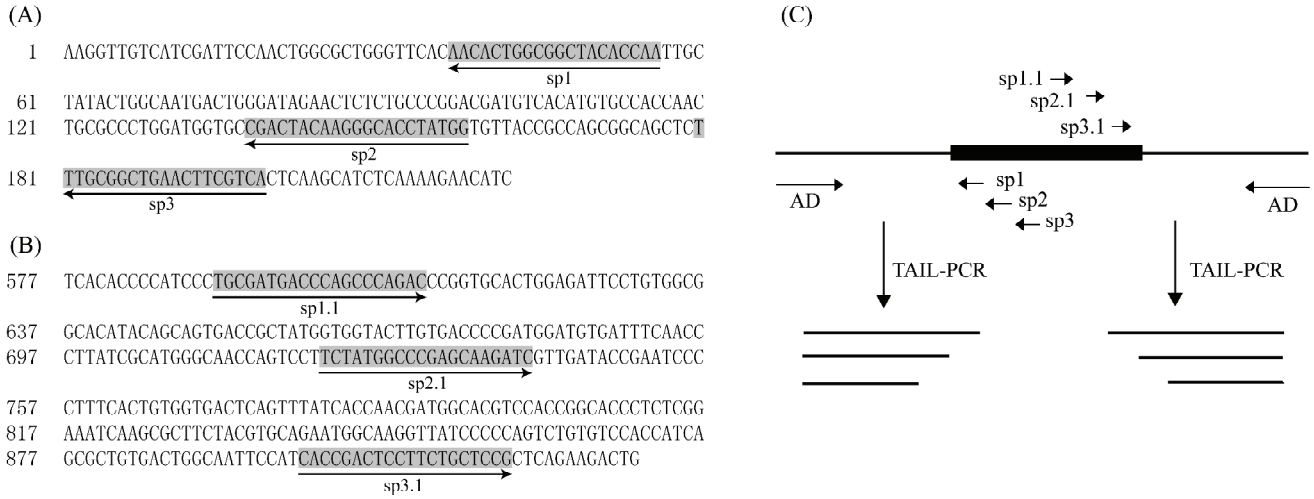

Fig. 1 Localization of the three 3′ gene-specific primers (A) and three 5′ gene-specific primers (B) for the *cbh1* gene and schematic outline of the procedures (C) used to isolate the full-length *cbh1* gene from *P. chrysogenum* FS010

The 3′ and 5′ specific primers were designed according to the sequence of degenerate PCR product (1062 bp).

(1062-bp PCR product) were synthesized [**Fig. 1(A)**, sp1, sp2 and sp3]. The tertiary PCR products were separated by electrophoresis on 1.0% agarose gels. The correct PCR product was purified, and then cloned into the pGEM-T vector. Its nucleotide sequence was determined.

According to the method described above, three interlaced specific sense primers according to the known nucleotide sequence were designed [**Fig. 1(B)**, sp1.1, sp2.1 and sp3.1] to isolate the 3′-end of *cbh1* fragment. Five arbitrary degenerate primers were also used (AD1, AD2, AD3, AD4 and AD5).

DNA manipulations and sequence analysis

DNA was sequenced by an ABI 377 automated DNA sequencer (ABI, Foster City, USA). Database similarity searches were performed using the National Centre for Biotechnological Information (NCBI) online program BLAST [28] against protein (BlastX) and nucleotide (BlastN) sequences stored in GenBank. Multiple sequence alignments were done by DNAMAN program. The protein sequence was analyzed by CBS Prediction Server [29, 30] and ExPASy server [31].

Southern and Northern blot analyses

Chromosomal DNA (5.0 μg) from *P. chrysogenum* FS010 was digested to completion overnight with *Bam*HI, *Eco*RV, *Pst*I and *Xho*I (TaKaRa, Dalian, China), separated on a 0.8% agarose gel, and transferred to Hybond-N^+ filter (Amersham, Piscataway, USA). The full-length *cbh1* gene was fluorescein-labeled using an ECL random prime labeling and detection system (Amersham), and used as a probe to determine the copy numbers of the *cbh1* gene of *P. chrysogenum* FS010. Total RNA was isolated from powdered mycelia with Trizol reagent (Sangon, Shanghai, China) according to the supplier's manual. For Northern blot analysis, 10 μg of total RNA was separated on a 1.2% agarose/formaldehyde gel. After capillary blotting to Hybond-N^+ membrane, the filter was probed with a fluorescein-labeled full-length *cbh1* cDNA probe. 18S rRNA was used as a loading control. Southern hybridization and Northern hybridization were performed according to the supplier's instructions. The signal intensity was determined by the GeneTool software (Cambridge, UK).

Construction of a shuttle expression vector and transformation of *S. cerevisiae*

Total RNA induced by filter paper was isolated using SV Total RNA Isolation System (Promega). The *cbh1* cDNA gene was amplified from *P. chrysogenum* first-strand cDNA, using primers, corresponding to the putative amino-terminal and carboxyl-terminal sequences from the 5′ and 3′ TAIL-PCR products, *cbh1* sense primer 5′-GCGCGAATTCATGGCTTCCACTTCTCCTTCA-AGA-3′ and the *cbh1* anti-sense primer 5′-GCGC-CTCGACTACAGGCACTGCGAGTAGTAATCA-3′. The following PCR cycling parameters were used: 94 ºC for 5 min (1 cycle); 94 ºC for 1 min, 59 ºC 30 s, 72 ºC for 1.5 min (35 cycles); and 72 ºC for 10 min. The amplified PCR product was digested with these two enzymes, and then purified by PCR Clean-Up system. The

DNA fragment of approximately 1.6 kb containing the *cbh1* cDNA gene was cloned downstream of the PGK promoter of *Eco*RI/*Xho*I treated pAJ401. The recombinant plasmid was designated as pAJ401-cbh1. pAJ401-cbh1 was transformed into $CaCl_2$ competent *E. coli* DH5α cells. After propagation in *E. coli*, the transformant plasmid (5 μg) was purified and transformed into the yeast *S. cerevisiae* strain H158 by electroporation (Bio-Rad, Hercules, USA) according to the manufacturer's instructions. The yeast transformants were selected on synthetic complete medium lacking uracil (SC-URA) medium plates. The pAJ401-cbh1 transformant identified by yeast colony PCR was grown in liquid SC-URA for 3 d at 30 ºC. After the incubation, cell-free extracts from cell pellets were prepared and analyzed with 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The preparation of cell-free extracts was carried out as described by Kushnirov [32].

SDS-PAGE analysis

12% SDS-PAGE was performed as described by Laemmli [33]. Protein concentrations were determined by Bradford method [34] with bovine serum albumin as the standard.

Activity assay

Hydrolytic activities produced by recombinant yeast cells were assayed based on the method of Takashima *et al.* [35]. *p*-nitrophenyl-β-*D*-cellobioside (pNPC) (Sigma-Aldrich) was used to as a substrate. One unit of CBHI was defined as the amount of protein that produces 1.0 μmol of pNP per minute under the standard assay conditions.

Nucleotide sequence accession number

The genomic and cDNA sequence of *cbh1* gene have been deposited in GenBank under the accession number AY790330 and AY973993, respectively.

Results and Discussion

Cloning and analysis of the primary structure of the *P. chrysogenum cbh1* gene

Under experimental conditions described using degenerate oligonucleotide primers with homology to other *cbh1* genes, a specific fragment of 1062 bp was amplified from *P. chrysogenum* chromosomal DNA. Sequence analysis confirmed that the PCR product was homologous to the *cbh1* gene family (data not shown). Based on the sequence of the gene fragment, a modified TAIL-PCR was performed to clone the entire *cbh1* gene (**Fig. 1**). The full-length *cbh1* was successfully and rapidly isolated. To examine whether the sequence obtained from TAIL-PCR reactions and sequencing was correctly deduced, a 2940-bp DNA fragment, including 1316-bp of the region upstream of the putative initiator ATG and 34-bp of the region downstream of the stop codon, was amplified by PCR as a continuous fragment using genomic DNA as a template and sequenced.

The nucleotide sequence of the 2940-bp DNA fragment was determined for both strands. One open reading frame (ORF) was located between nucleotides 1 and 1590, and its molar G+C content was 56.04%. The sequence of the 5′ flanking region of the *cbh1* gene was determined to nt −1316. The putative translation start site was designated as +1. A putative TATA box was found at nt −41, and three putative CAAT box was found at nt −207, −412 and −589 respectively. Two putative binding sites were found at nt −418 and −809 for the transcriptional activator ACEII [36]. In the 5′ upstream region of the *cbh1* gene, seven carbon catabolite-repressor binding consensus sequences (5′-SYGGRG-3′) [37] that possibly mediate carbon catabolite repression by a CREA-homologue were found at nt −230, −232, −252, −259, −296, −572 and −776 respectively. Sequence analysis showed that the *cbh1* promoter region from *P. chrysogenum* FS010 has no homology with those *cbh1* promoter from *T. reesei* [37], *Thermoascus aurantiacus* [38] and *Trichoderma koningii* [39], suggesting that the cloned *cbh1* promoter is a novel promoter. The *cbh1* promoter would be useful for development of a high efficient regulated expression system for *P. chrysogenum*.

The TAIL-PCR approach developed by Liu and Whittier [40] is a simple and efficient technique for genomic walking in plant molecular biology [27,41], which does not require any restriction or ligation steps. But to the best of our knowledge, it has never been employed for the isolation of full-length genes from fungi. In this paper, a modified TAIL-PCR method in combination with degenerate PCR was recruited to clone the CBHI encoding gene and the *cbh1* promoter from a cold-adaptive *P. chrysogenum* FS010. Our results indicated that bioinformatics analysis in combination with TAIL-PCR protocol would facilitate the fungal full-length gene cloning and the development of filamentous fungi molecular biology.

Structure of the CBHI protein

The ORF encodes a protein of 529 amino acid residues, with a deduced molecular mass of about 56 kDa. At the

N terminus of the deduced sequence, a putative signal sequence was identified by the SignalP 3.0 server system (http://www.cbs.dtu.dk/services/), with cleavage predicted to occur after amino acid 26 of the pre-protein. Three potential N-glycosylation sites were found at Asn-295, Asn-442, and Asn-505. Comparison of the deduced CBHI amino acid sequence from *P. chrysogenum* with those available on databases reveals identity values of 70.37%, 66.91%, 62.08%, 62.00% and 56.69% respectively with the CBHI from *P. janthinellum* (GenBank accession No. CAA41780), *A. aculeatus* (GenBank accession No. BAA25183), *Penicillium occitanis* (GenBank accession No. AAT99321), *T. emersonii* (GenBank accession No. AAL89553) and *Trichoderma viride* (GenBank accession No. AAQ76092). All of the homologous sequences belong to Family 7 of the glycosyl hydrolase, which suggests that CBHI of *P. chrysogenum* is also a member. Among the conserved residues, the amino acid equivalent to Glu-237 was identified as a potential nucleophile in the displacement reaction and that equivalent to Glu-242 was identified as a potential proton donor [35]. An alignment of the deduced polypeptide sequence shows that the modular structure is conserved, with an N-terminal catalytic domain (aa 27–460) linked via a threonine rich linker (aa 461–493) region to the carboxyl terminal carbohydrate-binding module (aa 501–528). Prosite pattern search performed on the deduced FS010 protein sequence suggests a fungal cellulose-binding domain [42] signature pattern C-G-G-x(4,7)-G-x(3)-C-x(4,5)-C-x(3,5)-[NHGS]-x-[FYWM]-x(2)-Q-C (the four cysteine residues are involved in disulfide bonds) between amino acid 501 and 528.

Restriction analysis of the *P. chrysogenum cbh1* gene

In order to examine whether the *cbh1* gene is present in only one or multiple copies in the *P. chrysogenum* genome, Southern blotting was performed using total chromosomal DNA digested with different restriction enzymes (**Fig. 2**). A single hybridizing band is present in all the digestions. The hybridization result shows that *P. chrysogenum* has a single copy of the *cbh1* gene in its chromosomal DNA, which is the same as the reported findings in *T. reesei*, *T. viride*, and *T. aurantiacus* [37]. In contrast, *P. janthinellum* [4] has multiple copies of *cbh1* gene.

Northern blotting of *P. chrysogenum cbh1* transcription

To gain insight into the regulation of the *cbh1* gene in *P. chrysogenum* FS010, Northern blot was carried out by using the full-length *cbh1* gene probe, under high stringency. To ensure equal loading of each RNA sample,

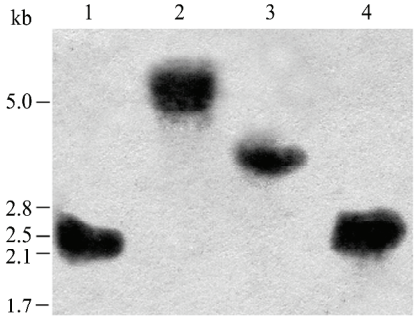

Fig. 2 Southern blot analysis of *cbh1*

Genomic DNA digested with the individual enzymes was fractionated and probed with fluorescein-labelled probe. 1, *Bam*HI; 2, *Eco*RV; 3, *Pst*I; 4, *Xho*I.

the membrane was rehybridized with 18S rRNA (approx. 1.5 kb) probe. The data obtained from the Northern blot analysis shown in **Fig. 3** indicated that Avicel strongly induces *cbh1* transcription. The signal intensity of Avicel induction was defined as 100%. The sophorose, cellobiose, gentiobiose, lactose and xylose induced 64%, 41%, 30%, 26% and 19% of *cbh1* expression. *D*-glucose, fructose and sorbitol could not induce any detectable levels of FS010 *cbh1* expression. Effects of various carbon sources to the *cbh1* transcription showed that CBHI of *P. chrysogenum* are inducible. Although sophorose, cellobiose, gentiobiose, lactose and xylose could induce the *cbh*1 transcription, the natural inducer of *P. chrysogenum* CBHI awaits further study. The addition of 1% glucose for 2 h to *P. chrysogenum* mycelia, previously cultured on Avicel (48 h), resulted in abolition of the *cbh1* signal (data not shown), indicating that the *P. chrysogenum* *cbh1* expression was subject to carbon catabolite repression.

Glucose repression in *Trichoderma* and *Aspergillus* species are mediated by the catabolite repressor Cre1 and CreA, respectively. These repressive proteins bound to specific target sequences in the promoters of cellulase genes and downregulated their transcription. Analysis of

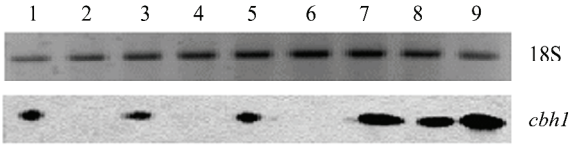

Fig. 3 Northern blot analysis of *P. chrysogenum cbh1* transcription with various carbon sources (1%, *W*/*V*)

1, lactose; 2, *D*-glucose; 3, xylose; 4, fructose; 5, gentiobiose; 6, sorbitol; 7, sophorose; 8, cellobiose; 9, Avicel cellulose.

the novel *cbh1* promoter from *P. chrysogenum* FS010 showed that six SYGGRG motifs are present. It is therefore likely that, as in other fungi, glucose repression in *P. chrysogenum* is also mediated through a CreA homologue.

Heterologous expression in *S. cerevisiae* H158

Although the amino acid sequence deduced from the nucleotide sequence of *cbh1* is homologous with other CBHs, whether it codes a CBH remained to be identified. Using RT-PCR, the *cbh1* cDNA was amplified and sequenced. The comparison of the *cbh1* cDNA sequence to the *cbh1* genomic sequence shows that *P. chrysogenum* FS010 *cbh1* gene is not interrupted by introns. The same result was obtained for the *A. aculeatus cbh1* gene [8], whereas all the other fungal *cbh1* genes sequenced, including from *P. janthinellum*, *T. reesei*, *A. nidulans* and *N. crassa* had their structural genes interrupted by introns at various positions. The expression plasmid was constructed as described above and designated as pAJ401-cbh1, which was introduced into *S. cerevisiae* H158.

A 62-kDa protein band from the cell-free extracts of H158-cbh1 was shown on SDS-PAGE, whereas no 62-kDa protein band from cell-free extracts of H158 harboring the plasmid pAJ401 was detected on SDS-PAGE (**Fig. 4**). Due to the hyperglycosylation in yeast, the molecular weight of recombinant CBHI (approx. 62 kDa) is different with the deduced size of the *cbh1* cDNA translate (53.5 kDa). The recombinant CBHI activity of cell extracts was measured against the pNPC under standard conditions. The specific activity of recombinant CBHI was 64.3 U/μg (total protein), suggesting that the *cbh1* cDNA from strain FS010 was successfully expressed in the *S. cerevisiae* H158. The comparison of the recombinant CBHI activity (64.3 U/μg) to the CBHI activity of *P. chrysogenum* FS010 (594.2 U/μg, total protein, unpublished data) showed that the CBHI activity in yeast transformant was low. No CBHI activity was detected in the supernatant of H158-cbh1cultures, suggesting that the recombinant CBHI could not be secreted into the medium.

To further confirm the 62-kDa protein was the product of the cDNA, the protein band was cut and analyzed by Edman degradation. The N-terminus amino acids analyzed were Q-V-G-T-S, identical to the deduced amino acid sequence from the 27th to 31st, indicating that the signal peptide of recombinant CBHI was recognized and cut in *S. cerevisiae* H158. Considering this 62-kDa recombinant protein had cellobiohydrolase activity and the N-terminal amino acid sequence, we concluded that *P. chrysogenum* FS010 *cbh*1 cDNA encoded a cellobiohydrolase.

Cellulase had been applied in a wide array of biotechnology ranging from biofuel production, paper making, food processing, biostoning, environmental bioremediation to stereoselective tools for separation of drug enantiomers [3,6,16,18]. Our research provides a new member for cellulase family and a novel experimental material for detailed research of the cellulase action mechanism. The investigation of *P. chrysogenum* cellulase gene and upstream regulatory sequence would be beneficial to the improvement of utilization of cellulosic substrate and research on the mechanism of cellulose degradation in *P. chrysogenum*. Further work will be needed to characterize the high-efficient expression of *cbh1* and the transcriptional factors in the *P. chrysogenum*.

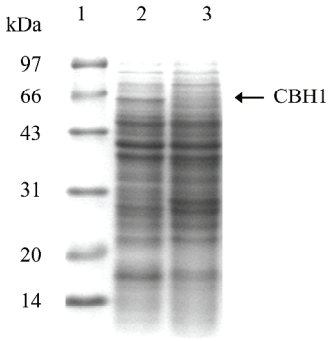

Fig. 4 SDS-PAGE of CBHI constitutive expressed in *S. cerevisiae* H158

1, molecular mass maker; 2, cell-free extracts from cultures of H158 cells harboring the plasmid pAJ401-cbh1; 3, cell-free extracts from cultures of H158 cells harboring the plasmid pAJ401.

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