Cloning of a novel levoglucosan kinase gene from *Lipomyces starkeyi* and its expression in *Escherichia coli*

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Abstract Levoglucosan, cellulosic pyrolysate, is converted to glucose-6-phosphate by a specific levoglucosan kinase in fungi. A novel cDNA of levoglucosan kinase gene (*lgk*) from yeast *Lipomyces starkeyi* YZ-215 was isolated by RACE method. The 1,445 bp cDNA fragment (*lgk*) harbouring the kinase gene exhibited one open reading frame (ORF) composed of 1,317 bp flanked by a 14 bp 5'-UTR and a 114 bp 3'-UTR, including a 25 bp poly(A) tail. The ORF encoded a 439 amino acid polypeptide with a 48.4 kDa predicted molecular mass. Analysis of amino sequence revealed that the kinase belonged to the bacterial anhydro-*N*-acetylmuramic acid kinase (AnmK) family, and kinase-like proteins existed in some fungi, especially in filamentous fungi such as *Aspergillus*. The kinase gene was transformed into *Escherichia coli* BL21 (DE3), recombinant *E. coli* could grow in M9 minimal medium with levoglucosan as a sole carbon source when induced by IPTG. In addition, the recombinant kinase was overexpressed, purified and characterized. The kinase was stable at pH 7–10 and showed maximum activity at 30°C and pH 9.0 as natural kinase, but presented higher thermostability. Kinetic constants (apparent K_m values) for LG and ATP

were 105.3 ± 12.5 and 0.20 ± 0.02 mM, respectively. Furthermore, the kinase showed substrate specificity for LG. This novel levoglucosan kinase gene would be useful in constructing recombinant microbial strains for the efficient bioconversion of cellulosic pyrolysate to ethanol.

Keywords Levoglucosan kinase gene ·
Lipomyces starkeyi YZ-215 · RACE · Cellulose pyrolysis

Introduction

Levoglucosan (1,6-anhydro- β -D-glucopyranose, LG) is very scarce in nature compared with other natural carbohydrates such as glucose, sucrose and fructose, and mainly exists in derivatives from natural pyrolysis of cellulosic materials such as woody and herbaceous biomass (Gao et al. 2003; Schauer et al. 2001). However, LG can be obtained in bulk under appropriate pyrolysis conditions using cellulosic materials (Brown et al. 2001; Prosen et al. 1993). LG can be used as a potential fermentation carbon and energy source in the fermentation industry, because LG can be metabolized directly by microorganisms or indirectly through mild acid hydrolysis to produce glucose (Nakagawa et al. 1984; Yu and Zhang 2003). Nakagawa et al. (1984) and Zhuang et al. (2001) have reported its use in itaconic acid and citric acid fermentation by *Aspergillus terreus* and *Aspergillus niger*, respectively.

As for direct bioconversion of LG, eukaryotic microorganisms have a different metabolic pathway from prokaryotic organisms. In *Arthrobacter* bacteria, LG is transformed into glucose through at least three enzymatic reactions (Nakahara et al. 1994). In yeasts and filamentous fungi, LG is directly converted to glucose-6-phosphate (one-step) by a specific levoglucosan kinase (LGK)

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(sense, corresponding to the amino sequence DDLVATT), P4: 5' IGTIGCIACIARRTCRTC 3' (antisense, corresponding to the amino sequence DDLVAT). For 3' RACE amplification, first PCR was carried out with P1 and GeneRacer™ 3' Primer, then Nested PCR with P3 and GeneRacer™ 3' Nested Primer; for 5' RACE amplification, first PCR was carried out with P4 and GeneRacer™ 5' Primer, then Nested PCR with P2 and GeneRacer™ 5' Nested Primer. The PCR products were ligated into pCR4®-TOPO® vector and sequenced. The primers of 3' end and 5' end of *lgk* gene were then synthesized, and the full-length cDNA of *lgk* gene was ultimately acquired.

Expression and purification of recombinant LGK

The coding region of *lgk* gene was ligated into pET-21a vector, and the resulted plasmid pET-*lgk*, carrying *lgk* gene under the control of the T7 promoter, was transformed into *E. coli* BL21 (DE3). Cells carrying pET-*lgk* were grown at 37°C with vigorous shaking in 100 ml liquid LB medium containing 100 µg of ampicillin/ml. After the culture grew to the mid-log phase, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and incubation was continued at 30°C for another 3 h. Aliquot culture about 1 ml was pelleted by centrifugation and total cell proteins were analyzed by SDS–PAGE. Remainder of culture was harvested by centrifugation and washed twice with 20 mM Tris–HCl buffer (pH 8.0) containing 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were then suspended in 20 ml buffer and ruptured by sonication. After centrifugation at 20,000g for 20 min at 4°C, the supernatants were collected for purification.

Purification works were carried out following the procedures as described by Ning et al. (2008). All enzyme purification procedures were performed at 4°C. In brief, the crude extract was treated by 40% ammonium sulfate and the supernatant was precipitated by 65% ammonium sulfate. The pellet was dissolved in 20 mM Tris–HCl buffer (pH 8.0, 1 mM EDTA, 1 mM DTT) and pretreated using an Amicon Ultra-15, 10 K centrifugal filter device (Millipore), followed by sequential chromatographic steps with HiTrap Desalting column, Resource Q and Superdex 200™ 200 10/300 GL columns (Amersham Biosciences, USA). During the chromatographic steps, fractions with high kinase activity were pooled, and the manipulations were performed on an Akta FPLC system (Amersham-Pharmacia Biotech).

Enzyme assays

Enzyme assays were carried out following the procedures as described by Ning et al. (2008). The reaction buffer was

50 mM Tris–HCl buffer (pH 9.0), and the reaction mixture contained 75 mM LG, 2 mM ATP, 0.2 mM NADP, 10 mM MgCl₂ and 1 U glucose-6-phosphate dehydrogenase. Total soluble protein concentration was determined by the Bradford method (Tiangen) using bovine serum albumin (BSA) as the standard.

Characterization of recombinant LGK

The effects of temperature between 15 and 45°C on enzyme activity and stability were determined. Thermostability was determined by incubating the enzyme for 30 min at the designated temperature in 50 mM Tris–HCl buffer (pH 9.0). The effects of pH on enzyme activity were determined by varying the substrate pH between 5.5 and 11.0. The buffers used were 50 mM sodium citrate for pH 5.5–6.5, 50 mM triethanolamine-HCl/NaOH for pH 7.0–9.0, and 50 mM glycine-NaOH for pH 9.0–11.0. To determine the pH stability of enzyme, the enzyme was incubated at the designated pH for 30 min at 30°C and the residual activity was then measured in 50 mM Tris–HCl buffer (pH 9.0). The apparent K_m values were measured by determining the enzymatic activity at substrate concentration ranging from 10 to 100 mM for LG and 0.2 to 2 mM for ATP. To determine the substrate specificity, the reaction mixture contained LG and its different analogs, and concentration was 25 mM, respectively.

Chemicals

Analogs of LG: 1,6-anhydro-β-D-glucosamine (anhGlcN) and 1,6-anhydro-N-acetyl-β-D-glucosamine (anhGlcNAc), both with 98% purity, were synthesized in Beijing Carbo-mex Biotech Co., Ltd.

Results and discussion

Cloning and characterization of *lgk* gene from *L. starkeyi* YZ-215

In order to isolate *lgk* gene, RT-PCR amplification was applied to clone the gene. Four degenerate primers were designed from two of three identified peptides (Ning et al. 2008), and 5' RACE and 3' RACE amplifications were conducted. Two fragments about 761 and 555 bp corresponding to the 5' and 3' region were obtained, respectively. The deduced amino sequence of 5' fragment contained the third identified peptide TGLTSLTDFR. The full-length cDNA was a 1,445 bp fragment containing a 1,317 bp open reading frame (ORF), which was flanked by a 14 bp 5'-UTR and a 114 bp 3'-UTR, including a 25 bp poly(A) tail. The stop codon was TAATGA with two

Fig. 2 Phylogenetic analysis of LGK from *L. starkeyi* YZ-215, LGK-like proteins from fungi and AnmK from *E. coli* MG1655. The phylogenetic relationship was analyzed by the ClustalX2.03 (Thompson et al. 1997) program and drawn by TreeView 1.6.6 (Roderic, D. M. Page, <http://taxonomy.zoology.gla.ac.uk/rod/rod.html>) program. Numbers show results from bootstrap analyses (1,000 bootstrap replicates). Protein accession numbers: [1], EDU45785; [2], EDN99772; [3], EDN25583; [4], EAQ93900; [5], EAW25579; [6], EAL84290; [7], EDP47186; [8], EAA29125; [9], EAW24529; [10], EDP50956; [11], EAL86194; [12], EDK40502; [13], ABN66269; [14], EAW22768; [15], EAQ84482; [16], EAA32985; [17], CAK44911; [18], EAU33314; [19], BAE65712; [20], EED45075; [21], EAW24693; [22], EDP50844; [23], EAL89335; [24], EAA59060; [25], CAP80499; [26], EED14977; LGK: ACE79748; AnmK, AAC74712

continuous stop codons and was followed by several other stop codons that were both in and out of frame. The ORF encoded a 439 amino acid polypeptide with a 48.4 kDa predicted molecular mass, which is similar to natural LGK's mass, 48 and 47.7 kDa, identified by SDS-PAGE and MALDI-TOF MS (Ning et al. 2008), respectively. This suggests that LGK would not be glycosylated in *L. starkeyi*. No signal peptide was found with SignalP 3.0 program (<http://www.cbs.dtu.dk/services/SignalP/>). The nucleotide sequence of *lgk* gene was deposited in the GenBank database under accession number EU751287.

Comparison and alignment of amino acid sequences

The deduced amino sequence of *lgk* gene was searched in NCBI (<http://www.ncbi.nlm.nih.gov/>) using the BLASTP program. The results indicated that LGK belonged to the bacterial anhydro-*N*-acetylmuramic acid kinase (AnmK)

family which is Clusters of Orthologous Groups (COGs). The AnmK family was sorted into two groups: one group was AnmK which existed widely in bacteria, and the other was LGK-like proteins which existed in some fungi, especially in filamentous fungi such as *Aspergillus*. Some fungi have multiple LGK-like proteins. For example, *Aspergillus fumigatus* has three variant multiples, *Neosartorya fischeri* has four. In addition, *A. niger* has LGK-like protein, yet it showed no similarity to the LGK reported in *A. niger* (Xie et al. 2005). In particular, it is worth noting that all sequenced yeast strains of *Saccharomyces cerevisiae* do not have LGK-like protein. This accounts for why only one strain of *S. cerevisiae* with LG-assimilating capability has been screened up to now (Prosen et al. 1993).

In the AnmK family, the function of AnmK was approved by experiments in *E. coli* (Uehara et al. 2005), whereas LGK-like proteins in fungi were hypothetical

proteins. Similar to the catalytic mechanism of LGK, AnmK cleaves the 1,6-anhydro bond of 1,6-anhydro-*N*-acetylmuramic acid (anhMurNAc) and simultaneously phosphorylates the sugar to form *N*-acetylmuramic acid-6-phosphate (MurNAc-P). LGK shares 33.0% amino acid identity to AnmK.

Multiple alignments of amino acid sequence were performed using the ClustalX2.03 program (Thompson et al. 1997). The results revealed that LGK had a variant identity from 29.9 to 77.5% to LGK-like proteins in fungi with the average of 52.3%, while AnmK had a variant identity from 20.4 to 37.1% to LGK-like proteins with the average of 27.1%. A phylogenetic tree of LGK, LGK-like proteins and AnmK based on multiple alignments is shown in Fig. 2.

At present, owing to limited fungi genomes decoded, only 16 species were presented LGK-like proteins. Considering the *N*-acetylmuramic acid (NAM) not existing in fungi, it is suggested that LGK-like proteins would be levoglucosan kinases in fungi.

Expression of *lgk* gene in *E. coli*

Expression plasmid pET-*lgk*, together with empty pET-21a as a control, was transformed into *E. coli*. The maximum activity of the crude extract was 7.9 U/mg. The analysis of cell free extract by SDS-PAGE shows that

Fig. 3 SDS-PAGE profile of recombinant LGK overexpressed in *E. coli* BL21 (DE3)/pET-*lgk*. The protein samples were separated on 12.0% SDS-PAGE with Coomassie blue R-250. Lane 1 protein molecular weight marker; lane 2 crude extract of *E. coli* BL21 (DE3)/pET-21a; lane 3 crude extract of *E. coli* BL21 (DE3)/pET-*lgk*; lane 4 purified recombinant LGK

Fig. 4 Growth of *E. coli* BL21 (DE3)/pET-*lgk* in M9 minimal medium plate. The top panel is *E. coli* BL21 (DE3)/pET-*lgk*, the lower panel are controls *E. coli* BL21 (DE3)/pTanmK and *E. coli* BL21 (DE3)/pET-21a. M9 minimal medium was modified with 1.0% LG instead of glucose; concentration of IPTG was 0.1 mM. *E. coli* BL21 (DE3)/pTanmK (Uehara et al. 2005), a kind gift from Professor Dr. James T. Park, carries *anmK* gene under the control of the T7 promoter of pGEM-T

Fig. 5 Effects of temperature (A) and pH (B) on the activity (■) and stability (●) of recombinant LGK from *E. coli* BL21 (DE3)/pET-*lgk*

Table 1 Growth comparison of pET-21a, pTanmK, pET-*lgk* and *L. starkeyi* in liquid MM

Strains	Carbon sources in MM				
	LG	anhGlcN	anhGlcNAc	GlcN	GlcNAc
pET-21a ^a	–	–	–	+++++	+++++
pTanmK ^a	–	–	+	+++++	+++++
pET- <i>lgk</i> ^a	+++++	+++	++	+++++	+++++
<i>L. starkeyi</i> ^b	+++++	–	–	–	–

“+++++” denotes stationary phase of growth. Concentration of carbon source was 1.0%, respectively. Temperature of cultivation was 30°C, time was about 72 h. The tests were conducted in small vials containing 5 ml liquid MM

^a M9 minimal medium

^b CD minimal medium

recombinant LGK is overexpressed and its molecular mass is similar to theoretic calculation 48.4 kDa (Fig. 3). Furthermore, in liquid M9 MM with 1.0% LG as a sole carbon source, recombinant pET-*lgk* would grow and reach stationary phase about 15 h under 0.1 mM IPTG. As shown in Fig. 4, pET-*lgk* can grow in M9 MM plate, while pTanmK and pET-21a cannot. This indicates that recombinant LGK has activity for LG and AnmK has not, although they have similar catalytic mechanism.

Properties of recombinant LGK

The optimum temperature and pH were 30°C and pH 9.0, respectively (Fig. 5), which was consistent with natural LGK (Ning et al. 2008). Compared with natural LGK, recombinant LGK seems to be more thermostable, for recombinant LGK still had 57% relative activity after incubation at 35°C, while natural LGK lost its activity rapidly. In addition, recombinant LGK nearly had no activity at pH 6.5, while natural LGK had 20% relative activity. Recombinant LGK was stable between pH 7–10 as natural LGK.

Recombinant LGK had a higher apparent K_m value for LG (105.3 ± 12.5 mM) than natural LGK (68.6 mM), whereas the K_m value for ATP was similar (0.20 ± 0.02 mM).¹ Presumably, natural LGK would be an oligomer containing binding partner that is important for the activity of LGK.

It was previously shown that LGK had no activity for various analogs of LG including D-glucosamine (GlcN), N-acetyl-D-glucosamine (GlcNAc), 1,6-anhydro-β-D-galactopyranose, 1,6-anhydro-β-D-mannopyranose, 1,6-anhydro-maltose and 2,7-anhydro-β-D-altro-sedoheptulose, and these compounds had no inhibitions to the enzymatic activity of LGK (Kitamura and Yasui 1991). We assumed that anhGlcN or anhGlcNAc might be the substrate of

LGK, for they are transitional structural analogs between LG and anhMurNAc. However, in vitro, anhGlcN or anhGlcNAc had no inhibition to the enzymatic activity of LGK. In vivo, *L. starkeyi* does neither utilize them, nor GlcN and GlcNAc, which suggests that *L. starkeyi* would have no metabolism pathways for them in the cell or they could not enter the cell. In addition, Table 1 indicates that LGK has low activity for them and AnmK has faint activity for anhGlcNAc. It's reasonably deduced that LGK should be more compatible with LG than with anhGlcN or anhGlcNAc and shows substrate specificity for LG, while AnmK shows stricter substrate specificity.

In contrast to the *lgk* genes previously isolated (Zhang and Zhang, 2002; Xie et al. 2005), in this study, the deduced amino sequence of *lgk* gene is homologous to that of bacteria's AnmK, whose catalytic mechanism is similar to that of LGK, in addition, the crude extract had high activity and recombinant *E. coli* could grow in M9 MM with LG as a sole carbon source. This implies that they are different *lgk* genes, and the *lgk* gene isolated in this study is a novel and more useful gene for the bioconversion of LG.

Owing to LGK's low affinity for LG, direct bioconversion of LG is apparently bottlenecked. Thus, it is necessary to reconstruct LGK to enhance the affinity for LG. Recently, in the AnmK family, the 3D structure of AnmK from *Shewanella oneidensis* MR-1 was resolved with the method of X-ray Diffraction (Protein Data Bank accession no. 3cgy). It is promising to elucidate the detailed catalytic mechanism of LGK by virtue of homolog-modeling and to devise a high affinity enzyme for LG, and to construct efficient genetic microbial strains to convert LG to useful products such as ethanol. These are in progress.

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¹ The K_m value of natural LGK for ATP was 0.68 mM by Ning et al. (2008). We measured it again and affirmed that it was 0.20 mM.

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