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Molecular cloning, characterization and expression of a gene encoding phosphoketolase from *Termitomyces clypeatus*

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ABSTRACT

A phosphoketolase (*pk*) gene from the fungus *Termitomyces clypeatus* (TC) was cloned and partially characterized. Oligonucleotide primers specific for the phosphoketolase gene (*pk*) were designed from the regions of homologies found in the primary structure of the enzyme from other fungal sources related to TC, using multiple sequence alignment technique. The cDNA of partial lengths were amplified, cloned and sequenced in three parts by 3′ and 5′ RACE and RT-PCR using these oligonucleotide primers. The full length ds cDNA was constructed next by joining these three partial cDNA sequences having appropriate overlapping regions using Overlap Extension PCR technique. The constructed full length cDNA exhibited an open reading frame of 2487 bases and 5′ and 3′ UTRs. The deduced amino acid sequence, which is of 828 amino acids, when analyzed with NCBI BLAST, showed high similarities with the phosphoketolase enzyme (Pk) superfamily with expected domains. The part of the TC genomic DNA comprising of the *pk* gene was also amplified, cloned and sequenced and was found to contain two introns of 68 and 74 bases that interrupt the *pk* reading frame. The coding region of *pk* cDNA was subcloned in pKM260 expression vector in correct frame and the protein was expressed in *Escherichia coli* BL21 (DE3) transformed with this recombinant expression plasmid. The recombinant protein purified by His-tag affinity chromatography indicated the presence of a protein of the expected size. *In vivo* expression studies of the gene in presence of different carbon sources indicated synthesis of Pk specific mRNA, as expected. Phylogenetic studies revealed a common ancestry of the fungal and bacterial Pk. The TC is known to secrete several industrially important enzymes involved in carbohydrate metabolism. However, the presence of Pk, a key enzyme in pentose metabolism, has not been demonstrated conclusively in this organism. Cloning, sequencing and expression study of this gene establishes the functioning of this gene in *T. clypeatus*. The Pk from TC is a new source for commercial exploitation.

1. Introduction

Fungi are capable of living on dead plant materials and consume necessary nutrition by degrading the complex carbohydrates like cellulose, hemicellulose, xylose, lignin etc. present in the biomass to their simpler forms. These complex carbohydrates are abundant in the biomass. However, in order for them to utilize abundant pentose resources in the biomass they should have the metabolic capability in the form of pentose utilization pathway. The enzyme phosphoketolase (Pk) is an important player in such a pathway. Phosphoketolases (EC 4.1.2.9, EC 4.1.2.22) are thiamine diphosphate (ThPP)-dependent enzymes found to be mainly involved in pentose phosphate pathway of heterofermentative and facultative homofermentative lactic acid bacteria and D-fructose-6-phosphate shunt of bifidobacteria and can act upon fructose-6-phosphate and xylulose-5-phosphate [1,2]. There are reports of the presence of Pk in some yeast and filamentous fungi like *Penicillium chrysogenum*, *Aspergillus nidulans* etc. and in the insect pathogen *Metarhizium anisopliae* [3–6]. Panagiotou et al. have reported the first evidence that the phosphoketolase pathway plays a significant role in the central carbon metabolism of filamentous fungi [6]. Studies of Thykaer and Nielsen in *P. chrysogenum* using ^{13}C-labeling analysis revealed an unrecognized pathway responsible for the formation of cytosolic acetyl-CoA [5]. Previously published ^{13}C data from *A. nidulans* also showed the same kind of trend. So, it was assumed that Pk activity could be contributing to the formation of acetyl-CoA in these fungi. From genomic sequencing information in *A. nidulans*, the targets for a pathway from xylulose-5-phosphate to acetate were identified [5].

Pk is also a very useful enzyme in the central carbon metabolism of filamentous fungi as well as bacteria from industrial point of view

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[6,7]. Chinen et al. have designed a pathway for efficient L-Glu production utilizing phosphoketolase to bypass the CO_2 releasing pyruvate dehydrogenase reaction [7]. They have cloned *pk* gene from *Bifidobacterium animalis* and over expressed in *Corynebacterium glutamicum*. In yet another study, utilization of phosphoketolase in the production of mevalonate, isoprenoid precursors and isoprene was done by Beck et al. [8]. Phosphoketolase pathway is absent in the preferred microorganism, the yeast *Saccharomyces cerevisiae*, for alcoholic fermentation. Because of this absence, *S. cerevisiae* is not capable of metabolizing the most abundant hemicellulosic pentoses such as arabinose and xylose [9]. These two sugars are present in the biomass in extremely large quantities, so much so that they could serve as cheap raw materials for low-cost bioethanol production. So, in the past decade, lots of researches were done to install pentose utilization capabilities in yeast [10–13]; and phosphoketolase pathway was installed to produce pentose utilizing yeast to establish an economically viable process of bio-ethanol production from non-starch substrates [14]. Our model organism TC is a filamentous fungi and an edible mushroom [15,16]. It is capable of producing several industrially important enzymes like Cellobiase, Cellobiose dehydrogenase, Amyloglucosidase, Xylanase etc. [16–18]. Many of these enzymes have been characterized biochemically earlier from this source. However, in spite of its economic potentials because of the natural secretions of several important carbohydrate degrading enzymes mentioned earlier, the pentose utilization through the Pk pathway in this fungus TC has not been studied in detail. Recently Pal et al. have mentioned about the possible presence of pentose phosphate pathway and the utilization of xylose via xylulose-5-phosphate [19] in this fungus. In the present study we report cloning and partial characterization of the *pk* gene from TC. We have also demonstrated its transcriptional expression in response to the presence of xylose in growth media which is a clear evidence of the presence of this pathway in TC.

2. Materials and methods

2.1. Microbial strains and growth conditions

The white-rot fungus TC was a kind gift from Dr. S. Sengupta from IICB, Kolkata. The microbial strains and plasmids used in this study are described in Table S1. For routine maintenance and DNA isolation, the fungus was grown in inorganic complex media containing glucose (5%) as described by Ghosh and Sengupta [20] and Mukherjee et al. [21] . For RNA isolation, TC cells were grown in an induction media [20,21] containing cellulose (1%) as the carbon source. The cells were grown routinely at 30 °C in a rotary incubator cum shaker at 120 rpm. *Escherichia coli* DH5α and BL21 (DE3) (Table S1) were maintained at 4 °C in LB (Luria–Bertani) agar slants or plates and grown at 37 °C with 180–200 rpm.

2.2. Primer designing

Pk enzyme sequences from different fungal sources like *Aspergillus clavatus*, *Aspergillus terreus*, *Botryotinia fuckeliana*, *Cryptococcus neoformans*, *M. anisopliae*, *Neosartorya fischeri*, *Talaromyces stipitatus* were collected from the NCBI database. Multiple sequence alignment was done with the above sequences by using the software Multalin [22]. From the alignment data, suitable regions of homologies were selected. Nucleotide sequences corresponding to these selected homologous regions were next obtained from NCBI database and aligned using the computer program Multalin [22]. From these nucleotide sequence alignment data, degenerate oligonucleotide forward and reverse primers specific for *pk* were designed manually following standard parameters such as GC contents, melting temperature, size etc. As the work progressed, additional primers were also designed from the obtained sequence data (primer walking) of TC *pk* in order to amplify and clone the full length cDNA. After obtaining the putative full length *pk* cDNA of TC, its 5′ and 3′ UTR sequence data were utilized to design primers needed for amplifying and cloning its genomic region. The details of the primers are listed in Table S1. The oligonucleotide primers were supplied by Operon, IDT, Chromous Biotech, Xcelris genomics and Sigma–Aldrich.

2.3. Molecular cloning and analysis of phosphoketolase cDNA, gene and protein

Standard methods were used for the cloning of the phosphoketolase cDNA and then the gene itself from the chromosome of TC. The cDNA was cloned in an expression vector and the His-tagged protein was analyzed in SDS-gel. The detailed methods are described in the supplementary Sections *2.3.1s, 2.3.2s, 2.3.3s* and *2.4s*. Phylogenetic analysis is described in supplementary Section *2.5s*.

2.4. DNA sequencing and analysis

Sequencing runs were carried out using dideoxy method [23] in ABI 3500XL/3730XL sequencers. Commercial service providers Chromas Biotech, GCC Biotech and Xcelris Genomics carried out the sequencing runs. All the sequence data were also checked by Multalin [22] for doing multiple sequence alignment with the *pk* gene sequences from the other fungal and bacterial sources. The sequence data was checked by NCBI ORF finder program to find out the open reading frame (ORF) and to deduce amino acid sequence from the nucleotide sequence. NCBI BLAST program was used to check whether the gene sequence contains any conserved domains specific for Pk. PROSITE was also used to further analyze the sequence to determine if there are any patterns within it which can be designated as Pk signature sequence [24,25].

2.5. Nucleotide sequence accession number

The deduced nucleotide sequence reported here for phosphoketolase of TC was deposited in GenBank with accession number KF690709.

2.6. Expression study of the native pk mRNA

Expression of *pk* gene within the cells in presence of different carbon sources was checked by RT-PCR as explained earlier [26,27]. TC was grown for 5 days in presence of sole carbon sources cellulose, glucose, ribose and xylose separately in the induction media described earlier. Total RNA was isolated from the cells grown in each of these induced cultures. cDNAs were synthesized from these total RNA preparations using the gene specific reverse primer 1-CH. Then PCR was done using primer 1-CH and another gene specific forward primer PCDH-f2 using these cDNA preparations as templates, separately (RT-PCR). For control, same primer was used to amplify the same region from the full length cDNA and *pk* genomic DNA separately. The primer pairs are supposed to amplify a fragment of length 704 bp from cDNA and 846 bp from the genomic DNA (because of the presence of two introns).

3. Results

3.1. Amplification and cloning of full length pk cDNA and sequence analysis

The full length cDNA of phosphoketolase from TC was cloned in three parts (Parts 1–3) from total RNA of *Termitomyces clypeatus* as

described in the Section 2. The primer information, locations, directions, sizes and overall cloning strategies are shown in Table S1 and Fig. S1. Identities of each of these three fragments were tested extensively with internal nested primers and in some cases by expected restriction site analyses. All the three parts were sequenced in both strands and had overlapping regions (Fig. S1). These overlapping regions were utilized for sequential joining of these three parts (Overlap Extension PCR) to obtain the full length cDNA sequence [28]. The integrity of the joined putative full length cDNA was confirmed by sequencing the two overlapping junction regions where the fragments were joined to each other. When analyzed, the sequence data of the full length cDNA exhibited a continuous open reading frame of 2487 bp and coded for a polypeptide of 828 amino acids (Fig. S2) with a calculated molecular mass of approximately 93408.3 Da. This molecular size was very close to the previously reported molecular masses of other Pk enzymes studied. Additionally, this amino acid sequence data showed high similarities with the Pk enzyme from different fungal as well as bacterial sources (Fig. S3). PROSITE analysis showed that the sequence contains consensus Pk signature 1, which was EGGELGY from amino acid 162–168 and Pk signature 2, which was GavfDnPdlIaltMvGDGE (TPP binding motif) from amino acid 175–193 (Fig. S3); [24,25,29]. Phosphoketolases contain a domain common to transketolases and other TPP containing enzymes. TPP is related to intermediary metabolism in many ways and acts as the coenzyme for different types of carbohydrate degrading reactions [29]. So identification of these major domains found in all other Pk confirmed the identity of the cloned cDNA being *pk* gene (Fig. S3). When analyzed with NCBI BLAST tool also, the deduced amino acid sequence showed that the gene is of *XFP* superfamily having regions belonging to *TPP* binding superfamily and *XFP* C-terminal superfamily (Fig. 1) like the other phosphoketolases. This was also a major proof of the cloned cDNA being that of *pk*. 3′ and 5′ untranslated regions of mRNA were identified before start codon and after the stop codon respectively. No poly-(A) signal of the sequence AAUAAA was identified as such, but poly-(A) signal like sequences (AACAAA and AAUAAC) were identified 19 bp and 29 bp upstream from the polyadenylation site respectively (Fig. 2B) [30].

3.2. Organization of the phosphoketolase gene of T. clypeatus

The part of the genomic DNA comprising the *pk* gene was also isolated, cloned and sequenced. For this purpose total genomic DNA of the fungus was isolated and amplified using two primers designed from the outermost parts of the 5′ and 3′ UTRs. The amplified products were further refined by nested PCR and the final amplified product (Fig. 2A) was cloned, sequenced and analyzed against the full length cDNA. The data revealed that the open reading frame of the cDNA is interrupted by 2 introns of 68 bp and 74 bp respectively (Fig. 2B, Fig. S4) in the genome of TC. Both of them had typical intron signals, identified at 5′-end by GTAAG (consensus for filamentous fungi: G/GTANG) and at 3′-end by TAG (consensus for filamentous fungi: (C/T) AG) [31]. A schematic presentation, based on our studies, of the genomic organization of the phosphoketolase from this fungus is presented in Fig. 2B.

3.3. Expression and purification of cloned phosphoketolase protein

The cloned full length cDNA was subcloned in an expression vector pKM260 with an in-frame 6-residues His-tag at the end of the cloned sequence (Fig. 3A) and transformed into appropriate *E. coli* strain. Following induction (Fig. 3B, lane 4) and purification of the expressed proteins using affinity chromatography, the expressed protein could be recovered as a single protein band of about the expected size (Fig. 3B, lane 6). This was a proof that the three parts of the cDNA were joined together properly to produce the full ORF and also of the expression of the cDNA at the protein level. As expected, the negative control did not show any protein band (Fig. 3B, lane 5).

3.4. RT-PCR analysis of mRNA expression of pk under different carbon sources

There was no prior information regarding the expression of *pk* gene in TC. Two primers 1-CH and PCDH-f2 were designed in such a way that when the *pk* cDNA was used as template, it would amplify a fragment of 704 bp (Fig. 4, lane 5); however, if the template was genomic DNA containing the *pk* gene locus, the amplified fragment would be of size 846 bp (Fig. 4, lane 6) due to the presence of two introns in it (Fig. 2B, Fig. S4). The total RNA were isolated from TC cultures induced with four different sugars as the carbon sources separately; the isolated total RNA was subjected to RT-PCR to make the cDNA in the first step using primer 1-CH and the resulting cDNA was subjected to PCR in the second step using 1-CH and PCDH-f2 primers to amplify the region in between. As seen in Fig. 4 (lanes 1–4), the RT-PCR product in all the induced cultures exhibited a

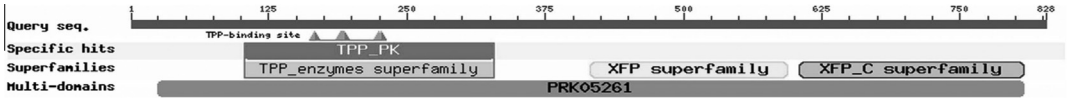

Fig. 1. Different domains specific for Pk enzyme, present in the Pk sequence from TC. It consists of the *TPP* binding domain, which is a major domain in all the *TPP* dependent enzymes and the XFP superfamily.

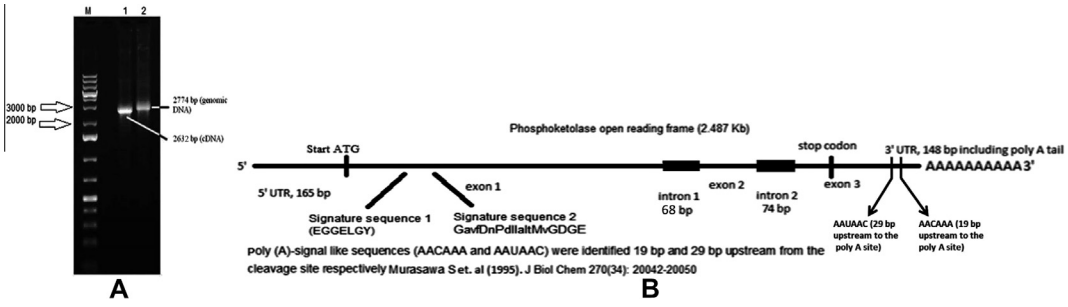

Fig. 2. (A) cDNA of phosphoketolase and the corresponding genomic DNA fragment of TC in 1% agarose gel, and (B) the organization of the phosphoketolase gene. (A) Lane M: 1 Kb DNA ladder (Fermentas), lane 1: cDNA of TC *pk* amplified using the primer pairs TCPK-f2 and TCPK-a, lane 2: genomic DNA amplified using the same primer pairs TCPK-f2 and TCPK-a. Genomic DNA fragment is showing slightly larger size in agarose gel because of the presence of two introns in it.

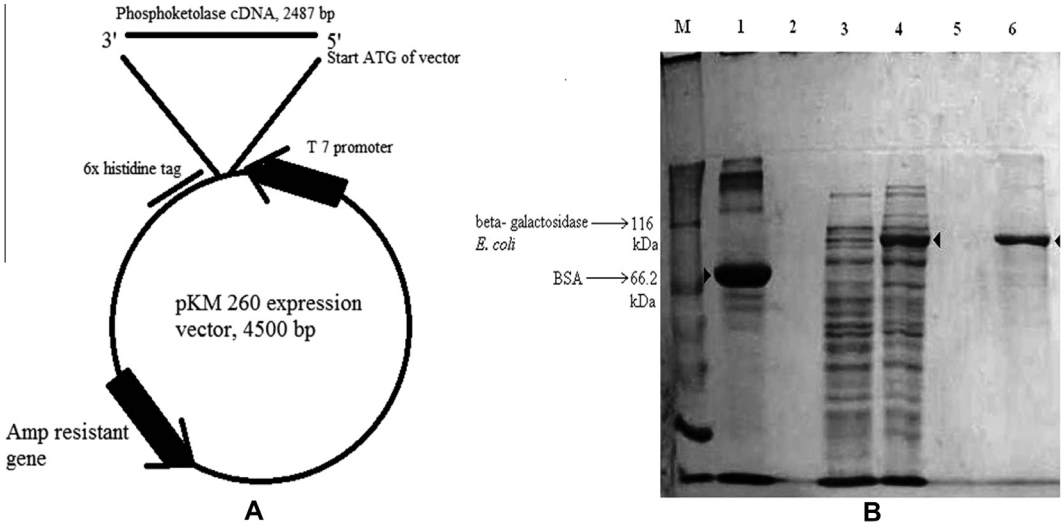

Fig. 3. (A) Cloning strategy of *pk* cDNA in expression vector pKM260. (B) Analysis of the expressed protein by SDS–PAGE. (B) Lane M: protein molecular weight standards, lane 1: Bovine Serum Albumin, lane 2 is blank, lane 3: cell lysate of uninduced *E. coli* containing recombinant pKM260 (XFP full-pKM260 (Table S1), lane 4: cell lysate of *E. coli*, induced with IPTG, containing recombinant pKM260 (XFP full-pKM260 (Table S1), lane 5: His-tag affinity matrix eluted sample from IPTG uninduced recombinant *E. coli* cells, lane 6: His-tag affinity matrix eluted sample from IPTG induced recombinant *E. coli* cells.

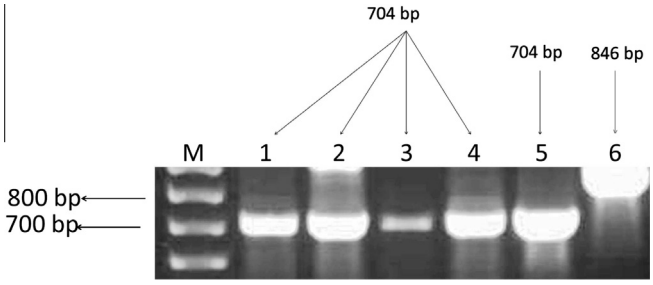

Fig. 4. Amplification of partial length *pk* cDNA. Lane M: 100 bp DNA ladder markers. Lane 1–4: 704 bp partial *pk* cDNA amplified using 1-CH and PCDH-f2 gene specific primers from the total RNA from cells grown in presence of cellulose, glucose, ribose, xylose respectively, lane 5: 704 bp fragment as control from the cloned *pk* cDNA using the same primers, lane 6: 846 bp fragment containing two introns from the genomic DNA using the same primers.

distinct and clear band at 704 bp and no band in the 846 bp region was visible in any of these four cases (Fig. 4, lanes 1–4). So, this was a clear proof of the RT-PCR products being part of the *pk* cDNA (without any of the introns). This in turn proved the presence of *pk* mRNA in the total RNA preparations of all the induced cultures (Fig. 4, lanes 1–4) which were reverse transcribed to *pk* cDNA (in the first step) and finally amplified to obtain the 704 bp fragment (second step). So, it was a clear indication that the *pk* gene was inducible by all of the above carbon sources. That the amplified bands actually did not originate from the genomic DNA but only from the specific expressed *pk* mRNA was confirmed because in the former case the band size would have been 846 bp (because of the inclusion of the two introns in it) and not 704 bp. The two primers chosen for this experiment intentionally encompassed the introns in genomic DNA locus specific for the *pk* gene; this was done to avoid misinterpretation that the amplified 704 bp fragment in the RT-PCR was from the genomic DNA contaminations. This finding that *pk* mRNA was inducible with ribose and xylose (Fig. 4, lanes 3, 4) was important because this furnished the proof that pentose utilizing pathway was operational in *T. clypeatus* (TC).

3.5. Phylogenetic analysis

Pks are present in both eukaryotes and bacteria [32]. Previous structural and phylogenetic analyses of proteins of TPP-dependent family had shown that their domains were common to that of transketolases and they were structurally and evolutionarily related [32–34]. The studies of the Duan revealed that there are two types of fungal Pk named SF I and SF II (Subfamily I and II) and the Pk from *M. anisopliae* comes under the SF I category [4]. Phylogenetic tree analysis of the TC Pk among different fungal as well as bacterial Pk revealed that the TC Pk was in the same clade with Pk of *M. anisopliae* while it appears to be distantly related to that of *Bifidobacterium* sp. (Fig. S5). Evolutionary relationship study also additionally confirms the identity of cloned cDNA from TC to be that of a phosphoketolase.

4. Discussion

Phosphoketolases are industrially important enzymes. It has uses in the industrial production of L-glutamic acid, mevalonate, isoprenoid precursors, isoprene, etc. [7,8]. Also, the phosphoketolase pathway is required in the utilization of pentose sugar. Absence of this pathway in *S. cerevisiae* makes this organism incapable of utilizing the most abundant pentose sugar xylose found in the biomass for the cheap production of biofuel ethanol [9]. For this reason, there have been many efforts to install a pentose utilizing system from heterologous systems into *S. cerevisiae* so that it acquires this capability [10–14]. In this study we have cloned the full length cDNA of *pk* from a new source, the basidiomycetes fungus *T. clypeatus* and sequenced it. The sequence analysis of the cloned cDNA showed the presence of phosphoketolase signature sequences, 3′ & 5′ UTR, start and stop codons and a poly A tail. Phylogenetic study showed the relatedness and divergence of the gene among the other fungal as well as bacterial sources. Upon analysis of the genomic DNA sequence it was found that there are two putative introns having consensus intron–exon junction sequences in the reading frame. The cDNA was cloned starting from the putative start codon in an expression vector under the control of T7 promoter and expressed in *E. coli*. SDS–PAGE revealed that the expressed protein was of the expected size observed for Pk from other organisms. It is also a confirmation of the putative start ATG as the true transcription start site. Upon induction with different carbon sources in culture media, it was found that the gene is inducible by cellulose, ribose, xylose and glucose. The finding that xylose is able to induce synthesis of the *pk* mRNA strongly indicate

the presence of the pentose phosphate like pathways operating in this fungus. In view of the industrial importance of this enzyme, further studies relating to the enhancement of the expression of this Pk under the control of specific promoters or by protein engineering in specific hosts could be undertaken for the betterment of the expressed protein.

Conflicts of interest

The authors report no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.054.

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