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BIOCATALYSIS

Discovery and characterization of a novel ATP/polyphosphate xylulokinase from a hyperthermophilic bacterium *Thermotoga maritima*

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Abstract Xylulokinase (XK, E.C. 2.7.1.17) is one of the key enzymes in xylose metabolism and it is essential for the activation of pentoses for the sustainable production of biocommodities from biomass sugars. The open reading frame (TM0116) from the hyperthermophilic bacterium *Thermotoga maritima* MSB8 encoding a putative xylulokinase were cloned and expressed in *Escherichia coli* BL21 Star (DE3) in the Luria–Bertani and auto-inducing high-cell-density media. The basic biochemical properties of this thermophilic XK were characterized. This XK has the optimal temperature of 85 °C. Under a suboptimal condition of 60 °C, the k_{cat} was 83 s^{-1}, and the K_m values for xylulose and ATP were 1.24 and 0.71 mM, respectively. We hypothesized that this XK could work on polyphosphate possibly because this ancestral thermophilic microorganism utilizes polyphosphate to regulate the Embden–Meyerhof pathway and its substrate-binding residues are somewhat similar to those of other ATP/polyphosphate-dependent kinases. This XK was found to work on low-cost polyphosphate, exhibiting 41 % of its specific activity on ATP. This first ATP/polyphosphate XK could have a great potential for xylose utilization in thermophilic ethanol-producing microorganisms and cell-free biosystems for low-cost biomanufacturing without the use of ATP.

Keywords Biomanufacturing · Cell-free biosystem · In vitro synthetic biology · Polyphosphate · Thermoenzyme · *Thermotoga maritima* · Xylulokinase

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Introduction

The economically viable production of biofuels and biochemicals from non-food biomass must utilize all biomass sugars—including hexoses and pentoses [18, 47]. D-Xylose is the most abundant pentose, a major component of plant cell walls. For biotransforming xylose to biocommodities, D-xylose is first converted to D-xylulose followed by phosphorylation mediated by xylulokinase (XK, EC 2.7.1.17). Xylulose-5-phosphate is further metabolized through the pentose phosphate pathway. Also, XK is essential in the metabolism of L-arabinose, another key sugar component of hemicellulose [31]. Due to its key role in pentose utilization, numerous XK genes have been cloned and expressed in cellulosic ethanol-producing microorganisms for efficient utilization of hemicellulose- and cellulose-derived sugars [1, 4, 13, 16, 18, 46].

XK catalyzes the ATP-dependent phosphorylation of xylulose to xylulose 5-phosphate (Xu5P) and ADP [2, 8]. Most XKs have been isolated and cloned from mesophilic sources [8, 9, 17, 20], while few were characterized from a

thermophilic bacterium *Saccharococcus caldoxylosilycus* [1] and a thermophilic yeast *Kluyveromyces marxianus* [38]. A hyperthermophilic bacterium *Thermotoga maritima* with an optimum growth temperature of ~80 °C is considered as a valuable source of thermophilic enzymes [14, 27, 40]. An open reading frame (ORF) TM0116 (1,479 bp) was annotated to encode a putative XK [27], but there is no report pertaining to its cloning, protein expression, and basic biochemical characterization.

Polyphosphate is a low-cost phosphoryl donor because it can be produced by the dehydration of concentrated phosphoric acid at an elevated temperature or from phosphate ions through biological linkage by polyphosphate-accumulating microorganisms, especially in wastewater treatment process [19, 21]. Because it is less costly and highly stable as compared to other phosphoryl donor compounds, such as, ATP, creatine phosphate, phosphoenolpyruvate, and acetyl phosphate, polyphosphate is an attractive phosphoryl donor for cell-free ATP generation in preparative-scale organic synthesis and biocatalysis [11, 15, 29, 49]. In addition, polyphosphate in extremophilic microorganisms could play an important role in energy utilization, adaptation process, and stress mitigation. For example, ancestral hyperthermophilic microorganisms may have utilized polyphosphate instead of ATP in their metabolisms since polyphosphate can be found in abundance in volcanic condensates and deep-oceanic steam vents [21]. We hypothesized that xylulokinase from the ancestral microorganism *T. maritima* might utilize polyphosphate to activate xylulose because (1) this microorganism can use polyphosphate to regulate the Embden–Meyerhof pathway [7], (2) it has a polyphosphate-dependent phosphofructokinase [7], and (3) ancestral enzymes might have low substrate selectivity [26]. The discovery of polyphosphate-dependent XK could be essential to activate xylose based on polyphosphate rather than ATP, especially in cell-free biosystems for biomanufacturing [11, 45]. Such cell-free biosystems could be used to produce high-yield hydrogen from biomass sugars [42, 48], alcohols [10], organic acids [41], and so on.

In this study, the *T. maritima* ORF TM0116 encoding a putative XK (TMTK) was cloned and expressed. The basic biochemical properties of XK were characterized. For the first time, this enzyme was found to work on polyphosphate.

Materials and methods

Chemical and strains

All chemicals were reagent grade, purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fischer Scientific (Pittsburg, PA, USA), unless otherwise noted. Polyphosphate purchased from Sigma-Aldrich was sodium hexametaphosphate (Sigma-Aldrich, Cat. No. 305553, +200 mesh, purity 96 %). The DNA polymerase used was Phusion high-fidelity DNA polymerase from New England Biolabs (Ipswich, MA, USA). The protein marker (2–212 kDa) was purchased from New England Biolabs (Ipswich, MA, USA). Primers were purchased from IDT (Coralville, IA, USA). The PCR thermocycler was Eppendorf temperature gradient Mastercycler (Hauppauge, NY, USA). *T. maritima* MSB8 (ATCC 43589) genomic DNA was purchased from the American Type Culture Collection (Manassas, VA, USA). *E. coli* DH5α was used for DNA manipulation; *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA, USA) and pET20b (+) (Novagen, Germany) were used for gene expression. *E. coli* strains were cultivated in the Luria–Bertani (LB) medium at 37 °C. Ampicillin at 100 μg/ml was added in the *E. coli* medium.

Plasmid construction

The DNA fragment containing the ORF TM0116 was amplified by PCR with a pair of primers IF (5′-**ACTTT AAGAA GGAGA TATAC ATATG** AACCT GTACG TGGGA CTCGA TGTGG-3′) and IR (5′-AGTGG TGGTG GTGGT GGTGC TCGAG **AGATC TGAAC ATCTC TTTGA GAGAT**-3′) based on genomic DNA of *T. maritima* MSB8. A linear vector backbone was amplified based on pET20b (+) with a pair of primers VF (5′-ATC TC TCAAA GAGAT GTTCA GATCT **CTCGA GCACC ACCAC CACCA CCACT-3′**) and the reverse primer VR (5′-**CCACA TCGAG TCCCA CGTAC AGGTT** CAT AT GTATA TCTCC TTCTT AAAGT-3′). VF and VR contain the last 25 bp of the 3′ terminus of the insertion sequence (underlined) and the first 25 bp of the 5′ terminus of the vector sequence (highlighted). IF and IR have reverse complementary sequences of VR and VF, respectively. The two PCR products were assembled by prolonged overlap extension PCR (POE-PCR). POR-PCR conditions were as followings: initial denaturation (30 s at 98 °C), 25 cycles of denaturation (10 s at 98 °C, annealing 10 s at 60 °C, and elongation 72 °C at a rate of 2 kb/min), and a final extension step (10 min at 72 °C). The POE-PCR product was transferred to *E. coli* DH5α, yielding plasmid pET20b-TmXylK. This restriction enzymes-and ligase-free subcloning was called Simple Cloning [44].

Expression and purification of recombinant protein

The recombinant plasmid pET20b-TmXylK was transferred into *E. coli* BL21 (DE3) and the expression of the recombinant protein was induced in the LB medium or in

high-cell-density auto-inducing medium [33]. For expression in the LB medium, the *E. coli* culture was grown at 37 °C in 250-ml Erlenmeyer flasks containing 50 ml of the LB medium plus 100 μg/ml of ampicillin. The expression of XK was induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) for 16 h at 16 °C, when the absorbency (A_{600}) of the culture at 600 nm reached ca. 0.6. For the expression in high-cell-density auto-inducing medium, the inoculum was grown at 37 °C in a non-inducing minimum medium (MDG) containing 5 g/l glucose, 2.5 g/l aspartate, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 2 mM $MgSO_4$, trace metals and 100 μg/ml of ampicillin [33]. When the cell density of inoculum reached an A_{600} of ca. 8 at 600 nm, 1 ml of inoculum cells was added into 100 ml of auto-inducing complex medium (ZYM-5052). The cell culture was grown at 300 rpm and 37 °C until the A_{600} reached ca. 6. Then the cell culture temperature was decreased to 20 °C for protein expression until the A_{600} was 19. The ZYM-5052 media contained 10 g/l of N-Z-amine, 5 g/l yeast extract, 5 g/l glycerol, 0.5 g/l glucose, 2 g/l lactose, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 2 mM $MgSO_4$, trace metals and 100 μg/ml of ampicillin [33].

The cells were harvested by centrifugation at 4 °C and washed twice with 50 mM of Tris–HCl buffer (pH 8.5) containing 0.5 M NaCl. The cell pellets were suspended in the same buffer and lysed by ultra-sonication in an ice bath (Fisher Scientific sonic dismembrator Model 500; 3 s pulse on and 8 s off, total 180 s at 30 % amplitude). After centrifugation, the soluble His-tagged XK in the supernatant was purified using a packed column of Ni-charged resin (Bio-Rad, Profinity IMAC Ni-Charged Resin). The other *E. coli* cellular proteins were washed away with a binding buffer (100 mM HEPES buffer (pH 7.5) containing 0.5 M NaCl and 10 mM imidazole). The recombinant XK was eluted with 100 mM HEPES (pH 7.5) buffer containing 0.5 M NaCl and 35 mM imidazole. The purity of the XK was analyzing using 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein marker was in the range of 2-212 kDa (New England Biolabs, Ipswich, MA, USA) and the protein bands were visualized by using the Bio-Rad Coomassie Blue 250 staining kit. The protein concentration was measured with the Bio-Rad Bradford protein kit with bovine serum albumin as a reference.

XK activity assay

XK activities at 37 °C or lower were measured by using the coupling enzyme method as described elsewhere [8, 9, 17, 32, 37, 38]. The reaction mixture contained 100 mM HEPES buffer (pH 7.5) containing 5 mM $MgCl_2$, 2 mM ATP, 1 mM xylulose, 2 mM phosphoenolpyruvate (PEP), 0.25 mM, 0.15 mM NADH, and 10 U/ml of each lactate dehydrogenase (LDH) and pyruvate kinase (PK). The reaction was started by the addition of the purified enzymes. The rate of NADH disappearance was monitored at 340 nm. XK activities at more than 37 °C were measured as followings. The reaction buffer containing 100 mM HEPES buffer (pH 7.5) containing 5 mM $MgCl_2$, 2 mM ATP, 1 mM D-xylulose was incubated for 5 min and then stopped by adding ice-cold $HClO_4$ (final concentration 0.18 M). After neutralization with K_2CO_3 (final concentration 0.19 M) at 4 °C, the insoluble salt $KClO_4$ and the deactivated proteins were removed with centrifugation. The amount of ADP formed, compared with a zero time control, was determined with the addition of PEP, NADH, and the mix of enzymes PK/LDH.

The XK activity on polyphosphate (i.e., sodium hexametaphosphate) at 37 °C was measured by a coupled enzymatic assay for the detection of Xu5P formation. The reaction mixture contained 100 mM HEPES buffer (pH 7.5), 5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 1 mM xylulose, polyphosphate (i.e., 12 mM phosphate unit equivalents) or 2 mM ATP, 2 mM ribose 5-phosphate (R5P), 0.15 mM NADH and 1 U/ml of transketolase (TK) from *T. maritima* [39], triose phosphate isomerase (TIM) from *Thermus thermophilus* [43], and glycerol dehydrogenase from rabbit muscle purchased from Sigma. The product Xu5P was quantified through the consumption of 0.15 mM NADH measured at 340 nm for 5 min. Note: TPP is a cofactor for TK. TPP cannot be utilized by the TK because no xylulose-5-phosphate was generated in the absence of polyphosphate or ATP, especially in the above high-temperature xylose utilizing experiments.

Kinetic study

The temperature effect was tested in the range of 25 to 95 °C in 100 mM HEPES buffer (pH 7.5) containing 5 mM $MgCl_2$, 1 mM xylulose and 2 mM ATP for 5 min. The kinetic parameters of XK were determined based on the initial reaction rate at the first 2 min at 37 and 60 °C. Xylulose concentration was changed from 0.1 to 5.0 mM while ATP concentration ranged from 0.2 to 5.0 mM. Each result was an average of at least three repetitions. For determining kinetic parameters of XK on polyphosphate at 37 °C, polyphosphate concentration was changed from 0 to 12 mM phosphate equivalents when xylulose concentration was 3 mM. Kinetic data were processed using SigmaPlot software (Systat Software Inc., San Jose, CA, USA).

Thermostability

The lifetime of the purified XK was studied at two enzyme concentrations (0.1 and 0.01 g/l) in 100 mM HEPES buffer

(pH 7.5) containing 5 mM $MgCl_2$ at 60 °C. The effect of the presence of 2 mM of xylulose was studied at an enzyme concentration of 0.01 g/l in the same buffer and temperature. Samples were taken at different times and the residual activities of XK were measured according to the XK activity assay described above. Each result was an average of at least three repetitions.

Results

Cloning, overexpression, and purification

The encoding protein sequence of ORF TM0116 (GenBank accession number: AAD35210.1) has the sequence similarities of 58, 19, 18, and 17 % compared to the confirmed XKs from *E. coli* (NCBI Reference number: YP_491870.1) [6], *Pichia stipitis* (GenBank accession number: EAZ63302.2) [8], *K. marxianus* (GenBank accession number: ADW23548.1) [38], and *Candida maltosa* (GenBank accession number: EMG46966.1) [9], respectively.

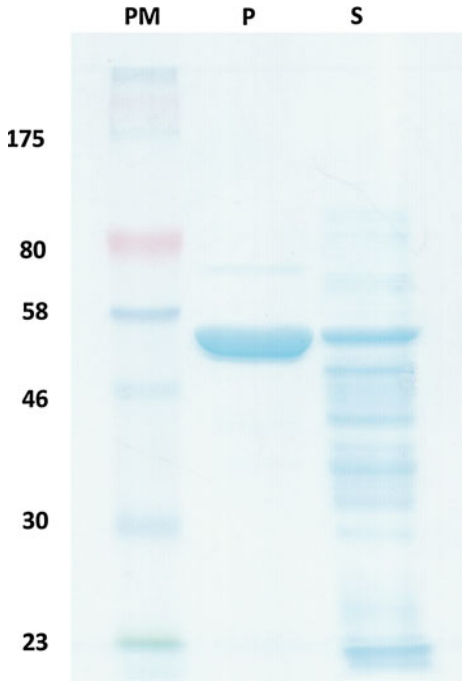

Fig. 1 SDS-PAGE gel analysis of protein expression and purification of *T. maritima* XK in *E. coli*. *PM* protein marker; *P* purified protein; *S* soluble proteins of cell lysate

The ORF TM0116 was subcloned into a 3.6-kb pET20b vector backbone, yielding 5.1-kb plasmid, pET20b-TmXylK. XK was expressed in the LB medium at 16 °C. A majority of the recombinant enzyme was present in the soluble fraction (Fig. 1, Lane S). The XK with a C-terminal His-tag was purified to homogeneity by using a nickel-resin (Fig. 1, Lane P). In SDS-PAGE gel, a single protein band had a molecular weight of approximately 60 kDa (Fig. 1) in agreement with the calculated molecular mass of 57.5 kDa from its deduced amino acid sequence. The purification yield and fold were 95 % and 57, respectively (Table 1). Approximately 42 mg of purified XK per liter of the cell culture were obtained in the LB medium (Table 1). The specific activity of the purified XK at 60 °C and pH 7.5 was 48.9 U/mg on ATP. Also, this enzyme was expressed well in a high-cell-density auto-inducing medium ZYM-5052 [33]. Approximately 300 mg of XK was purified per liter of the high-cell-density culture, suggesting the feasibility of low-cost XK production.

This enzyme was purified by using heat precipitation since some recombinant thermo enzymes expressed in *E. coli* can be easily purified by using heat precipitation [34]. A single protein band in SDS-PAGE was obtained after heat treatment of 80 °C for 20 min (data not shown) but its specific activity was less than a tenth that of purified one based on nickel charged resin. This result suggested that this enzyme was not so stable as other *T. maritima* enzymes [24, 34, 40].

Basic biochemical properties

Since it is known that XK requires magnesium as a cofactor [8, 28], 5 mM $MgCl_2$ was added for all enzymatic activity assays. The temperature effect on XK activity was measured in the range of 25 to 95 °C. Its activity increased drastically when the temperature was increased to 85 °C (Fig. 2). The optimum temperature of XK was 85 °C. The activation energy in the range of temperature of from 37 to 85 °C was 31.7 kJ/mol based on the Arrhenius plot.

Furthermore, XK was found to have a specific activity of 9.12 U/mg at 37 °C on polyphosphate, 41 % of its activity on ATP (22.5 U/mg). This first polyphosphate-dependent XK could have great potential application in high-speed enzymatic hydrogen generation from biomass sugars at elevated temperature. This hyperthermophilic enzyme has

Table 1 The purification of *T. maritima* XK from 250 ml of the *E. coli* cell culture growing on the LB medium

Fraction	Vol. (ml)	Protein (mg/ml)	Sp. activity (U/mg)	Total activity[a] (U)	Yield (%)	Purification fold
Cell lysate	75	8.51	0.53	544	100	1
Purified XK	7.3	1.45	48.9	518	95	57

[a] TK activity was measured at 60 °C

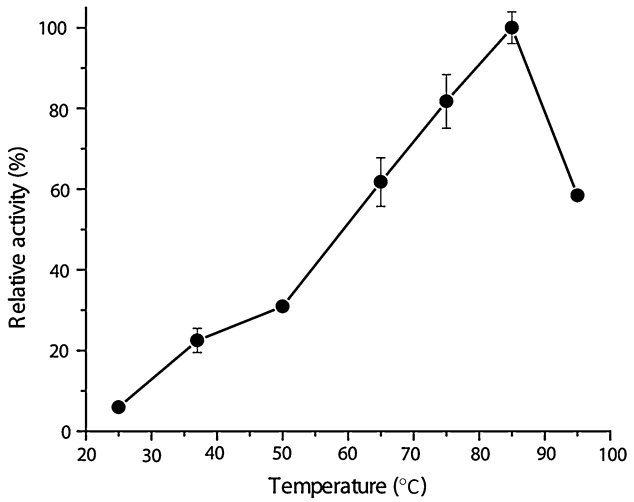

Fig. 2 The temperature profile of *T. maritima* XK in 100 mM HEPES buffer (pH 7.5) containing 5 mM $MgCl_2$

the same optimal temperature as the hyperthermophilic hydrogenase [50]. This XK activity on other phosphoryl donors, such as GTP, TTP, pyrophosphate, tripolyphosphate, and so on, was not tested because this enzyme will be used for low-cost cell-free biomanufacturing.

This XK displayed typical Michaelis–Menten kinetics. The kinetic constants were determined by fixing the concentration of one substrate and by changing the concentration of the other at 37 and 60 °C (Table 2). The K_m values for xylulose and ATP at 37 °C were 0.22 and 0.14 mM, respectively, while their values at 60 °C increased to 1.24 and 0.71 mM, respectively. The k_{cat} values at 37 and 60 °C were 26.0 and 83 s^{-1}, respectively. The K_m and k_{cat} values on polyphosphate were $17 \pm 5 \times 10^{-3}$ mM and 8.77 ± 0.02 s^{-1} at 37 °C, respectively (Table 2).

The thermostability of XK was studied at two enzymes concentrations. The degradation constants (k_d) at 0.1 and 0.01 g/l XK at 60 °C were −0.20 and −0.35 h^{-1}, respectively (Fig. 3a). The presence of 2 mM xylulose enhanced the thermostability of 0.01 g/l XK, increasing k_d to −0.29 h^{-1} (Fig. 3b), in agreement with the previous report [1]. Under its suboptimal conditions (60 °C, 0.01 g of XK/l and 2 mM xylulose), XK had a total turn-over number (TTN) of 1.03×10^6 mol of product per mol of enzyme, where TTN can be calculated as TTN = k_{cat}/k_d [30].

Discussion

The discovery and utilization of thermophilic enzymes is of great interest in cell-free biosystems for biomanufacturing, especially in the production of low-value biocommodities [50]. As compared to other reported XK enzymes (Table 3), the *T. maritima* XK is among the most active XKs and has the highest optimum temperature reported (85 °C), suggesting its potential application in high-speed enzymatic hydrogen generation at elevated temperature, which was compatible with the hyperthermophilic hydrogenase [50]. The utilization of xylose could be very important to low-cost generation from biomass sugars [42], where xylose cannot be economically separated from cellulosic materials. By using this polyphosphate xylulokinase, we designed a non-natural synthetic pathway comprised of 13 enzymes for high-yield hydrogen production from xylose [22]. This enzyme cocktail was able to convert xylose and polyphosphate instead of ATP to dihydrogen and carbon dioxide with approaching 100 % theoretical yield (9.6 mol of dihydrogen per mole of xylose) at 50 °C and 1 atmosphere pressure [22]. The stoichiometric reaction was $C_5H_{10}O_5 + 6H_2O + (P_i)_n \rightarrow 10H_2 + 5CO_2 + (P_i)_{n-1} + P_i$.

The economic analysis suggested that enzyme costs in cell-free biosystems could be minimal when all the enzymes have TTN values of more than 10^7–10^8 mol of produce per mole of enzyme [39]. Although the TTN value of this XK was one order of magnitude lower than the above requirement, further development in enzyme immobilization and/or protein engineering could enhance its TTN value greatly, such as phosphoglucose isomerase [25], polyphosphate glucokinase [21], and glucose isomerase.

It was interesting to learn why this new XK can work on polyphosphate. The crystalline structure of the *Arthrobacter* sp. strain KM polyphosphate/ATP-dependent glucomannokinase (PGMK) (Fig. 4c) [23] taught us that up to 11 residues—Asp18, Thr22, Lys25, Arg42, Thr81, Pro83,

Table 2 Summary of biochemical parameters for *T. maritima* XK at 37 and 60 °C

Parameter	Temperature				
	37 °C			60 °C	
	Xylulose	ATP	Polyphosphate	Xylulose	ATP
k_{cat} (s^{-1})	26 ± 2	24 ± 2	8.77 ± 0.02	78 ± 13	83 ± 10
K_m (mM)	0.22 ± 0.05	0.14 ± 0.03	$17 \pm 5 \times 10^{-3}$	1.24 ± 0.05	0.70 ± 0.07
k_{cat}/K_m (mM^{-1} s^{-1})	116	171	516	62	119

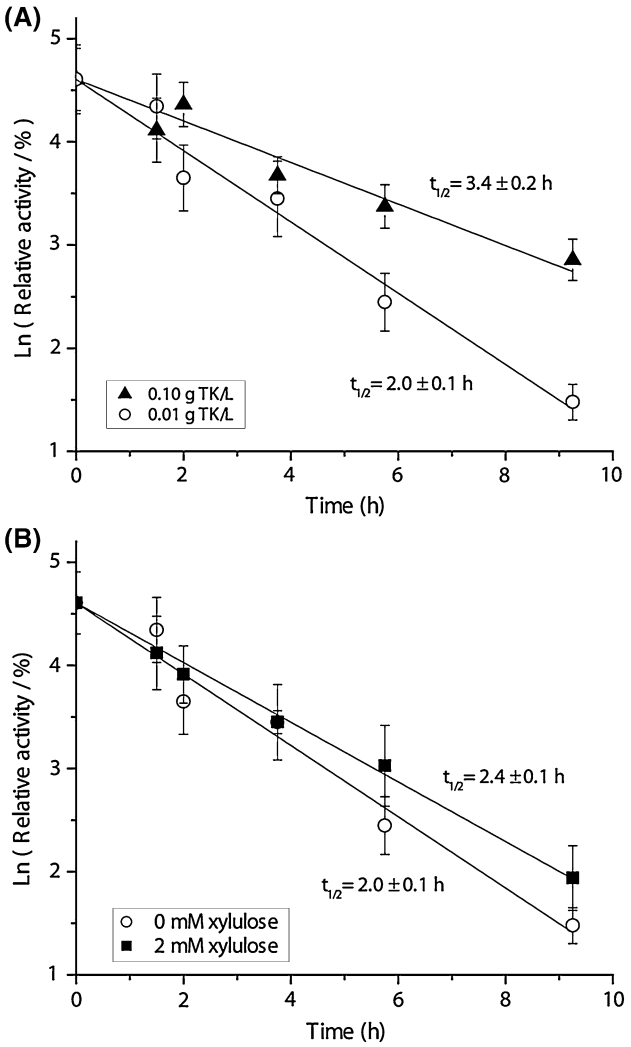

Fig. 3 Thermo inactivation of the purified *T. maritima* XK at 60 °C at two enzyme concentrations (0.1 and 0.01 g/l) (**a**), and in the presence of the substrate xylulose (2 mM) at an enzyme concentration of 0.01 g/l (**b**)

Asn96, Asp123, Thr151, Asn248, and Ile252—may be responsible for the binding with polyphosphate, because the side chain of these resides point to the polyphosphate-binding pocket. The homology structure model of TMXK was constructed based on the template of *E. coli* XK (PDB ID: 2ITM) by using the Swiss model [3] (Fig. 4a). The amino acid sequences of TMXK was compared to the *Thermobifida fusca* YX strictly polyphosphate-dependent glucokinase (TFGK) (Fig. 4b) [21], which has 11 similar binding residues responsible for binding to polyphosphate. TMXK has something in common with the polyphosphate-binding pocket of TFGK: the four same residues (i.e., Asp8, Lys15, Asp 241, and Thr263, shown by solid black arrows) and two similar residues (i.e., Thr12 and Arg 32, shown by solid gray arrows) (Fig. 4). In contrast, TMXK had two different residues (i.e., Glu429 and Ser432 shown by dotted black arrows) and three non-matched residues (i.e., Leu206, Pro207, and Asn218 shown by dotted gray arrows), which were far away from the putative polyphosphate-binding pocket. The above analyses of homology structure modeling and sequence alignment implied that this newly discovered XK could work with polyphosphate somewhat but not so efficiently as polyphosphate-dependent kinases. This hypothesis was in good agreement with that the catalytic coefficient (k_{cat}/K_m) of TMXK (516 mM^{-1} s^{-1}) was two orders of magnitude less than the reported value of the *T. maritima* polyphosphate-dependent phosphofructokinase (i.e., 6.5×10^4 mM^{-1} s^{-1}) [7] and the *T. fusca* polyphosphate-dependent glucokinase (i.e., 8.8×10^4 mM^{-1} s^{-1}) [21]. The above speculation pertaining to the substrate promiscuity of this enzyme could be worth further investigation through site-directed mutagenesis.

XK plays an important role in the fermentation pathway of xylose [8, 9]. For example, the overexpression of xylulokinase gene in a genetically modified *Saccharomyces cerevisiae* was shown to drastically increase the xylose uptake rates as well as ethanol production rate [36]. This thermophilic XK enzyme may be applied to develop hyperthermophilic ethanol-producing microorganisms that can convert hexoses and pentoses to ethanol and separate ethanol in situ at high temperature fermentation [5, 35].

Table 3 The comparison of xylulokinase from different sources reported in the literature

Microorganism	Temp (°C)	Specific activity (U/mg)	k_{cat} (s^{-1})	K_m on ATP (mM)	K_m on xylulose (mM)	References
C. maltosa	30	0.07	NA	0.15	NA	Guo et al. [9]
Bos taurus	37	4.1	NA	N/A	4	Hickman and Ashwell [12]
E. coli	25	N/A	255	0.15	0.29	Di Luccio et al. [6]
P. stipitis	30	21.4	112	0.28	0.52	Flanagan and Waites [8]
K. marxianus	37	0.024	NA	NA	NA	Wang et al. [38]
Saccharococcus caldoxylosilyticus	25	60	117	0.16	0.09	Ahmad and Scopes [1]
T. maritima	37	22.1	26	0.14	0.22	This study
	60	48.9	83	0.71	1.24	

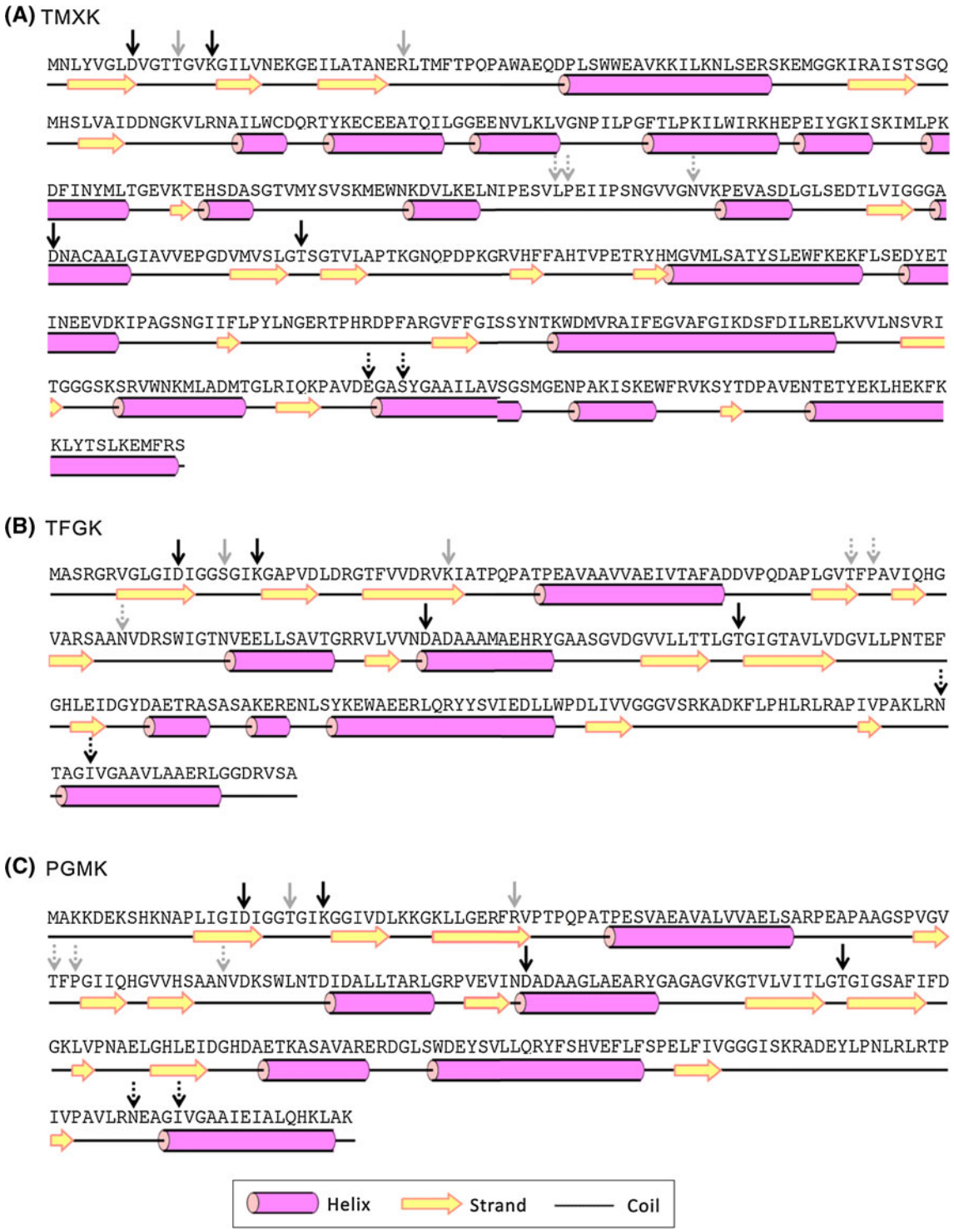

Fig. 4 The predicted secondary structures of the *T. maritima* XK (TMXK), *T. fusca* strictly polyphosphate-dependent glucokinase (TFGK), and *Arthrobacter* sp. KM polyphosphate/ATP-dependent glucomannokinase (PGMK). The residues shown by *arrows* are the putative polyphosphate binding sites. *Solid black arrows* point to the same residues (i.e., Asp8, Lys15, Asp 241, and Thr263 in TMXK) based on the sequence alignment of TMXK, TFGK, and PGMK; *solid gray arrows* point to similar residues (i.e., Thr12 and Arg 32 in TMXK); *dotted black arrows* point to different residuals (i.e., Glu429 and Ser432 in TMXK); and *dotted gray arrows* point to unmatched residues (i.e., Leu206, Pro207, and Asn218 in TMXK)

In summary, this first ATP/polyphosphate XK was discovered, to our limited knowledge. This enzyme has the highest optimum temperature, implying its potential application in high-speed in vitro enzymatic hydrogen production without the use of costly ATP [22] and high-temperature biomass-to-ethanol microbial fermentation.

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