TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2014) 38: 633-639
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doi:10.3906/biy-1401-92

Cloning, purification, and characterization of a thermophilic ribulokinase from *Anoxybacillus kestanbolensis* AC26Sari

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Received: 31.01.2014 • **Accepted:** 29.05.2014 • **Published Online:** 05.09.2014 • **Printed:** 30.09.2014

Abstract: The gene encoding ribulokinase *araB* from *Anoxybacillus kestanbolensis* AC26Sari was cloned and sequenced. The recombinant protein was expressed in *Escherichia coli* BL21 under the control of isopropyl-β-D-thiogalactopyranoside-inducible T7 promoter. The enzyme, designated as AC26RK, was purified with the MagneHis Protein Purification System. The molecular mass of the native protein, as determined by SDS-PAGE, was about 61 kDa. AC26RK was active throughout a broad pH (pH 5.0–10.0) and temperature (50–75 °C) range, and it had an optimum pH of 9.0 and optimum temperature of 60 °C. The enzyme displayed about 90%–100% of its original activities after a 30-min incubation at a pH interval of 5.0–10.0. The enzyme exhibited a high level of D-ribulose activity with apparent K_m, V_{max}, and K_{cat} values of 0.94 mM, 3.197 U/mg, and 3.31 s^{-1}, respectively. AC26RK activity was strongly inhibited by Zn^{2+} but increased by Mg^{2+}. The effects of some chemicals on the ribulokinase activity revealed that *Anoxybacillus kestanbolensis* AC26Sari does not need metallic cations for its activity. In this paper, we describe for the first time the cloning and characterization of a thermophilic ribulokinase from thermophilic bacteria.

Key words: *Anoxybacillus*, ribulokinase, thermophilic, expression

1. Introduction

Anoxybacillus is a relatively new genus compared to the well-studied genera *Geobacillus* or *Bacillus*. The genus *Anoxybacillus* represents aerobic or facultatively anaerobic, neutrophilic, obligately thermophilic, endospore-forming bacteria (İnan et al., 2011). Most of the reported data have revealed that the members of this genus produce interesting enzymes that are thermostable and tolerant to alkaline pH. Some of the well-studied enzymes were discovered through partnerships with industry; for example, the raw starch-degrading amylase was discovered by a Novozyme team (Viksø-Nielsen et al., 2006), and the BflI RE was discovered by New England Biolabs (D'Souza et al., 2004; Goh et al., 2013).

L-ribulokinase (RK; EC 2.7.1.16) is 1 of 3 major enzymes of the arabinose catabolic pathway. L-arabinose is 1 of the major polysaccharide components in plant cell walls and among the most abundant monosaccharides in nature. Furthermore, its utilization pathway in bacteria has been investigated extensively (Zhang et al., 2012). The arabinose regulon is 1 of many gene systems in *Escherichia coli* and the regulon consists of 4 operons, araBAD, araC, araE, and araFGH, which are responsible for L-arabinose catabolism, gene regulation, low-affinity transport, and high-affinity transport, respectively (Englesberg and Wilcox, 1974; Lichenstein et al., 1987). In the low-affinity transport system, the transporter, the araE gene product, is bound to the inner membrane and utilizes the electrochemical potential to transport arabinose. The araFGH genes encode arabinose-specific components of a high-affinity transport system, ABC transporters. These are 3 proteins of the ATP-binding cassette transporter family. AraF is the periplasmic arabinose-binding protein, AraG is the ATP-binding component, and AraH is the membrane-bound component (Schleif, 2010). AraC acts directly as an inducer or an activator of gene expression.

The araBAD operon encodes 3 different enzymes required for catabolism of L-arabinose, which are responsible for the conversion of L-arabinose into D-xylulose-5-phosphate. AraA, as an isomerase (L-arabinose isomerase), converts arabinose to L-ribulose; AraB, as a kinase (L-ribulokinase), phosphorylates L-ribulose; and AraD, as an epimerase (L-ribulose-5-phosphate 4-epimerase), converts L-ribulose-phosphate

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to D-xylulose-phosphate. This final product is then transferred to the pentose phosphate pathway (Schleif, 2010; Agarwal et al., 2012).

L-ribulokinase is unusual among kinases since it phosphorylates all four 2- ketopentoses (L- or D-ribulose and L- or D-xylulose) with almost the same K_{cat} values (Lee et al., 2001). Despite the central role of L-ribulokinase in arabinose catabolism, its 3-dimensional structure and mechanism of action as well as the bases of this broad substrate selectivity have not been elucidated (Agarwal et al., 2012).

This is the first report on thermophilic L-ribulokinase. In this paper, we describe for the first time the cloning and characterization of thermophilic L-ribulokinase from *Anoxybacillus kestanbolensis* AC26Sari.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Dulger et al. (2004) isolated and identified *Anoxybacillus kestanbolensis* AC26Sari from the *Çamköy* mud hot spring in the province of Çanakkale, Turkey. *A. kestanbolensis* AC26Sari was used as the source of chromosomal DNA and thermophilic L-ribulokinase. *E. coli* strains used in this study were BL21 (DE3):pLysS (Novagen) and JM101 (NEB). pGEM-T Easy (Promega) and pET 28a(+) vectors (Novagen) were used for cloning and overexpression.

A. kestanbolensis AC26Sari was grown aerobically at 55 ºC and pH 7.0 in either Luria-Bertani medium (LB) or minimal medium with the addition of 2 g L^{-1} casamino acid (M9CA) (Sambrook et al., 1989). All *E. coli* strains containing recombinant plasmids were cultured in LB medium supplemented with 50 µg/mL ampicillin or kanamycin for selection, as appropriate, at 37 °C and pH 7.4, unless otherwise stated.

2.2. Cloning and sequencing of the ribulokinase gene

A ribulokinase gene was amplified directly from the genomic DNA of *A. kestanbolensis* AC26Sari using a pair of primers (RibkF: 5'-CGC TAG CAT GGG GAA AAA GTA TGT CAT TGG-3'; RbikR: 5'-CAA GCT TAA TCA CGA TAA ACT TAT AGA TTT TTT C-3') designed according to the sequence of ribulokinase from *Anoxybacillus flavithermus*. The restriction sites *Nhe*I and *Hind*III were incorporated into the forward and reverse primer sequences, respectively. Taq DNA polymerase was used to perform PCR with *A. kestanbolensis* AC26Sari genomic DNA as the template. The PCR conditions were as follows: 1 initial denaturation step at 95 °C for 2 min, 36 cycles at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 2 min, except in the final cycle, where the extension proceeded for 5 min. The PCR fragment was cloned into the pGEM-T Easy vector and sequenced by Macrogen (Amsterdam, the Netherlands). Similarity analyses of the sequence were carried out with the advanced BLAST program of GenBank (NCBI, NIH, Washington, DC, USA).

2.3. Overexpression and purification of the ribulokinase

A 1695-nt fragment was released from pGEM-T digested with *Nhe*I and *Hind*III restriction enzymes and then was ligated into pET28a(+) having overhanging ends digested with *Nhe*I and *Hind*III. The ligation products were transformed into *E. coli* BL21 (DE3):pLysS. The recombinant plasmid was designated pAC26RK and expressed in the same cell in 1 L of LB containing 50 µg/mL kanamycin. The expression products were designated as in AC26RK and contained a His tag at the N-terminal. The transformed cells of *E. coli* BL21 were incubated up to an optical density of about 0.6 at 600 nm. The expression of recombinant proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the strain was cultured for 4 h at 37 °C. The cells were harvested by centrifugation at 11,000 rpm for 10 min, and were then resuspended in 50 mM phosphate buffer (pH 7.0) followed by sonication with a Sartorius Labsonic M (70% amplitude, 0.6 cycles for 5 min) to release intracellular proteins. The cell-free extract was centrifuged at 14,800 rpm for 15 min to remove cell debris. The purification procedure was performed at room temperature. Crude extract was heated at 65 °C for 15 min, and the precipitated proteins were removed by centrifugation at 14,800 rpm for 15 min. The ribulokinase protein containing a His tag was purified with the MagneHis Protein Purification System (Promega, Madison, WI, USA).

2.4. Ribulokinase activity assay

Ribulokinase activity was assayed by modified cysteine-carbazole reaction. The activity system (200 µL) contained 1.5 mM ribulose, 50 mM Tris-HCl buffer (pH 9.0), 12 mM ATP, 20 mM magnesium acetate, 1 mM NaF, 20 mM β-mercaptoethanol, and the enzyme preparation. After incubation at 60 °C, 0.4 mL of 5% $ZnSO_4.7H_2O$ and 0.4 mL of 0.3 N $Ba(OH)_2$ were added to the reaction mixture. The precipitate containing phosphorylated sugar was removed by centrifugation, and the supernatant was assayed for free ribulose by the cysteine-carbazole method. Next, 0.9 mL of 70% H_2SO_4 was added to 0.2-mL aliquots of the incubation mixture, and the solution was subjected to the cysteine-carbazole reaction at room temperature for 20 min (Lim and Cohen, 1966). After this ribulokinase activity assay, all experiments were carried out in triplicate and the average values were taken.

2.5. Optimum temperature and thermostability of ribulokinase

The optimum temperature was measured by performing the ribulokinase activity assay for 20 min at temperatures ranging from 50 to 75 °C at pH 8.0 with an increment of 5 °C. The effect of temperature on ribulokinase stability was determined by measuring the residual activity (%) at 60 °C

after 120 min of preincubation ranging from 50 to 85 °C with an increment of 5 °C.

2.6. Optimum pH and pH-dependent stability of ribulokinase

The optimum pH of the enzyme was measured at 60 °C using buffer solutions with different pH values, and relative activity (%) was also measured. The following buffers (50 mM) were used: 50 mM sodium acetate (pH 5.0–6.0), 50 mM potassium phosphate (pH 6.0–7.0), 50 mM Tris-HCl (pH 7.0–9.0), and 50 mM glycine buffer (pH 9.0–10.0).

In order to identify the stability of the enzyme at pH intervals of 5.0 to 10.0, a variety of buffer systems such as 50 mM sodium acetate (pH 5.0–6.0), 50 mM potassium phosphate (pH 6.0–7.0), 50 mM Tris-HCl (pH 7.0–9.0), and 50 mM glycine buffer (pH 9.0–10.0) were tested. Preincubation was performed for each pH value at the optimum temperature for 120 min. The residual activities (%) were measured in Tris-HCl (pH 9.0) at 60 °C for 20 min.

2.7. Effect of metal ions and other reagents

To determine of the effect of metal ions and other reagents on ribulokinase activity, metal ions were removed from the purified ribulokinase by treatment with 5 mM EDTA at 4 °C for 1 h followed by dialysis against 50 mM Tris-HCl (pH 8.5) overnight with several changes of buffer. The dialyzed enzyme was centrifuged at 14,800 rpm for 15 min. The effect of various ions and other reagents on the transferase activity were assayed at 60 °C (pH 9.0) using D-ribulose as substrate for ribulokinase, and then 1 mM metal ions were added to the reaction. The ribulokinase activity of the enzyme without metal ions was defined as the 100% level. The residual activity (%) was assayed spectrophotometrically.

2.8. Kinetic parameters

The values of Michaelis–Menten kinetic parameters, V_{max} and K_m, of the enzyme were calculated using D-ribulose as the substrate. V_{max}, K_m, and K_{cat} parameters were calculated using OriginPro 8.1 (OriginLab Data Analysis and Graphing Software, Northampton, MA, USA).

2.9. Nucleotide sequence accession number

The nucleotide sequence of the *A. kestanbolensis* AC26Sari ribulokinase gene was deposited in GenBank under accession number KC905743.

3. Results and discussion

3.1. Cloning of the ribulokinase gene

PCR fragments amplified by using the previously described primers were cloned into the pGEM-T Easy vector and then sequenced. A BLAST search revealed that the amplified fragment is a ribulokinase-encoding gene. The analysis of the whole gene revealed the presence of a 1695-bp open reading frame encoding a hypothetical 564-amino acid protein with a molecular mass of 61 kDa (calculated by ProtParam, www.expasy.org), and the protein was identified by a database enquiry (BLAST program) as a ribulokinase (Figure 1).

A. kestanbolensis AC26Sari ribulokinase was found to have 99% amino acid and 99% DNA identity with *Anoxybacillus flavithermus* WK1 ribulokinase, 96% amino acid and 90% DNA identity with *Geobacillus thermodenitrificans* NG80-2 ribulokinase, and 95% amino acid and 86% DNA identity with *Geobacillus kaustophilus* HTA426 DNA ribulokinase. Based on the sequence similarities, 14 key residues responsible for the catalysis and substrate-binding interactions of the enzyme were identified as conserved: Asp10, Gly12, Thr13, Thr93, Ala94, Met97, Trp124, Glu158, Lys206, Asp272, Ala273, Thr294, Cys297, and Glu327. These data strongly supported the conclusion that AC26RK should be included in the FGGY family of carbohydrate kinases (FGGY carbohydrate kinase domain-containing).

As previously stressed, AC26RK reveals similarities to the reported ribulokinases based on amino acid and nucleotide sequences, yet there are some differences among them. The putative ATP-binding motif of *Bacillus halodurans* ribulokinase was recently specified as ^{447}GGLPQK^{452} (Agarwal et al., 2012). This motif is also conserved in AC26RK and positioned in a slightly different location (between 445 and 450 amino acids instead of 447 and 452 amino acids). On the other hand, O1, O2, O3, and O4 of L-ribulose are responsible for interaction with the Glu329, Lys208, Ala96, and Asp274 residues of *Bacillus halodurans* ribulokinase, respectively (Agarwal et al., 2012). These residues were located in the AC26RK aa sequence as Glu327, Lys206, Ala94, and Asp272, respectively.

3.2. Expression of ribulokinase gene and purification of the recombinant ribulokinase

pAC26RK, which places the ribulokinase gene under the control of a T7 promoter, was transformed into *E. coli* BL21 (DE3). The overexpression of the ribulokinase protein induced by the addition of IPTG resulted in a high expression pattern of soluble ribulokinase, with activity of 2.17 U/mg; no activity was identified from the control cells harboring only the empty vector pET28a(+).

The ribulokinase expressed in the *E. coli* BL21 (DE3) strain was purified with the MagneHis Protein Purification System (Promega) (Figure 2). The purified enzyme yield was 90%. The molecular weight (MW) of the protein was 61 kDa through SDS-PAGE. Parallel to *A. flavithermus* WK1 ribulokinase (accession number: YP_002314899) and *Deinococcus maricopensis* DSM 21211 ribulokinase (accession number: YP_004169580), which both consist of 564 amino acids and have 61 kDa MW, AC26RK protein also has 61 kDa MW.

TGGGGAAAAAGTATGTCATTGGTATCGACTATGGGACGGAATCGGGACGCGCCGTCCTCGTTGATCTGGAAG
GGAACGAAATTGCGGACCACGTCACCCCGTATCATCACGGAGTCATTGATGAAGTGTTGCCGGAATCGAACG
TACAACTAGAACCAGACTGGGCACTGCAGCACCCGGGCGATTATATCGAAGTGTTGGCGACCGCTGTTCCAG
CTGTATTGCAAAAGTCAGGGGTTAACCCGGCGGATGTGATCGGGGTCGGAATTGATTTTACAGCTTGTACGA
TGTTGCCGATCTCTGGCTCCGGCGAGCCGCTTTGCTTGAAGCCCGAATTCAAGCATCGCCCACACAGCTGGG
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CCGTTGCAGTCGGAAATGTCGATGCTCATGCGGCCGTACCGGGTGTCGGGGTCGTTGAACCGGGCAAAATGG
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GGGTTGTCGAGGATGGCATCATTCCGGGATATTTCGGCTATGAAGCCGGGCAGTCAGCGGTGGGCGACATTT
TCGCTTGGTATGTTGAACAAAGTGTTCCGGCCTATGTGAAAGAAGCAGCGGAAAAAGAGGGAGTTAGTGTCC
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ACGGAAACCGTTCAGTATTGGTCGACACGGATTTAACCGGGTTGATCATCGGTTATACGTTGCTGACGAAAC
CGGAAGAAATTTACCGTGCATTGCTTGAAGCAACGGCCTTCGGTACACGGAAAATTATCGACGCCTTTGTTG
GAAGCGGTATCAATGTGGATGAGCTGTATGCATGTGGCGGATTGCCACAAAAAAATAAGTTACTCATGCAAA
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TGTTCGCGGCGGTCGCGGCCGGCAAAGAAAACGGTGGGTATGAATCAATTATTGAAGCGGCACGAAACATGG
GGAAAGTACGGGAAGAAACATTCAAGCCAATTCCGGAGAATGTCGCCATTTATGAACAGTTGTATCAAGAAT
ATACGAAGCTGCATGACTATTTTGGTCGTGGGGAAAACGATGTGATGAAGCGGCTTAAACATTGGAAAGAGA
CCGCACGGGCGGTGAAAAAATCTATAAGTTTATCGTGA

MGKKYVIGIDYGTESGRAVLVDLEGNEIADHVTPYHHGVIDEVLPESNVQLEPDWALQHPGDYIEVLATAVP
AVLQKSGVNPADVIGVGIDFTACTMLPISGSGEPLCLKPEFKHRPHSWVKLWKHHAAQDEANLLNEMAAKRG
EAFLPRYGGKISSEWMIAKIWQILNEDPDIYDQTDLFLEATDWVIFKMTGQLVRNSCTAGYKSIWHKQDGYP
SKEFFRALDPRLEHLTESKLRGSIVPLGTRAGVLTKEMAAMMGLLPGTAVAVGNVDAHAAVPGVGVVEPGKM
VMAMGTSICHMLLGTEEKYVEGMCGVVEDGIIPGYFGYEAGQSAVGDIFAWYVEQSVPAYVKEAAEKEGVSV
HEWLEKRAAAYRPGETGLLALDWWNGNRSVLVDTDLTGLIIGYTLLTKPEEIYRALLEATAFGTRKIIDAFV
GSGINVDELYACGGLPQKNKLLMQIYADVTNREIKIAASKQTPAVGAAMFAAVAAGKENGGYESIIEAARNM
GKVREETFKPIPENVAIYEQLYQEYTKLHDYFGRGENDVMKRLKHWKETARAVKKSISLS

Figure 1. Gene and protein sequences of ribulokinase of *Anoxybacillus kestanbolensis* AC26Sari.

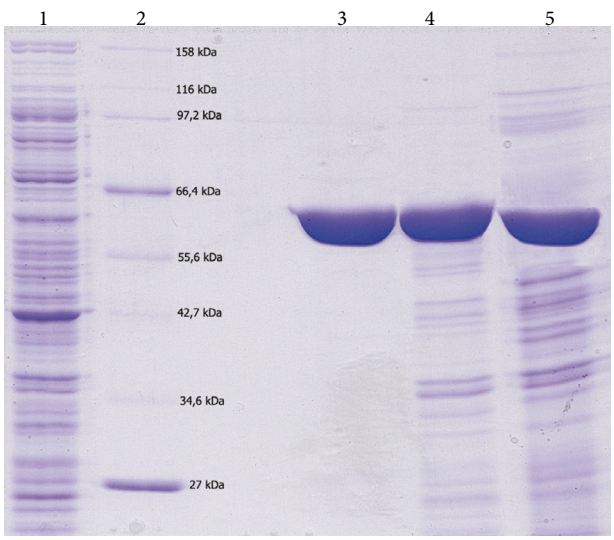

Figure 2. SDS-PAGE analysis of AC26RK. Lane 1: Crude extract from *E. coli* BL21 DE3 including only pET28a(+) vector, lane 2: protein marker, lane 3: AC26RK after heat shock and Ni column, lane 4: AC26RK after heat shock, lane 5: crude extract from *E. coli* BL21 DE3 including pAC26RK.

3.3. Characterization of the cloned ribulokinase

3.3.1. Effects of temperature on activity and stability

Based on the effect of temperature on ribulokinase activity, AC26RK exhibited optimal activity at 60 °C (Figure 3). The enzymatic activity decreased significantly at temperatures below 50 °C and above 75 °C (Figure 4). The enzyme was active in a broad temperature range (50–75 °C). The thermostability assays indicated that the enzyme revealed about 45%–50% of its original activity after 30 min at 50, 55, and 60 °C, whereas incubations at 65–85 °C for 30 min led to a loss of activity around 90%. The enzyme displayed about 40% residual activity at 50, 55, and 60 °C after 1 h of incubation, yet the enzyme activities were about 1% of their original activity at other temperatures for the same incubation period (Figure 5).

In the literature, it was reported that *E. coli* (Lee and Bendet, 1967) and *Lactobacillus plantarum* (Burma and Horecker, 1957) ribulokinases revealed optimum activity at 37 °C. The optimum temperature for AC26RK activity, however, was determined at 60 °C. To the best of our knowledge, there is no previously reported ribulokinase with such a high optimum temperature.

3.3.2. Effects of pH on activity and stability

When assayed at various pH values at 60 °C, it was found that the recombinant ribulokinase reveals higher activity in alkaline conditions. The optimum pH for enzyme activity was 9.0 (Figure 4). However, the enzyme maintained its high activity across a broad pH range, with >80% of maximum activity observed at pH values of 8.0 to 9.5.

The recombinant ribulokinase stabilities were studied between pH 5.0 and 10.0 at 60 °C, and the enzyme exhibited about 90%–100% of its original activity after 30 min. The enzyme had 70% residual activity at pH 10.0, and the enzyme activities were about 80%–95% of their original activity at other pH levels tested after 1 h of incubation. Moreover, in 24 h the enzyme maintained its activity and exhibited about 90% stability at pH 6.0–7.0 and about 40%–80% stability at pH 7.5–10 (Figure 6).

It was reported that the pH optima of *E. coli* and *Lactobacillus plantarum* ribulokinases were 7.0 and 7.5, respectively (Burma and Horecker, 1957; Sedlak and Ho, 2001). The optimum pH of AC26RK was 9.0. Thus, we suggest that AC26RK operates at higher pH conditions than other ribulokinases.

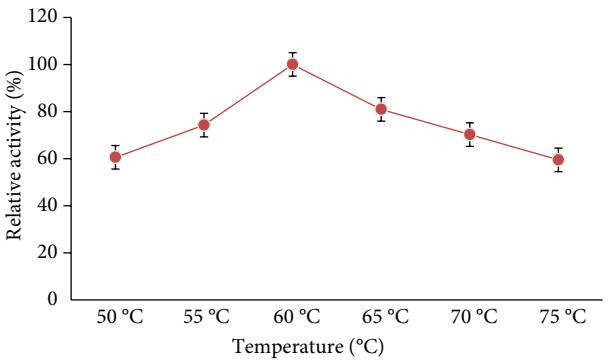

Figure 3. Optimum temperature for activity of AC26RK.

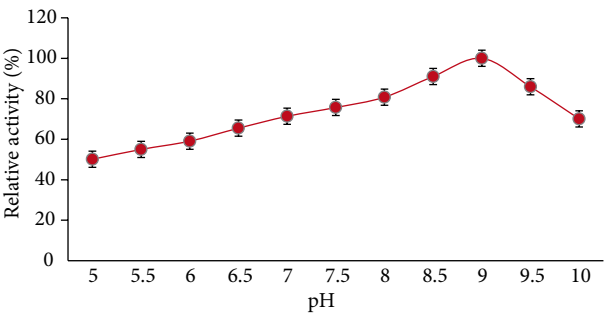

Figure 4. Determination of optimum pH for activity of AC26RK.

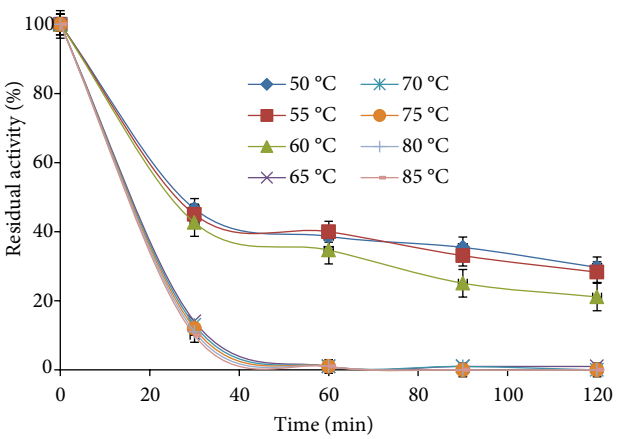

Figure 5. Effect of temperature on thermostability of AC26RK.

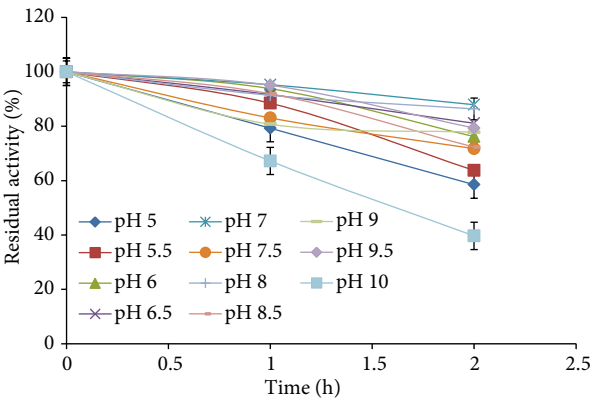

Figure 6. Effect of pH on thermostability of AC26RK.

3.3.3. Substrate specificity

The substrate specificity of the enzyme was determined by performing the assay with different substrates. AC26RK enables phosphorylation of D-ribulose, L-ribulose, D-xylulose, and L-xylulose. The phosphorylation assay results indicated that AC26RK exhibits maximal activity toward D-ribulose and decreasing affinities to L-ribulose, D-xylulose, and L-xylulose (Figure 7).

Substrate saturation curves for D-ribulose showed that *A. kestanbolensis* AC26Sari ribulokinase follows a simple Michaelis–Menten kinetic pattern. The substrate saturation curve was obtained by decreasing substrate concentrations gradually.

3.3.4. Kinetic parameters

Kinetic parameters were measured by employing a cysteine-carbazole assay, which requires the use of D-ribulose as a substrate. The initial rates were calculated by measuring the absorption at 540 nm for several concentrations of D-ribulose over a range of 0–1 mM. The ribulokinase exhibited simple Michaelis–Menten kinetics for D-ribulose (Figure 8). The values of K_m and V_{max} were 0.94 mM and 3.197 U/mg, respectively. However, the K_{cat} value for *E. coli* was 3.31 s^{-1} and the K_m value was 0.39 mM (Lee et al., 2001). The analysis results suggest that AC26RK exhibits better or moderate K*m* values compared to *E. coli* ribulokinase.

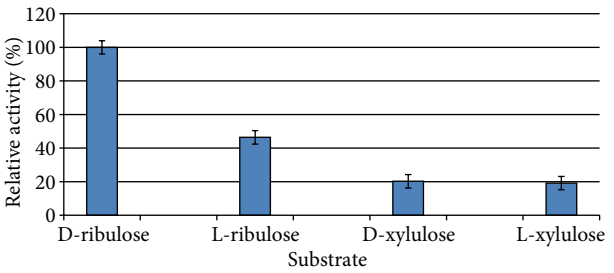

Figure 7. Substrate specificity of AC26RK.

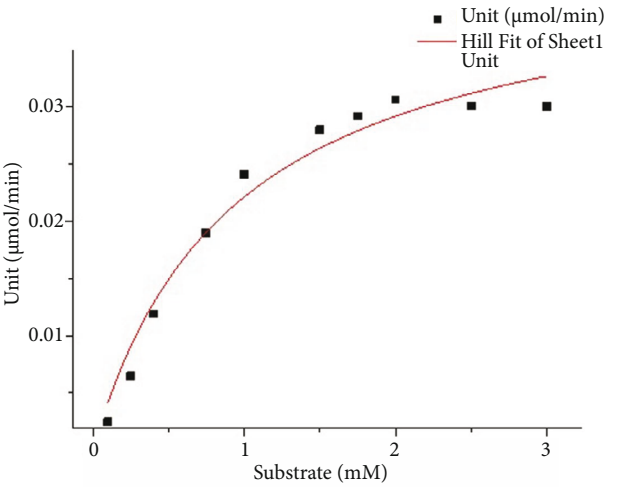

Figure 8. Michaelis–Menten plot. The measurements were performed under optimum pH and temperature conditions.

3.3.5. Effect of metal ions and other reagents

The effects of various metal ions and other reagents on phosphorylation activity of ribulokinase were assayed at 60 °C and pH 9.0 using D-ribulose as a substrate. The activity measured without additional metal ions and reagents was considered as 100%. Independent Mg^{2+} addition increased ribulokinase activity up to 633%. Mg^{2+} ion plays a role in the stability of all polyphosphate compounds in the cells (Bachelard, 1971). An independent Mg^{2+} addition stabilizes ATP, which is a substrate of ribulokinase, so that it induces the activity of ribulokinase. Treatment of AC26RK with β-mercaptoethanol induced activity by approximately 175%. Cysteine, which favors an S-S to -SH interchange, suggests that ribulokinase is an enzyme that does not contain disulfide linkages (Ay et al., 2011). On the other hand, Zn^{2+} strongly inhibited AC26RK activity. K^+, Li^+, Ca^{2+}, and EDTA additions did not induce any variation in AC26RK activity (Figure 9). The chelating agent EDTA

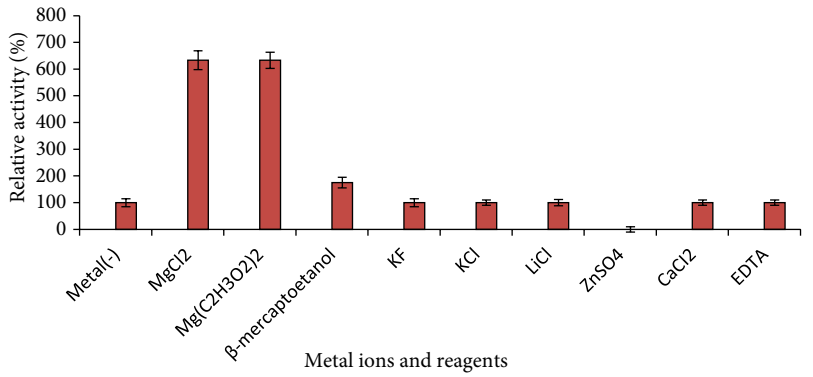

Figure 9. Effect of some metal ions and reagents on AC26RK activity. In the presence of metal ions or reagents, the enzyme activities were compared with a control including no metal ion or reagent whose activity was considered 100%.

did not affect the activity of AC26RK, suggesting that the enzyme has good activity without metallic cations and that metallic cations are not necessary for its activity.

This study provides the first report on the cloning, purification, and characterization of a ribulokinase from thermophilic bacteria. In conclusion, AC26RK reported from thermophilic bacteria is the first thermophilic and alkaline ribulokinase belonging to the FGGY family of the carbohydrate kinase classification system.

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