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Biochemical characterization of a novel glucose isomerase from *Anoxybacillus gonensis* G2^T that displays a high level of activity and thermal stability

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ABSTRACT

In the continuing search for novel enzymes suitable for the production of high fructose corn syrup (HFCS), a new glucose isomerase (GI) from the thermophile *Anoxybacillus gonensis* G2^T is described. The gene encoding this GI (AgoG2GI) was cloned and then engineered for heterologous expression in *Escherichia coli*. The recombinant enzyme was purified from the heat treated cell-free extract by anion exchange chromatography followed by hydrophobic interaction chromatography. The purified enzyme showed optimal activity at 85 °C and pH 6.5. The steady state parameters of K_m and k_{cat} with D-glucose were found to be 146.08 ± 9.50 mM and 36.47 ± 2.01 (1/s), respectively. L-arabinose, D-ribose and D-mannose also served as substrates for the enzyme with comparable kinetic parameters. AgoG2GI requires the divalent cations of Co^{2+}, Mn^{2+} and Mg^{2+} for its maximal activity and thermostability. The results reported here are indicative of a new GI with desirable kinetics and stability parameters for the efficient production of HFCS at industrial scale.

1. Introduction

Xylose isomerase (D-xylose ketol-isomerase E.C 5.3.1.5) catalyzes the isomerization of D-xylose to xylulose on the first step of xylose metabolism in many microorganisms [1]. Interconversion between xylose and xylulose provides a nutritional requirement in saprophytic bacteria that flourish on rotting plant matter and also assists in the bioconversion of hemicellulose to ethanol [2]. Xylose isomerase is also known as glucose isomerase (GI) due to its involvement in the reversible isomerization of glucose to its respective ketose (fructose) in the presence of divalent metal ions (Fig. 1). This isomerization reaction is important in industrial processes such as the production of high fructose corn syrup (HFCS) in the food industry [3,4]. In the existing processes for HFCS production, non-thermostable xylose (glucose) isomerases from mesophilic microorganisms are utilized in an immobilized enzyme reactor producing 40% to 42% fructose syrup at an operating temperature of 50 °C and 58 °C with residence times of <1 h [5]. In this process, additional expensive chromatographic steps are necessary to obtain the required fructose syrup concentration of 55%. It has been theorized that isomerization at elevated temperature (at about 95 °C) and lower pH (at about 4.5–5.5) are required to achieve the desired fructose level in the syrup due to the isomerization reaction equilibrium [6]. If this could be achieved, additional concentration steps would be unnecessary [7]. For this reason, researchers have sought to isolate the microorganisms that are capable of producing GI with high levels of activity and stability at elevated temperature. In this context, several genes encoding these enzymes have been reported from *Escherichia coli* [8], *Bacillus subtilis* [9], *Clostridium* species [10], *Streptomyces* species [11], *Ampullariella* species [12], *Actinoplanes missouriensis* [13] and *Thermus thermophilus* [14]. Furthermore, GI is also an ideal enzyme for studying structure–function relationships from an academic perspective [2].

In brief, an industrially valuable GI must have a lower pH optimum, a higher temperature optimum, a resistance to inhibition by Ca^{2+}, and an elevated affinity for glucose over currently employed enzymes. Therefore, there is currently significant research effort devoted to identifying a thermostable and acid stable GI with higher affinity for glucose. What is more, advances in recombinant DNA technology and protein engineering have opened new and encouraging possibilities for development of an economically viable commercial process.

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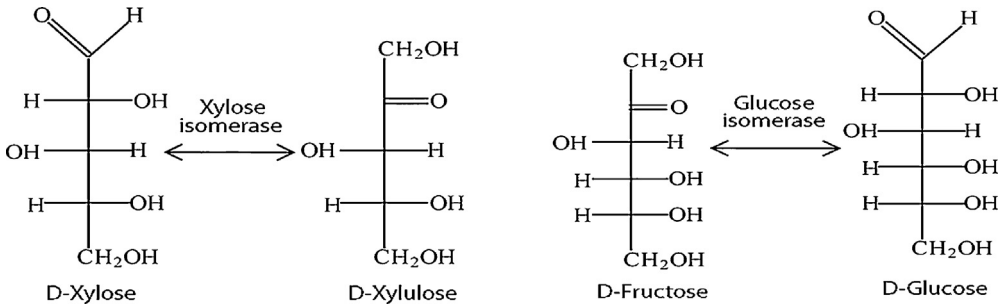

Fig. 1. Reactions catalyzed by GI.

Further in this direction, we report here, for the first time, a novel glucose isomerase (named as *Ago*G2GI) from the thermophile *Anoxybacillus gonensis* G2^T, a xylanolytic, sporulating, Gram-positive, rod-shaped, facultative anaerobe and moderately thermophilic bacterium growing naturally at 55 °C to 60 °C in the thermal spring at Balıkesir, Turkey [15]. This study describes the biochemical characterization of *Ago*G2GI together with its kinetic parameters with various substrates. The potential application of *Ago*G2GI in the food industry is also discussed. We believe that the identification of a thermophilic GI along with recently developed recombinant systems for heterologous gene expression, with the possibility for further enhancement of enzyme activity by protein engineering, will be of interest to both industry and academia.

2. Materials and methods

2.1. Substrates and chemicals

The chemicals were purchased commercially from Merck A.G. (Darmstadt, Germany), Sigma Chem. Co. (St. Louis, MO, USA), Fluka Chemie A.G. (Buchs, Switzerland), Acumedia Manufacturers, Inc. (Baltimore, Maryland, USA), and Aldrich-Chemie (Steinheim, Germany). The Wizard Genomic DNA Purification Kit, Wizard Plus SV Minipreps DNA Purification System, MagneHis Protein Purification System, *Taq* DNA Polymerase, dNTP, and all of the restriction enzymes were purchased from Promega Corp. (Madison, WI, USA). All chemicals were reagent grade, and all solutions were made with distilled and deionized water.

2.2. Strains, vectors, and media

A. gonensis G2^T NCIMB 13933^T, *E. coli* BL21 (DE3):pLysS was purchased from Novagen (Madison, WI, USA) and XL1-Blue from Stratagene (La Jolla, CA, USA). The plasmid vectors used were pGEMT-Easy (Promega) and pET28 (a-c)+ (Novagen). All *E. coli* strains containing recombinant plasmids were cultured in Luria broth (LB) medium supplemented with 50 μg/ml ampicillin or kanamycin, as appropriate, at 37 °C and pH 7.4, unless otherwise stated.

2.3. Plate assays for detection of thermostable glucose isomerase in G2^T

Plate assay was carried out according to Lee et al. [16]. The plates were incubated for 5 to 6 days at 55 °C for *A. gonensis* G2^T. The same plates containing 1% xylose were also used of xylose utilization for *E. coli xyl-5* mutants (*E. coli* HB101) at 37 °C. Glucose isomerase activities were determined as a dark brown halo around the colonies.

2.4. Cloning of the xylA gene

PCR was used to amplify a portion of the *A. gonensis G2^T xylA* gene with degenerate primers (Table 1). Degenerate primers F1, F2, R1, R2 and R3 were designed based on the conserved sequence of the other xylose isomerases. Genomic DNA was purified using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. *Taq* DNA polymerase was used to perform PCR with *A. gonensis* G2^T genomic DNA as the template. The PCR fragment was subsequently cloned into a pGEM-T vector system I and sequenced by Macrogen (Seoul, Korea). Sequence analysis revealed the PCR produced a 534 bp fragment of the *xyl* gene.

Inverse PCR was performed to clone the complete *xyl* gene according to the methods described by Trigilia et al. [17]. Two and a half micrograms of genomic DNA were separately digested with restriction enzymes (NcoI, MboI, HinfI, AvaII, BamHI, HindIII, SacI, PstI, Sa3AI, SalI, PspGI, AvaI, BfaI, EcoRI, or XbaI), which did not digest the cloned 534 fragment. Digestions were performed according to the manufacturer's instructions overnight in a final volume of 50 μl. The enzymes were heat-inactivated at 70 °C for 10 min. The mixtures were then self-ligated overnight at 16 °C with 1 U/μl T4 DNA ligase in a total volume of 400 μl. The ligation mixture was ethanol precipitated. The primers for inverse PCR (Table 1) were designed according to the partial sequence encoding *xylA* gene and to the inverse PCR fragments for the next inverse PCR. The PCR products were checked by agarose gel electrophoresis and cloned into pGEM-T easy vector and then sequenced as described above.

The *xylA* gene was amplified directly from genomic DNA of *A. gonensis* G2^T using a pair of primers (Xyla_Ex_F1-Xyla_Ex_R1 or Xyla_Ex_F1-Xyla_Ex_R2) designed according to the sequence of xylose isomerase from *A. gonensis* G2^T. The restriction sites NcoI and BamHI or HindIII (for HisTag) were incorporated into the forward and reverse primer sequence, respectively. *Taq* DNA polymerase was used to perform PCR with *A. gonensis* G2^T genomic DNA as the template.

The 1326 bp fragment was cloned into pET28(a-c)+ to generate p*Ago*G2GI or p*Ago*G2GI-his. The recombinant plasmids were then used to transform the expression strain *E. coli* BL21 (DE3):pLysS.

Molecular Evolutionary Genetics Analysis version 5.05 (MEGA 5.05) was used for multiple sequence alignments and for construction of a phylogenetic tree using the neighbor-joining method.

2.5. Overexpression of the enzyme

E. coli BL21 cells harboring p*Ago*G2GI or p*Ago*G2GI-his were grown to an optical density (OD) at 600 nm of about 0.6. Heterologous gene expression was then induced by addition of 1 mM iso-propyl-β-D-thiogalactopyranoside (IPTG) and the culture was continued for a further 4 h. Cells were harvested by centrifugation

Table 1
Degenerate, inverse-PCR and expression primers which successfully amplified *xylA* gene of *A. gonensis* G2^T.

Primers	Sequence	Remarks
F1 (degenerate primer)	5′-gTVYTBTggggYggVMgHgARgg-3′	For partial cloning of *xylA* gene
F2 (degenerate primer)	5′-ATHgARCCNAARCCNAWRgARCC-3′	
R1 (degenerate primer)	5′-ggRAAYT**C**RTCBgTRTCCCAKCC-3′	
R2 (degenerate primer)	5′-NgCRTCRAARTTBANNCCDCC-3′	
R3 (degenerate primer)	5′-SYRTCCATBSMDSCDAYRTg-3′	
XylaIF01	5′-AgACgCggTTCgTTTgAACC-3′	Inverse-PCR
XylaIF02	5′-ACgTTTCATAgCCTTCACg-3′	
XylaIF03	5′-AgCAgCAAAggTgAAAAAAgg-3′	
XylaIF04	5′-TggACTAAAAgTggCgTATCg-3′	
XylaIF05	5′-ATCATTgATgggAAggCCgAC-3′	
XylaIR01	5′-gCATAgTCAACCgCCATATgC-3′	
XylaIR02	5′-AggCggATTgAACTTTgATgC-3′	
XylaIR03	5′-TgCCARTAVgCRAYVgMRADDCg-3′	
XylaIR04	5′-TCCAAAgCAACTTggTTTTgC-3′	
XylaIR05	5′-TgCACCATgTACAAAACgAgg-3′	
XylaIR06	5′-gCgCAAATgCTCCTCCATCg-3′	
XylaIR07	5′-ggCCAATTCCTTCTgTATAgC-3′	
Xyla_Ex_F1	5′-CgAgCTCCATggCgTATTTTgAAAACg-3′ (NcoI)	For cloning into pET28(a)+
Xyla_Ex_R1	5′-CCAAgCTTACgAgCTACACAAACTTC-3′ (HindIII)	
Xyla_Ex_R2	5′-CggATCCgTTACTATCATTAACgAgC-3′ (BamHI)	

at 11,000 rpm for 5 min. The cells were disrupted using a Sartorius Labsonic M sonicator at 0.6 cycle scale (80% amplitude) to release intracellular proteins. The cell-free extract was clarified by centrifugation at 14,800 rpm for 15 min to remove cell debris, and assayed for glucose isomerase activity.

A. gonensis G2^T for expression of glucose isomerase was induced with 0.5% final D-xylose concentration at OD 0.6 (600 nm). The expressed enzyme from type stain G2 was designated as *Ago*G2GI-wt (wild-type enzyme), whereas the enzyme expressed from *E. coli* BL21/p*Ago*G2GI or p*Ago*G2GI-his was designated as *Ago*G2GI-rec or *Ago*G2GI-recHis.

2.6. Purification of the enzymes

The purification procedure was performed at room temperature. A crude extract of *Ago*G2GI-wt (30 ml) was heated at 75 °C for 15 min and precipitated proteins were removed. In the first step of purification of *Ago*G2GI-wt, ammonium sulfate precipitation was carried out as follows: solid $(NH_4)_2SO_4$ was added to the heat-treated extracts to give 60% saturation, and the precipitate was removed by centrifugation (12,500 rpm for 30 min). $(NH_4)_2S0_4$ was added further to the supernatant to give 70% saturation. This precipitate was collected and dialyzed overnight against the same buffer. In the subsequent purification step, the dialyzed enzyme preparation was loaded onto a diethylaminoethyl (DEAE)-Sepharose anion-exchange column. After loading the sample the column was extensively washed to remove unbound material. Bound proteins were then eluted on a linear salt gradient from 0 M to 0.6 M NaCl in 20 mM MOPS buffer (pH 6.5) at a flow rate of 1 ml/min. Fractions containing *Ago*G2GI-wt were pooled, concentrated by ultrafiltration (Sartorious, 10,000 MWCO filters) and loaded onto a Phenyl-Sepharose-6 Fast Flow hydrophobic interaction column (Sigma) equilibrated in buffer containing 1.3 M $(NH_4)SO_4$. Proteins were eluted on a linear gradient from 1.3 M to 0 M $(NH_4)SO_4$ at a flow rate of 0.5 ml/min.

*Ago*G2GI-rec (crude extract 13 ml) was purified according to the procedures described above, except for the ammonium sulfate precipitation.

The enzyme purified directly from *A. gonensis* was designated as *Ago*G2GI-wt while the enzyme with and without HisTag from pET28(a-c)+ in *E. coli* designated as *Ago*G2GI-recHis and *Ago*G2GI-recHis, respectively. The protein, which contained a His tag (*Ago*G2GI-recHis), was purified using the MagneHis^{TM} Protein Purification System (Promega).

Protein concentration was determined using the method of Bradford [18]. Bovine serum albumin was used as a standard in this procedure.

2.7. Activity assay of GI

Cell extracts prepared by sonication and purified preparations were used as enzyme sources. Glucose isomerase activity was measured by incubating a reaction mixture that contained 10 mM $MgSO_4$, 1 mM $CoCl_2$, 0.2 M glucose and the enzyme in 50 mM MOPS buffer (pH 6.5) at 85 °C for 30 min in 100 μl reaction volume. The reaction was stopped with the addition of 100 μl 0.5 M perchloric acid, 40 μl of 1.5% cysteine hydrochloride, and 40 μl of 0.12% carbosol, respectively. After 1.2 ml of 70% sulfuric acid was added, the mixture was vortexed and then incubated at room temperature for 30 min. Absorbance was measured at 560 nm for fructose. The amount of fructose formed after the enzyme reaction was estimated using the cysteine–carbazole–sulfuric acid method [19]. One unit of activity was defined as the amount of enzyme that released 1 μmol of fructose/minute under the assay conditions described above.

2.8. Determination of K_m and V_{max} values

The kinetic parameters V_{max} (μmol/min/mg), K_m (Michaelis constant, mM) and k_{cat} (1/s) were determined from Michaelis–Menten plots of specific activities at various substrate (D-glucose, L-arabinose, D-mannose, D-ribose) concentrations using the OriginPro8.1 program (OriginLab Data Analysis and Graphing Software). Typically, duplicate measurements at 6 to 10 different concentrations of substrate spanning 0–700 mM were used to determine the value of K_m.

2.9. Determination of submolecular mass by SDS-PAGE

SDS-PAGE was performed as described by Maniatis et al. [20]. Subunit molecular mass values were estimated by SDS-PAGE using marker proteins (Promega).

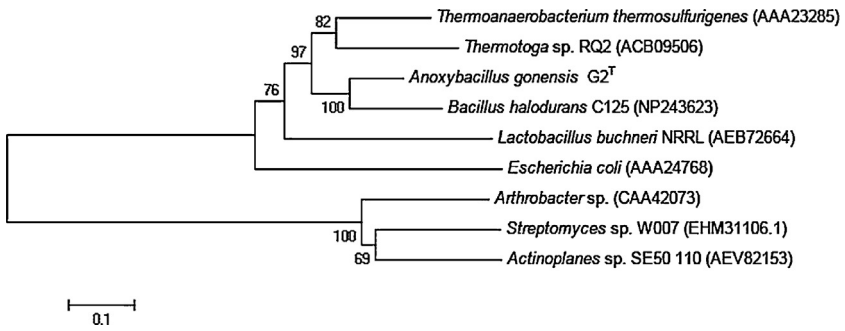

Fig. 2. Phylogenetic analysis of *Ago*G2GI and other amino acid sequences of the tested XIs reported in the GenBank database. The phylogenetic tree was constructed using MEGA 5.05 software with the neighbor-joining method. Bar, 0.1 substitutions per nucleotide.

2.10. Determination of submolecular mass by MALDI-TOF

Mass spectrometry of *Ago*G2GI-rec was performed by MALDI-TOF using a MicroFlex LT (Bruker). Protein sample was diluted in 0.1% formic acid to approximately 0.05 mg/ml and mixed with an equal volume of matrix (saturated solution of sinapinic acid in 50% acetonitrile, 0.1% formic acid) on a stainless steel surface. The samples were air dried at room temperature to crystallize. The machine was operated in positive ion mode and calibrated with bovine serum albumin.

2.11. Determination of molecular mass with HPLC

The apparent molecular mass of the native enzyme was determined by high performance liquid chromatography (HPLC) under the following conditions: column, Superdex^{TM} 200 10/300 GL Gel filtration column (Amersham Pharmacia Biotech); mobile phase, 50 mM phosphate buffer; flow rate, 0.5 ml/min. The eluted compound was detected by UV index. Carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and apoferritin (443 kDa) were used as the molecular mass marker proteins.

2.12. Effects of temperature on activity and stability

The effects of temperature on AgoG2GI-wt and AgoG2GI-rec activities were determined spectrophotometrically using D-glucose as the substrate. The enzymatic reactions at various temperatures over the range of 25 °C to 100 °C were performed using the enzyme activity assay described previously, and the results were expressed as relative activity (%) obtained at optimum temperature.

The effect of temperature on AgoG2GI-wt and AgoG2GI-rec stability was determined by measuring the residual activity (%) after pre-incubation in 50 mM MOPS (pH 6.5) at 4 °C, 30 °C and 85 °C. Samples of assay mixtures were taken at intervals and residual enzyme activity was determined. The percentage of residual activity of glucose isomerase was calculated by comparison with untreated enzyme.

2.13. Effects of pH on activity and stability

The optimum pH of the enzyme was measured at 85 °C and 560 nm by using buffer solutions of different pH values. Their relative activities (%) were measured, and the results were expressed as relative activity (%) obtained at pH. Other parameters were determined in optimum pH conditions.

To determine the stability of the enzyme at pH values between 5.0 and 10.5, pre-incubation was performed at each pH value at 4 °C for 300 h and at 85 °C for 8 h. Samples of assay mixtures were taken at intervals, and residual enzyme activity was determined. The percentage of residual activity of glucose isomerase was calculated by comparison with untreated enzyme.

The following buffers (200 mM) were used: sodium acetate (pH 5.0–6.0), potassium phosphate (pH 6.0–7.0), tris–HCl (pH 7.0–9.0), and glycine–NaOH buffer (pH 9.0–10.0).

2.14. Effect of metal ions

It is reported that Co^{2+}, Mn^{2+}, and Mg^{2+} bivalent metal ions are activators of glucose isomerases [2]. The activator effects of various metal ions on the glucose isomerase activity of *Ago*G2GI-wt and *Ago*G2GI-rec was assayed at optimum reaction conditions. To determine the effect of these metal ions on glucose isomerase activity, metal ions were removed from the purified glucose isomerase by treatment with 5 mM EDTA at 60 °C for 1 h followed by dialysis against 50 mM MOPS buffer (pH 6.5) overnight with several changes of buffer [21]. MOPS buffer containing 0.1 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, 10 mM, 20 mM, 50 mM and 100 mM of bivalent metal ions such as Co^{2+}, Mn^{2+}, and Mg^{2+} chloride salts were added separately to the dialyzed enzyme preparation and assayed glucose isomerase activity as described above. The glucose isomerase activity of the enzyme without metal ion was defined as 100%. The residual activity (%) was assayed spectrophotometrically.

The inhibitor effects of various metal ions on the glucose isomerase activity of *Ago*G2GI-wt and *Ago*G2GI-rec were assayed at optimum reaction conditions. The metal ions Cd^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} were added as chloride or sulfate salts to the enzyme solution in amounts of 0.1 mM, 1 mM, 5 mM, and 10 mM. The glucose isomerase activity of the enzyme without metal ion was defined as the 100% level. The residual activity (%) was assayed spectrophotometrically.

The nucleotide sequence has been submitted to the GenBank/EMBL Data Bank with accession number JQ768452.

3. Results

3.1. Detection of thermostable glucose isomerase in *Anoxybacillus gonensis G2*^{T}

The presence of glucose isomerase activity in the *A. gonensis* G2^T and *E. coli* HB101 strains was studied by plate assay. *A. gonensis* G2^T showed a dark brown halo around the light colonies, indicating glucose isomerase activity. The *E. coli* HB101 strain had no activity.

3.2. Cloning of AgoG2 gene

In this work, using two degenerate primers, we obtained a 534 bp fragment of the *XylA* gene; the remaining portion of the gene sequence was subsequently obtained by using inverse PCRs. The whole gene was analyzed by using the Advanced Blast Program

Table 2
Summary of purification steps: (a) AgoG2GI-wt (b) AgoG2GI-rec.

(a)

Purification step	Volume (ml)	[Protein] (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Cell extract	30	11.53	345.9	423	1.22	100	1
Heat treatment	24	2.50	60.1	255	4.25	60.2	3.47
$(NH4)_2SO_4$ sedimentation	19	1.80	34.2	175	5.11	41.3	4.18
DEAE-Sepharose	45	0.24	10.8	147	13.61	34.7	11.12
Phenyl-Sepharose 6 Fast flow	17	0.33	5.5	116	20.98	27.4	17.14

(b)

Cell extract	13	17.38	225.9	1730	7.66	100.0	1.00
Heat treatment	12	5.74	68.8	1251	18.17	72.3	2.37
DEAE-sepharose	33	1.21	39.8	1045	26.25	60.4	3.43
Phenyl-sepharose 6 fast flow	30	0.85	25.5	752	29.50	43.5	3.85

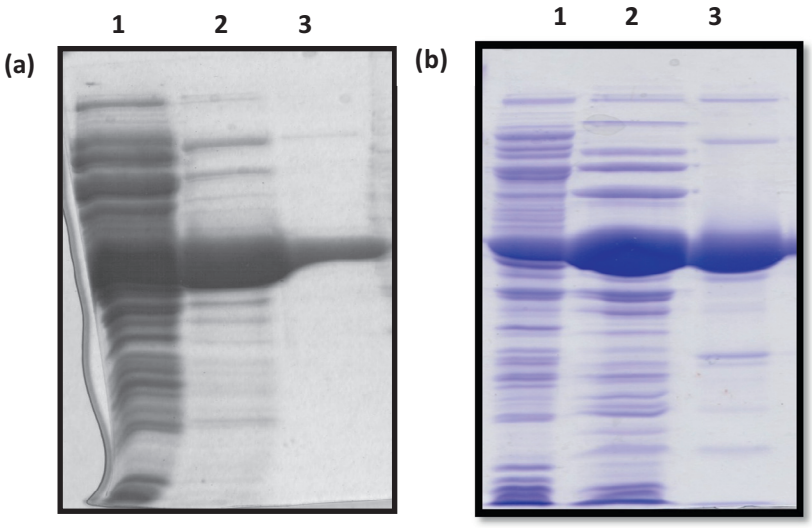

Fig. 3. (a) SDS-PAGE showing purified recombinant AgoG2GI-rec enzyme contained HisTag tail. (1) Crude cell extract of *E. coli* BL21 DE3 expressing recombinant AgoG2GI-rec enzyme. (2) AgoG2GI-rec after heat shock. (3) The recombinant enzyme purified by MagneHis Protein Purification System. (b) SDS-PAGE showing purified recombinant AgoG2GI-rec enzyme without HisTag tail. (1) Crude cell extract of *E. coli* BL21 DE3 expressing recombinant AgoG2GI-rec enzyme. (2) AgoG2GI-rec after heat shock (3) AgoG2GI-rec after ion-exchange column and hydrophobic interaction chromatography.

of GenBank (NCBI, NIH, Washington DC) and analysis revealed the presence of a 1326 bp open reading frame (ORF) encoding a hypothetical 441 amino acid protein with a molecular mass of 49,976 Da (calculated by ProtParam, www.expasy.org), identified by a database enquiry (BLASTP).

3.3. Comparison of the protein sequences

The phylogenetic tree of *Ago*G2GI and other glucose isomerase amino acid sequences indicates that *A. gonensis* $G2^T$ glucose isomerase clearly belongs to xylose isomerase family II, which includes the enzymes from *E. coli, Bacillus* sp., *Lactobacillus* sp., *Lactococcus* sp., *Thermoanaerobacterium thermosulfurigenes* and *Thermotoga* sp. [22]. Family I glucose isomerases are shorter by 40 to 50 residues at the N-terminal end; *Streptomyces* sp., *Actinoplanes* sp., *Ampullariella* sp., *Arthrobacter* sp. and *T. thermophilus* are the examples of family I (Fig. 2) [23].

3.4. Expression and purification of glucose isomerase gene

The gene encoding *Ago*G2GI was engineered for expression to give p*Ago*G2GI-rec or p*Ago*G2GI-recHis. In both cases, expression of the glucose isomerase gene was under the control of a T7 promoter. The two constructs were used to transform *E. coli* BL21(DE3). Overexpression of the cloned glucose isomerase induced by the addition of IPTG resulted in a high expression of soluble glucose isomerase activity of 7.66 U/mg for *Ago*G2GIrec but a low expression at 0.05 U/mg for *Ago*G2GI-recHis. Due to the low activity of *Ago*G2GI-recHis, purification studies were initiated with *Ago*G2GI-rec.

In *A. gonensis* $G2^T$, the glucose isomerase gene was not expressed unless D-xylose was present in the medium. *Ago*G2GI-wt was expressed in *A. gonensis* $G2^T$ with specific activity of 1.22 U/mg in the presence of 0.5% xylose. The purification procedures applied for both enzymes (*Ago*G2GI-rec and *Ago*G2GI-wt) were the same except for ammonium sulfate precipitation and are described under Section 2. Because the enzyme was stable for several hours at room temperature, all purification applications were performed at room temperature. Table 2 shows the effects of all purification techniques on the specific activities, fold purification and yield. Because of the thermostability of the recombinant enzyme, heat treatment of the cell-free extract at 75 °C for 15 min was a very efficient purification step for thermostable glucose isomerase from either *E. coli* or *A. gonensis* $G2^T$. Indeed, *Ago*G2GI-wt and *Ago*G2GI-rec were estimated to be approximately 60% and 72% pure after this heat treatment step, respectively. A 17.14 and 3.85-fold purification and a yield of 27.4% and 43.5% for *Ago*G2GI-wt and *Ago*G2GI-rec, respectively, were obtained from the cell-free extracts. Fig. 3 gives the SDS-PAGE analysis of *Ago*G2GI-rec and *Ago*G2GI-wt.

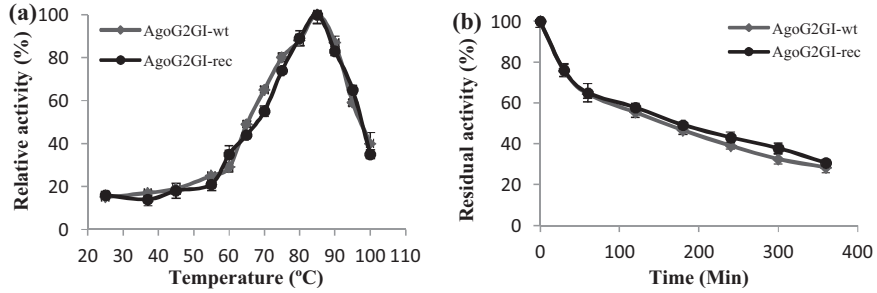

Fig. 4. (a) Effect of temperature on activity of the purified glucose isomerases. (b) Effect of temperature on stability of the purified glucose isomerases. The percentage relative enzyme activity was calculated by comparison with unincubated enzyme.

3.5. Determination of molecular mass

Analysis of the soluble fractions on SDS-PAGE revealed a band below the 50 kDa protein marker that did not correspond to the anticipated molecular mass of 50 kDa for *Ago*G2GI. The discrepancy between the theoretical mass and observed mass was resolved by MALDI-TOF analysis. The molecular mass of *Ago*G2GI-wt and *Ago*G2GI-rec were determined as 50.047 kDa. The apparent solution molecular mass of *Ago*G2GI was determined to be 180 kDa according to gel filtration analysis using a Superdex^{TM} 200 10/300 GL column (Amersham Pharmacia Biotech).

3.6. Characterization of the cloned glucose isomerase

The native (*Ago*G2GI-wt) and expressed glucose isomerases (*Ago*G2GI-rec) displayed identical molecular weights and compositions, pH and temperature activity optima, thermostabilities, and metal ion requirements.

The glucose isomerases exhibited simple Michaelis–Menten kinetics. Based on the Michaelis–Menten equation, the apparent enzyme K_m, V_{max}, and k_{cat} values for D-glucose were determined as 146.08 ± 9.50 mM, 43.72 ± 1.01 μmol/min/mg protein and 36.47 ± 2.01 (1/s), respectively, for *Ago*G2GI-wt, and 138.37 ± 7.63 mM, 40.51 ± 0.81 μmol/min/mg protein and 33.79 (1/s), respectively for *Ago*G2GI-rec. The K_m, V_{max} and k_{cat} values did not significantly change for the recombinant and native enzymes. The initial rates were calculated by measuring the absorption at 560 nm for several concentrations of glucose over a range of 0 mM to 700 mM.

The K_m and V_{max} values for *Ago*G2GI-rec toward different substrates, L-arabinose, D-ribose and D-mannose, were determined to be 58.74 ± 9.5 mM, 0.0413 μmol/min/mg; 80.32 ± 10.06 mM, 0.85 μmol/min/mg, and 121.8 ± 25.33 mM, 0.0276 μmol/min/mg, respectively. k_{cat} values for *Ago*G2GI-rec toward L-arabinose, D-ribose, and D-mannose, were determined to be 0.0344, 0.709 and 0.023 (1/s), respectively.

3.7. Effects of temperature on activity and stability

The effects of temperature on glucose isomerase activity and stability were tested spectrophotometrically using D-glucose as substrate. The optimum temperature for glucose isomerase activity was observed to be at 85 °C (Fig. 4a).

*Ago*G2GI-rec and *Ago*G2GI-wt retained 100% of their activity at room temperature and 4 °C (data not shown). The enzymes lost more than 50% of their activity at 85 °C after 3 h (Fig. 4b). The enzymes also require Co^{2+} or Mg^{2+} and Mn^{2+} for high thermal stability (data not shown).

3.8. Effects of pH on activity and stability

The optimum pH for the glucose isomerase activities of *Ago*G2GI-rec and *Ago*G2GI-wt were observed to be 6.5 (Fig. 5a).

The enzymes were active and stable in the broad pH range of 5.0 to 9.0 at 4 °C. When assayed at various pH values at 85 °C, the purified enzymes showed the most stability at the pH range of 8 to 9. Fig. 5b shows the effect of pH on stability of AgoG2GI-rec enzyme. The effect of pH on stability of AgoG2GI-wt was not shown since the results of AgoG2GI-wt are in accordance with AgoG2GI-rec.

3.9. Effect of metal ions

The effects of various metal ions on glucose isomerase activity was assayed at 85 °C (pH 6.5) using D-glucose as substrate for AgoG2GI-wt and AgoG2GI-rec. Co^{2+} was the most required metal ion for glucose isomerase activity, whereas Mg^{2+} and Mn^{2+} slightly enhanced enzyme activity (Table 3). A minimum concentration of 1 mM $CoCl_2$ was required to achieve maximum glucose isomerase activity. Higher Mg^{2+} or Mn^{2+} concentrations did not increase the activity to the level of 1 mM $CoCl_2$. In the absence of metal ions (Co^{+2}, Mg^{+2}, and Mn^{+2}) the enzyme lost all of its original activity at 85 °C over a 2 h period. However, in the presence of 1 mM Co^{2+} or

Table 3
The activator effects of various metal ions on the glucose isomerase activity of *Ago*G2GI-wt and *Ago*G2GI-rec.

Concentration of metal ions (mM)	*Ago*G2GI-wt relative activity (%)			*Ago*G2GI-rec relative activity (%)		
	Co^{+2}	Mg^{+2}	Mn^{+2}	Co^{+2}	Mg^{+2}	Mn^{+2}
0	0	0	0	0	0	0
0.1	64 ± 1.7	11 ± 1.4	21 ± 2.4	61 ± 2.1	10 ± 1.3	19 ± 1.1
0.5	83 ± 3.2	10 ± 1.5	35 ± 2.1	82 ± 1.4	15 ± 0.7	35 ± 2.6
1	100 ± 1.5	10 ± 1.1	36 ± 3.4	100 ± 1.5	14 ± 2.1	43 ± 2.9
2	98 ± 4.5	12 ± 0.3	35 ± 7.5	93 ± 8.9	13 ± 0.8	34 ± 1.3
4	96 ± 6.3	18 ± 2.7	24 ± 2.1	93 ± 7.5	17 ± 0.9	28 ± 0.4
10	83 ± 4.6	24 ± 3.2	26 ± 1.2	88 ± 5.8	28 ± 2.1	28 ± 0.7
20	59 ± 10.1	22 ± 2.1	21 ± 0.4	55 ± 3.5	32 ± 2.7	26 ± 1.1
50	57 ± 8.7	26 ± 0.6	21 ± 0.7	45 ± 2.1	25 ± 2.6	31 ± 2.1
100	39 ± 9.8	24 ± 1.2	17 ± 1.1	32 ± 1.6	26 ± 1.4	20 ± 0.4

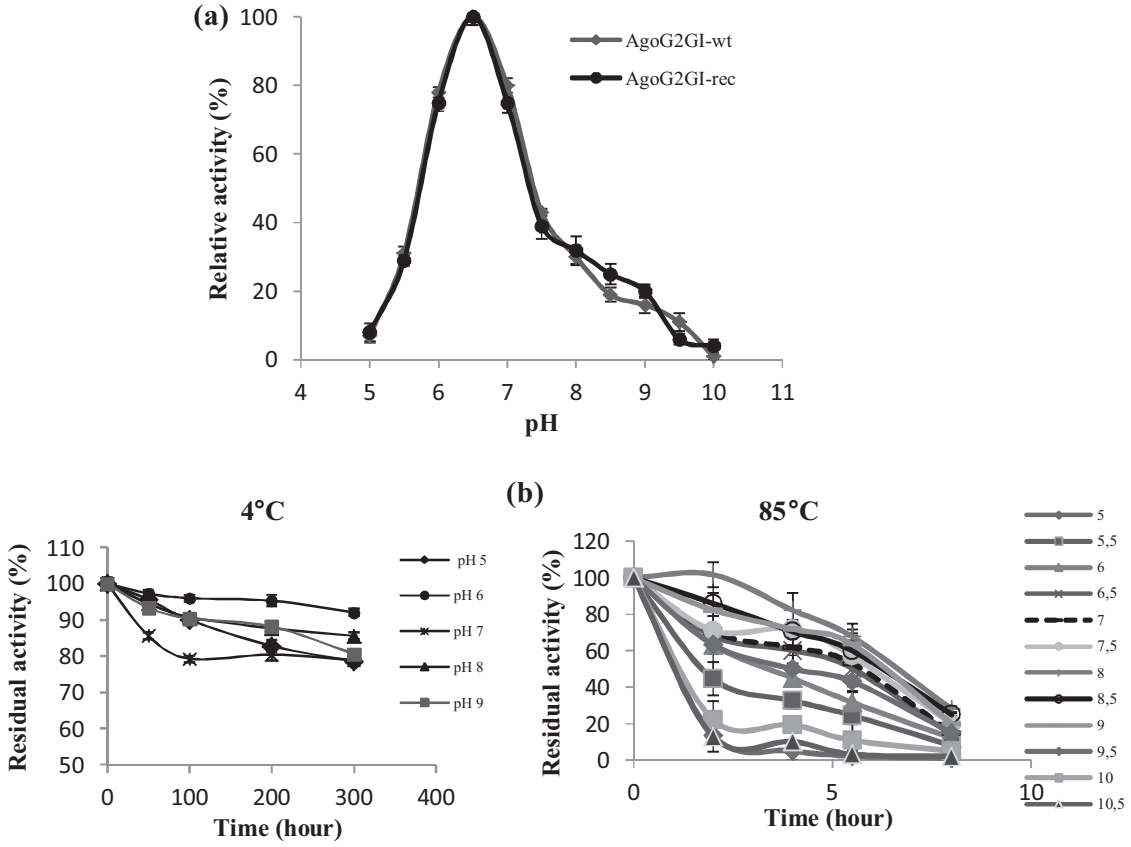

Fig. 5. (a) Effect of pH on the activity of the purified glucose isomerases. (b) Effect of pH on stability of AgoG2GI-rec enzyme at 4 °C and 85 °C. The percentage relative enzyme activity was calculated by comparison with unincubated enzyme.

Mn^{2+}, the enzymes retained 100% and 36% (or 43%) of their original activities, respectively. The activities of purified glucose isomerases were dependent mainly on Co^{2+} rather than Mn^{2+} or Mg^{2+} at 85 °C.

Mg^{2+} and Mn^{2+} are determined as the activators of *Ago*G2GI-wt and *Ago*G2GI-rec as with other GIs [24]. *Ago*G2GI-wt and *Ago*G2GI-rec were inhibited by the addition of chloride and sulfate salts of Cd^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} bivalent metal ions (Table 4).

4. Discussion

The screening method, based on a specific assay that detected conversion of glucose to fructose on agar plates, facilitated the identification of glucose isomerase activity in *A. gonensis* $G2^T$ [16]. Here, we have described the cloning, expression, purification and biochemical characterization of the *A. gonensis* $G2^T$ glucose isomerase gene (*xylA*). This is the first example of GI from *A. gonensis* $G2^T$ and the genus *Anoxybacillus*.

The *A. gonensis* $G2^T$ glucose isomerase clearly belongs to glucose isomerase family II, which includes the enzymes from *Bacillus stearothermophilus* (91%), *Geobacillus kaustophilus* HTA426 (90%), *Geobacillus* sp. C56-T3 (89%), *Bacillus halodurans* C-125 (79%), *Bacillus megaterium* DSM 319 (77%) and *Bacillus coagulans* 36D1 (75%), with the indicated amino acid sequence similarity, and over 70% for many other bacteria. Enzymes of family I are shorter than those of family II by 40 to 50 residues at the N-terminal end. The *A. gonensis* $G2^T$ enzyme is different from the enzymes belong to family I glucose isomerases. According to phylogenetic analysis of *Ago*G2GI and other glucose isomerase amino acid sequences, the *A. gonensis* $G2^T$ glucose isomerase clearly belongs to xylose isomerase family II.

Heterologous proteins overproduced in *E. coli* are often generated in the form of insoluble inclusion bodies [25]. Whether a protein precipitates in the cytoplasm or remains soluble, however, depends also on the nature of the protein being overproduced [26]. In the case of the recombinant thermostable glucose isomerase, the enzyme remained soluble, which may be an important factor in attempts to scale up its production [16]. However, expressing enzymes from hyperthermophiles in mesophilic hosts raises questions about the properties of the recombinant enzyme versus the native enzyme. In addition to the problems normally encountered in expressing recombinant proteins, the correct folding of a hyperthermophilic protein at significantly lower temperatures is a key concern [23]. Therefore, we purified the enzyme not only from *E. coli* BL21/pAgoGI-rec but also from *A. gonensis* $G2^T$ and compared the kinetic parameters of both enzymes. We found no significant differences, suggesting that the host organism did not affect the refolding of the enzyme and that both enzymes are of the same size.

Glucose isomerase is a tetramer, and the asymmetric unit of the crystal contains a dimer. Each subunit contains two domains. The main domain is a parallel-stranded α β barrel. The C-terminal domain is a loop structure consisting of five helical segments and is involved in intermolecular contacts between subunits [2]. AgoG2GI-recHis have the His tag at the C-terminus in the resulting recombinant enzyme. Overexpression of the cloned glucose isomerase resulted in a high expression of soluble glucose isomerase activity for *Ago*G2GIrec but a low expression for AgoG2GI-recHis. These results suggest that the C-terminal region is important for intersubunit contacts, which may explain why the His tag is unfavorable for enzyme activity. Due to the low activity of AgoG2GI-recHis, characterization studies were initiated with *Ago*G2GI-rec.

Table 4
The inhibitor effects of various metal ions on the glucose isomerase activity of *Ago*G2GI-wt and *Ago*G2GI-rec.

Concentration of metal ions (mM)	*Ago*G2GI-wt residual activity (%)							*Ago*G2GI-rec residual activity (%)						
	Cd^{2+}	Ca^{2+}	Hg^{2+}	Ni^{2+}	Zn^{2+}	Fe^{2+}	Cu^{2+}	Cd^{2+}	Ca^{2+}	Hg^{2+}	Ni^{2+}	Zn^{2+}	Fe^{2+}	Cu^{2+}
0	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5	100 ± 1.5
0,1	27 ± 1.2	99 ± 2.1	1 ± 0.1	98 ± 5.7	20 ± 2.1	63 ± 3.6	7 ± 0.2	21 ± 1.6	95 ± 7.8	0	90 ± 5.4	15 ± 1.3	52 ± 4.1	0
1	9 ± 1.2	83 ± 3.5	0	61 ± 3.2	10 ± 0.2	18 ± 1.6	0	0	94 ± 5.4	0	49 ± 3.9	10 ± 0.4	5 ± 0.2	0
5	2 ± 0.1	52 ± 2.6	0	20 ± 1.3	0	0	0	0	77 ± 3.7	0	22 ± 1.4	1 ± 0.1	0	0
10	0	40 ± 3.2	0	21 ± 2.8	0	0	0	0	52 ± 4.5	0	5 ± 0.3	0	0	0

The molecular mass estimated from SDS-PAGE analysis was inconsistent with the anticipated value based on the corresponding protein sequence. Such a discrepancy may be due to the nature of the protein under investigation. Sizing accuracy of SDS-PAGE depends on the protein characteristics, such as amino acid sequence, isoelectric point, structure, and the presence of certain side chains or prosthetic groups. Consequently, discrepancies may arise for certain proteins, such as glycosylated proteins, which do not truly migrate according to their molecular weight [27]. The apparent inconsistency between theoretical mass and observed mass estimated from SDS-PAGE was resolved by MALDI-TOF analysis, which confirmed the size of both *Ago*G2GI-wt and *Ago*G2GI-rec to be 50.047 kDa.

*Ago*G2GI-wt and *Ago*G2GI-rec showed optimal activity at 85 °C when incubated with D-glucose for 30 min. Glucose isomerases described in the literature ~~are~~ generally operate at an optimum temperature between 60 °C and 80 °C with a few exceptions: [2] the optimum temperature for *Streptomyces* sp.[28] and *Bacillus* sp.[29] was observed to be 85 °C; the optimum temperature of the glucose isomerase of *Thermotoga neapolitana* was reported as 95 °C [23], the highest T_{opt} glucose isomerase. The thermodynamic equilibrium fructose/glucose ratio increases with temperature so commercial processes are carried out with immobilized enzymes at around 60 °C, which is the effective upper limit of their thermostability. These processes yield 45% fructose/55% glucose syrups, but 55% fructose syrups are desirable for food and soft drinks use, so the former are fortified with chromatographically purified fructose. This expensive step would be superfluous if the isomerization could be performed at 90–95 °C where 55% fructose syrups could, in theory, be produced directly. Hence, there has been a widespread search for glucose isomerases that are stable at these elevated temperatures [6]. We can conclude that AgoG2GI is among the highest T_{opt} glucose isomerases described to date. AgoG2GI has activity of more than 50% at 90–95 °C. This finding has great potential in biotechnological terms because the higher temperatures drive the equilibrium of the isomerization of glucose toward fructose formation. Thus, the T_{opt} 85 of AgoG2GI makes it one of the favored enzymes for HFCS production [30].

The AgoG2GIs lost 50% of their original activity at 85 °C after 3 h. However, full activity was retained after an extended period of storage at +4 °C. *Bacillus* sp. glucose isomerase retained 100% of its activity at the temperature range of 50 °C to 80 °C and lost its activity above 80 °C within 30 min [29]. *Bacillus* TX-3 glucose isomerase lost more than 20% of its activity at 80 °C within 10 min [31]. The half-life of *Thermoanaerobacterium* sp. glucose isomerase is 1 h at 82 °C [21]. Based on these data, it is suggested that AgoG2GI is more stable than these glucose isomerases at 85 °C.

The activity of glucose isomerase was tested in the pH range of 5.0 to 11.0. The optimum activities of many glucose isomerases were observed at pHs higher than 6.5, ranging generally between pH 7.0 and 9.0 [2]. Glucose isomerases from *Streptomyces* sp., *T. thermophilus*, and *Clostridium thermosulfurogenes* showed pH optimum at 7.0 for D-glucose substrate [14,16,28]. However, commercial application of glucose isomerase requires an acidic pH optimum to enable starch liquefaction and isomerization to be performed in a single step. In addition, the acidic conditions in industrial processes decrease the formation of undesired compounds like browning products (mannose, psicose and other acidic compounds). Fig. 5 shows the pH dependence of the enzymatic activity, measured after 30 min at 85 °C for D-glucose substrate, demonstrating highest activities around pH 6.5. This enzyme can be thought suitable for industrial applications, like *Lactobacillus brevis* having a lower pH optimum between 6.0 and 7.0 [2]. To determine the stability of the enzyme at pH values of 5.0 and 10.5, pre-incubation was performed at each pH value at 4 °C for 300 h and at 85 °C for 8 h. The enzymes were active and stable in a broad pH range between 5.0

and 9.0 at 4 °C (Fig. 5). When assayed at various pH values at 85 °C, the purified enzymes showed the most stability at the pH range of 8 to 9 (Fig. 5). The pH-optima of activity and stability are different for AgoG2GI and this difference occurs in some cases according to Talley and Alexov [32].

The pH stability of an enzyme is very important to predict its storage condition, because the enzyme can lose activity after being stored at different pH values. According to these results, glucose isomerase can be stored in a broad range of pH conditions for a long period of time without significant loss of activity.

The high thermostability of the *A. gonensis* G2^T glucose isomerase made it possible to use heat treatment of crude cell extracts as one of the most efficient purification steps when the enzyme was produced in mesophilic hosts. Thermal treatment caused the denaturation of mesophilic host proteins. The presence of metal ions (Mg^{2+} and Co^{2+}) and a high protein concentration in cell extracts were essential for optimal recovery of the enzyme during heat treatment [33]. The activity of xylA from G2 was more dependent on Co^{2+} than on Mn^{2+} or Mg^{2+} at 85 °C. Co^{2+} increased thermal stability effectively, whereas Mg^{2+} had a low effect and Mn^{2+} decreased thermal stability (data not shown). Co^{2+} is supposed to be more important for stabilization of the multimeric structure, resulting in thermostability of the enzyme [34].

It has long been known that all glucose isomerases require either Co^2, Mg^{2+} or Mn^{2+} ions for activity and stability. We can now see that all of these enzymes have essentially similar metal ion requirements, but the activity and stability depend on pH, temperature, substrate and the presence of other ions [6]. AgoGI showed no measurable activity in the absence of metal ions. In the presence of Co^{+2}, AgoGI has the highest activity, as is the case for glucose isomerases from *B. coagulans* [35,36], *Bacillus* sp. [29,37], *Thermus aquaticus* [38] and *Streptomyces violaceoruber* [39]. The effects of Mg^{2+}, Co^{2+} or Mn^{2+} on AgoGI are consistent with Vieille et al. for glucose isomerase from *Bacillus licheniformis* [24].

The behavior of *Ago*GIs for some metal ions was examined by using chloride and sulfate salts of each metal at concentrations of 0 mM to 10 mM. *Ago*GIs were inhibited by divalent ions of heavy metals, strongly by Cd^{2+}, Hg^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+}, but only moderately by Ni^{2+} and a competitive inhibitor, Ca^{2+}, which is in accordance with the results of Lehmacher and Bisswanger [38], Rangarajan and Hartley [40], Smith et al. [41], Suekane et al. [42], Chen and Anderson [43] and Kwon et al. [37].

The domestic HFCS market in 2011 sustained the strong momentum of demand, production and sales from 2010. The industry developed rapidly and the production recorded 2.36 million tons in 2011, whereas it had been merely 400,000 t in 2006. The total amount of HFCS produced by glucose isomerase exceeds a million tons per year [44]. Acid-stable glucose isomerases, which are resistant to inhibition by Ca^{2+}, are useful in a uni-pH process. The combination of saccharification and isomerization is an ideal development in the progress of HFCS production, and it is likely to be in operation once an acid-stable, thermostable and Ca^{2+}-tolerant GI is discovered. Ca^{2+} showed slight inhibition on AgoGI and has high optimum temperature but the optimum pH for AgoGI was observed to be 6.5. This can be improved by protein engineering and can be used for commercial production of HFCS [2].

The AgoG2GI-wt or AgoG2GI-rec glucose isomerase has kinetic characteristics for glucose isomerization similar to those of other glucose isomerases with similar amino acid sequences. With respect to glucose, however, the AgoG2GI-wt or AgoG2GI-rec enzymes have very low K_m and a higher V_{max} (146.08 mM and 43.72 ± 1.01 μmol/min/mg protein) than most thermophilic glucose isomerases. The K_m values for GIs from *Arthrobacter* sp., *A. missouriensis*, *Bifidobacterium adolescentis*, *Streptomyces* sp. and *Bacillus* sp. were 210 mM [41], 290 mM [45], 398 mM [46], 400 mM [28] and 142 mM [29], respectively.

AgoG2GI has very high k_{cat} value for glucose. The k_{cat} value of AgoG2GI was determined to be 36.47 ± 2.01 (1/s), which is greater than that of *Streptomyces chibaensis* (30.72/s) [47], *A. missouriensis* (25.3/s) [48], *Arthrobacter* sp. (19.9/s) [41] and *T. thermophilus* (0.833/s) [49]. In general, the k_{cat} value of AgoG2GI is the second best among the published k_{cat} values of other species except the species of *Bacillus* (53/s) [29]. This is almost the highest turnover rate among any of the wild-type glucose isomerases characterized so far. Moreover, AgoG2GI shows a better catalytic efficiency with respect to glucose. It remains to be seen if the catalytic efficiency for the industrial substrate can be improved by site-directed mutagenesis, as has been done for other xylose isomerases [24].

Substrate orientation, especially the hydroxyl group (–OH) of sugar is speculated to define the preferential substrate for isomerases [50]. In addition to D-glucose, *Ago*G2GI also isomerizes D-ribose, L-arabinose, and D-mannose, which are non-preferential substrates for glucose isomerases. k_{cat} values for *Ago*G2GI-rec toward L-arabinose, D-ribose and D-mannose were determined to be 0.0344, 0.709 and 0.023 (1/s), respectively. *Ago*G2GI has a higher k_{cat} value for glucose than the other substrates. According to these results, L-arabinose, D-ribose and D-mannose are non-preferential substrates for this glucose isomerase.

5. Conclusion

In conclusion, a new D-glucose isomerase (AgoG2GI) was isolated, cloned and characterized from *A. gonensis* G2^T. Our analysis indicates that this enzyme is among the best D-xylose–glucose isomerases described so far. Special features of AgoG2GI is its relatively high catalytic activity toward the substrate D-glucose with relatively high V_{max}, low K_m, high k_{cat}, and high k_{cat}/K_m values. The enzyme preferentially uses glucose as a substrate.

Given the promising characteristics of AgoG2GI, it may compete with the enzymes currently used in industry. However, further experiments are needed before practical industrial utility can be attained i.e., lowering the T_{opt} to 60 °C, which is the temperature used in the industrial process.

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References

[1] M.G. Wovcha, D.L. Steuerwald, K.E. Brooks, App. Environ. Microb. 45 (1983) 1402–1404.
[2] S.H. Bhosale, M.B. Rao, V.V. Deshpande, Microbiol. Rev. 60 (1996) 280–300.
[3] V.J. Jensen, S. Rugh, Methods Enzymol. 136 (1987) 356–370.
[4] A. De Raadt, M. Ebner, C.W. Ekhart, M. Fechter, A. Lechner, M. Strobi, A.E. Stutz, Catal. Today 22 (1994) 549–561.
[5] W.D. Crabb, J.K. Shetty, Opin. Microbiol. 2 (1999) 252–256.
[6] B.S. Hartley, N. Hanlon, R.J. Jackson, M. Rangarajan, Biochim. Biophys. Acta 1543 (2000) 294–335.
[7] E.B. Da silva, A.U. De souza, S.G. De Souza, A.E. Rodrigues, Adsorption 11 (2005) 847–851.
[8] G.D. Schellenberg, A. Sarthy, A.E. Larson, M.P. Backer, J.W. Crabb, M. Lidstrom, B.D. Hall, C.E. Furlong, J. Biol. Chem. 259 (1984) 6826–6832.
[9] M. Wilhelm, C.P. Hollenberg, Nucleic Acids Res. 13 (1985) 5717–5722.
[10] K. Dekker, H. Yamagata, K. Sakaguchi, S. Udaka, Agric. Biol. Chem. 55 (1991) 221–227.
[11] G.J. Joo, J.H. Shin, G.Y. Heo, Y.M. Kim, I.K. Rhee, J. Microbiol. 43 (2005) 34–37.
[12] G.C. Saari, A.A. Kumar, G.H. Kawasaki, M.Y. Insley, P.J. O'Hara, J. Bacteriol. 169 (1987) 612–618.
[13] R. Amore, C.P. Hollenberg, Nucleic Acids Res. 17 (1989) 75–115.
[14] K. Dekker, H. Yamagata, K. Sakaguchi, S. Udaka, J. Bacteriol. 173 (1991) 3078–3083.

[15] A.O. Belduz, S. Dulger, Z. Demirbag, Int. J. Syst. Evol. Microbiol. 53 (2003) 1315–1320.
[16] C.Y. Lee, L. Bhatnagar, B.C. Saha, Y.E. Lee, M. Takagi, T. Imanaka, M. Bagdasarian, J.G. Zeikus, App. Environ. Microb. 56 (1990) 2638–2643.
[17] T. Trigilia, M.G. Peterson, D.J. Kemp, Nucleic Acids Res. 16 (1988) 81–86.
[18] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[19] Z. Dische, E. Borenfreund, J. Biol. Chem. 192 (1951) 583–587.
[20] T. Maniatis, E.F. Fritsch, J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982, pp. 545.
[21] S.Y. Liu, J. Wiegel, F.C. Gherardini, J. Bacteriol. 178 (1996) 5938–5945.
[22] C. Vieille, J.M. Hess, R.M. Kelly, J.G. Zeikus, Appl. Environ. Microbiol. 61 (1995) 1867–1875.
[23] J.M. Hess, V. Tchernajenko, C. Vieille, J.G. Zeikus, R.M. Kelly, Appl. Environ. Microbiol. 64 (1998) 2357–2360.
[24] C. Vieille, K.L. Epting, R.M. Kelly, J.G. Zeikus, Eur. J. Biochem. 268 (2001) 6291–6301.
[25] F. Baneyx, M. Mujacic, Nat. Biotechnol. 22 (2004) 1399–1408.
[26] G. Bueli, N. Panayotatos, in: W. Reznikoff, L. Gold (Eds.), Mechanism and Practice, Maximizing Gene Expression, Butterworths Publishers, Boston, 1986, pp. 345–363.
[27] H. Goetz, M. Kuschel, T. Wulff, C. Sauber, C. Miller, S. Fisher, C. Woodward, J. Biochem. Biophys. Methods 60 (2004) 281–293.
[28] C.U. Inyang, U. Gebhart, S.K.C. Obi, H. Bisswanger, Appl. Microbiol. Biotechnol. 43 (1995) 632–638.
[29] J. Chauthaiwale, M.B. Rao, Appl. Environ. Microbiol. 60 (1994) 4495–4499.
[30] L.D. Kluskens, J. Zeilstra, A.C. Geerling, W.M. De vos, J. Van der Oost, Environ. Technol. 31 (2010) 1083–1090.
[31] M. Kitada, Y. Dobashi, K. Horikoshi, Agric. Biol. Chem. 53 (1989) 1461–1468.
[32] K. Talley, E. Alexov, Proteins 78 (2010) 2699–2706.
[33] C.Y. Lee, M. Bagdasarian, M.H. Meng, J.G. Zeikus, J. Biol. Chem. 265 (1990) 19082–19090.
[34] J. Cha, Y. Cho, R.D. Whitaker, H.L. Carrell, J.P. Glusker, P.A. Karplus, C.A. Batt, J. Biol. Chem. 269 (1994) 2687–2694.
[35] G. Danno, Agric. Biol. Chem. 34 (1970) 1805–1814.
[36] G. Danno, Agric. Biol. Chem. 35 (1971) 997–1006.
[37] H.J. Kwon, M. Kitada, K. Horikoshi, Agric. Biol. Chem. 51 (1987) 1983–1989.
[38] A. Lehmacher, H. Bisswanger, J. Gen. Microbiol. 136 (1990) 679–686.
[39] M. Callens, H. Kersters-Hilderson, W. Vangrysperre, C.K. Debruyne, Enzyme Microb. Technol. 10 (1988) 695–700.
[40] M. Rangarajan, B.S. Hartley, Biochem. J. 283 (1992) 223–233.
[41] C.A. Smith, M. Rangarajan, B.S. Hartley, Biochem. J. 277 (1991) 255–261.
[42] M. Suekane, M. Tamura, C. Tomimura, Agric. Biol. Chem. 42 (1978) 909–917.
[43] W.P. Chen, A.W. Anderson, Appl. Environ. Microbiol. 38 (1979), 1111-111.
[44] T. Johannes, M.R. Simurdiak, H. Zhao, in: S. Lee (Ed.), Biocatalysis, Encyclopedia of Chemical Processing, New York, 2006, pp. 101–110.
[45] J. Jenkins, J. Janin, F. Rey, M. Chiadmi, H. Van Tilbeurgh, I. Lasters, M. DeMaeyer, D. VanBelle, S.J. Wodak, M. Lauwereys, P. Stanssens, N.T. Mrabet, J. Snauwaert, G. Matthyssens, G.A. Lambeir, Biochemistry 31 (1992) 5449–5458.
[46] Y. Kawai, H. Konishi, H. Horitsu, H. Sakurai, K. Takamizawa, T. Suzuki, K. Kawai, Biosci. Biotechnol. Biochem. 58 (1994) 691–694.
[47] G.J. Joo, J.H. Shin, G.Y. Heo, Y.Y. Kwak, J.H. Choi, I.K. Rhee, Agric. Chem. Biotechnol. 44 (2001) 113–118.
[48] J. Karimaki, T. Parkkinen, H. Santa, O. Pastinen, M. Leisola, J. Rouvinen, O. Turunen, Protein Eng. Des. Sel. 17 (2004) 861–869.
[49] A. Lonn, M. Gardonyi, W. Van Zyl, B. Hahn-Hagerdal, R.C. Otero, Eur. J. Biochem. 269 (2002) 157–163.
[50] D.H. Patel, E.J. Cho, H.M. Kim, I.S. Choi, H.J. Bae, Protein Eng. Des. Sel. (2012) 1–6.