J. Biochem. **141,** 817–825 (2007)
doi:10.1093/jb/mvm085

Cloning, Expression, Purification and Characterization of Fructose-1,6-bisphosphate Aldolase from *Anoxybacillus gonensis* G2

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Received February 14, 2007; accepted February 19, 2007; published online March 29, 2007

The fructose-1,6-bisphosphate aldolase gene from the thermophilic bacterium, *Anoxybacillus gonensis* G2, was cloned and sequenced. Nucleotide sequence analysis revealed an open reading frame coding for a 30.9 kDa protein of 286 amino acids. The amino acid sequence shared ~80–90% similarity to the *Bacillus* sp. class II aldolases. The motifs that are responsible for the binding of a divalent metal ion and catalytic activity completely conserved. The gene encoding aldolase was overexpressed under T7 promoter control in *Escherichia coli* and the recombinant protein purified by nickel affinity chromatography. Kinetic characterization of the enzyme was performed at 60°C, and K_m and V_{max} were found to be 576 μM and 2.4 μM min^{-1} mg protein^{-1}, respectively. Enzyme exhibits maximal activity at pH 8.5. The activity of enzyme was completely inhibited by EDTA.

Key words: *Anoxybacillus gonensis*, cloning, fructose-1,6-bisphosphate aldolase, sequencing, thermophilic.

Abbreviations: FBP, fructose-1,6-bisphosphate; IPTG, isopropyl β-D-thiogalactopyranoside.

Carbon–carbon bond formation is one of the most important reactions for organic chemistry. The enzymes catalyze C–C bond formation are used as biocatalysts in organic reactions because of their ability to catalyze stereoselective reactions (*1*). In this area, aldol condensations catalyzed by aldolases are widely used in the synthesis of sugars, polyhydroxy antibiotics, and many other biologically active compounds (*2*). Therefore, aldolases are increasingly attractive as biocatalysts and especially important for carbohydrate biosynthesis. Among the most studied representatives of this group enzymes are fructose-1,6-bisphosphate (FBP) aldolases (*3*, *4*). FBP aldolase (EC 4.1.2.13) that is an essential enzyme for glycolysis, gluconeogenesis, and Calvin cycle catalyses the reversible aldol condensation or cleavage of FBP into glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate (*5*).

There are two classes of FBP aldolases based on the reaction mechanism. Class I FBP aldolases form a Schiff-base intermediate between the C-2 carbonyl group of the substrate (dihydroxyacetone-phosphate) and the ε-amino group of a lysine residue in the active site. The class I enzymes are mainly found in higher organisms (animals, plants), in green algae and in few prokaryotes (*5–7*). This class of enzyme forms tetramer in eukaryotes, but can be monomeric to decameric in prokaryotes (*8–10*). Class II FBP aldolases are metalloenzymes hence they are EDTA sensitive and require a divalent metal cation (usually Zn^{2+}) for their activities. They use the divalent metal cation as electrophile in their mechanism and their activity can be enhanced by monovalent metal cations. Class II FBP aldolases are found in bacteria, yeast, fungi and some green algea (*5*, *8*). But, some exceptions have been found that class I aldolases were found in bacteria and class II aldolases in eukaryotes (*9*, *11*). It is also known that the few organisms have both classes of FBP aldolases like *Mycobacterium tuberculosis* and *Escherichia coli* (*12*, *13*). However, only one type of FBP aldolases is usually functional in most organisms (*14*). According to the amino acid sequence, class II aldolases could be divided into two different groups, referred to as type A and type B (*8*). Type A enzymes characterized are dimeric, whereas type B can be dimeric, tetrameric or octameric (*7*, *15*).

Much less is known about class II aldolases, but they are more stable than class I aldolases and offer new opportunities in bio-transformation chemistry if an understanding of mechanism and selectivity can be attained (*4*). Since class II aldolases are not found in animals, it has been suggested that they represent a target for antibacterial drugs (*16*, *17*). Although the subunit molecular masses of these two classes of FBP aldolases are similar (~40 kDa), and they have the same overall $(\alpha/\beta)_8$ barrel fold, and catalyze the same enzymatic reactions, they neither share significant homologies in their primary structures (*14*, *18*), nor common catalytic residues and the location of their active site is also different (*16*).

Enzymes from thermophilic organisms offer attractive possibilities both for practical biocatalysis and understanding structure–function relationships in protein catalysis (*19*). The lack of commercial availability of aldolases has caused some limitations for the particular development of industrial scale aldolase-catalyzed

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synthetic processes. So, it is important to produce the aldolases by using economically feasible processes. For this aim, they have been cloned in *E. coli* and expressed as fusion proteins with a histidine tag under the control of an inducible promoter (*20*).

Anoxybacillus gonensis G2 strain is a xylanolytic, sporulating, Gram positive, rod-shaped, facultative anaerobe and moderately thermophilic bacterium that grows naturally at 55–60°C in thermal springs in Gönen, Balikesir, Turkey (*21*). In this study, cloning and sequencing of *A. gonensis* G2 FBP aldolase gene are reported. The procedures for the overexpression, purification and its characterization are also described. So far, no report is published about cloning and characterization of *fba* gene from *Anoxybacillus* sp., therefore we expect that this study may be interesting.

MATERIALS AND METHODS

Materials—All reagents used were of analytical grade. FBP, NADH and coupling enzymes used in the activity assays (triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase) were obtained from Sigma Co. (St Louis, MO, USA).

Anoxybacillus gonensis G2 strain was grown in LB medium at 55°C and used as a DNA donor. *Escherichia coli* JM101 strain, pUC18 (Promega, Madison, USA) and pGEM-T (Promega, Madison, USA) plasmids were used for DNA manipulation and sequencing. *Escherichia coli* BL21 (DE3)pLysS (Promega, Madison, USA) and pET-28a(+) (Novagen, Madison, WI, USA) were used for gene expression. *Escherichia coli* strains were cultivated in LB medium at 37°C. Ampicillin and kanamycin were added to the mediums at a concentration of 50 μg/ml when needed. Amino acid alignments were performed by using the on-line BLAST search engine at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Genomic and plasmid DNAs were purified using Wizard Genomic DNA Purification Kit (Promega, Madison, USA) and Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, USA), respectively. Restriction and modification enzymes were purchased from Promega (Madison, USA) and NBI Fermentas (Vilnius, Lithuania). Extraction of the DNA from the agarose gel was performed using DNA Extraction Kit (NBI Fermentas, Vilnius, Lithuania). DNA sequence analysis were performed by Macrogen Inc. (Seoul, Korea). MagneHis Protein Purification System (Promega, Madison, USA) was used for purification of the recombinant protein.

Genomic DNA Library Construction—The genomic DNA from *A. gonensis* G2 was used as a template to construct the library in *E. coli*. Genomic DNA was digested with *Eco*R I restriction enzyme and DNA fragments were inserted into pUC18 plasmid vector digested with *Eco*R I. Recombinant vectors were transformed into *E. coli* JM101 strain (*22*) and sequenced under BigDyeTM terminator cycling conditions, purified by ethanol precipitation, and run on an Applied Biosystems 3730xl sequencer by Macrogen Inc. (Seoul, Korea).

Inverse PCR—Two primers (forward; ALD F1 5′-GTGGTGCGCGAAGTATTAGC-3′ and reverse; ALD R1 5′-CTGCTAAAATTGCTTGTGTCC-3′) were designed from the known sequence of *A. gonensis* G2 FBP aldolase gene and inverse PCR was performed to find the missing DNA sequence of the aldolase gene (*23*).

Two micrograms of genomic DNA was separately digested with rare cutting site restriction enzymes (*Eco*R I, *Hin*d III and *Hin*f I) in a final volume of 50 μl for overnight at 37°C. Digestions were performed according to the manufacturer. The enzymes were inactivated at 65°C for 15 min. The mixtures were then ligated overnight at 16°C with 3 μl (5 U/μl) T4 DNA ligase in a total volume of 500 μl. After the ligation, DNA was absolutely precipitated with ethanol, washed with 70% ethanol and air-dried. The pellet was resuspended in 30 μl distilled water. PCRs were performed using 2–5 μl of the digestion with the primer set ALD F1 and ALD R1. The programme was as follows: 95°C 2 min, 36 cycles of 94°C 1 min, 57°C 1 min, 72°C 2 min and a final extension at 72°C at 5 min.

Cloning and Expression of FBP Aldolase Gene from A. Gonensis G2—To get the whole DNA sequence of the gene, two primers (forward; ALD F2 5′-CCATATG CCTTTAGTTTCAATGACGG-3′ and reverse; ALD R2 5′-GGGATCCCTCACTTACAACGCTTTGCCGG-3′, with the underlined sequences showing *Nde* I and *Bam*H I sites, respectively) were designed. PCR experiments were performed with these primers. The program was as follows: 95°C 2 min, 36 cycles of 94°C 1 min, 55°C 1 min, 72°C 1.5 min and a final extension at 72°C at 5 min.

The DNA fragments obtained from PCR were digested with *Nde* I and *Bam*H I restriction enzymes, and then inserted into the pET-28a(+) expression vector that had been digested with the same enzymes. The resulting recombinant plasmid, pET28a-*fba*, was transformed into *E. coli* BL21(DE3)pLysS. The recombinant *E. coli* was grown in LB medium contained 50 μg/ml kanamycin at 37°C and expression of the gene was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After induction for 3 h at 37°C, the cells were harvested by centrifugation. The recombinant enzyme containing His-tag was purified by using a manual procedure according to the manufacturer by MagneHis Protein Purification System containing paramagnetic precharged nickel particles. Protein concentrations were determined by the Lowry method using bovine serum albumin as the standard (*24*).

Characteristics of the Enzyme—Enzyme assay

Aldolase cleavage activity towards the FBP substrate was assayed by the spectrophotometric measurement of NADH oxidation at 340 nm (*25*). Activity was measured by an end point assay due to the thermal denaturation of the coupling enzymes at assay temperatures (*7*). Kinetic parameters (V_{max} and K_m) were obtained by using FBP at different concentrations and all characterization studies were performed using 50 mM of the buffer at the optimum temperature and pH. The assay mixture was preheated for 4 min at the reaction temperature. After the enzyme was added, the reaction was allowed to proceed for 3 min. The reaction mixture was stopped by chilling on ice. Then NADH was added at a final concentration of 0.17 mM and the reaction mixture was warmed to room temperature. Five units of the coupling enzymes triose-phosphate

isomerase and α-glycerol-3-phosphate dehydrogenase were added to the assay mixture (total volume 1 ml) and NADH oxidation was monitored for 1 min on an ATI Unicam UV2-100 double beam UV-Vis spectrophotometer. One unit of enzyme activity is defined as 1 μmol of FBP cleaved per min at 60°C.

SDS and native gel electrophoresis

SDS-PAGE was performed according to Sambrook *et al*. (*22*) in 12% acrylamide gels. PageRuler Protein Ladder (Fermentas) which is a mixture of 14 recombinant proteins from 10 kDa to 200 kDa was used as marker proteins. A non-denaturating gel of 8% acrylamide was used to analyse the quaternary form of the enzyme. Proteins both on denaturating and non-denaturating gels were visualized with Coomassie brillant blue R-250.

Activity staining of FBP aldolase on native gel

Activity staining specific for FBP aldolase activity (*26*) was used to detect FBP aldolase in non-denaturing gels. Following electrophoretic migration, the polyacrylamide gel was placed on a Whatman paper saturated with an activity staining solution made up of 50 mM Tris–HCl, pH 7.3; 2.2 mM NAD^+; 10 mM Na_2HAsO_4; 10 mM FBP and 125 μg/ml glyceraldehyde-3-phosphate dehydrogenase; 0.1 mg/ml phenazine methosulfate; 1 mg/ml nitro blue tetrazolium. Purple colour on the gel was developed where aldolase was present.

pH/activity profile

The effect of pH on the aldolase activity was determined by using 4 mM FBP with the following buffers (50 mM) at the indicated pH; acetate buffer (pH 5.0 and 5.5), phosphate buffer (pH 6.0 and 6.5), Tris–HCl buffer (pH 7.0–9.0) and glycine-NaOH buffer (pH 9.5 and 10.0). The reaction was performed for 3 min at 55°C after a suitable aliquot of the enzyme was added to the reaction mixture. Then, the standard enzyme assay described previously was used. The optimum pH obtained was used for determining thermal properties and other parameters (*27*).

Temperature/activity profile and thermal stability

To determine the optimum temperature for the aldolase activity, enzymatic reactions at various temperatures over the range 30–80°C were performed in 4 mM FBP, 50 mM Tris–HCl buffer pH 8.5, using the procedure described previously.

In order to determine the thermal stability of the enzyme, aliquots of enzyme in Eppendorf tubes were incubated from 30 min to 3 h at various temperatures of 30–70°C with 10°C increments. After incubation, the tubes were rapidly cooled in an ice bath and then brought to room temperature. The residual activity was determined with the enzyme assay described previously. The percentage residual aldolase activity was calculated by comprasion with unincubated enzyme (*28*).

Enzyme kinetics

Enzymatic reactions were performed by using different concentrations of FBP (0.05–4 mM) in Tris–HCl buffer (50 mM, pH 8.5). The reaction was performed for 3 min at 60°C after a suitable aliquot of the enzyme was added. Then the standard procedure described previously was used.

Optimum substrate concentration was determined from substrate saturation curve. The kinetic data were plotted as reciprocals of activities versus substrate concentrations. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values were determined as the reciprocal absolute values of the intercepts on the *x*- and *y*-axes, respectively, of the linear regression curve (*29*).

Effect of metal ions and EDTA on aldolase activity

The aldolase activity was measured under the standard conditions in the presence of Zn^{2+}, Ca^{2+}, Co^{2+}, Cr^{2+}, Cd^{2+}, Cu^{2+} and K^+ at 1 mM final concentration in the reaction mixture. The remaining percentage activities were determined by comparison with the standard assay mixture with no metal ion added (*27*).

To investigate the effect of EDTA that is the general inhibitor of class II aldolases on the aldolase activity, EDTA-sodium salt solution was incubated with FBP substrate for 4 min. Then, the standard procedure described previously was performed. The final concentration of EDTA was 1 mM in 1 ml final reaction volume. The results were compared with the standard assay mixture without EDTA.

RESULTS AND DISCUSSION

Molecular Cloning and Sequence Analysis of the Aldolase Gene—The genomic DNA library were constructed with pUC18 plasmid in *E. coli* JM101, a set of recombinant pUC18 plasmids containing ~3–4 kb were sequenced and the DNA sequences were transformed to the amino acid sequences. These sequences were compared to the other sequences using the on-line BLAST program. The alignment results showed that 832 bp of clone 123 (pUC-123) that contains 3.6 kb genomic fragment shared ~80–90% similarity to the class II FB aldolase of *Bacillus* sp. The missing sequence of the gene were obtained by performing inverse PCR with ALD F1 and ALD R1 primers designed from the known sequence of the gene. Before inverse PCR, genomic DNA was separately digested with *Eco*R I, *Hin*d III and *Hin*f I restriction enzymes. DNA fragments were ligated and amplified with inverse PCR. Only *Hin*f I digestion products formed ~750 bp DNA fragment and this fragment was cloned into pGEM-T vector and sequenced. Subsequently, missing 26 bp of the gene was determined. The *fba* gene were amplified by using FBA F2 and FBA R2 primers (Fig. 1), cloned into pET-28a(+) expression vector and transformed into *E. coli* BL21(DE3)pLysS for expression. The nucleotide sequence of *fba* gene was submitted to GeneBank (accession number EF151166).

Comparison of A. gonensis *G2 Aldolase Sequence with other Class II Aldolases*—The *fba* gene from *A. gonensis* G2 (GenBank accession no. EF151166) consists of 861 bp coding for a polypeptide of 286 amino acid (excluding the initiator methionine) (Fig. 2) with a calculated molecular

mass of 30.9 kDa. The molecular mass of the 6xHis-tagged FBP aldolase was calculated to be 33.3 kDa. The deduced protein shares 92, 91, 84, 80, 78, 78, 46 and 44% similarity with class II B FBP aldolases from *Geobacillus kaustophilus* HTA426 (GenBank accession no. BAD77671), *Geobacillus stearothermophilus* (GenBank accession no. P94453), *Bacillus cereus* ATCC10987 (GenBank accession no. AAS44365), *Bacillus licheniformis* ATCC 14580 (GenBank accession no. AAU25399), *Bacillus subtilis* (GenBank accession no. AAA16803), *Staphylococcus epidermidis* ATCC 12228 (GenBank accession no. AAO05322), *Thermus aquaticus* (GenBank accession no. AAF22441) and class II A FBP aldolase from *E. coli* (GenBank accession no. P11604), respectively.

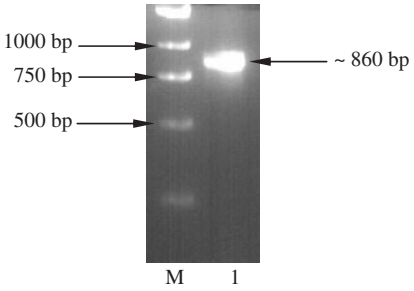

Fig. 1. **Agarose gel electrophoresis of *A. gonensis* G2 *fba* gene that was amplified by ALD F2 and ALD R2 primers.** Lane M: 1 kb standard, Lane 1: *fba* gene amplified by PCR.

Clustal W program was used for sequence aligment of class II FBP aldolases for determining conserved residues (Fig. 3). It was indicated that sequence aligment of *A. gonensis* G2 FBP aldolase had numerous conserved residues (shown in Fig. 3 by stars): H86, H181, H209 and E135 which are responsible for binding of the divalent metal ion (*30*), R258 interacting with the C6 phosphate of FBP or C3 phosphate of glyceraldehyde-3-phosphate (*31*), E137, E145 and N231 which take place in the catalytic reaction (*32*) and D85 which may polarize the C4 carbonyl group of glyceraldehyde-3-phosphate in the condensation direction and deprotonise the C4 hydroxy

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   1  GTACATGTGAAAGGCGCTTTCGGATAGTGAATTATGGCTACATTTAAGGAGGATTCAGCG

  61  ATGCCTTTAGTTTCAATGACGGAAATGCTTAAACAAGCATTGGCAGGCAAGTATGCGGTC
   1   M  P  L  V  S  M  T  E  M  L  K  Q  A  L  A  G  K  Y  A  V

 121  GGTCAATTCAATATTAACAACTTAGAATGGACACAAGCAATTTTAGCAGCGGCGGAAGAA
  21   G  Q  F  N  I  N  N  L  E  W  T  Q  A  I  L  A  A  A  E  E

 181  GAAAAATCTCCTGTCATTCTCGGTGTGTCAGAAGGGGCAGCTCGCTACATGGGCGGCTTT
  41   E  K  S  P  V  I  L  G  V  S  E  G  A  A  R  Y  M  G  G  F

 241  AAAACTGTCGTGAACATGGTGAAAGGGTTAATGGAAGACATGAACATTACTGTTCCTGTC
  61   K  T  V  V  N  M  V  K  G  L  M  E  D  M  N  I  T  V  P  V

 301  GCTATTCACATTGATCACGGTTCAAGCTTTGAAAAATGTAAAGCAGCGATCGACGCTGGA
  81   A  I  H  I  D  H  G  S  S  F  E  K  C  K  A  A  I  D  A  G

 361  TTTACATCAGTCATGATCGATGCATCGCATCATCCGTTTGAAGAAAACGTTGAAATTACA
 101   F  T  S  V  M  I  D  A  S  H  H  P  F  E  E  N  V  E  I  T

 421  TCAAAAGTTGTCGAGTACGCTCATGCGCGTGGTGTATCGGTTGAAGCGGAGCTCGGAACT
 121   S  K  V  V  E  Y  A  H  A  R  G  V  S  V  E  A  E  L  G  T

 481  GTTGGTGGACAAGAAGATGATGTGGTGGCAGAAGGGATCATTTATGCAAATCCAGATGAG
 141   V  G  G  Q  E  D  D  V  V  A  E  G  I  I  Y  A  N  P  D  E

 541  TGCGAAGAGCTTGTGAAGCGCACAGGCATTGACTGTTTAGCGCCAGCGCTCGGTTCTGTA
 161   C  E  E  L  V  K  R  T  G  I  D  C  L  A  P  A  L  G  S  V

 601  CACGGTCCATACAAGGGAGAACCGAAATTAGGTTTTAAAGAGATGGAAGAAATTCGCGAT
 181   H  G  P  Y  K  G  E  P  K  L  G  F  K  E  M  E  E  I  R  D

 661  CGTACAGGTGTACCGCTCGTATTACACGGTGGCACAGGTATTCCGACAGAACAAATTCAA
 201   R  T  G  V  P  L  V  L  H  G  G  T  G  I  P  T  E  Q  I  Q

 721  CGTGCTATTTCGCTCGGTACATCAAAAATTAACGTCAATACAGAAAACCAAATGGCGTTT
 221   R  A  I  S  L  G  T  S  K  I  N  V  N  T  E  N  Q  M  A  F

 781  ACAAAAGTGGTGCGCGAAGTATTAGCAAAAGATGAAAAAGTGTACGATCCGCGCAAATTC
 241   T  K  V  V  R  E  V  L  A  K  D  E  K  V  Y  D  P  R  K  F

 841  CTCGGCCCTGGCCGCGATGCGATTAAAGAAACAGTGATTGGCAAAATGCGCGAATTCGGT
 261   L  G  P  G  R  D  A  I  K  E  T  V  I  G  K  M  R  E  F  G

 901  TCTTCCGGCAAAGCGTTGTAAGGCAAGCCGCACCGTTTCAGCCTTGCTGAAGCGGGCGGT

 281   S  S  G  K  A  L ***
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Fig. 2. **Nucleotide sequence of *A. gonensis* G2 FBP aldolase and the deduced amino acid sequence of the encoded protein.** The numbers correspond to the nucleotide and amino acid sequences in *A. gonensis* G2 aldolase. The initial and stop codons (represented by three stars) of the gene are underlined.

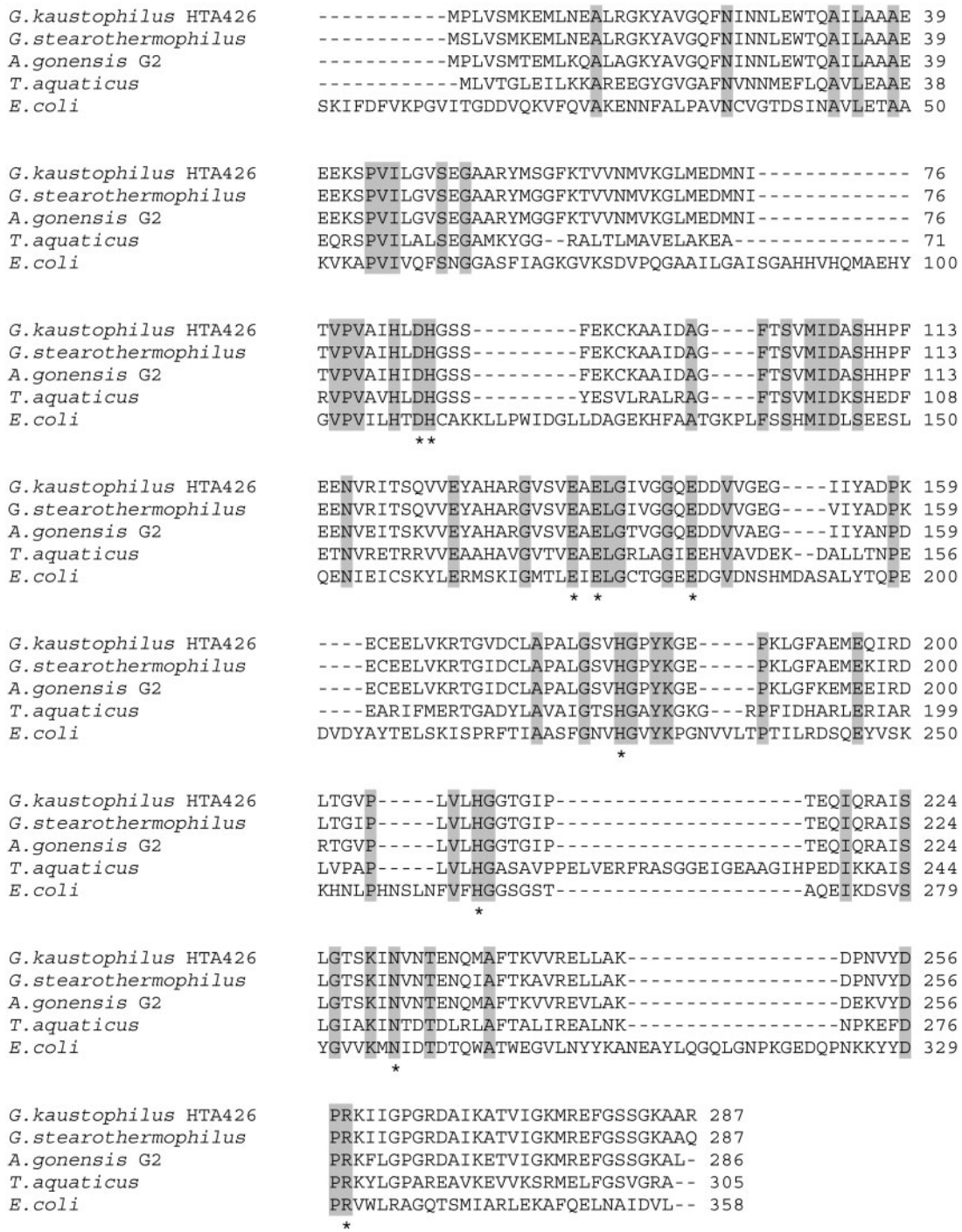

Fig. 3. **Alignment of the amino acid sequence of *A. gonensis* G2 aldolase with class II B aldolases from *G. kaustophilus* HTA426** (GenBank accession no. BAD77671), *G. stearothermophilus* (GenBank accession no. P94453), *T. aquaticus* (GenBank accession no. AAF22441) and class II A aldolase from *E. coli* (GenBank accession no. P11604). The stars indicate the conserved amino acids mentioned in the text.

proton in the cleavage direction (*1*). The motifs that are responsible for the binding of a metal ion were completely conserved. This result showed that the FBP aldolase from *A. gonensis* G2 is a member of the class II aldolases.

According to the sequence alignment shown in Fig. 3, *A. gonensis* G2 FBP aldolase can belong to group B, because it is very similar in amino acid sequence to class II B FBP aldolases except *Taq* aldolase. Whereas, when *A. gonensis* G2 FBP aldolase was compared with class II A FBP aldolase from *E. coli*, sequence similarity declines from 80–90% to 44%. Aldolases from *A. gonensis* G2, *G. kaustophilus* and *G. stearothermophilus* do not contain an insertion of 21 residues; however,

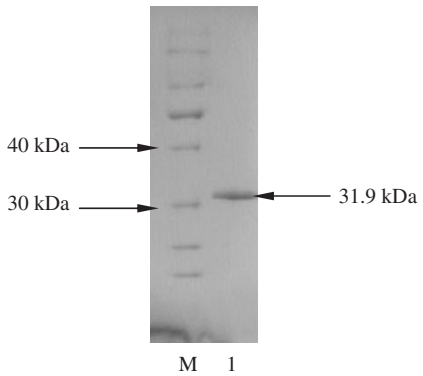

Fig. 4. **SDS-PAGE showing purified recombinant *A. gonensis* G2 FBP aldolase.** Lane M: contains the protein molecular weight markers, Lane 1: The recombinant enzyme contained His-tag and purified by MagneHis Protein Purification System.

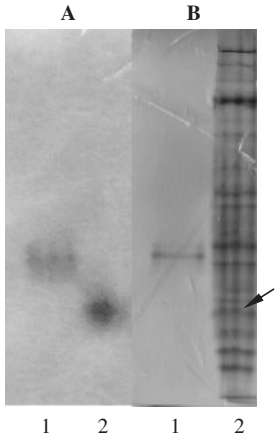

Fig. 5. **Non-denaturating native PAGE showing purified recombinant *A. gonensis* G2 FBP aldolase.** (A) Activity satining, (B) Coomassie brillant blue staining. Lane 1: The recombinant enzyme contained His-tag and purified by MagneHis Protein Purification System, Lane 2: original *A. gonensis* G2 as a positive control.

Taq aldolase has this insertion part found immediately after Ser-211 in the *Taq* aldolase sequence. This 21-residue insertion is unique to class II B aldolases from the subclass of extremophiles consisting of *Thermus*, *Thermotoga*, *Aquifex* and *Helicobacter* (*7*).

Protein Expression and Purification—Recombinant protein was expressed under the control of T7 RNA polymerase promoter with 6xHis-tag in the C-terminal of the protein. Expression of the aldolase was successfully achieved in *E. coli* BL21(DE3)pLysS harbouring pET28a-*fba* following induction by IPTG. The recombinant enzyme contained His-tag was purified by MagneHis Protein Purification System (Fig. 4). Paramagnetic precharged nickel particles were used to isolate polyhistidine-tagged protein directly from the crude cell lysate using a manual procedure according to the manufacturer.

Characterization—Electrophoresis analysis

SDS-PAGE analysis of the purified aldolase contained 6xHis-tag in the C-terminal of protein showed a single band with a subunit molecular mass of 31.9 kDa as shown in Fig. 4, which was in a good agreement with the molecular mass calculated from predicated amino acid sequence. This subunit molecular mass lies in the range of 30.5–40 kDa previously observed for class II aldolase subunits (*33*–*35*).

On non-denaturating native PAGE gel, colorimetric detection yielded a purple band (Fig. 5A) at the same electrophoretic mobility as developed from Coomassie blue staining (Fig. 5B). The purple band showed that *A. gonensis* G2 FBP aldolase contained a C-terminal His-tag was overproduced under the control of T7 RNA polymerase promoter in *E. coli* and the recombinant protein expressed was active. Additionally, this colorimetric assay is specific for FBP aldolase activity therefore forming the purple band proved the existence of FBP aldolase (*7*).

Effect of pH on aldolase activity

Activity-pH profile of the enzyme shown in Fig. 6 exhibited a sharp peak with an optimum at pH 8.5.

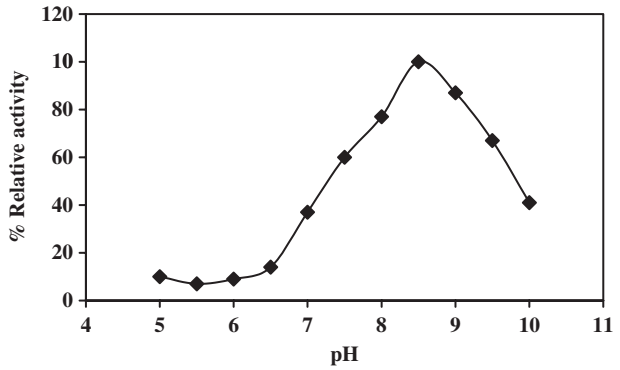

Fig. 6. **Effect of pH on activity of *A. gonensis* G2 FBP aldolase.** Assays were performed in 50 mM of different buffer systems at indicated pH; acetate buffer (pH 5.0 and 5.5), phosphate buffer (pH 6.0 and 6.5), Tris–HCl buffer (pH 7.0–9.0) and glycine-NaOH buffer (pH 9.5 and 10.0) at 55°C. Each point represents the relative V_{max} of the enzyme at each pH value.

This optimum is similar with the optimum at pH 8.5–8.6 of the class II FBP aldolase from *B. stearothermophilus* (*36*). However, *Taq* FBP aldolase belongs to class II aldolases exhibited a broadened maximum at pH 6.5. This optimum pH is lower than the optimum pH of all class II aldolases purified so far (*37*). The purified class II FBP aldolase from *M. tuberculosis* has also a sharp pH optimum at 7.8. Class II aldolases indicate a narrow peak at pH 7.0–9.0, while class I aldolases have a broad pH optimum at pH 7.0–9.0 (*38*). The fact that *A. gonensis* G2 FBP aldolase has a narrow peak reflects a general characteristic of class II aldolases.

Effect of temperature on aldolase activity and thermal stability

Thermal activity data are shown in Fig. 7. FBP aldolase from *A. gonensis* G2 exhibits a maximum activity

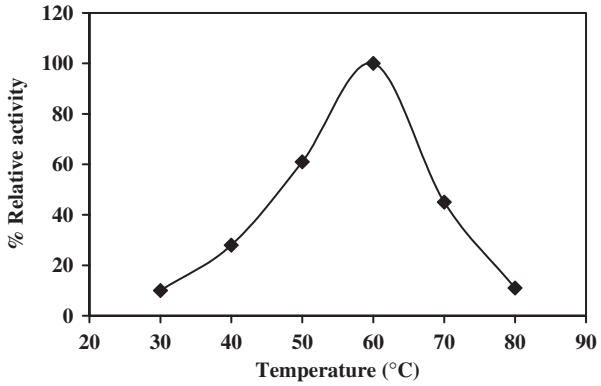

Fig. 7. **Effect of temperature on *A. gonensis* G2 FBP aldolase activity.** The activity reactions were performed in 4 mM FBP, 50 mM Tris–HCl buffer pH 8.5 at different temperatures from 30 to 80°C.

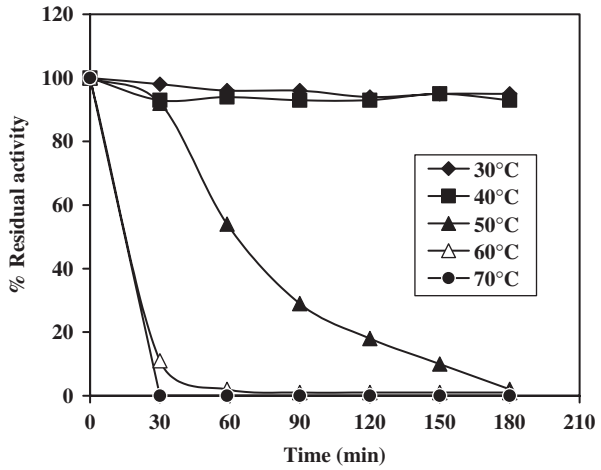

Fig. 8. **Thermal stability of *A. gonensis* G2 FBP aldolase.** Enzyme solutions were incubated from 30 min to 3 h at various temperatures of 30–70°C with 10°C increments and residual enzyme activities were determined by using standard assay procedure in 50 mM Tris–HCl buffer pH 8.5 at 60°C.

at 60°C. Figure 8 shows the loss of activity of the purified enzyme incubated for different lengths of time at various temperatures. The enzyme remained stable at temperatures 30 and 40°C when incubated at pH 8.5 for 3 h. However, the enzyme is unstable at temperatures above 50°C. The enzyme almost fully lost its activity by heating 60 and 70°C for 30 min, while ~5% of the activity is lost at 50°C. Class II FBP aldolase from *B. stearothermophilus* characterized previously shows the optimum temperature at 70°C and is stable on exposure up to 45°C. Slight inactivation is observed on treatment of the enzyme at 55–65°C, and exposure to 75°C for 30 min results in almost complete inactivation (*36*).

Enzyme kinetics

Kinetic parameters for the aldol cleavage reaction catalyzed by *A. gonensis* G2 FBP aldolase were determined at various concentrations of FBP. Optimum substrate concentration was determined as 1 mM from

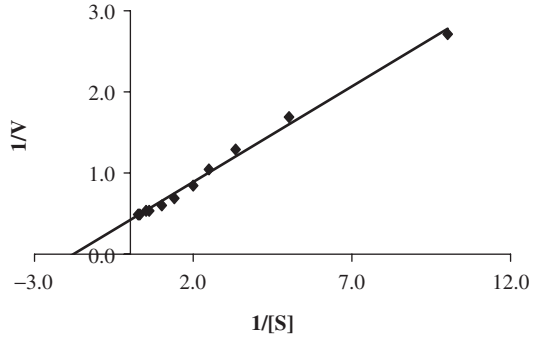

Fig. 9. **Lineweaver–Burk plot of *A. gonensis* G2 FBP aldolase toward FBP as a substrate at 60°C.**

Table 1. **Effect of various metal ions and EDTA on *A. gonensis* G2 FBP aldolase activity.**

Metal ion	% Residual activity	Metal ion	% Residual activity
None	100	Cr^{2+}	64
K^+	130	Cd^{2+}	100
Zn^{2+}	289	Cu^{2+}	43
Ca^{2+}	182	EDTA	0
Co^{2+}	163		

the Michaelis–Menten graphic (data not shown). The Lineweaver–Burk plot (Fig. 9) analysis of this enzyme showed 567 μM K_m and 2.4 μM min^{-1} mg $protein^{-1}$ V_{max} values at 60°C. The apparent catalytic parameters of both class II FBP aldolases have wide variations (*38*). The calculated K_m values of characterized class II A aldolases from *M. tuberculosis*, *E. coli*, *Euglena gracilis* and *Saccharomyces cerevisiae* are 20.3, 170, 175 and 370 μM, respectively (*38*, *5*, *39*, *40*). Among the class II B FBP aldolases caharacterized, the apparent Michaelis constants also show considerable variance. The class II FBP aldolases from *Pseudomonas putida*, *B. stearothermophilus*, *Synechocystis* sp. PCC6803 and *B. subtilis* have an apparent K_m of 30, 4.55, 8 μM and 2 mM, respectively (*41*, *34*, *42*). A number of other class II B enzymes have K_m between 160 μM and 300 μM for FBP cleavage (*5*, *7*, *15*, *38*). Maximal specific activities (V_{max}) of characterized FBP aldolases from *E. coli*, *T. aquaticus* and *M. tuberculosis* were found 5.9, 46 and 35.1 μM min^{-1} mg $protein^{-1}$, respectively (*15*, *7*, *38*).

Effects on metal ions and EDTA on aldolase activity

The effects of various metal ions on the activity of *A. gonensis* G2 aldolase are presented in Table 1. The final concentrations of all metal ions tested were 1 mM and the enzyme activity was assayed under standard conditions. While K^+ stimulated *A. gonensis* G2 aldolase by 30%, Cr^{2+} and Cu^{2+} decreased the activity by 36 and 57%, respectively. *Anoxybacillus gonensis* G2 aldolse was activated by the other metal ions, Zn^{2+}, Ca^{2+} and Co^{2+}. Aldolase activity increased approximately three times in the presence of Zn^{2+}. Since metal ions may have different coordination numbers and geometries in their coordination compounds, and potentials as Lewis acids, they may behave

differently towards proteins as ligands. These differences may also result in metal binding to different sites, and therefore, perturb the enzyme structure in different ways (*43*, *44*). Inhibition with divalent metal chelating agent, EDTA, is a general feature of class II aldolases (*5*). It was reported previously that FBP aldolase activity of *B. stearothermophilus* was strongly inhibited by EDTA at 65°C (*36*). In the case of FBP aldolases from *Streptomyces galbus* DSM40480 when EDTA was added to the enzyme assay, the enzymatic activity was completely inhibited (*45*). We found that EDTA (1 mM) completely inhibited *A. gonensis* G2 FBP aldolase activity (Table 1).

In summary, *A. gonensis* G2 possesses a class II FBP aldolase with a theoretically molecular weight 30.9 kDa, 8.5 optimum pH, 60°C optimum temperature, and 567 μM K_m and 2.4 μM min^{-1} mg $protein^{-1}$ V_{max} values. Aldolase activity is increased by Zn^{2+} while EDTA caused completely inhibition of enzyme activity. The amino acid sequence had ~80–90% similarity to the *Bacillus* sp. class II aldolases. This results show that *A. gonensis* G2 aldolase shares similar properties with some other microbial class II aldolases. So, this thermostable enzyme may be improved to use industrial applications.

This work was supported by a research grant to AC from the Research Fund of Karadeniz Technical University, Turkey (Pr. Nr. 2002.111.002.2).

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