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Characterization of Hyperthermostable Fructose-1,6-Bisphosphatase from *Thermococcus onnurineus* NA1

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To understand the physiological functions of thermostable fructose-1,6-bisphosphatase (TNA1-Fbp) from *Thermococcus onnurineus* NA1, its recombinant enzyme was overexpressed in *Escherichia coli*, purified, and the enzymatic properties were characterized. The enzyme showed maximal activity for fructose-1,6-bisphosphate at 95°C and pH 8.0 with a half-life ($t_{1/2}$) of about 8 h. TNA1-Fbp had broad substrate specificities for fructose-1,6-bisphosphate and its analogues including fructose-1-phosphate, glucose-1-phosphate, and phosphoenolpyruvate. In addition, its enzyme activity was increased five-fold by addition of 1 mM Mg^{2+}, while Li^+ did not enhance enzymatic activity. TNA1-Fbp activity was inhibited by ATP, ADP, and phosphoenolpyruvate, but AMP up to 100 mM did not have any effect. TNA1-Fbp is currently defined as a class V fructose-1,6-bisphosphatase (FBPase) because it is very similar to FBPase of *Thermococcus kodakaraensis* KOD1 based on sequence homology. However, this enzyme shows a different range of substrate specificities. These results suggest that TNA1-Fbp can establish new criterion for class V FBPases.

Keywords: *T. onnurineus* NA1, thermostability, fructose-1,6-bisphosphatase, hyperthermophilic Archaea, fructose-1,6-bisphosphate

Hyperthermophiles are microorganisms that can optimally grow in extreme environmental temperatures above 80°C. Hyperthermophiles are useful sources for the study of bacterial adaptation to high temperatures and screening of thermostable enzymes (Kelly and Adams, 1994; Conners *et al.*, 2006). Recently, several hyperthermostable marine *Archaea* were studied to understand their hyperthermostable mechanisms and potential applications of their enzymes (Klenk *et al.*, 1997; Cohen *et al.*, 2003; Fukui *et al.*, 2005). Genome sequencing of these stains revealed their basic metabolic characteristics and proteomic studies accelerated the elucidation of protein functions in *Aeropyrum pernix* K1 (Yamazaki *et al.*, 2006).

Thermococcus onnurineus NA1 is a hyperthermophilic archaeon that was isolated from a deep-sea hydrothermal vent area of the East Manus Basin (Bae *et al.*, 2006). Genomic and proteomic studies showed that *T. onnurineus* NA1 induced heterotrophic and carboxydotrophic growth according to different carbons sources (Lee *et al.*, 2008; Kwon *et al.*, 2009). In our previous study, we applied shotgun proteomic to screen hyperthermostable proteins of *T. onnurineus* NA1 and identified 150 hyperthermostable candidate proteins including fructose-1,6-bisphosphatase (TON_1497). Hyperthermostable protein sets were enriched and fractionated by a heat-treatment and centrifugation method (Prosinecki *et al.*, 2006).

Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) is an essential regulatory enzyme in the gluconeogenic pathway (Shieh and Chiang, 1998). FBPase catalyzes the hydrolysis of D-fructose-1,6-bisphosphate (FBP) to D-fructose-6-phosphate (F6P) and inorganic phosphate. This is the reverse reaction catalyzed by phosphofructokinase (EC.2.7.1.11) in glycolysis, and the product, F6P, is an important precursor for various biosynthetic pathway (Pilkis and Claus, 1991). FBPases are categorized into five groups (class I-class V) based on amino acid sequences (Donahue *et al.*, 2000; Nishimasu *et al.*, 2004; Hines *et al.*, 2006). Many FBPases from thermophilic *Archaea* and bacteria belong to class V (Nishimasu *et al*., 2004) whose members have narrow and strict substrate specificities.

In this study, we over-expressed and purified a recombinant FBPase (TON_1497) to characterize its enzymatic properties and understand its physiological function in *T. onnurineus* NA1. Despite having a significant sequence similarity up to 90% with the class V FBPase of *Thermococcus kodakaraensis* KOD1 (Rashid *et al.*, 2002), the FBPase of *T. onnurineus* NA1 showed broad substrates specificities and different inhibitor effects. Our results suggest that the FBPase of *T. onnurineus* NA1 is a class V FBPase, but has a range of substrate specificities different from previously characterized members of class V FBPases.

Materials and Methods

Chemicals, plasmids, and growth conditions

The *E. coli* strain DH5α was used for subcloning gene fragments and DNA manipulation. TOPcloner Blunt kit (Enzynomics, Korea) was used for subcloning. For gene overexpression, *E. coli* BL21-Codon-Plus(DE3)-RIL cells (Stratagene, USA) were used as a host and a pET28a vector (Novagen, USA) was used for gene expression. *E. coli* strains in the present study were cultured in Luria-Bertani (LB) medium containing the appropriate antibiotics at 37°C. Fructose 1,6-

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bisphosphate (FBP) and its analogues were purchased from Sigma (USA).

DNA manipulation

Restriction enzymes and DNA polymerase were purchased from NEB (USA) and Enzynomics (Korea). The small-scale preparation of plasmid DNA from *E. coli* cells was performed with a LaboPass Mini kit (COSMO, Korea). The QIAquick Gel Extraction kit (QIAGEN, Germany) was used to recover DNA fragments from agarose gels.

Cloning and expression of fructose-1,6-bisphosphatase

The full-length TON_1497 encoding the putative FBPase of *T. onnurineus* NA1 was amplified by PCR using genomic DNA and two primers (sense [5′-CATATGGCCATTGGAGAGAAAATAACGA-3′] and antisense [5′-GGATCCTCACTCAATGTCC TCAAAGCGCT-3′]; the underlined sequences indicate *Nde*I and *Bam*HI sites, respectively). The amplified DNA fragment (1,128 bp) of the FBPase gene was cloned into a TOPcloner Blunt V2 plasmid (Enzynomics, Korea) and used to transform *E. coli* DH5α cells. The FBPase gene from TOPcloner Blunt V2 was excised by *Nde*I and *Bam*HI digestion and ligated into a pET-28a(+) plasmid (Novagen, USA) that was used to transform *E. coli* DH5α cells. The true transformants were selected by colony PCR and the nucleotide sequences of their plasmids were confirmed. These plasmids were introduced into *E. coli* BL21-CodonPlus(DE3)-RIL. Transformants were grown to an optical density of 0.6 at 600 nm at 37°C in LB medium containing 50 μg/ml kanamycin. The culture was then cooled on ice and isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 100 μM. Cells were grown for an additional 4 h at 37°C.

Purification of recombinant TNA1-Fbp

Cells harvested by centrifugation were resuspended in lysis buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole] and were sonicated using a Vibra-Cell VC130 (Sonics and Materials, USA). After centrifugation at 13,000 rpm for 20 min, the soluble fraction was heat-treated for 10 min at 80°C to remove thermo-labile proteins from the host *E. coli*, followed by a second centrifugation (13,000 rpm, 20 min). The supernatant was applied to a column of nickel nitrilotriacetic acid (Ni-NTA) gel (QIAGEN, Germany) and purified according to the product manual. The recombinant FBPase protein was eluted with buffer containing 250 mM imidazole. Protein concentrations were determined by the Pierce BCA protein assay kit (Thermo Scientific, USA) with bovine serum albumin as a standard. The molecular weight of purified protein was calculated by gel filtration on a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare, USA) using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and albumn (67 kDa) as standard proteins.

Enzyme activity assay

FBPase enzymatic activities of the recombinant proteins were measured by a spectrophotometric assay coupled with phosphoglucose isomerase (PGI) and NADP-dependent glucose-6-phosphate dehydrogenase (G6PD) (Rashid *et al*., 2002). PGI and G6PD were purchased from Sigma. The reaction buffer (100 mM Tris-HCl; pH 8.0 and 20 mM $MgCl_2$) was incubated at 95°C for 5 min before the enzyme activity assay. 5 μg of FBPase and 5 mM FBP were added into the reaction buffer and the mixture was incubated for 1 min at 95°C. After the incubation, the reaction mixture was cooled on ice for 5 min, then 0.4 mM $NADP^+$, 0.5 units of PGI, and 0.5 units of G6PD were added to initiate the coupling reaction. Incubation was carried out at room temperature for 3 min. Generation of NADPH was quantified by measuring the absorbance at 340 nm using a ProteomeLab DU 800 (Beckman Coulter, USA).

Alternatively, we performed another enzyme activity assay for FBPase activity using Malachite Green. In this assay, inorganic phosphate (P_i) was released and measured (Geladopoulos *et al.*, 1991). The reaction was performed in 100 μl of reaction mixture containing 100 mM Tris-HCl buffer (pH 8.0), 20 mM $MgCl_2$, and 10 mM of the substrates. The reaction mixture was pre-incubated at 95°C for 3 min, and then FBPase was added. The reaction was stopped after 1 min by rapid cooling on ice for 5 min. To measure released inorganic phosphate, 400 μl of Malachite Green ammonium molybdate solution was added to 100 μl of the reaction mixture. The preparation of Malachite Green ammonium molybdate solution was previously described (Geladopoulos *et al*., 1991). The release of inorganic phosphate was determined by measuring the absorbance at 630 nm using a ProteomeLab DU 800 (Beckman Coulter, USA).

Results

Purification of recombinant protein TNA1-Fbp

To confirm the thermostablity of TNA1-Fbp, we over-expressed *tna1-fbp* (TON_1497) in *E. coli* and purified the recombinant TNA1-Fbp protein (Fig. 1). The molecular weight of recombinant TNA1-Fbp estimated by 12.5% SDS-PAGE was 40 kDa which is similar to the predicted molecular weight based on the amino acid sequence. The relative molecular mass of the native form of TNA1-Fbp was also shown to be 300 kDa by gel filtration chromatography (data not shown). This indicated that native TNA1-Fbp has an octameric structure similar to Tk-Fbp of *T. kodakaraensis* KOD1 (Rashid *et al*., 2002) and St-Fbp of *Sulfolobus tokodaii* strain 7 (Nishimasu *et al*., 2004).

Effects of temperature, pH, and metal ions on TNA1-Fbp enzymatic activity, and its catalytic properties

Enzyme activity of TNA1-Fbp was measured according to

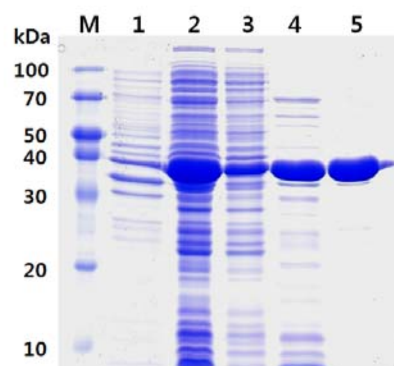

Fig. 1. Purification of recombinant TNA1-Fbp from *E. coli* BL21-codonplus(DE3)-RIL. Lanes: M, molecular weight markers; 1, precipitated fraction of crude extract after centrifugation at 13,000 rpm; 2, soluble fraction of crude extract after centrifugation at 13,000 rpm; 3, precipitated fraction after heat treatment at 80°C for 10 min and centrifugation at 13,000 rpm; 4, soluble fraction after heat treatment at 80°C for 10 min and centrifugation at 13,000 rpm; 5, purified TNA1-Fbp after Ni-NTA column chromatography.

Table 1. Substrate specificities of TNA1-Fbp

Substrate	Relative activity (%)
Fructose-1,6-bisphosphate	100
Fructose-1-phosphate	21.9
Fructose-6-phosphate	17.2
Glucose-1-phosphate	26.7
Glucose-6-phosphate	18
Glycerol-2-phosphate	17.3
Phosphoenolpyruvate (PEP)	19.6
5'-AMP	13.9
6'-ADP	18
7'-ATP	27.2

Assays for *T. onnurineus* NA1 FBPase was performed in 100 μl of reaction mixture containing 100 mM Tris-HCl buffer (pH 8.0), 20 mM $MgCl_2$, and 10 mM of the substrates at 95°C.

temperature variation (60-100°C) by a coupled assay with phosphoglucose isomerase and glucose-6-phosphate dehydrogenase (Rashid *et al*., 2002). TNA1-Fbp activity was increased in proportion to the increase of temperature, with 90-100°C being the temperature range for optimal enzyme activity (Fig. 2A). The thermostability of TNA1-Fbp was assayed in a 50 mM Tris-HCl (pH 8.0) buffer containing $MgCl_2$ at 95°C. The half-life ($t_{1/2}$) of the enzyme activity was about 8 h, as shown in Fig. 2B. The effect of pH and metal ions on the FBPase activity of TNA1-Fbp was investigated by measuring the release of inorganic phosphate as previously described (Geladopoulos *et al*., 1991). TNA1-Fbp displayed maximal activity at pH 8.0 (Fig. 2C). Significant metal ion effect on enzyme activity was observed in buffer including Mg^{2+} (Fig. 2D). Enzyme activity was increased five-fold by addition of 1 mM Mg^{2+}. Interestingly, high concentrations (up to 30 mM) of Mg^{2+} still maintain increased enzymatic activity. However, other metal ions such as Zn^{2+}, Mn^{2+}, and Ni^{2+} had no stimulative effect at high concentration (>5 mM) even though they increase enzyme activity at low concentration (<5 mM). Ca^{2+} and Li^{+} did not increase enzyme activity at all (Fig. 2D). Additionally, kinetic analysis of TNA1-Fbp was performed. The K_m value of TNA1-Fbp using FBP as substrate was 0.235 mM, and the k_{cat} value was 14.84 s^{-1} $subunit^{-1}$ at 95°C. The k_{cat}/K_m (s^{-1} mM^{-1}) value was 63.15, which was a little less than previously reported values for Tk-Fbp of *T. kodakaraensis* KOD1 (170.0) and St-Fbp of *Sulfolobus tokodaii* strain 7 (92.59) (Rashid *et al*., 2002; Nishimasu *et al*., 2004).

Substrate specificity and inhibition assay

The specific activities of TNA1-Fbp for FBP and its analogue substrates were determined at 95°C by measuring the release of inorganic phosphate (Geladopoulos *et al*., 1991). The highest activity of TNA1-Fbp was obtained by using FBP as a substrate. As shown in Table 1, other related substrates including fructose-1-bisphosphate, fructose-6-phosphate, glucose-1-phosphate, glucose-6-phosphate, glycerol-2-phosphate, phosphoenolpyruvate, 5′-AMP, 6′-ADP and 7′-ATP were also

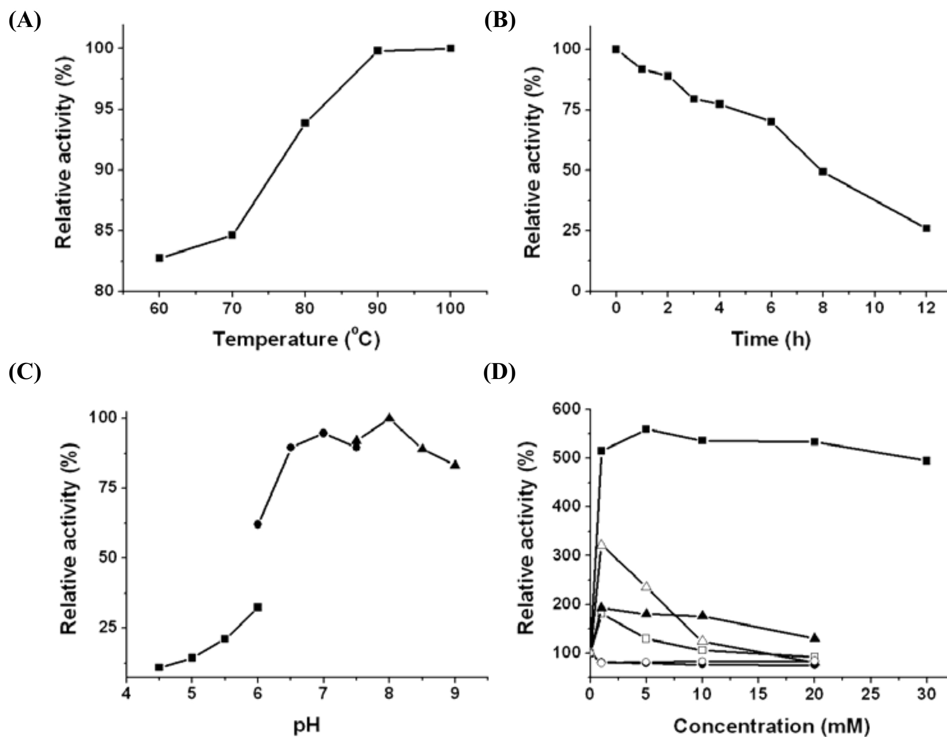

Fig. 2. Characterization of TNA1-Fbp of *T. onnurienus* NA1. (A) Optimal temperature of TNA1-Fbp activity was measured over the range of 60-100°C. (B) Thermostability of TNA1-Fbp was assayed at 95°C in 50 mM Tris-HCl buffer (pH 8.0) in the presence of 5 mM $MgCl_2$. (C) Effect of pH on the FBPase activity of TNA1-Fbp. The following buffers were used: closed squares, citrate buffer (pH 4.5-6.0); closed circles, MOPS buffer (pH 6.0-7.5); closed triangles, Tris-HCl buffer (pH 7.5-9.0). (D) Effect of major metal ions. The enzyme activity was examined at 95°C. Mg^{2+}, closed squares; Mn^{2+}, open squares; Ni^{2+}, closed triangles; Zn^{2+}, open triangles; Li^{+}, closed circles; Ca^{2+}, open circles.

dephosphorylated by TNA1-Fbp although the activities for these substrates were relatively low (13.9-27.2%). These results indicate that TNA-Fbp has a broad specificity for substrates unlike Tk-Fbp of *T. kodakaraensis* KOD1. The effect of inhibitors on the activity of TNA1-Fbp was investigated by adding cations and metabolites (0 to 100 mM) to the standard enzyme assay mixture (10 mM FBP, 2.5 μg of enzyme) at 95°C. TNA1-Fbp was inhibited by ATP, ADP, and PEP, but AMP up to a concentration of 100 mM did not affect FBPase activity (Table 2). Li^+ ions, which have been shown to inhibit FBPase I and IV in only *P. furiosus* (Zhang *et al.*, 1996; Verhees *et al.*, 2002), did not affect the enzyme activity of TNA1-Fbp at concentrations up to 100 mM (Table 2). However, Ca^{2+} ions effectively inhibited the enzyme activity of TNA1-Fbp in a manner similar to FBPase IV (AF453319) of *P. furiosus* (Verhees *et al.*, 2002).

Discussion

Characterization of recombinant thermostable TNA1-Fbp of *T. onnurineus* NA1 was performed in this study. In a previous study, we identified about 150 candidate proteins including TNA1-Fbp (TON_1497) which was found to have hyper-thermostability through a heat-treated precipitation and LC-based protein identification methods. TNA1-Fbp was examined to confirm its thermostability by over-expression since TNA1-Fbp was predicted to be a low copy protein according to previous proteomic analysis (Kwon *et al.*, 2009). TNA1-Fbp was shown to be thermostable with an 8 h half-life at 95°C. Sequence homology analysis demonstrated that TNA1-Fbp has significant sequence similarities with FBPases of *T. kodakaraensis* KOD1 (Tk-Fbp, 90%), *P. furiosus* DSM 3638 (Q8U359, 87%), and *P. horikoshii* OT3 (O58501, 86%) which are all homologous to class V FBPases.

Generally, phosphatases require divalent cations for dephosphorylation activity (Bone *et al.*, 1994). In our experiment, the enzymatic activity of TNA1-Fbp was enhanced by addition of high concentrations (up to 30 mM) of Mg^{2+} in addition to low concentrations (1 mM). This effect of Mg^{2+} ions on enzymatic activity is nearly identical to that of Tk-Fbp and St-Fbp (Rashid *et al.*, 2002; Nishimasu *et al.*, 2004). St-Fbp has maximum activity at 5 mM Mg^{2+}, identical to TNA1-Fbp (Nishimasu *et al.*, 2004), while Tk-Fbp shows maximum activity at a higher concentration of 20 mM Mg^{2+} (Rashid *et al.*, 2002). It was reported that four Mg^{2+} ions surround the 1-phosphoryl group of the FBP molecule penetrating into St-Fbp, and the residues involved in Mg^{2+} ion binding are highly conserved among class V FBPase enzymes (Nishimasu *et al.*, 2004). These results indicate that Mg^{2+} is essential for the catalytic activity of class V FBPase enzymes.

Table 2. Inhibitors of TNA1-Fbp activity

Effector	[1]IC_{50} (mM)
[2]Li^+	-
Ca^{2+}	5
[3]AMP	-
ADP	40
ATP	10
PEP	40

[1] IC_{50} is defined as the concentration of the effectors causing the decrease of enzyme activity to fall below to 50%. [2,3] AMP and Li^+ had no inhibitory effects on FBPase up to 100 mM.

Recently, the crystal structure of a class V FBPase (St-Fbp) from the *S. tokodaii* strain was elucidated and metal-binding residues (six Asp, one His, and one Gln) and substrate recognizing residues (two Tyr, one Gly, one Asn, one Lys, and one Arg) were reported (Nishimasu *et al.*, 2004). In general, class V FBPases are considered to have strict substrate specificities similar to Tk-Fbp of *T. kodakaraensis* KOD1 (Rashid *et al.*, 2002). However, our study revealed that substrate specificity is not a good criterion for classifying FBPases. Unlike Tk-Fbp of *T. kodakaraensis* KOD1, TNA1-Fbp has broad substrate specificities toward FBP and its analogues (Table 2). We considered two possible explanations for the different substrate specificities. One possibility is that the different substrate specificity is based on differences in active amino acid residues. However, sequence similarity between the two enzymes was nearly 90%. Until now, we could not identify any known active amino acid residues related to different substrate specificities in the protein sequence. The second possibility is that different enzyme activity assays and reaction temperatures were used for our experiments. In the present study, we assayed the activity of TNA1-Fbp by measuring released inorganic phosphate using UV/visible spectrometry as previously described (Geladopoulos *et al.*, 1991). However, enzyme activity of Tk-Fbp of *T. kodakaraensis* KOD1 was assayed by measuring FBP substrates recovered from an activity assay using HPLC (Rashid *et al.*, 2002). The two assays were performed at 95°C and 50°C, respectively. Since the optimal temperature range of the two enzymes is 90-100°C, we believe that the assay for measuring substrate specificities should be performed at 95°C for greater accuracy. In the case of class IV FBPase of *P. furiosus* (Verhees *et al.*, 2002), the enzyme assay was performed at 85°C although optimal temperature of this enzyme is approximately 100°C (Verhees *et al.*, 2002).

T. onnurineus NA1 has two putative FBPases (TNA1-Fbp; TON_1497 and TON_1045). TON_1045 is a class IV FBPase. Based on identification of two enzymes by shotgun proteomic analysis, we believe that two enzymes were induced in our culture conditions (data not shown). However, after the heat-treatment and enrichment at 100°C, only TNA1-Fbp (Ton_1497) was identified by proteomic analysis suggesting that TNA1-Fbp is more thermostable. Despite this, we do not know which enzyme is the major FBPase in a natural environment. In conclusion, recombinant TNA1-Fbp of *T. onnurineus* NA1 was successfully purified from an *E. coli* expression system and its physicochemical properties were characterized. This enzyme is a class V FBPase but has a substrate specificity range different from that of Tk-Fbp in *T. kodakaraensis* KOD1. Therefore, we propose that the criterion for defining class V FBPases should include broad substrate specificity as well as sequence homology.

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