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Cloning, expression, and characterization of a novel CTP synthase gene from *Anoxybacillus gonensis* G2

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Abstract: The cytidine-5'-triphosphate (CTP) synthase (EC 6.4.3.2) gene (*pyrG*) was cloned and sequenced from the thermophilic bacterium *Anoxybacillus gonensis* G2 (*Ago*). The gene is 1590 bp in length and encodes a protein of 530 amino acids, with a molecular mass of 59.5 kDa. The amino acid sequence of CTP synthase shares approximately 90%–94% similarity to *Bacillus* sp., and it belongs to the triad glutamine amidotransferases, which utilize a Cys–His–Glu triad for activity. Multiple sequence alignments revealed that the enzyme includes conserved amino acids responsible for catalytic activity and the binding of a divalent metal ion (Mg^{+2}). *Ago*CTP synthase (*Ago*G2CTPs) was overproduced in *Escherichia coli* BL21 (DE3) pLysS as recombinant and purified by nickel affinity chromatography. Its biochemical characterization showed that the enzyme had maximal activity at pH 9.0–10.0 and 65 °C. K_m, V_{max}, and k_{cat} were found to be approximately 12.415 mM, 0.381 U/L, and 0.762 s^{-1} at 65 °C, respectively. CTP synthase promotes the formation of CTP in dividing cells and is a recognized target for anticancer and antibacterial drugs. The results obtained from this study can be improved upon with the use of different species and substrates.

Key words: *Anoxybacillus gonensis*, cytidine 5'-triphosphate synthase, thermophilic, NH_3-dependent characterization

1. Introduction

All organisms need nucleotides for the synthesis of DNA, RNA, and various coenzymes. These requirements can be met in 2 paths, either by de novo synthesis of nucleotides or by exploiting nucleotides, nucleosides, and nucleobases taken up from the surroundings through the salvage pathways. The de novo synthesis of pyrimidines seems to be universal. The pathway consists of 6 enzymatic reactions leading to uridine monophosphate (UMP), which is finally converted into uridine triphosphate (UTP) and cytidine-5'-triphosphate (CTP) (Wadskov-Hansen et al., 2001).

Glutamine amidotransferase (GATase) enzymes catalyze the removal of the ammonia group from glutamine and then transfer this group to a substrate to form a new carbon-nitrogen group (Buchanan, 1973). The glutamine amidotransferase is a single polypeptide chain composed of 2 domains. The C-terminal glutamine amide transfer (GAT) domain catalyzes the hydrolysis of glutamine and originating NH_3 derived from this glutaminase activity, which is transferred to the N-terminal synthase domain, where the amination of UTP is catalyzed (Weng and Zalkin, 1987). Amino acid sequence similarities between GAT domains have been used to classify amidotransferases into 2 well-characterized families (Zalkin and Smith, 1998). CTP synthase [CTPS; EC 6.3.4.2; UTP: ammonia ligase (ADP-forming)] is a part of the triad family of glutamine amidotransferases that utilize a Cys–His–Glu triad to catalyze glutamine hydrolysis. CTP synthase catalyzes the final step of cytidine triphosphate synthesis, and it also has a principal role in phospholipid synthesis. CTP synthase also catalyzes the ATP-dependent formation of CTP from UTP using either L-glutamine or NH_3 as the nitrogen source. CTP acts as a feedback inhibitor by binding to the synthase domain (Long and Pardee, 1967). GTP is required as a positive allosteric effector to increase the impact of glutaminase activity and Gln-dependent CTP synthesis (Levitzki and Koshland, 1972; Bearne et al., 2001), but it inhibits CTP synthesis at concentrations of >0.15 mM ATP. UTP acts synergistically to promote tetramerization of the enzyme to its active form (Levitzki

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and Koshland, 1972). CTP synthase has been studied as a target for antiviral (Dereuddre-Bosquet et al., 2004), antineoplastic (Verschuur et al., 2000), and antiparasitic (Hofer et al., 2001) drug development.

CTP synthases from different organisms should be characterized because of the importance of this enzyme in pyrimidine biosynthesis. In this study, we describe the cloning and functional characterization of the *Anoxybacillus gonensis* (*Ago*) *pyrG* gene. *Ago* is a thermophilic gram-positive bacterium. Based on the 16S rRNA, DNA–DNA hybridization analyses, and phenotypic features, *Anoxybacillus* was proposed as a new genus of the Bacillaceae; the name *Anoxybacillus* was proposed based on its anaerobic property (Pikuta et al., 2009). To date, a total of 19 species have been reported from this new genus, and several enzymes, mostly related to the metabolism of carbohydrate, have been characterized from this genus (Goh et al., 2013). On the other hand, many enzymes have been characterized from the *Bacillus* genus (Tekin et al., 2012; Elleboudy et al., 2013).

2. Materials and methods

2.1. Construction of DNA library and determination of the *pyrG* gene sequence

The nucleotide sequence of the CTP synthase gene was determined by the shotgun cloning method (Messing et al., 1981). Briefly, the genomic DNA of *Ago* was used as a template to construct the library in *E. coli* JM109 using pUC18 plasmid as a cloning vector**.** *Ago* and *E. coli* JM109 cells harboring pUC18 were grown in LB medium for 16 h at 55 and 37 °C, respectively. The cells were precipitated via centrifugation for 5 min at 10,000 rpm at room temperature. Genomic DNA and plasmid DNA were purified using genomic DNA purification kits and a plasmid purification system (Promega, Madison, WI, USA), and then 2 µg of purified genomic DNA of *Ago* and 2 µg of pUC18 plasmid vector were digested separately with 20 U of *Eco*RI restriction enzyme (Fermentas) in a 50-µL reaction volume at 37 °C for 2 h. The digested DNA fragments and pUC18 were purified using QIAGEN PCR Purification Kits, and the digested DNA fragments were ligated into pUC18 plasmid vector at 16 °C for 16 h using 10 U of T4 DNA ligase (Fermentas). The ligation mixture was transformed into *E. coli* JM101 strain prepared according to Sambrook et al. (1989), which was grown on agar plates containing ampicillin (50 µg/mL) and X-gal (40 µg/mL). Colonies that appeared white were subjected to plasmid purification using the Promega Plasmid Purification Kit. Purified plasmids were sequenced (Macrogen, Amsterdam, the Netherlands), and the obtained sequences were analyzed by BLAST searches using the NCBI GenBank database (Altschul et al., 1990; Benson et al., 2012) to determine the ORF in our shotgun library. It was found that one of the recombinant plasmids contained the *pyrG* gene in its full length. The nucleotide sequence of this new *pyrG* gene was translated into an amino acid sequence using the Translate Tool (ExPASY Bioinformatics Resource Portal) and compared to the amino acid sequences of CTP synthases deposited in the NCBI GenBank database. The nucleotide sequence was submitted to the GenBank database.

2.2. Cloning of *pyrG* gene into expression vector

The *pyrG* gene of *Ago* was cloned into the pET-15b expression vector and overexpressed in *E. coli* BL21 (DE3) pLysS for biochemical characterization. Briefly, the *pyrG* gene was amplified by 2 primers, CTP_F_*Xho*I_5'CCGCTCGAGATGACAAAATATATTTTT GTAACAGGTGGCGTCG-3' and CTP_R_*Xho*I_5'GGCCTCGAGTTATTTTTGCAACGATGCGCGAATAAATTCGCGG-3', that have *Xho*I restriction sites (underlined) to allow in-frame ligation into the pET-15b expression vector (Novagene). The PCR reaction was carried out in a 50-µL reaction volume containing 1X polymerase buffer, 5 ng of genomic DNA, 3 mM $MgCl_2$, 200 µM of each dNTP, 10 µM primers, and 1.5 U of Expand High Fidelity Taq DNA polymerase (Fermentas). Amplification was performed with a thermal cycler (Bio-Rad) using the following cycling parameters: 1 cycle at 94 °C for 3 min followed by 32 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, and then the final extension at 72 °C for 10 min. The amplified product was resolved in 1.4% agarose gel (1X TBE) with a 1-kb DNA ladder (as molecular marker; New England Biolabs, Evry, France), stained with ethidium bromide (10 µg/mL), and photographed under UV light. The obtained PCR fragment was purified with a QIAquick column (QIAGEN, Courtaboeuf, France). The pET-15b expression vector was grown and purified as mentioned for the pUC18 plasmid. The purified PCR product and 2 µg of pET-15b expression vector were digested separately by 10 U of *Xho*I restriction enzyme (Fermentas) in a 50-µL reaction volume at 37 °C for 2 h. The digested PCR products and pET-15b expression vector were purified using QIAGEN PCR purification kits again, and ligation reaction was carried out in a 20-µL reaction volume using 10 U of T4 DNA ligase (Fermentas) at 16 °C for 16 h. The ligation mixture was transformed into the *E. coli* JM101 strain prepared according to Sambrook et al. (1989), which was grown on agar plates containing ampicillin (50 µg/mL). Plasmids were purified from several colonies and positive clones were determined by sequencing (Macrogen). The resulting recombinant plasmid, named p*Ago*CTP, was transformed into *E. coli* BL21 (DE3) pLysS for overexpression. *E. coli* cells harboring the p*Ago*CTP vector were grown to an optical density at 600 nm of about 0.6 in LB medium containing ampicillin (50 µg/mL) at 37 °C, and expression was induced by the addition of 1

mM isopropyl β-D-thiogalactopyranoside for 3 h at 37 °C. The cells were collected by centrifugation and exploded by sonication. The recombinant enzyme containing His-tag was purified by using a manual procedure according to the manufacturer's protocol with a MagneHis protein purification system containing paramagnetic precharged nickel particles (Promega). Upon affinity purification, the crude extract was heated at 52 °C for 20 min and centrifuged (12,000 rpm). The resulting enzyme solution was dialyzed into HEPES buffer (50 mM, pH 8.0) (Lunn and Bearne, 2004) and visualized on 12% SDS-PAGE. Protein concentrations were measured with a NanoDrop Spectrophotometer 2000.

2.3. Biochemical characterization assays

The CTP synthase activity of recombinant enzyme was measured under standard CTP synthesis assay conditions (Lunn and Bearne, 2004). Reaction mixtures containing 60 mM HEPES buffer (pH 8.0), 0.5 mM EDTA, 10 mM $MgCl_2$, 1 mM UTP, and 1 mM ATP were incubated for 30 min at 65 °C. Reactions were initiated by the addition of 60 mM NH_4Cl and 5 ng of *Ago*CTP synthase (*Ago*G2CTPs) into prewarmed reaction mixture at 37 °C. Reactions were terminated by the addition of 15 mM EDTA, and CTP synthase activity was measured at 291 nm by spectrophotometer (SpectraMax M5, Molecular Devices) to determine the conversion of UTP into CTP. In addition, the ionic strength was maintained by the addition of 0.25 M KCl in all spectrophotometric assays. The optimum temperature of *Ago*G2CTPs was determined by measuring the CTP synthase activity in a temperature range of 22 to 80 °C for 30 min under standard assay conditions. In order to test thermostability, 50 ng of enzyme was portioned to individual tubes and incubated at 60, 65, 70, 75, and 80 °C for 5 min. These heat-treated enzymes were tested for residual CTP synthase activity as described above. The optimum pH of *Ago*G2CTPs was determined by measuring the CTP synthase activity in HEPES buffer with different pH levels (5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0) at 65 °C for 30 min, and CTP synthase activity was measured as described above. In order to determine the K_m and V_{max} values of *Ago*G2CTPs, the reactions were carried out in the standard assay conditions for 30 min with various amounts of the substrate from 0 to 200 mM. The reactions were terminated by the addition of 15 mM EDTA and measured as above.

2.4. Sequence comparison

Amino acid sequences of different species such as *Anoxybacillus gonensis* (ACD45983.1), *Anoxybacillus flavithermus* WK1 (YP_002317070.1), *Bacillus halodurans* C-125 (NP_244659.1), *Brevibacillus brevis* NBRC100599 (YP_002774973.1), *Geobacillus kaustophilus* HTA426 (YP_149242.1), *Bacillus licheniformis* DSM 13 (YP_081040.2), *Escherichia coli* (WP_001456462.1), *Thermus aquaticus* (WP_003044229.1), and *Oceanobacillus iheyensis* HTE831 (NP_693929.1) were obtained from GenBank. These sequences were aligned and compared by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3. Results

After the shotgun construction of the genomic DNA library from *Ago* on pUC18 plasmid in *E. coli* JM101 strains using *Eco*RI restriction enzyme, we obtained many clones that had different sizes of fragments of the *Ago* genome. Genetic information in each clone was determined by sequencing analysis, and an approximately 4-kb DNA fragment was determined on 1 of the recombinant pUC18 plasmids. When this fragment was completely sequenced, the entire nucleotide sequence of the *pyrG* gene was identified and submitted to GenBank at NCBI under accession number EU675940. The nucleotide sequence of the *pyrG* gene from *Ago* is 1590 bp in length (excluding the stop codon) and encodes a protein of 530 amino acids with a calculated molecular mass of 59.5 kDa (Figure 1). The DNA sequence was transformed into the amino acid sequence, and this sequence was compared to the other sequences in GenBank using the online BLAST program. The alignment results showed that the CTP synthase had approximately 90%–94% similarity to the CTP synthases of *Bacillus* sp.

Recombinant protein was expressed under the control of T7 RNA polymerase promoter with 6× His-tag in the N-terminal of the protein. Induction of CTP synthase expression was successfully achieved in *E. coli* BL21 (DE3) by IPTG. The recombinant enzyme containing His-tag was subsequently purified with the MagneHis Protein Purification System. The purified *Ago*G2CTPs (61.6 kDa

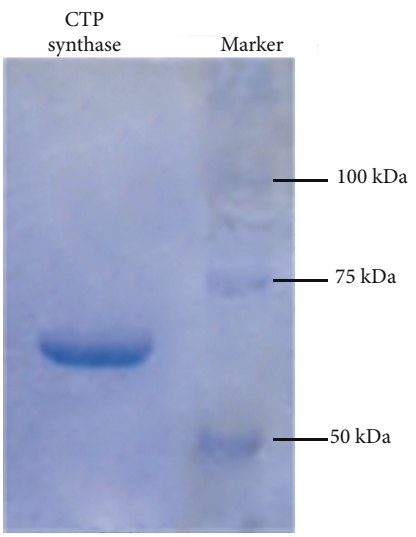

Figure 1. SDS-PAGE analysis of the purified *Ago*G2CTPs.

with histidine tag) was homogeneous as judged by SDS-PAGE analysis (Figure 1), and 0.510 mg/mL of purified enzyme was obtained from 1 L of cell culture.

The maximum synthesis activity of pure *Ago*G2CTPs was observed at 65 °C (Figure 2A). The thermostability of the enzyme was also investigated after heat treatment of the protein with temperatures ranging from 60 to 80 °C for 5 min. The heat-treated enzymes were used for synthesis activity assays at 65 °C. We found that *Ago*G2CTPs was stable at 60 and 65 °C for up to 5 min (Figure 2B). However, *Ago*G2CTPs showed 63%, 50%, and 8% synthesis activity at 70 °C, 75 °C, and 80 °C, respectively. The optimum pH range of the enzyme was measured to be between 5 and 11; it was found that *Ago*G2CTPs exhibited more activity at pH 10 (Figure 3). The kinetic constants were determined from a Hanes–Woolf plot of cytidine triphosphate activity in the presence of 0–200 mM NH_4Cl, and the apparent K_m and V_{max} values were determined by linear regression analysis. K_m and V_{max} were calculated as approximately 12.415 mM and 0.381 U/L, respectively. k_{cat} was calculated as approximately 0.762 s^{-1}. Comparisons of amino acid sequences of CTP synthase among bacterial species show that sequences responsible for catalytic activity are conserved in *Ago*G2CTPs (Figure 4).

4. Discussion

In this study, the molecular cloning and the biochemical characterization of the novel *pyrG* gene of thermophilic *Ago* were reported for the first time. Cleaving the entire genome of a cell with a specific restriction endonuclease and cloning each fragment into a vector is called the "shotgun cloning method". This technique can produce a very large number of DNA fragments, which will generate different colonies of transfected bacterial cells. Each of these colonies harbors many copies of a particular stretch of the fragmented genome. Genomic DNA is cut into fragments at random; only some fragments contain genes (Alberts et al., 2002). By using this method, the *pyrG* gene was identified from the *Ago* genomic DNA library. Sequence analysis showed that the *pyrG* gene is not a part of the *pyr* operon in *Ago*; the gene lies upstream of the *rpoE* and fructose-1,6-bisphosphate aldolase gene and it is transcribed in the same direction. We compared the *pyrG* gene from other bacteria such as *Anoxybacillus flavithermus* WK1, *Bacillus halodurans* C-125, *Brevibacillus brevis*

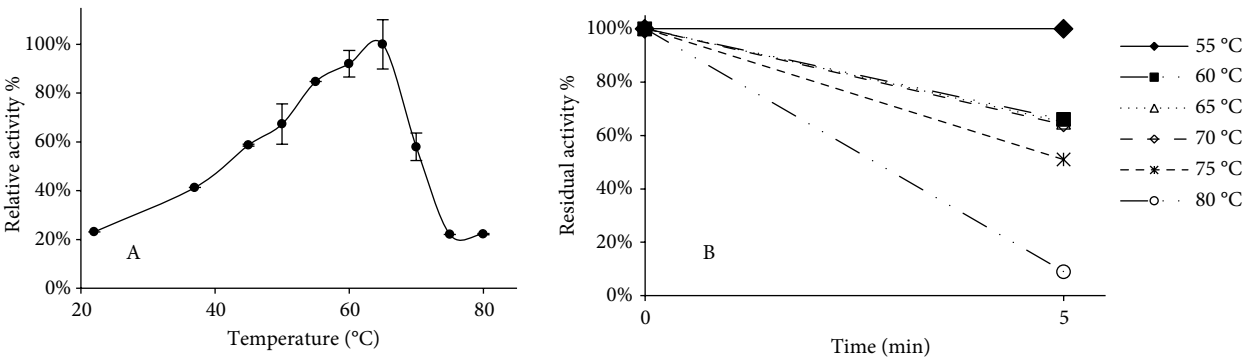

Figure 2. Determination of optimum temperature for maximum activity (A) and thermal stability (B) of *Ago*G2CTPs.

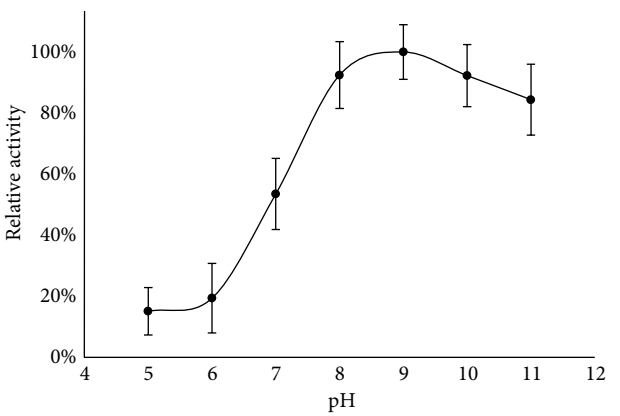

Figure 3. Determination of optimum pH for maximum activity of *Ago*G2CTPs.

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Agon        GAHSAEIDPS TPHPIIDLLPEQKDI----- -----EDLGGTLRLGLYPCK
Afla        GAHSAEIDPS TPHPIIDLLPEQKDI----- -----EDLGGTLRLGLYPCK
Bhal        GAHSAEINPD TPHPIIDLLPEQKDV----- -----EDMGGTLRLGLYPCK
Brev        GANSSEINPN TAYPVIDLLPEQKDI----- -----EDKGGTMRLGLGPTK
Gkau        GAHSSEFDPN TPHPIIDLLPEQKDV----- -----EDLGGTLRLGLYPCK
Blic        GAHSAEIDPS TPYPIIDLLPEQKDI----- -----EDLGGTLRLGLYPCK
Ecol        NANSTEFVPD CKYPVVALITEWRDENGNVE VRSEKSDLGGTMRLGAQQCQ
Taqu        GANSTEFDPY TPHPVIDLMPEQLEVEG--- -------LGGTMRLGDWPMR
Oihe        QAHSAENPHT PHP--VDLLPEQKDE----- ------DLGGTLRLGAYPCK
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Figure 4. Conserved GxxxRLG sequence of 9 different species: *Ago* [*Anoxybacillus gonensis* (ACD45983.1)], *Afla* [*Anoxybacillus flavithermus WK1* (YP_002317070.1)], *Bhal* [*Bacillus halodurans* C-125 (NP_244659.1)], *Brev* [*Brevibacillus brevis* NBRC 100599 (YP_002774973.1)], *Gkau* [*Geobacillus kaustophilus* HTA426 (YP_149242.1)], *Blic* [*Bacillus licheniformis* DSM 13 (YP_081040.2)], *Ecol* [*Escherichia coli* (WP_001456462.1)], *Taqu* [*Thermus aquaticus* (WP_003044229.1)], and *Oihe* [*Oceanobacillus iheyensis* HTE831 (NP_693929.1)].

NBRC100599, *Geobacillus kaustophilus* HTA426, *Bacillus licheniformis*, *Escherichia coli*, *Thermus aquaticus*, and *Oceanobacillus iheyensis*. In all compared species, and many other species not mentioned here, the localization of *pyr*G is different and manifests differences among the compared bacteria. In the glutaminase domain of CTP synthase proteins, the GxxxRLG sequence is highly conserved between many prokaryotic and eukaryotic species (Simard et al., 2003) [(*Giardia intestinalis* (AAB41453.1), *Synechococcus elongatus* (Q54775), *Spiroplasma citri* (P52200), *Mycobacterium bovis* (AAB48045.1), *Methanocaldococcus jannaschii* (Q58574), *Chlamydia trachomatis* (Q59321), *Haemophilus influenzae* (P44341), *Nitrosomonas europaea* (AAC33441.1), *Azospirillum brasilense* (P28595), *Helicobacter pylori* (O25116), *Borrelia burgdorferi* (O51522), *Cricetulus griseus* (P50547), *Mus musculus* (P70698), *Homo sapiens* (P17812), *Arabidopsis thaliana* (AAC78703.1), *Saccharomyces cerevisiae* (URA-8, P38627), *Lactococcus lactis* (CAA09021.2), and *Escherichia coli* (AAA69290.1)]. This sequence was found to be conserved in *Ago*G2CTPs (Figure 4). In earlier studies, it was determined that Lys^{306} residue is not essential for ATP binding, but acts by bringing about the conformational changes that mediate interactions between the ATP and UTP sites and between the ATP-binding site and the glutamine amide transfer domain. The fact that Lys^{306} is not fully conserved between species also suggests that interaction of Lys^{306} with ATP is not essential (MacLeod et al., 2006). Sequence comparison shows that Lys^{306} is not conserved in *Ago*. Phe^{353} is fully conserved among CTP synthases, and examination of the *Thermus thermophilus* and *E. coli* CTPS crystal structures suggests that the phenyl ring of Phe^{353} packs between bound glutamine and GTP (Endrizzi et al., 2004). Lys^{187} residue in the synthase domain is extremely conserved among CTP synthases from different organisms. This region, between residues 116 and 229, has been suggested to constitute the CTP/UTP-binding site (Long and Pardee, 1967; Ostrander et al., 1998). Sequence comparison shows that Lys^{187} and Phe^{353} are fully conserved in *Ago*G2CTPs. These data show that the critical residues are conserved in *Ago*G2CTPs.

For biochemical characterization of CTP synthase enzymes, enzymes and nucleotides were generally preincubated together for 2.5 min at 37 °C, and then the reaction was initiated by the addition of NH_4Cl (MacDonnell et al., 2004). In our study, NH_4Cl was used as a substrate in all assays, and we observed increased enzyme activity when we initiated the reaction by the addition of the enzyme in place of NH_4Cl. The optimum temperature of *Ago*G2CTPs agrees well with the temperature at which *Ago* grows (65 °C), but the enzymes listed in the Table show higher activity at 37 °C. The highest optimum temperatures among CTP synthases were seen in *Ago*G2CTPs; these properties of *Ago*G2CTPs may have potential applications biotechnologically when high temperature is necessary. The K_m value of *Ago*G2CTPs was seen to be higher than that of some other species. *Bos taurus* and *Rattus norvegicus* CTP synthases have the same K_m value as *Ago*G2CTPs. There is negative correlation between the K_m value and the affinity of the enzyme for substrate. This shows that *Ago* has low CTP pools. Specific activity values listed in the Table are similar to specific activity of *Ago*G2CTPs, and CTP synthase of *E. coli* shows optimum activity at a pH range of 7.3–10.3. CTP synthases from other organisms show more activity around these pH levels. Therefore, these values further demonstrated that the pH range between 7.3 and 10.3 may be the common range for CTP synthases.

In conclusion, in this study we expressed, purified, and characterized a novel CTP synthesis protein from *Ago* with higher activity at higher temperatures than other CTP synthases for the first time. This enzyme is important for the biosynthesis of phospholipids and nucleic acids and plays a key role in cell growth, development, and tumorigenesis. Knowledge of the molecular and biochemical properties of this enzyme is important for the development of new drugs. According to sequence comparison, CTP synthases

Table. Comparison of biochemical parameters of CTP synthases from various organisms.

	K_m (mM)	Specific activity (μmol min^{-1} mg^{-1})	pH range	Optimum temperature	Storage stability	References
B. taurus	0.07	0.015				Weinfeld et al. (1978)
E. coli	0.424 Gln	5.8	7.3–10.3	37 °C	–20 °C	Anderson (1983), Lunn and Bearne (2004), MacLeod et al. (2006), Roy et al. (2010)
L. lactis	0.259 Gln				–20 °C	Willemoes (2004)
Mus musculus		0.06	7.3–9.5			Kizaki et al. (1985)
R. norvegicus	0.07		7–9			Williams et al. (1978)
S. cerevisiae	0.04	0.66	7.5–9		–80 °C for 6 months	Chang and Carman (2008), Pappas et al. (1999), Park et al. (2003), Yang et al. (1994)
Trypanosoma brucei	0.26 Gln	0.5515	7.3	37 °C		Fijolek et al. (2007)
C. trachomatis	0.097					Wylie et al. (1996)
A. gonensis G2	12.415 NH_4Cl	0.156–0.341	9–10	65 °C	–20 °C	This study

of bacteria and of humans are very similar to each other structurally, and the results obtained from *Ago*G2CTPs by further analysis may have potential application on CTP synthases from different organisms.

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