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ARAŞTIRMA MAKALESİ RESEARCH PAPER

Cloning and Determination of Kinetic Activity of Thermophilic GCH-I from *Anoxybacillus flavithermus* DSM 2641^{T} [*]

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Abstract: Phenylketonuria (PKU) is a disease caused by phenylalanine hydroxylase enzyme deficiency in newborn infants and is the most important cause of treatable mental retardation. One of the causes of the disease comes from the defects of the PTPS in the metabolic pathway of phenylalanine metabolisms. Treatment of the disease is not feasible, and life-time tetrahydrobiopterin loading is performed in chronic patients. Today, tetrahydrobiopterin is chemically synthesized. Biological production is a different point of view due to the long duration of chemical synthesis, costs, and exposure to chemical pollutants. NH_2TP is a difficult substance to obtain in studies that are difficult to synthesize and need to be used as a substrate. In this study, it was aimed to clone the *gch*-I gene from the thermophilic *A. flavithermus* bacterium and to investigate the activity of the enzyme for the enzymatic conversion of NH_2TP, which is the substrate of many enzymes and is difficult to synthesize chemically. Fort his purpose, it was aimed to clone the *gch*-I gene from a thermophilic bacteria, *Anoxybacillus flavithermus*, and investigate the kinetic activity for enzymatic conversion from GTP as an alternative to the production of NH_2TP. For this reason, the *gch*-I gene from the thermophilic *A. flavithermus* DSM 2641^{T} was identified by PCR method. We cloned the *gchI* gene that was 603 bp and its open reading frame has 200 amino acids. The gene was cloned into pET28a(+) expression vector with 6xHis tags and transform in *E. coli* BL21(DE3)pLys host cells to express with 1 mM IPTG induction. After purification with Ni-NTA resin, we determined that GCH-I is 24 kDa, its optimum pH is 8.0 and temperature is 65°C. Under optimal conditions, GCH-I exhibited enzymatic activity with K_m- and V_{max}- values of 243 ± 23.25 µM and 100.93 ± 3.5 nM/min/mg protein, respectively.

Keywords: *A. flavithermus* DSM 2641^{T}, BH4, GCHI, PKU, thermophilic bacterium.

Anoxybacillus flavithermus DSM 2641^{T} Bakterisinin Termofilik GCH-I Enziminin Klonlanması ve Kinetik Aktivitesinin Belirlenmesi

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Öz: Fenilketonüri (FKU) yenidoğan bebeklerde fenilalanin hidroksilaz enzim eksikliğinin neden olduğu bir hastalıktır ve tedavi edilebilir zihinsel geriliğin en önemli nedenidir. Hastalığın nedenlerinden biri, fenilalanin metabolizmasının metabolik yolundaki PTPS'nin kusurlarından kaynaklanmaktadır. Hastalığın tedavisi mümkün değildir ve kronik hastalarda yaşam boyu tetrahidrobiyopterin yüklemesi yapılır. Bugün, tetrahidrobiyopterin kimyasal olarak sentezlenir. Biyolojik üretim, kimyasal sentezin zaman alıcı olması, maliyetler ve kimyasal kirleticilere maruz kalma nedeniyle farklı bir bakış açısıdır. NH_2TP, kimyasal sentezi güç olan ve substrat olarak kullanılması gereken çalışmalarda temini zor bir maddedir. Bu çalışmada, bir çok enzimin substratı olan ve kimyasal yolla sentezlenmesi güç olan NH_2TP'nin eldesinde alternatif olarak GTP'den enzimatik dönüşümü için termofilik *A. flavithermus* bakterisinden *gch*-I geninin klonlanması ve enzimin aktivitesinin araştırılması amaçlanmıştır. Bu amaç doğrultusunda, termofilik *A. flavithermus* DSM 2641^{T}'den *gchI* geni PCR yöntemi ile tanımlandı. 603 bp olan *gchI* genini klonlandı ve açık okuma çerçevesi 200 amino aside sahip olduğu tespit edildi. *AfgchI* geni, 6xHis etiketleri ile pET28a(+) ekspresyon vektörüne klonlandı ve 1 mM IPTG indüksiyonu ile eksprese etmek için *E. coli* BL21(DE3)pLys konakçı hücrelerine transforme edildi. Ni-NTA afinite kromatografisi ile saflaştırıldıktan sonra GCH-I'in 24 kDa, optimum pH'sının 8,0 ve optimum sıcaklık isteminin 65°C olduğu belirlendi. Optimal koşullar altında GCH-I, K_m- ve V_{max}- değerleri sırasıyla 243 ± 23,25 µM ve 100,93 ± 3,5 nM/dak/mg protein olarak tespit edildi.

Anahtar kelimeler: *A. flavithermus* DSM 2641^{T}, BH4, GCHI, FKU, termofilik bakteri.

[*] *This study was produced from the master thesis prepared.* *Bu çalışma yüksek lisans tezinden üretilmiştir.*

INTRODUCTION

Currently, phenylketonuria (PKU, or hyperphenylalanemia, HPA) is the most studied congenital metabolic disease, characterized by the absence of phenylalanine hydroxylase enzyme activity, characterized by severe motor and mental retardation when not treated early (Muntau et al., 2002; Özer, 2004). One of the primary causes of PKU or HPA formation is the impairment of tetrahydrobiopterin (BH4) metabolism (Hizal, 2013; Kure et al., 1999; Muntau et al., 2002; Spaaen et al., 2001). These disorders can also be described as dihydropteridine reductase, GTP cyclohydrolase-I (GCHI), pterin-4–8-carbinolamine dehydratase, and 6-pyrovyl tetrahydropterin synthase (PTPS) defect and are affected by liver, kidneys, brain and blood tissue (Blau & Burgard, 2005). The overall incidence of diseases due to metabolism disorders of tetrahydrobiopterin, this ratio is 15% in Turkey, while 1-3% of the World (Hizal, 2013). This result shows that PKU is quite common in our country. According to available data, 58% of BH4-dependent defects in congenital diseases are caused by defects in *ptps*, 4% in *gchI*, and 0.9% in *spr* genes (Ponzone et al., 2004). BH4 is an essential cofactor for many enzymes including nitric-oxide synthase and phenylalanine hydroxylase (Wang et al., 2011). In addition, this cofactor has been implicated in a vast array of physiological roles including hyperphenylalaninaemia, cellular proliferation, vascular dysfunction, and various neurological disorders. Cells form BH4 in two different ways. In the *de novo* biosynthesis pathway, GTP is used as a precursor, whereas in the Salvage path, dihydropterins which are already present in the medium are used. The *de novo* biosynthesis pathway takes place with three major enzymes, and the same is true *in vivo* and *in vitro*. These enzymes are respectively GCHI, PTPS, and SPR (Moens & Kass, 2006; Celik, 2018).

The chemical synthesis of BH4 is very difficult, the complexity of the methods used, the application of many synthetic steps, the cost of using expensive materials is quite high. Due to the difficulties of chemical synthesis, BH4 production can be achieved by bacterial pathways. The work to be done in this direction is to make bacterial production using homologs of enzymes involved in this pathway in humans.

The GCHI enzyme (EC: 3.5.4.16) is the precursor enzyme of BH4 biosynthesis (Blau et al., 2001). It was obtained from many different organisms (bacteria, fungi, flies, chicken, human) and examined. The enzyme converts GTP to dihydroneopterin triphosphate (Fig. 1). GCHI is an enzyme coded from a single copy with 30 kbp in size in human. The enzyme containing 250 amino acids is similar in other living things (Blau et al., 2001; Woo et al., 2002). Dihydroneopterin triphosphate (NH_2TP), the product of GCH-I, is the substrate of 6-carboxytetrahydropterine synthase, dihydroneopterin triphosphate diphosphatase, dihydroneopterin triphosphate aldolase, PTPS, and dihydroneopterin triphosphate 2'-epimerase.

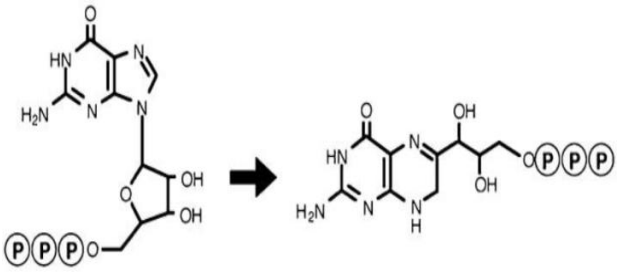

Figure 1. Reaction pathway of GCH-I (revised form Maita et al., 2002).

As an alternative to the chemical synthesis of BH4, the production of microorganisms using target genes can be demonstrated. The first step is to find the *gchI* gene. The only study on this subject was by Yamamoto et al. (2003). In this study, *ptps* and *spr* genes were cloned and evaluated together with a homologous gene instead of *gchI*. The genes source from mice for PTPS and SPR, and from *B. subtilis* for GCHI analog (*mtrA*) were used. This can be achieved at a lower cost by means of a recombinant plasmid to be formed. As in the example here, thermophilic bacteria, *A. gonensis* G2, which has many advantages in industrial applications, were used as a gene source for the bacterial production of BH4. Today, thermophilic organisms are frequently used in biotechnological applications. The resistance of enzymes in thermophilic bacteria to harsh industrial applications is a preferred element. These factors can be listed as follows: reactions occurring at elevated temperatures, reduced risk of general mesophilic contamination of the environment, no unwanted by-product formation, increased solubility of substrates and products, and increased stability by generating preferred equilibrium changes in endothermic reactions (Celik, 2018; Kalemci, 2020).

Anoxybacillus flavithermus DSM 2641^T is an thermophilic bacteria with optimum growth contidions of 55-60°C (Saw et al., 2008). This bacteria is a facultatively aerobic heterotroph. *Anoxybacillus* species have become very popular in industrial research (Celik, 2018). In this report, we describe the expression and characterization of GCHI from the thermophilic bacteria *Anoxybacillus flavithermus* DSM 2641^T in *E. coli* BL21(DE3)pLys. In this study, cloning and biochemical characterization of the GCH-I enzyme from a thermophilic bacterium for the production of NH_2TP was performed.

MATERIAL AND METHOD

Microorganism, Chemicals and Other Materials: *Anoxybacillus flavithermus* DSM 2641^T, *E. coli* DH5α, and BL21(DE3)pLys were obtained from our laboratory stocks. Modifying enzymes such as DNA polymerase, DNA ligase, restriction endonucleases, and dNTPs were purchased from ThermoScientific (USA). The purification kits for PCR

products or DNA restriction fragments from agarose gels were obtained from Wizard® SV Gel and PCR Clean–Up System (Promega Co., Medison, USA). Plasmid and genomic DNA isolation kits were purchased from Thermo Sci. (GeneJet Plasmid Miniprep Kit) and Promega Co. (Wizard® Genomic DNA Purification Kit), respectively. All other chemicals and reagents were obtained from Sigma & Aldrich Inc. (St. Louis, MO, USA) and Merck GmbH (Germany). All primers for PCR were synthesized by Macrogen Inc. (Amsterdam, Netherlands).

Strains and Their Growth Conditions: *Anoxybacillus flavithermus* DSM 2641^T was cultivated under aerobic conditions at 55°C in Luria-Bertani (LB) medium containing 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl. *Escherichia coli* DH5α and BL21(DE3)pLys used as a host for cloning and expression studies were grown in LB medium. And also, LB medium with ampicillin (100 µg/mL) or kanamycin (30 µg/mL) was used in clonal selection and expression studies, respectively.

Cloning of AfgchI gene: Genomic DNA isolation from *A. flavithermus* was performed by Promega Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. To obtain *AfgchI* gene by PCR from *A. flavithermus*, we designed a pair of primers (forward, adding with *Nco*I restriction site, AfgchF: 5'-CCATGGTAGAATCGGATTACG-3' and reverse, adding with *Xho*I restriction site, AfgchR: 5'-CTCGAGCTATTTAATCAGCGC-3') according to NCBI GenBank data of *gchI* gene from *A. flavithermus* WK1 (NCBI Reference Sequence: CP000922). The PCR conditions were as follows: one cycle of the first denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, extension for 72°C for 1 min, and final extension at 72°C for 4 min. After gel extraction with Wizard® SV Gel and PCR Clean-Up System (Promega Co., Medison, USA), the 603 bp PCR product of *Afgchl* was ligated into pGEM®-T/Easy Vector (Promega Co., Medison, USA) according to manufacturer's protocol. This vector was transformed into chemically competent *E. coli* DH5α host cells. The obtained colonies containing the recombinant plasmids were identified and confirmed by restriction digestion with suitable enzymes, PCR, and DNA sequencing. The recombinant plasmid was obtained from the same enzyme and cloned into pET28a(+) and introduced into *E. coli* BL21(DE3)pLys for expression.

Expression and Purification of Recombinant Enzyme: One colony was inoculated into 3 mL LB broth containing kanamycin (30 µg/mL) and incubated overnight at 37°C with shaking at 150 rpm. A sufficient aliquot of the overnight culture was added into 400 mL LB medium with 30 µg/mL kanamicin and incubated at the same conditions. When the optical density at 600 nm of the cultures reached 0.5, cells were induced with 1 mM IPTG. After 4 hours, the pellet of the cultures was obtained by centrifugation at 8000 rpm for 10 min at 4°C. The pellet was resuspended and purified His Link™ Protein Purification System (Promega, Medison, WI, USA) according to the manufacturer's protocol. Then purified protein was checked by SDS-PAGE (12% w/v). Then, the protein concentration of the dialyzed sample was determined by the Bradford methods (Bradford, 1976).

Enzyme Assays and Determination of Kinetic Parameters: Enzyme activity was determined using Tanaka et al. (2005) with minor modification. In this method, the incubation temperature was used as 55°C. The reaction mixture (100 µL) comprised 50 mM Tris-HCl (pH 8.0), 0.1 M KCl, 2 mM $MgCl_2$, 1 mM GTP and about 0.2 µM enzyme (AFGCHI). Reactions were performed at 55°C for 1 hour and terminated by the addition of an oxidation solution comprising 1% I_2 and 2% KI in 1 N HCl for 15 min in dark condition. The product (NH_2TP) was oxidized to neopterin triphosphate by the iodine. Excess iodine was reduced by the addition 15 µL 2% ascorbic acid. The fluorescence of neopterin triphosphate was measured with excitation at 350 nm and emission at 450 nm using a SpectraMax spectrofluorimeter (Molecular Devices, CA, USA). We use a neopterin as standart.

Characterization of AFGCHI

Effects of pH and temperature on AFGCHI activity: The pH and temperature effects on the activity were determined in the pH range of 5.5-9.0 and 37-85°C. The buffers were MES for pH 5.5 and 6.0, potassium buffer for pH 5.8, 6.6, 7.2, and 8.0, Tris-HCl for pH 7.5, 8.0, and 8.8, and also glycine-NaOH for pH 9.0. After determining the optimum pH, the reaction was done between 37-85°C for optimum temperature experiments.

Determination of Kinetic Parameters: The K_m and V_{max} values of the AfGCHI, were determined using 10-50, 100-500 µM, and 1, 3, 5, and 10 mM GTP by the optimized conditions. The kinetic values were determined from the results using Origin 8.0 program.

RESULTS AND DISCUSSION

Cloning of AfgchI: In this study, we completed the characterization of AFGCHI enzyme belonging to *A. flavithermus* bacteria which is a thermophilic character. Data from the gene encoding the enzyme was obtained using NCBI data from *A. flavithermus* WK1 bacteria, whose genome analysis was completed previously. Based on these data, the primers designed by PCR were found to be 603 base pairs as in the original strain. It has a total size of 200 amino acids. Cloning the gene, p6xHis-*Afgchl* was constructed and successfully expressed in *E. coli* BL21(DE3)pLys cells. After expression with 1 mM IPTG at appropriate time according to the manufacturer's protocol, the cells were collected and sonicated. A heat treatment of cell extract at 60°C at 30 min eliminated most of the host cell's proteins. Then, a Ni-NTA affinity chromatography was used to purify the AfGCHI and dialysis was performed. SDS-PAGE analysis (12%) showed that the purified protein was homogeneous with a molecular mass of 24 kDa (Fig 2).

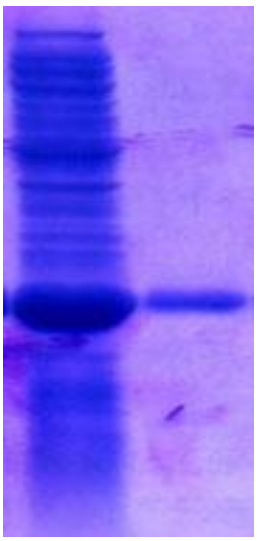

Figure 2. 12% SDS-PAGE profiles of AfGCH-I (Crude extract on the left after IPTG induction, Ni-NTA purified AFGCH-I on the right).

The aminoacid sequences of the gene have similar to *A. kamchatkensis*, *A.* sp. DT3-1, and *A.* sp. SK3-4 (98%), *Geobacillus* sp. WCH70 (89%), *G.* sp. Y4.1MC1, *G. thermodinitrificans* NG80-2, and *G. thermoglucosidasius* (87%), *Bacillus cereus, B. thrungiensis*, *G. kaustophilus* HTA426, *G. thermoleovorans* CCB_US3_UF5 and *G. caldoxylosilyticus* (86%), *B. cytotoxicus* NVH 391-98, *B. thuringiensis* HD-771, *B. cereus* ATCC 14579, and *B. thuringiensis* str. Al Hakam (85%), and *B. megaterium* WSH-002 (83%). These organisms are closely related to *A. flavithermus*. Comparing the amino acid numbers, *Bacillus anthracis* strain A0248, *B. cereus* strain Q1 and AH820, *B. weihenstephanensis* strain KBAB4, *B. huringiensis* subsp. *konkukian* strain 97-27 and 189, *B. amyloliquefaciens* strain FZB42, *B. subtilis* strain 168 has 190 amino acids, *G.* sp. strain WCH70 has 188, *B. halodurans* strain ATCC BAA-125 has 299 amino acids. In the other thermophilic bacteria, we see different numbers of amino acids of the gene, for examples, *Thermotoga maritima* strain ATCC 43589, *Thermotoga neapolitana* strain ATCC 49049, and DSM 4359 have 259, *Thermodesulfovibrio yellowstonii* strain ATCC 51303 and DSM 11347 have 188, *Thermoanaerobacter pseudethanolicus* strain ATCC 33223 has 188 (BRENDA data, see https://www.brenda-enzymes.org/enzyme.php?ecno=3.5.4.16).

The optimal pH for AfGCHI was 8.0 with GTP as substrate. The enzyme shows <10% of the activity at pH 5.8 (Fig 3a). According to BRENDA data, this value was 8.0 for *G. sterothermophilus*, 8.0 for *B. subtilis*, 8.5 for *Thermus thermophilus*, and 6.5 for *Methanococcus janashii* (Suzuki et al., 1979; De Saizieu et al., 1995; Tanaka et al., 2005; Grochowski et al., 2007; respectively). The optimal temperature for AfGCHI was 65°C. At the optimum bacterial growth temperature (55°C), the enzyme shows 75% of the original activity (Fig 3b). In other studies, we see that the optimum temperature value differs depending on how it works, *i.e.* 42°C for *E. coli*, 37-78°C for *G. sterothermophilus*, and 37°C for *B. subtilis*, (Yim & Brown, 1976; Suzuki et al., 1979; De Saizieu et al., 1995, respectively).

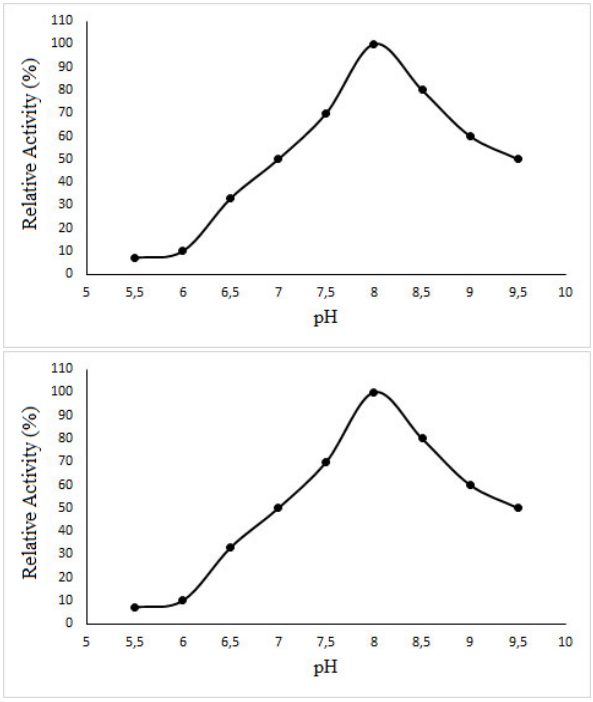

Figure 3. a. Determination of optimal pH of AfGCHI, **b.** Determination of optimal temperature of AfGCHI.

Kinetic futures of AfGCHI: The enzyme had the V_{max} and K_m for 100.93 ± 3.5 nM/min/mg protein and 243 ± 23.25 µM for GTP (Fig 4). Compared to the literature, K_m values of *Thermotoga maritima* COG1469 are 0.0023-0.0053 µM (El Yocoubi *et al.*, 2006), 2.9 µM in *E. coli* (Yim & Brown, 1976), 4.2 µM of *Thermus thermophilus* (Tanaka et al., 2005), 4.0 µM of *B. subtilis* (De Saizieu et al., 1995), 80 µM of *Streptomyces tubercidicus* (Yo et al., 1998), and 980 µM for *Geobacillus stearothermophilus* (Suzuki et al., 1979). As it is known, as an enzyme's interest in the substrate increases, its K_m value decreases. According to the literature data, the K_m value of our enzyme is low in the substrate when compared to GCH-I enzymes obtained from different sources studied. It is high only compared to the same enzyme of *G. stearothermophilus*, which has a similar thermophilic character.

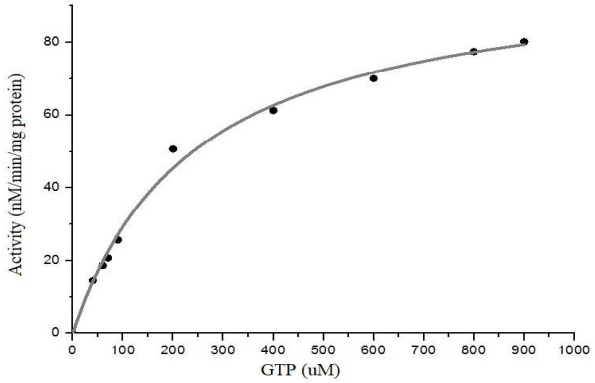

Figure 4. Lineweaver-Burk plots of AfGCHI under optimal conditions.

DISCUSSION AND CONCLUSION

As a result, AFGCHI is one of the enzymes that must be used to make bacteria as an alternative to the chemical synthesis of BH4, which is used in the treatment of phenylketonuria disease, which has a prevalence of 1/4500 in our country and one of 20-25 children is caught each year. The enzymes are thermophilic character previously mentioned reasons it is preferred for industrial applications (Andrade et al., 1999; Hartley et al., 2000; Taylor et al., 2004; Hızal, 2013). This study showed that the human homologous GCHI enzyme can be cloned from a thermophilic bacterium, thus NH_2TP can be obtained without chemical synthesis. The pathway can be completed by similarly cloning and characterizing other enzymes.

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