International Journal of Biology Research
ISSN: 2455-6548, Impact Factor: RJIF 5.22
www.biologyjournal.in
Volume 2; Issue 2; April 2017; Page No. 07-12

Cloning, sequencing and expression of L-Asparaginase II gene from Citrobacter freundii 1101

[1] Elham Bahreini, [2]Azam sabaghi, *[3] Khosrow Aghaiypour

[1]Department of Biochemistry, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2,3] Department of Genomics and Genetic Engineering, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

Abstract
L-Asparaginase II from bacteria has been used in treatment of acute lymphoblastic leukemia. In this study, ASNase II gene from Citrobacter freundii1101 was sequenced and cloned in E.coli DH5α. For this purpose, three pairs of primers were designed to amplify different fragments of the gene. After sequencing ansB by DNAMAN software, the full length of the gene was amplified by PCR. ASNase II gene was cloned into the pET22b plasmid and transformed into E. coli BL21pLysS (DE3)-competent cells by the heat shock method. The expression of periplasmic recombinant ASNase II was induced via IPTG (1mM) in transformed E.coli cells. Periplasmic ASNase II was extracted using an alkaline lysis method. The extracted protein was purified by one-step DEAE-Sepharose fast-flow chromatography. ASNase II activity was considered as an index for the protein expression. Bioinformatics analysis of Citrobacter freundii1101- ASNase II gene showed some similarity with the therapeutic enzyme from E.coli K12 and Erwinia. BLAST analysis showed more than 91% and 99% similarity in nucleotide and amino acid sequences, respectively, with other strains of Citrobacters; and also a similarity of 82.52% and 92.82% in nucleotide sequence and amino acid sequences, respectively, with E.coli K12. The antigenicity of the protein was predicted and compared with the therapeutic enzyme using semi-empirical method. These results indicated other potent bacteria source of ASNase II as a candidate for anticancer consideration.

Keywords: L-Asparaginase II, acute lymphoblastic leukemia, Citrobacter freundii 1101, pET22b, gene expression, heat shock method, E.coli BL21

Introduction

L-Asparaginase II (ASNase II: EC 3.5.1.1) catalyzes the hydrolysis of L-asparagine to L-aspartate and ammonia (Pasut *et al.,* 2008) [1]. It is one of the most biotechnologically and biomedically important group of therapeutic enzymes and is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system. Although, ASNase II has been found in number of organisms like serum of guinea pig and rodents, chicken liver, yeast, molds, plants and number of bacteria, not all of these enzymes are clinically active (Pasut *et al.,* 2008; Narta *et al.,* 2007; Verma *et al.,* 2007; Geckil *et al.,* 2004) [1-4]. Only ASNase II obtained from E.coli and Erwinia chrysanthemi have been used in human.

Most malignant lymphoblastic cells and some other suspected tumor cells are unable to synthesize the non-essential amino acid asparagine, whereas normal cells are able to make their own asparagines. Thus these tumor cells require high amount of asparagines and depend on circulating asparagine. The conversion of blood asparagine to aspartic acid by ASNase II deprives the leukemic cell from circulating asparagine, which leads to cell death(Narta *et al.,* 2007; Deokar *et al.,* 2010) [2, 5].

In addition to treatment, the most common use of ASNase II is as a processing aid in the manufacture of food. Marketed under the brand names Acrylaway and PreventASe, ASNase II can effectively reduce the level of acrylamide up to 90% in a range of starchy foods without changing the taste and appearance of the end product (Pedreschi *et al.,* 2008; Ros´en *et al.,* 2002) [6, 7]. Acrylamide is often formed in the cooking of starchy foods. During heating the amino acid asparagine, naturally present in starchy foods, undergoes a process called the Maillard reaction, which is responsible for giving baked or fried foods their brown color, crust and toasted flavor. Suspected carcinogens such as acrylamide and some heterocyclic amines are also generated in the Maillard reaction. By adding ASNase II before baking or frying the food, asparagine is converted into another common amino acid, aspartic acid, and ammonium. As a result, asparagine cannot take part in the Maillard reaction, and therefore the formation of acrylamide is significantly reduced (Pedreschi *et al.,* 2008; Ros´en et al 2002; Taeymans *et al.,* 2005; Dhanam *et al.,* 2013; Tareke *et al.,* 2002) [6-10].

The therapeutic effect of ASNase II from Escherichia coli and Erwinia chrysanthemi is accompanied by side effects. The main side effect is an allergic or hypersensitivity reaction; anaphylaxis is a possibility. Additionally, it can also be associated with a coagulopathy as it decreases protein synthesis, including synthesis of coagulation factors (e.g. progressive isolated decrease of fibrinogen) and anticoagulant factor (generally antithrombin III; sometimes protein C&S as well), leading to bleeding or thrombotic events such as stroke. Bone marrow suppression is common but only mild to moderate, rarely reaches clinical significance and therapeutic consequences are rarely required (Pieters *et al.,* 2011; Rizzari *et al.,* 2013; Truelove *et al.,* 2013) [11-13]. Therefore, it is desirable to search for other ASNase II producing sources with novel properties that can produce an enzyme with less adverse effects. In the present study,

ASNase II gene from Citrobacter freundii 1101 (C. freundii 1101) was amplified and sequenced; then it was used to produce recombinant protein in E.coli expression host.

Materials and Methods

Reagents

Purchased reagents included restriction enzymes, NdeI and HindIII (New England Biolab), calf intestinal alkaline phosphatase, T4 DNA ligase (Roche), protein and DNA molecular weight markers (Fermentas), Pfu Turbo DNA polymerase (Stratagene). Unless otherwise specified, all other chemicals were from Merck.

Microorganism media and growth conditions

The lyophilized C. freundii 1101 was inoculated in TSB medium (Tryptic soy broth) enriched by horse serum. After 8 hour of incubation, the TSB medium was cultured on MacConkey agar, blood agar and TSA to obtain single colonies.

Genomic DNA extraction

Single colony was inoculated in TSB medium and incubated till logarithmic growth cell density of 0.6. TSB medium was centrifuged (1200×g) and bacteria pellet was obtained. The pellet was washed two times by PBS buffer. The genomic DNA was isolated and purified by phenol-chloroform method (Sambrook *et al.,* 1989) [14].

Separating of ansB from C. freundii

Before the present study, there were no reports of C. freundii 1101-ASNase II gene (ansB) in GenBank. Thus, the ansB was amplified from genomic DNA using primers designed based on other ASNase II gene sequences from GenBank. The sequence alignments of the considered ansB were performed by DNAMAN software (version 4.13) sequence alignment program and three pair primers were designed by Oligo software (version 5.0): First pair primers: Ans-com-U576 (GGG CGC TTT TTG CGG GTT), Ans-com-L1138(GAT GGT CGG CGC RAT GCG); Second pair primers: Ans-com-L1558 (GCG TTC ACR TAA CTG GAG), Ans-com-U576 (GGG CGC TTT TTG CGG GTT); Third pair primers:Ans-com-L1138(GAT GGT CGG CGC RAT GCG), Ans-com-U197(GGT GAC CAG GTG GCC TGA). The primers were custom synthesized by MWG Biotech. PCR was performed in a final volume of 25µL containing PCR amplification buffer (1X), Hot-Start Taq polymerase (1U/µl), dNTPs mixture (2.5mM), $MgCl_2$ (2.5mM) primers (5µM) and template DNA (1ng/µl). Amplification conditions were, initial denaturation at 95°C for 5 min followed by 30 cycles at 95°C for 1 min, 52°C by first and third pair primers and 42°C by second pair primers for 1 min, extension at 72°C for 2 min and with a final extension of 72°C for 10 min. The resulting PCR product was purified using gel extraction kit (Roche).

Vector cloning and bacteria transformation

Purified primer-extended PCR products were ligated into pTZ57 R/T vector using InsTAclone™ PCR Cloning Kit (Fermentase). A cloning reaction containing 7 µL of PCR product, 3 µL of 10x ligation buffer, 3 µL of pTZ57R/T, 3 µL PEG solution, 0.75µL BSA, and 5u T4 DNA Ligase was mixed gently and incubated for overnight in 37°C.

The resulting recombinant plasmid was transformed into competent cells of E.coli DH5α (Invitrogen, Carlsbad, CA) by heat shock method (Bahreini *et al.,* 2014) [15]. Screening of transformed bacteria was carried out by blue-white colonies on LB agar containing ampicillin 50µg/ml (Sambrook *et al.,* 1989) [14] and treated with IPTG and X-Gal. White colonies selected as recombinant bacteria and used for amplifying recombinant plasmids. Amplified plasmids were purified using High Pure Plasmid Isolation Kit (Roche).

C. freundii 1101-ansB sequencing by software analysis

The fragments cloned in pTZ57 R/T vector were sequenced by MWG German Company. Determined sequences were analyzed by DNAMAN software. Considering the overlap sequences, single sequence was determined for C. freundii 1101-ansB.

Construction of expression plasmid

The expression vector of pET 22b (Cat. No. 69744-3) was used for C. freundii 1101-ansB expression. The pET 22b vector carries an N-terminal pelB signal sequence for potential periplasmic localization; thus, signal peptide sequence of C. freundii 1101ansB was ignored as 58 nucleotide fragment at 5´ end which was determined by comparing C. freundii 1101-ansB sequence with known C. koseri asnB sequence in GenBank. Considering the restriction sites for NdeI and Hind III at 5´ ends of forward and reverse primers, respectively, the expression primers were designed as Nde I-Cit-ans (GGA ATT CCA TAT GGA GTT TTT CAA GAG AAC), Hind III-Cit-ans (CCC AAG CTT AAT ACT GAT TAA ACA TCG). PCR was performed by Pfu DNA polymerase. Purified asnB containing restriction sites of NdeI and Hind III was ligated into pET22b vector digested with same restriction enzymes. An insert to pET22b vector ratio of 2:1 was subjected to 2 µL of reaction buffer, and 1µL of T4 DNA ligase (Invitrogen, Carlsbad, CA) was brought to a total volume of 20 µL with dH2O. The reaction was carried out overnight at a temperature of 16° C.

Recombinant gene expression in E.coli BL21 (DE3)

Construction expression plasmid, pET22b-ansB, was transformed into competent cells of E. coli BL21 (DE3) by heat shock method (Bahreini *et al.,* 2014) [15]. Transformed E.coli BL21 (DE3) was inoculated into 100µl LB broth and then, it was cultured on LB agar containing ampicillin (50µg/ml). Bacteria colonies were inspected for pET22b-ansB by PCR using the forward and reverse expression primers.

A single colony of bacteria containing pET22b-ansB was inoculated into 5ml LB broth (ampicillin 50µg/ml) and incubated overnight at 37°C with shaking at 200 rpm. On the next day, the overnight culture was transferred into 100 mL of LB broth (ampicillin 50µg/ml) and incubated at the same conditions until the optical density at 600 nm reached 0.6. At this point isopropyl-1-thio-β-galacto-pyranoside (IPTG) was added to a final concentration 1 mM to induce ansB gene expression. Sampling was carried out before and hourly after induction (up to 24 hr) for future analysis (i.e. enzyme activity assay, SDS-PAGE, Western Blot).

Extraction of ASNase II from E. coli BL21 (DE3)

The peripelasmic ASNase II was extracted from the bacteria

pellet using alkaline lysis. In this method, the bacteria pellet was resuspended in 4 volume double-distilled water. Under gentle stirring, 0.1M NaOH was slowly added until pH of the solution reached about 11.5. Stirring continued for 5 min and then pH of the solution was returned to 7.0 by slowly adding 0.1M phosphoric acid. The solution was centrifuged at 35000×g for 30 min at 4ºC. The supernatant was filtered through a 0.45µm sterile filter and prepared for anion-exchange chromatography. Before applying to the column, the enzyme activity and protein content of the filtrate were determined.

Purification of ASNase II

The resulting filtrate was applied to the DEAE-Sepharose fast flow column (1cm×7cm) pre-equilibrated with phosphate buffer (0.01mM, pH 7.0). The unbound proteins were removed from the column by passing phosphate buffer. The bound proteins were eluted from the column using stepwise increases in NaCl (50–200mM NaCl in phosphate buffer) at a flow rate of 2ml/min. The collected fractions were analyzed for enzyme activity (U/ml) and protein content (mg/ml). ASNase II purity was judged using SDS-PAGE (15%) stained with Coomassie Brilliant Blue. The fractions with the higher ASNase II activity were pooled togather.

The purified solution from the previous step was desalted using a Sephadex G-75 column (1.0×30 cm) pre-equilibrated with 0.01M phosphate buffer (pH 7.0), at a flow rate of 2.5 ml/min. Using mannitol (0.1mg/ml) as a protein stabilizer, the most active fractions were pooled and concentrated by lyophilization (-50ºC) and stored at 4ºC.

Estimation of ASNase II Activity

ASNase II activity was assayed using the Nessler method (Imada *et al.,* 1973) [17]. The reaction mixture contained 0.5ml Tris-HCl buffer (0.1 M, pH 8.5), 0.25 ml L-asparagine (10mM in Tris-HCl buffer) and 25 ml of the enzymatic solution. After 15 min of incubation at 37°C, the reaction was terminated by addition of 0.25 ml of 15% trichloro acetic acid (TCA). The liberated ammonia was determined by adding 0.25mL Nessler's reagent. The absorbance was recorded at 425nm after 10 min. Absorbance values were converted to micromoles ammonia from the standard curve prepared with ammonium sulfate. One unit of enzyme activity (IU) was defined as the amount of enzyme required to release 1mM ammonia per minute under standard assay conditions.

Bioinformatics analysis

The protein sequences were multiple aligned and homology analysis was done using BioEdit software. Secondary structure of the protein was predicted through SOPMA program which determined the role of individual amino acids in the building of the secondary confirmation of protein. The 3D protein structure was predicted by homology modeling using Swiss model workspace. The molecular weight and isoelectric point (pI) was predicted using ExPasy tool. The antigenicity of the protein was analysed and compared by semi-empirical method (Kolaskar and Prasad, 1990) [16].

Results and Discussion

The unknown C. freundii 1101-ansB sequence was amplified by three pairs of primer. The amplicon DNA fragments were gel checked on agarose gel and three bands were visualized (Figure 1) in the regions of about 980bp, 940bp and 600bp. Sequencing of the fragments was carried out by MWG German Company in triplicates. Considering the overlap parts, the full length of C. freundii 1101-ansB was successfully determined by DNAMAN software as a 1047 bp fragment that codes the monomer of ASNase II which is 348 amino acids long (Figure 2).

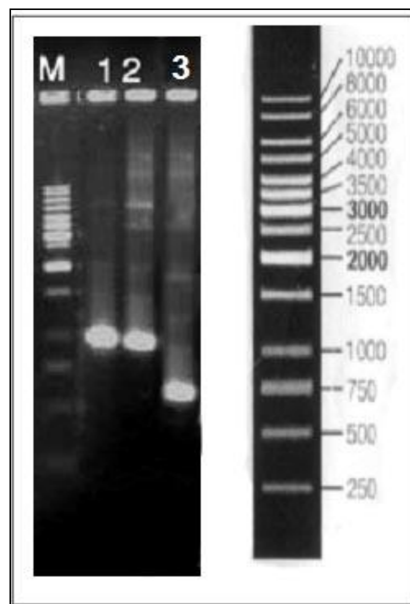

Fig 1: Electrophoresis pattern of PCR product by pair primers on 1% agarose gel. A: lane 1: PCR product of ~980bp, lane 2: PCR product of ~940bp, lane 2: PCR product of ~600bp. M: 1-kb DNA ladder; B: 1-kb DNA ladder with defined bands.

Fig 2: The full length of Citrobacter freundii 1101 ansB determined by DNAMAN software as a 1047 bp fragment

Approvment of ansB gene

In order to use of pET22b signal peptide, the first 57 nucleotide fragment from 3' OH end of C. freundii 1101 ansB was ignored by designed expression primers and PCR reaction. The agarose gel electrophoresis of the amplicon revealed an approximately 990 bp fragment that reinforced the results obtained from gene sequencing (Figure 3).

The purified *ansB-pET22b* vector was used for gene expression in E.coli BL21 (DE3). Screening of bacteria colonies containing transformed E.coli BL21 (DE3) with *ansB-pET22b* was carried out by PCR and expression

primers. Electrophoresis of PCR products detected some colonies containing pET22b-asnB through 990bp fragment which is expected to code the monomer of ASNase II with 330 amino acids long.

ASNase II expression

Transformed E.coli BL21 (DE3) by *ansB-pET22b* was induced by IPTG and sampling was done hourly. Enzyme activity was considered as an index for active protein expression. All known types of ASNase II are active as homotetramers with molecular mass of about140 kD (Swain *et al.,* 1993; Michalska and Jaskolski, 2006) [18, 19]. The closest interactions between the A and C subunits (as well as between the B and D) lead to the formation of two intimate dimmers within which the four non-allosteric catalytic centers are created. Such formations of tetramers, for reasons that are not completely clear, appear to be essential for the catalytic ability of ASNase II (Michalska and Jaskolski, 2006; Mezentsev *et al.,* 2012) [19, 20]. Figure 4 illustrates ASNase II activity/expression level during 4 hr. High activity levels was observed after 2 hours.

SDS-PAGE analysis (Figure 5) revealed deep color bands equal to 36.5KD. Because ASNase II is a homotetramer protein, its molecular weight would be 146 KD. The estimated molecular weight is matched with data obtained from ExPasy software.

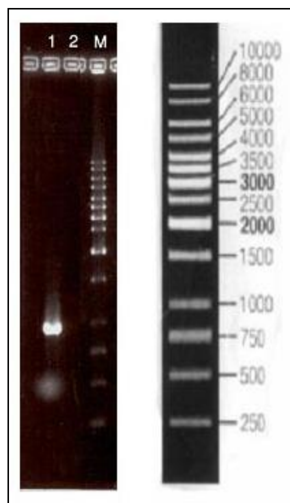

Fig 3: asnB gene amplified from C. freundii 1101. M:1kb DNA ladder (Fermentas), Lane1: ansB gene (~ 990bp)

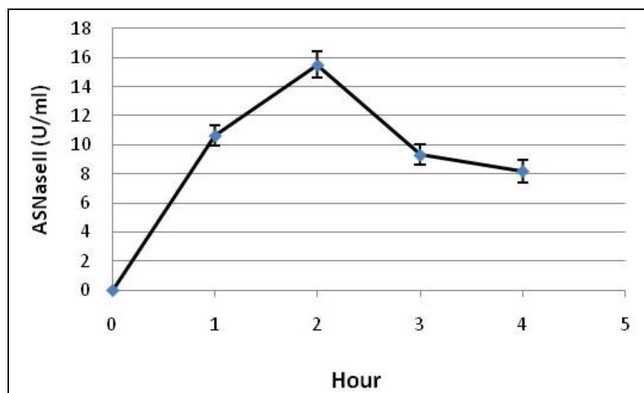

Fig 4: ASNase II activity-expression levels (as an index for protein expression) during 4 hr.

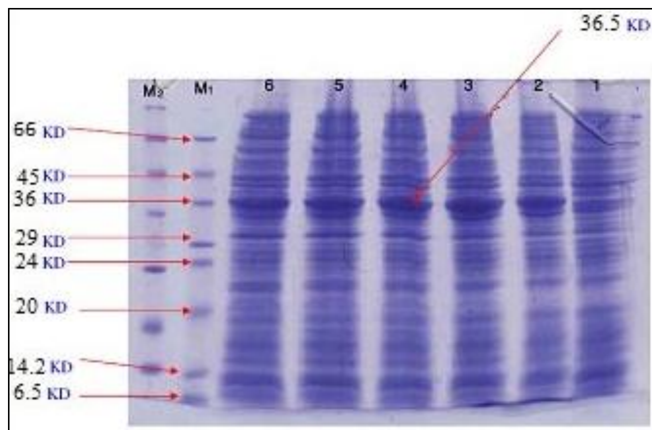

Fig 5: Gene expression assessment in E.coli BL21 by SDS-PAGE and Coomassie staining. Lane1: negative control (before induction); Lane2-6: asparaginase production in bacteria from second to sixth hour after induction; M1: protein marker.

Purification of ASNase II

Extraction and purification of periplasmic ASNase II were carried out from the bacteria pellet obtained from the protein expression step. The purification was carried out through one step of DEAE-Sepharose fast flow column chromatography. The bound recombinant protein was eluted from the column by NaCl 100mM. The purification was examined with SDS-PAGE following Coomassie Brilliant Blue staining. It revealed only a single distinctive protein band for the pure preparation of ASNase II with an apparent molecular mass of 36.5 kD (Figure 6) and as a homotetramer, a molecular mass of 140 kD. This homotetrameric structure is necessary for enzyme activity.

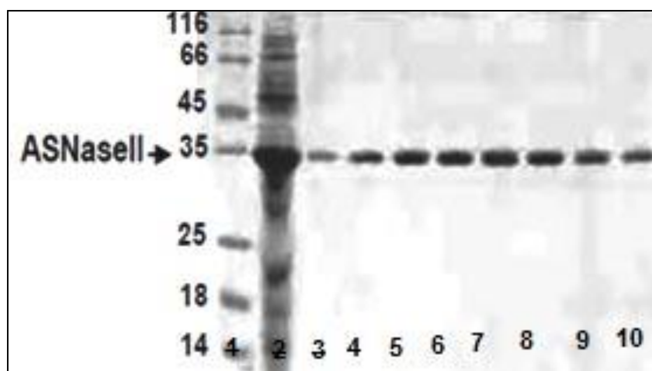

Fig 6: SDS-PAGE (15%) analysis of ASNase II purification using DEAE-sepharose. Lane 1: protein marker. Lane 2: Crude extract of *E. coli* by alkaline lysis. Lane 3-10: purified ASNase II eluted from the DEAE-Sepharose column in selected fractions.

Protein structure and bioinformatics prediction

Three therapeutic ASNase II preparations are available; the native ASNase II derived from E.coli K12 (E.coli-asparaginase), a pegylated form of this enzyme (PEG-asparaginase) and a product isolated from Erwinia chrysanthemi, i.e. Erwinia ASNase II. Clinical hypersensitivity reactions and silent inactivation due to antibodies against E.coli-ASNase II, lead to inactivation of E.Coli ASNase II in up to 60% of cases. Current treatment protocols include E.coli-ASNase II or PEG-ASNase II for first-line treatment of ALL. Typically, patients exhibiting

sensitivity to one formulation of ASNase II are switched to another product to ensure they receive the most efficacious treatment regimen possible. Erwinia-ASNase II is used as a second- or third-line treatment in European and US protocols. Despite the universal inclusion of ASNase II in such treatment protocols, there is much debate regarding the optimal formulation and dosage of these agents (Pieters *et al.,* 2011; Tong *et al.,* 2014; Asselin, 2016) [21-23].

The C. freundii 1101 ansB sequence was multiple aligned with E.coli K12 and Erwinia chrysanthemi by ClustalW2. The nucleotide sequence showed a maximum similarity of 58.67% with Erwinia chrysanthemi and 82.52% with E.coli K12. The protein sequence showed a similarity of 48.4% with Erwinia chrysanthemi and 92.82% with E.coli K12. Genscript software estimated 21% hydrophilic and 43% hydrophobic residues in the monomer of ASNase II from C. freundii 1101. The primary analysis by SIG-Pred software also suggests that the monomer has a fragment of 19 or 22 amino acids (MEFFKRTALAALVMGFSGA|ALA) at the N-terminal as signal peptide and the next 329 or 326 fragment is active mature peptide. Figure 7 describes the secondary structure of the protein predicted through SOPMA program. The results showed that 31.9% of amino acids are present in alpha helix region, 23.56% in extended strands, 12.36% in beta turn and 35.18% present in random coil, respectively.

The 3D protein structure was predicted by homology modeling using Swiss model workspace (Figure 8).The theoretical isoelectric point (pI) and molecular weight of the protein were determined through ExPasy tool and found to be 6.12 and 147.4KDa, respectively.

The antigenicity of the ASNase II from C. freundii 1101 was predicted using semi-empirical method and the obtained score was 1.028. It is comparable with the antigenicity of ASNase II from E. coli K12 (1.0275) and partially with the antigenicity of ASNase II from Erwinia chrysanthemi (1.0385).

BLAST analysis showed 98% similarity with C. koseri FDAARGOS_86 and 91-94% similarity with other strains of C. freundii 1101 in nucleotide sequence; and 99% similarity with most of Citrobacters in amino acid sequences. The sequence was submitted to Gen Bank as Citrobacter freundii1101under the accession number EU624347.

Fig 7: Secondary structure of ASNase II monomer from C. freundii 1101 using SOPMA tool: h-alpha helix, e-extended strand, t-beta turn, c-random coil

Fig 8: 3-D structure of ASNase II monomer designed using Swiss Model workspace

Conclusion

In the present study, ASNase II from C. freundii 1101 was cloned, sequenced and expressed in E.coli host. The results confirmed the potential of the enzyme to develop into a good anticancer drug. In the next study we are going to optimize C. freundii 1101ansB gene expression in host E.coli and also assay its effect on Leukemia cell line. Apart from the clinical use, this recombinant enzyme can be used in fried starchy food preparations to reduce the acrylamide content, a potent carcinogen formed during the baking process by the reaction of asparagine and sugar at high temperature. So it is beneficial to exploit potential ASNase II from C. freundii 1101 and construct suitable recombinants to over produce the enzyme to meet the ever increasing clinical and industrial demands**.**

Acknowledgments

This work was supported by Razi Vaccine and Serum Research Institute (Iran). Thanks to the members of the Biotechnology Department of Razi Vaccine and Serum Research Institute for their favors in improving this study.

References

1. Pasut G, Sergi M, Veronese FM. Anti-Cancer PEG-Enzymes: 30 Years Old, but Still a Current Approach. Adv Drug Deliv Rev. 2008; 60(1):69-78.

2. Narta UK, Kanwar SS, Azmi W. Pharmacological and Clinical Evaluation of L-Asparaginase in the Treatment of Leukemia. Cri Rev Oncol Hematol. 2007; 61(3):208-21.
3. Verma N, Kumar K, Kaur G, Anand S. Escherichia coli K-12 asparaginase based asparagine biosensor for leukemia. Artif Cells Blood Substit Immobil Biotechnol. 2007; 35(4):449-56.
4. Geckil H, Gencer S. Production of L-Asparaginase in Enterobacteraerogenes Expressing Vitreoscilla Hemoglobin for Efficient Oxygen Uptake. Appl Microbiol Biotechnol. 2004; 63(6):691-7.
5. Deokar VD, Vetal MD, Rodrigues L. Production of intracellular L-asparaginase from Erwiniacarotovoraand its statistical optimization using response surface methodology (RSM). Int J Chem Sci and Applications. 2010; 1(1):25-36.
6. Pedreschi F, Kaack K, Granby K. The effect of asparaginase on acrylamide formation in French fries. Food Chem. 2008; 109(2):386-92.
7. Ros´en J, Hellen¨as KE. Analysis of acrylamide in cooked foods by liquid chromatography tandem mass spectrometry. Analyst. 2002; 127(7):880-2.
8. Taeymans D, Anderson A, Ashby P, Blank I, Gonde P, van Eijck P *et al.* Acrylamide: update on selected research activities conducted by the European food and drink industry. J AOAC Int. 2005; 88(1):234-41.
9. Dhanam JG, Kannan S. L-asparaginase- Types, Perspectives and Applications. Adv Bio Tech. 2013; 13(5):01-05.
10. Tareke E, Rydberg P, Karlsson P, Eriksson P, Ornqvist MT. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. J Agric Food Chem. 2002; 50(17):4998-5006.
11. Pieters R, Hunger SP, Boos J, Rizzari C, Silverman L, Baruchel A *et al.* L-Asparaginase treatment in acute lymphoblastic leukemia: a focus on Erwinia asparaginase. Cancer. 2011; 117(2):238-49.
12. Rizzari C, Conter V, Starý J, Colombini A, Moericke A, Schrappe M. Optimizing asparaginase therapy for acute lymphoblastic leukemia. Curr Opin Oncol. 2013; 25(Suppl-1):S1-9.
13. Truelove E, Fielding AK, Hunt BJ. The coagulopathy and thrombotic risk associated with L-Asparaginase treatment in adults with acute lymphoblastic leukaemia. Leukemia. 2013; 27(3):553-9.
14. Sambrook, Fritsch, Maniatis, Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press. 1989.
15. Bahreini E, Aghaiypour K, Abbasalipourkabir R, Goodarzi MT, Saidijam M, Safavieh SS. An optimized protocol for overproduction of recombinant protein expression in Escherichia coli. Prep Biochem Biotechnol. 2014; 44(5):510-28.
16. Kolaskar AS, Prasad CT. A semiempirical method for the prediction of antigenic determinants on protein antigens. FEBS Lett. 1990; 276(1):172-4.
17. Imada A, Igarasi S, Nakahama K, Isono M. Asparaginase and Glutaminase Activities of Micro-Organisms. J Gen Microbiol. 1973; 76(1):85-99.
18. Swain AL, Jasko´ lski M, Housset D, Rao JK, Wlodawer A. Crystal Structure of Escherichia coli L-Asparaginase, an Enzyme Used in Cancer Therapy. Proc Natl Acad Sci USA. 1993; 90(4):1474-8.
19. Michalska K, Jaskolski M. Structural Aspects of L-Asparaginases, Their Friends and Relations. Acta Biochim Pol. 2006; 53(4):627-40.
20. Mezentsev YV, Molnar AA, Sokolov NN. Specificity of Molecular Recognition in Oligomerization of Bacterial L_Asparaginases. Biochemistry (Moscow) Suppl Ser B. 2012; 5(2):124-34.
21. Pieters R, Hunger SP, Boos J, Rizzari C, Silverman L, Baruchel A *et al.* L-Asparaginase treatment in acute lymphoblastic leukemia: a focus on Erwiniaasparaginase. Cancer. 2011; 117(2):238-49.
22. Tong WH, Pieters R, Kaspers GJL, Maroeska D, Bierings MB, van den Bos C *et al.* A prospective study on drug monitoring of PEG-asparaginase and Erwinia asparaginase and asparaginase antibodies in pediatric acute lymphoblastic leukemia. Blood. 2014; 123(13):2026-33.
23. Asselin B. Immunology of infusion reactions in the treatment of patients with acute lymphoblastic leukemia. Future Oncol. 2016; 12(13):1609-21.