

Original Research Paper

Intracellular L-Asparaginase from *Bacillus* sp. PG02: Purification, Biochemical Characterization and Evaluation of Optimum pH and Temperature

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Abstract: Bacterial L-asparaginases are amidohydrolases that act on L-asparagine and produce L-aspartate and ammonia. These enzymes have been used in treatment of lymphoblastic leukemia. In the present study, a novel strain, *Bacillus* sp. PG02 was explored for the production of intracellular L-asparaginase enzyme. The nitrogen source for L-asparaginase production was L-asparagine. New intracellular L-asparaginase was purified using ion exchange chromatography and the purity was assessed using SDS-PAGE. Kinetic parameters k_m and V_{max} and thermal properties were studied using L-asparagine as the substrate. SDS-PAGE analysis showed apparent molecular weight of approximately 38 kDa. The enzyme was active in a wide pH ranges (5-10) and it was maximally active at pH 7.5. *Bacillus* PG02 L-asparaginase was optimally active at 40°C. Thermal inactivation studies exhibited $t_{1/2}$ of 32.5 min in 37°C. Also T_{50} and ΔG of inactivation were measured. The results revealed that the enzyme had appropriate characteristics and thus could be a potential candidate for medical and basic investigations.

Keywords: L-Asparaginase, *Bacillus* PG02, Thermal Stability, Kinetics

Introduction

L-asparaginase (EC.3.5.1.1; L-asparagine amidohydrolase) acts on L-asparagine as substrate and produces aspartate and ammonia. This enzyme is usually used for the treatment of acute lymphoblastic leukemia and non-Hodgkin lymphomas (Muller and Boos, 1998). The L-asparaginase anti-leukemic effect arises from the complete elimination of the circulating L-asparagine. Since most cancer cells are unable to produce asparagine, they are dependent on an exogenous source of this amino acid for survival (Miller *et al.*, 1969). Normal cells are able to synthesize l-asparagine, therefore less affects are seen in these cells upon treatment by L-asparaginase enzyme. Protein synthesis would be impaired in tumor cells because of asparagine deficiency and this would be followed by inhibition in DNA and RNA synthesis (Verma *et al.*, 2007). Hence tumor cell functions impairs, which results in cell death (Gentili *et al.*, 1996). In fried and oven-cooked foods such as potato chips, formation of acrylamide is

probable. L-asparaginase enzyme can be used to reduce this reaction. Since, acrylamide formation in heated foods is mainly due to the reaction of free asparagine and reducing sugars, deamination of asparagine prevents acrylamide formation (Oza *et al.*, 2011; Friedman, 2003). L-asparaginase is widely distributed among microorganisms, plants and animals (Mishalska *et al.*, 2006; El-Bessoumy *et al.*, 2004) but it has been proved that microorganisms are better source for producing L-asparaginase due to their economic production, ease of optimization and purification and large-scale production (Ferrara *et al.*, 2006; Geckil *et al.*, 2004; Kumar *et al.*, 2011a). So far different aspects of the L-asparaginase activity like thermal stability and their optimum pH and temperature have been investigated but most of them did not show an appropriate condition for use as therapeutic agent. For instance, purification and characterization of L-asparaginase from *Cladosporium* sp. was reported. The enzyme was optimally active at pH 6.3 the optimum temperature of the enzyme activity was 30°C (Mohan Kumar and Manonmani, 2013). L-asparaginase

produced by *Aspergillusterreus* and *Bacillus licheniformis* RAM-8 were both optimally active at pH 9.0 and 40°C (Mahajan *et al.*, 2014; Loureiro *et al.*, 2012) and the enzyme from *Pectobacterium carotovorum* MTCC showed optimum pH and temperature of 8.5 and 40°C (Kumar *et al.*, 2011b). Although there are different reports about L-asparaginase producing microorganisms, but the enzyme from *Erwinia chrysanthemi* and *Escherichia coli* are clinically used (Mashburn and Wriston, 1964; North *et al.*, 1969). Unfortunately, some evidences of toxicity are shown by patients. Allergic responses happen as side effects and the use of L-asparaginases from other organisms can reduce this effect (Narta *et al.*, 2007). Therefore, novel sources of the enzymes could be beneficial, providing an alternative anti-tumor agent with fewer side effects. In this study purification and characterization of intracellular L-asparaginase was performed. This enzyme is produced by a novel *Bacillus PG02* (NCBI accession number: KF150761) isolated from Persian Gulf (Qeshmi *et al.*, 2014). We have studied kinetic and thermal parameters including $t_{1/2}$, T_{50} and ΔG^* of the enzyme. Also optimum pH and temperature of the enzymatic activity have been evaluated.

Materials and Methods

Chemicals

L-asparagine, Trichloroacetic acid (TCA), Sodium Dodecyl Sulfate (SDS), Nessler reagent chemicals (HgI₂, KI and sodium hydroxide) and Tris were purchased from Sigma (St. Louis, MO, USA). DEAE Cellulose was obtained from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

Bacterial Strain, Growth Media and Culture Conditions

The Bacterial strain used in this study was *Bacillus PG02* (Accession number: KF150761). This strain was previously isolated from coastal location in north of Persian Gulf (Hormozgan province), Iran and identified based on nucleotide sequence of *16S rRNA* gene (Qeshmi *et al.*, 2014). The strain was maintained on Nutrient agar media at 37°C for 24 h. The grown culture were stored at 4°C and subcultured monthly. L-asparaginase production by *Bacillus PG02* was carried out in M9 medium including: Maltose 20% as a sole carbon source; Na₂HPO₄ 6.0(g/L); KH₂PO₄ 3(g/L); NaCl 0.5(g/L); CaCl₂ 0.011(g/L); MgSO₄.7H₂O 0.12(g/L); pH 7.0(g/L). The inoculum was prepared by adding a loop full of 24 h old pure culture grown on slant into 10 ml of sterile medium in a 100 ml Erlenmeyer flask. The culture flask was incubated at 37°C and 180 rpm in an orbital shaking incubator for 10-12 h. Following growth until the midlogarithmic

phase (OD at 600 nm = 0.6-0.8), the culture was inoculated (2% v/v) into a flasks containing production medium (100 in 500 mL flasks). Incubation was carried out at 37°C at 180 rpm for 24 h (Qeshmi *et al.*, 2014).

Cell Disruption by Ultra-Sonication

The ultra-sonication was used to prepare the intracellular L-asparaginase extract. After 24 h inoculation, cells were harvested by centrifugation at 8000×g (4°C) for 10 min. Cells were washed once with 50 Mm Tris buffer (pH 7.5) and suspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), PMSF (1mM final concentration) and 5% glycerol. The cells were cooled on ice and were ultra-sonicated at 20 MHz, 40% amplitude, thirty times 10 sec ultrasonic pulses at 15 sec intervals (Ultrasonic processors 750W, Sigma-Aldrich, Germany). The lysate was centrifuged at 15,000×g for 10 min (4°C). The clear supernatant was used as the enzyme source.

Ammonium Sulfate Precipitation

All precipitation steps were performed at 0-4°C. After ultra-sonication, finely powdered ammonium sulfate was added to the clear supernatant with constant stirring and incubated overnight. Maximum L-asparaginase precipitation observed at 70-80% saturation. Centrifugation was performed (10,000×g) for 30 min to collect the precipitate. The precipitate was then dissolved in a minimal amount of 50 mM Tris-HCl buffer (pH 9) and dialyzed against the same buffer for 24 h.

DEAE Cellulose Chromatography and Molecular Weight Determination

All purification steps were performed at 0-4°C. OD 280 nm was measured for all chromatographic eluted samples to monitor protein.

About 50 mM Tris-HCl (pH 9) used as equilibrium buffer and the sample was loaded on equilibrated diethylaminoethyl (DEAE) cellulose column (4×60 cm). The column was washed with two column volume of the equilibrium buffer and the protein was eluted using a gradient of NaCl (50-1000mM) in 50 mM Tris-HCl (pH 9). The active fractions were collected, dialyzed in Tris-HCl (50 mM and pH 7.5) and concentrated in 4°C using centrifugal concentrator (Vivaspin 15, cut-off 5 kDa) according to the manufacturer. About 10 mL active fractions were added to the concentrator and centrifugation was performed until the final volume of 500 µL was reached. This preparation was used in the subsequent steps. SDS-PAGE was performed using 10% separating acrylamide gel (pH 8.8) and a 4% stacking gel (pH 6.8). Separated proteins in the gel were stained with Coomassie Brilliant Blue R-250. Standard molecular weight markers were used to determine approximate molecular weight of L-asparaginase (Laemmli, 1970).

Enzyme Activity, Protein Concentration and Enzyme Kinetics

L-asparaginase activity was measured by the known method of (Imada *et al.*, 1973). The purified enzymes fractions were mixed with 40 mM L-asparagine dissolved in 50 mM Tris-HCl buffer, pH 7.5. Enzymatic reaction was measured after incubation for 15 min at 37°C. 250 μ L of 1.5 M Trichloro acetic acid (TCA) was added to stop the reaction and the reaction mixture was centrifuged at 10,000 \times g for 5 min at 4°C before the addition of Nessler's reagent to measure the released ammonia after L-asparagine hydrolysis. Absorbance was measured at 450 nm (spectrophotometer UV/Vis Unico, USA). One unit of L-asparaginase activity was defined as the amount of enzyme that released 1 μ mol of ammonia (with 10 μ M-10mM ammonium sulfate as the standard) per minute under the assay conditions specified. Protein concentration was also measured using bovine serum albumin as the standard (Bradford, 1976). In order to determine kinetic constants for L-asparaginase, measurements were carried out over different substrate concentration including 10, 20, 30, 40 and 80 mM and the final concentration of the enzyme was 0.2 mg mL⁻¹. Different blanks were used for each L-asparagine concentration. Kinetic parameters, K_m and V_{max} for L-asparaginase enzymes were determined from a series of initial rates. Experimental data were analyzed graphically and numerically using the Lineweaver-Burk equation.

Effects of pH and Temperature on L-asparaginase Activity

The pH profile was determined at room temperature in various pH using 0.5 mg mL⁻¹ of enzyme solution. The buffers used in this study were potassium phosphate (50 mM, pH 5-7.5) and Tri-HCl (50 mM, pH 8.0-10). The activity of purified enzyme was determined at several temperatures (from 25 to 50°C) in 50 mM Tris-HCl buffer, pH 7.5.

Thermal Stability Assay

Half-lives were determined at two temperatures (37, 40°C). For half-life measurement, 0.5 mg mL⁻¹ enzyme solution in 50 mM Tris-HCl buffer (pH 7.5) was used. 10 μ L aliquots of the enzyme solution were prepared and incubated for various time intervals. Then each sample was chilled on ice for 60 min and diluted into 20 μ L of an assay solution (L-asparagine 40mM) for the measurement of residual activity at 37°C for 15 min. Plots of the log of residual activity versus time were drawn to determine the order of the inactivation reaction. The plots were linear indicating first-order decay. Another parameter which gives information about thermal stability was T_{50} which is the temperature of

incubation at which 50% of the initial enzyme activity is lost during 30 min incubation (Rahimzadeh *et al.*, 2012). Temperatures from 30 to 50°C were used for T_{50} measurements. The ΔG^* parameter of the L-asparaginase enzyme was determined as follows (Rahimzadeh *et al.*, 2012):

$$\Delta G^* = RT \ln(kBT/h) - RT \ln k$$

Results and Discussion

L-asparaginase Purification and Molecular Weight Determination

Most of the microbial L-asparaginases are intracellular enzymes in nature. Intracellular L-asparaginase was harvested from *Bacillus* PG02 by sonication and bacterial cell disruption. The purification steps of L-asparaginase are given in Table 1. After each step of purification, the specific activity of the enzyme increased. Specific activity of the purified enzyme after ion exchange step was 17.1 U/mg (Table 1). In DEAE-Cellulose anion-exchange chromatography step, total proteins of the eluted fractions were monitored at 280 nm and L-asparaginase activity was measured by nesslerization. Figure 1a shows the eluted fractions that had L-asparaginase activity. Samples were pooled, dialyzed and concentrated by ultrafiltration. After purification procedure, SDS-PAGE was performed for the fractions with the enzyme activity. The estimated molecular weight of the purified enzyme was 38 kDa. Figure 1b. However, exact estimation of the L-asparaginase enzymes needs to be evaluated by native page, because most of these enzymes have oligomer structures.

Effect of pH and Temperature on L-Asparaginase Activity

pH and temperature of enzyme incubation with the substrate is one of the important factors which influences the enzymatic reaction rates. The optimum pH and temperature of the enzyme is usually beyond which the reduction in activity is observed. Here, activities of the enzyme were determined in a pH range from 5 to 10. The results showed that *Bacillus* PG02 L-asparaginase was active over broad pH ranges from 5 to 10. The enzyme activity gradually increased until pH 7.5 at which the maximum activity was observed (Fig. 2a). This result showed that the optimum pH of the enzyme activity was near to that of the growth condition. There are very few reports in which the L-asparaginase enzymes showed optimum pH near physiologic pH range. Alkaline pH optima (8.0-9.0) were shown by the majority of L-asparaginases from *Erwinia* sp. whereas the enzyme from *E. coli* exhibited an acidic pH optimum

of 5.0-6.0 (Capizzi *et al.*, 1971; Whelan and Wriston, 1969). Currently, L-asparaginase enzyme produced by *Erwinia carotouora* is used for acute lymphoblastic leukemia treatment (Gentili *et al.*, 1996; North *et al.*, 1969). However, Kamble *et al.* (2006) found that the optimum pH of the enzyme from *E.carotouora* was 8.6. Our results revealed that the L-asparaginase produced by *Bacillus* PG02 was maximally active at pH 7.5 which is near to the physiologic pH. Since the physiological optimum pH is one of the main requirements for antitumor activity (Mannan *et al.*, 1995), hence this desirable characteristic makes the enzyme suitable and attractive for both the structure-function research studies and the industrial and pharmacological processes.

Similarly, the optimum temperature of the activity of L-asparaginase was studied in 20 to 50°C at 5°C intervals. Maximum activity was obtained at 40°C (Fig. 2b). However the enzyme was active at a wide range of temperatures from 30 to 45°C and was nearly 90% active in 37°C. This property makes the enzyme more suitable for complete elimination of asparagine in patients suffering from leukemia. Mannan *et al.* (1995) found 37°C to be the optimum temperature for the enzyme activity. Mesas *et al.* (1990) reported 40°C as the optimum temperature of the L-asparaginase from *Corynebacterium glutamicum*. Also Jia *et al.* (2013) reported L-asparaginase enzyme produced from *Bacillus subtilis* B11-06 with similar optimum pH and temperature.

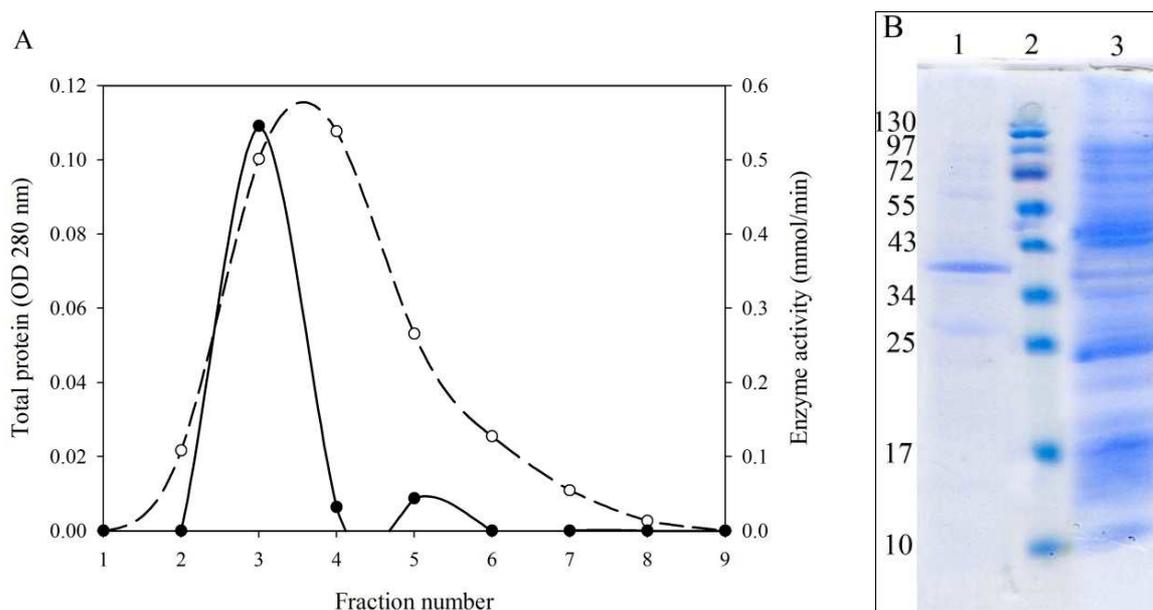


Fig. 1. Ion exchange chromatography and SDS-PAGE of L-asparaginase. The crude enzyme extract was chromatographed on DEAE-cellulose. Total protein was monitored at 280 nm, (A) Dashed chromatogram represents OD 280 nm, Solid chromatogram represents enzyme activity, (B) SDS-PAGE of the purified L-asparaginase. *Bacillus* PG02 L-asparaginase after purification (lane 1), Molecular weight marker (lane 2), L-asparaginase crude enzyme extract (lane 3)

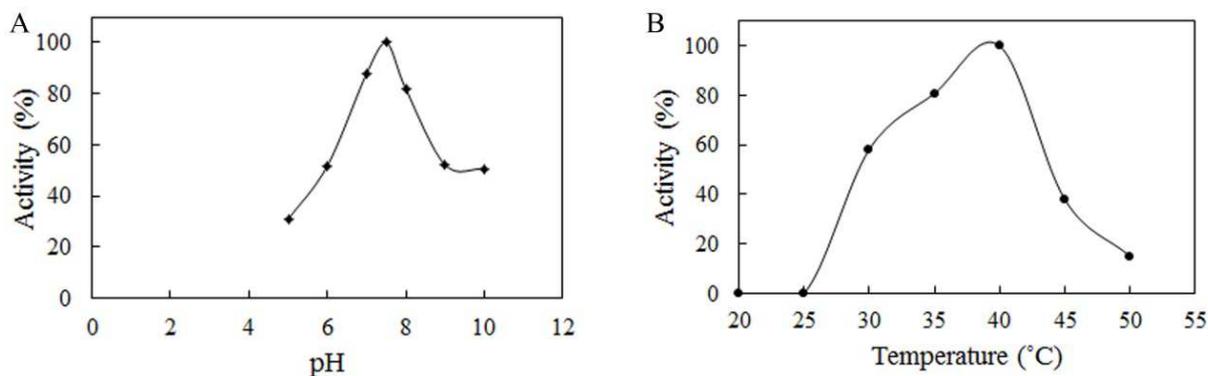


Fig. 2. (A) Effect of pH and (B) temperature on activity of *Bacillus* PG02 L-asparaginase. Optimum pH was determined by assaying the enzyme activity at various pHs (5 to 10 using 50 mM potassium phosphate and 50 mM Tris-HCl at room temperature. Optimum temperature was determined at 25 to 50°C in 50 mM Tris-HCl buffer, pH 7.5

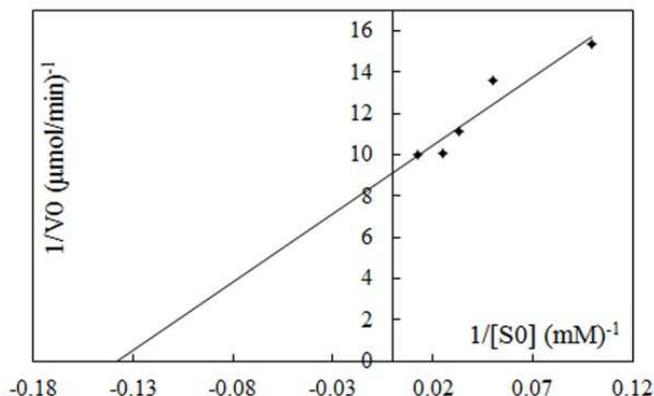


Fig. 3. Lineweaver-Burk plot of L-asparaginase activity. Five different concentrations of L-asparagine were used

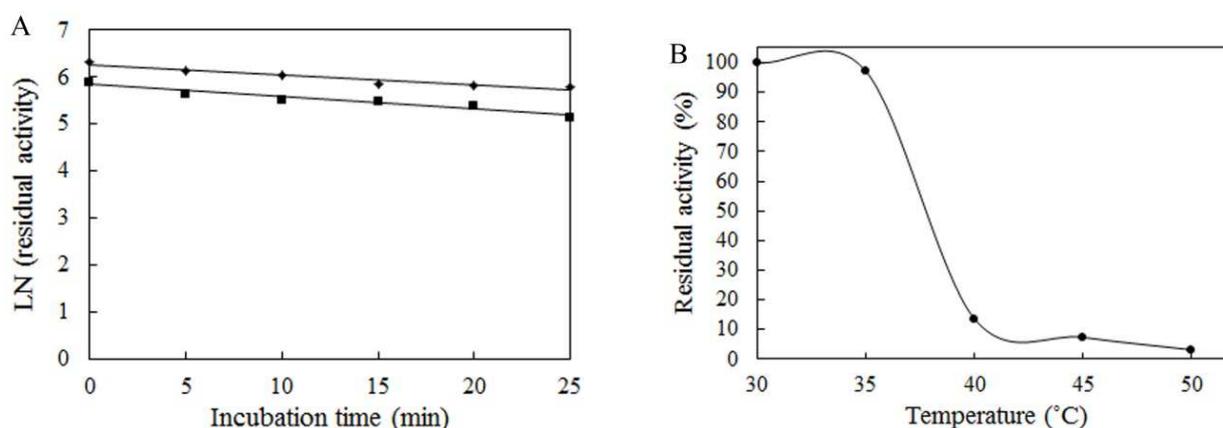


Fig. 4. Thermal stability studies of the L-asparaginase from *Bacillus PG02*, (A) Half-life curves at 37°C (closed diamonds) and 40°C (closed squares) using 0.5 mg mL⁻¹ enzyme solution in 50 mM Tris-HCl buffer (pH 7.5), (B) T50 curve of the L-asparaginase after enzyme incubation at different temperatures (30 to 50°C) using in 50 mM Tris-HCl buffer (pH 7.5)

Table 1. Purification of L-asparaginase from *Bacillus PG02*

Steps	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Fold purification	Yield (%)
Crude extract	91.3	50.3	1.8	1.0	100.0
(NH ₄) ₂ SO ₄ precipitation	74.2	34.2	2.2	1.2	81.3
DEAE cellulose column	41.1	2.4	17.1	9.4	45.0

Table 2. Kinetic constants and the thermostability parameters of the enzyme

Enzyme	K_m^a (mM)	V_{max}^a (µmol/min)	pH^b	T_{opt}^c (°C)	T_{50}^c (°C)	$t_{1/2}^d$ 37°C (min)	$t_{1/2}^d$ 40°C (min)	ΔG^{*d} (kcal/mol)
Bacillus PG02	7.2	0.11	7.5	40	39	32.5	26.5	4.97

Values are the averages of three experiments,

a Standard errors are less than ±0.07.

b The error margin amounts to approximately 0.5.

c The standard errors are less than ±1.

d Standard errors are less than ±0.5.

Kinetic Properties of the Enzyme

L-asparagine was used as the substrate for determining activity of the L-asparaginase enzyme. The enzyme revealed Michaelis-Menten kinetics. Based on

the Line weaver-Burk analysis K_m and V_{max} values for L-asparagine was 7.2 mM and 0.11 µmol/min, respectively (Table 2 and Fig. 3). Different substrate affinities have been reported for L-asparaginases from different microorganisms and therefore these enzymes probably

played different physiological roles. Higher and lower K_m values have been reported for different L-asparaginase enzymes (El-Bessoumy *et al.*, 2004).

Thermal Stability Properties

In order to measure the heat inactivation rate constant of L-asparaginase, the enzyme aliquots were incubated at desirable temperatures and then were cooled on ice before measurement of the residual activity (Declerck *et al.*, 1997; Kumar *et al.*, 2011a). After incubation at 37 and 40°C, the half-lives ($t_{1/2}$) of *Bacillus* PG02 L-asparaginase were measured (Table 2). k_i (inactivation rate constant) values were estimated from the plots of Log of residual activity versus incubation time. As is shown in Fig. 4a, inactivation process was a first order decay. Although the optimum temperature of the enzyme activity was 40°C, but the enzyme showed higher half-life in 37°C compared to 40°C. *Bacillus* PG02 L-asparaginase half-life was showed to be 32.5 min in 37°C. In 40°C still the enzyme was active, although the reduction in half-life of the enzymes was clearly observed compared to 37°C. This means that although the optimum activity of the enzyme occurs in 40°C, but the enzyme retains active for longer time in 37°C. Since the normal body temperature is near 37°C, this could be an appropriate characteristic of *Bacillus* PG04 L-asparaginase enzymes for future uses. In naturally occurring enzymes the highest stability could be seen at temperatures near that of growth of an organism. This results from the relationship between conformational stability and enzyme activity (Kumar *et al.*, 2011b; Daniel, 1996). Together these results showed that the purified enzyme could be regarded as an interesting target for thermal stability improvement investigations. Another criterion of thermal stability is T_{50} . The results of the T_{50} measurements showed that the enzyme retained 50% of its activity after 30 min incubation in 39°C (Fig. 4b and Table 2). This is consistent with the results of half-life studies. Free energy changes of inactivation (ΔG^*) was evaluated using the inactivation rate constant at 37°C. ΔG^* value of inactivation indicates the distance between the native state and the transition state and is a benchmark of the required input energy for enzyme inactivation (Table 2).

Conclusion

Bacillus PG02 L-asparaginase had several beneficial properties required for a therapeutic and industrial enzyme. The enzyme had a broad temperature range of activity with maximum activity at 40°C and at physiologic pH. These features of the enzyme were very important for the future therapeutic uses. Kinetic

properties of the enzyme showed that the enzyme had approximately appropriate affinity for L-asparagine.

On the other hand, thermal stability measurements showed that the enzyme could be a potential target for thermal stability improvement studies. Due to these characteristics, in our future studies, further enzymatic studies including glutaminase activity, structural analysis and determination of encoded gene sequence of this L-asparaginase will be carried out in order to understand the mechanism of its activity and to improve kinetic and stability properties of this therapeutic target by protein engineering methods. Although the enzyme from *Bacillus subtilis* B11-06 showed similar molecular weight and optimum pH and temperature (30), but thermal characteristics and kinetic properties (K_m) of that enzyme was different from the enzyme produced by *Bacillus* PG02. Anyway, N-terminal sequencing of the enzyme could be beneficial to more clarify the differences of this enzyme with other similar enzymes.

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Author's Contributions

Mahsa Rahimzadeh: Developed the idea and wrote the manuscript.

Fatemeh Izadpanah Qeshmi and Manijeh Poodat: Performed the experiments.

Sedigheh Javadpour: Read and improved the manuscript and advised in experimental methods.

Conflict of Interest

The authors declare that they had no conflict of interest.

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