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THERMOKINETIC STUDIES OF ANTI-LEUKEMIC L-ASPARAGINASE OF *PSEUDOMONAS FLUORESCENS*

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Abstract

L-Asparaginase (ASNase) is a well-known chemotherapeutic drug which has been effectively used in the treatment of acute lymphocytic leukaemia, a childhood cancer from several decades. L-asparagine is an essential amino acid for the growth of tumor cells, whereas the growth of normal cells is independent of its requirement. ASNase with high asparaginase activity and negligible glutaminase activity are reported to be less troublesome during the course of anti-tumor therapy. Hence there is a dire need to explore new sources of ASNase with different immunological characteristics which lead to fewer side effects. The thermodynamic parameters also have its importance in understanding the probable denaturation mechanism, which is very important in enzymatic processes. Therefore, in the present report, ASNase from *Pseudomonas fluorescens* were produced and purified and its thermostability as well as thermodynamic parameters, *i.e.* enthalpy, entropy and Gibbs free energy, of inactivation was determined. The determination of thermal stability of the enzyme at different temperatures, its deactivation studies as well as its thermodynamic parameter was investigated. The enzyme showed highest stability at room temperature (RT) as compared to higher temperatures with a half life of 46 hrs which is quite significant. Its deactivation energy was found to be 50.54 kJ/mol. The value of thermodynamic parameters including enthalpy (ΔH), entropy (ΔS) and Gibbs free energy of inactivation (ΔG) was found to be -47.98kJ/mol, 0.078kJ/mol.K and 71.22-72.78 kJ/mol respectively. ASNase from *Pseudomonas fluorescens* exhibited quite higher thermostabilities thus making it suitable for both chemotherapeutic and food processes industries with a better understanding of its structure-function relationship.

Keywords: L-Asparaginase, *Pseudomonas fluorescens*, thermodynamic, half life, thermostabilities, thermokinetic

Introduction

L-Asparaginase (ASNase) is a well-known chemotherapeutic drug which has been effectively used in the treatment of acute lymphocytic leukaemia, a childhood cancer from several decades. ASNase inhibits protein synthesis in T-cells by catalyzing the conversion of ASNase to L-aspartate and ammonia, and this catalytic reaction is essentially irreversible under physiological conditions (Jha *et al.*, 2012) and also to a lesser extent, the hydrolysis of L-glutamine to L-glutamate (Ebrahiminezhad *et al.*, 2011). Its anti-tumour properties were first revealed in the guinea pig serum which leads to regression of the Gardner lymphosarcoma in mice (Kidd, 1953b; Broome, 1961). Subsequently a potent source of ASNase was found in *Escherichia coli* (Mashburn and Wriston, 1964). Since then interest in ASNase arose. L-asparagine is an essential amino acid for the growth of tumor cells, whereas the growth of normal cells is independent of its requirement. Most normal tissues synthesize L-asparagine in amounts sufficient for their metabolic needs with their own enzyme, L-asparagine synthetase, but the malignant cells requires an external source of L-asparagine for growth and multiplication. In the presence of ASNase, the tumour cells get deprived of an important growth factor and hence, cannot survive. This fact suggested the development of this enzyme as a potent anti-tumor or anti-leukemic drug (Savitri *et al.*, 2002). This enzyme is usually considered to be cell cycle phase nonspecific and also reported to arrest the cell cycle in the G1 phase in the murine and human T- lymphoblastoid cell lines (Allison *et al.*, 1972; Shimizu *et al.*, 1992; Ueno *et al.*, 1997) and lead to apoptosis. Recent advances in food technology have demonstrated the potential application of ASNase in the prevention of acrylamide formation in fried potatoes and similar food products (Kuilman and Wilms, 2007;

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Pedreschi *et al.*, 2008). In this regard, ASNase from *Aspergillus oryzae* and *A. niger* are used in baking industries (Morales *et al.*, 2008). These enzymes work optimally at temperature 40-60^{0}C and pH 6.0-7.0 (Sinha *et al.*, 2013).

However, ASNase showed a distinct toxicity profile, ranging from acute hypersensitivity (immunological sensitization) and hyperglycaemia to hepatocellular dysfunction and pancreatitis (inhibition of protein synthesis) (Oettgen *et al.*, 1970). The toxicity is partially attributable to the glutaminase activity of these enzymes (Oza *et al.*, 2011). ASNase with high asparaginase activity and negligible glutaminase activity are reported to be less troublesome during the course of anti-tumor therapy (Hawkins *et al.*, 2004). Hence there is a dire need to explore new sources of ASNase with different immunological characteristics which lead to fewer side effects.

One of the important properties of an enzyme is its stability as it can provide information on the structure of the enzyme and facilitate an economical production design. ASNase deactivation plays a vital role in cancer therapy (Narta *et al.*, 2007). Rapid inactivation may constrain the efficiency of the therapeutic process. An improved knowledge of enzyme deactivation kinetics is needed to enhance the feasibility of therapeutic use. Since enzymes have highly defined structures, deactivation mechanisms can be complex. Even the slightest deviation from their native form can affect their specific activity. Hence a good knowledge about the enzyme stability is required to help optimize the profitability of enzymatic processes. The thermodynamic parameters also have its importance in understanding the probable denaturation mechanism, which is very important in enzymatic processes (Ustok *et al.*, 2010). There are very few papers in literature describing the thermokinetic and thermodynamic characterization of the enzyme ASNase. Therefore, in the present report, ASNase from *Pseudomonas fluorescens* were produced and purified and its thermostability as well as thermodynamic parameters was determined.

Materials and method

Microorganism and culture conditions:

The bacterial isolates of *Pseudomonas fluorescens* (NCIM 2100) was obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The isolates were sub-cultured on Luria-Bertani agar (Himedia, Mumbai, India) slants and stored at 4^{0}C for further studies.

Production and purification of L-asparaginase:

Seed culture was prepared in 50 ml of Luria broth supplemented with 0.1% L-Asparagine. The broth was inoculated from freshly sub-cultured *Pseudomonas fluorescens* from LB agar slants (not more than 1 day old). The culture flask was incubated at 37^{0}C and 120 rpm for 24 hours (Sinha *et al.*, 2015). The batch production of ASNase was carried out by transferring 10 % (v/v) innoculum to the production media containing glucose, 1.5 g/L; K_2HPO_4, 1g/L; Yeast Extract, 7g/L; Tryptone, 7g/L and L-Asparagine, 0.5% (w/v) with pH 7 as optimized by Taguchi DOE method (Jha *et al.*, 2014). The fermentation process was carried out at 37^{0}C and 180 rpm agitation for a fermentation period of 48 hours. Then the media were transferred to several centrifuge tubes and centrifuged at 10,000 RPM for 10 minutes. After centrifugation, the pellet was discarded and supernatant was collected and treated as crude enzyme. The crude enzyme was subjected to 80% saturated ammonium sulphate precipitation according to Distasio *et al.*, (1976).

Assay for ASNase:

The enzyme activity was determined by the Nesslerization method as reported by Bergmeyer and Beutler, (1985) by measuring the ammonia released from the hydrolysis of L-asparagine using Nessler's reagent. One unit of enzyme releases one micromole of ammonia per minute at 37^{0}C and pH 8.6 under the specified conditions.

Thermo-stability and estimation of half-life and deactivation energy of the enzyme:

Thermal stability of the enzyme was studied by incubating the enzyme at 25^{0}C, 37^{0}C and 45^{0}C in the absence of substrate. Aliquots were withdrawn at periodic intervals and assay was performed as described above. The residual activity was expressed as percent of the initial activity.

From the slope of semi-natural logarithmic plot of the residual activity vs. time, the deactivation rate constants (K_d) were calculated and the half-lives were estimated using Eq. 1. The half-life ($t_{1/2}$) is defined as the time taken for the residual activity to reach 50 %.

$$t_{½} = \frac{-\ln(0.5)}{K_d} \qquad (1)$$

The analysis of temperature dependence of K_d was done using Arrhenius plot and the deactivation energy was estimated from the Arrhenius equation as follows (Eq. 2):

$$\ln K_d = \ln A - \frac{E_d}{R}\left(\frac{1}{T}\right) \qquad (2)$$

where E_d is the deactivation energy of the transition state of enzyme deactivation, A is a constant and R the universal gas constant. The value of the deactivation energy (E_d) was estimated from the slope of the plot *of ln* (K_d) against *1/T.*

Determination of thermodynamic parameters:

From the absolute reaction rate, Gibbs free energy of inactivation (ΔG^*), enthalpy (ΔH^*) and entropy (ΔS^*) of deactivation can be estimated. The temperature dependency of the deactivation rate constant can be expressed as (Eq. 3 and 4):

$$K_d = \frac{kT}{h} exp\left(\frac{\Delta S^*}{R}\right) exp\left(\frac{\Delta H^*}{R}\right) \quad (3)$$

$$\ln\left(\frac{K_d}{T}\right) = \ln\left(\frac{k}{h}\right) + \frac{\Delta S^*}{R} - \left(\frac{\Delta H^*}{R}\right)\frac{1}{T} \quad (4)$$

where R is the universal gas constant, T the absolute temperature, h the Planck's constant (6.6262·10–34 J/s) and k the Boltzmann constant (1.3806·10–23 J/(Mol·K)).

The values of ΔH^* and ΔS^* were calculated from the slope and intercept of the plot of *ln* (K_d/T) vs. *1/T*, respectively, and ΔG^* was estimated from the following relationship (Eq. 5):

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (5)$$

Results

Thermostability studies and estimation of half-life and deactivation energy of the enzyme:

Thermostability of ASNase was studied at temperatures 25^{0}C, 37^{0}C and 45^{0}C. It was found that the enzyme remained slightly more stable at 25^{0}C (fig.1).

The deactivation rate constants (K_d) of ASNase from *Pseudomonas fluorescens* for the temperature of 25^{0}C, 37^{0}C and 45^{0}C were calculated from the slope of the semi-natural logarithmic plot of residual activity vs. time (fig.2). At the same time, the half-life ($t_{1/2}$) values were

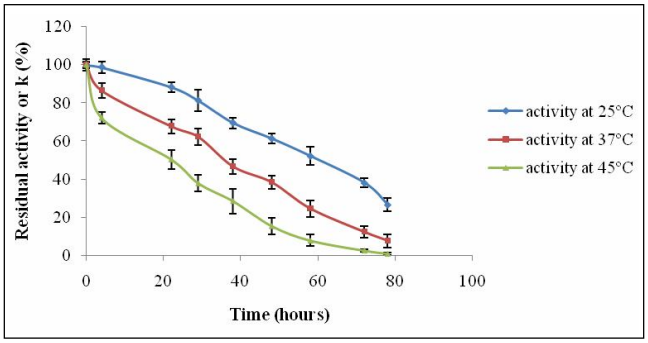

Fig. 1: Thermostability of L-asparaginase at 25^{0}C, 37^{0}C and 45^{0}C.

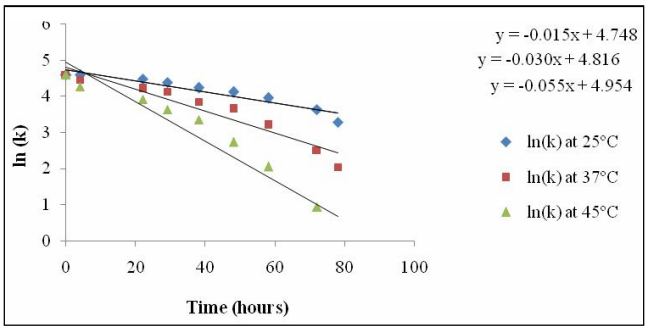

Fig. 2 : Semi-logarithmic plot of residual activity of the enzyme *vs.* time.

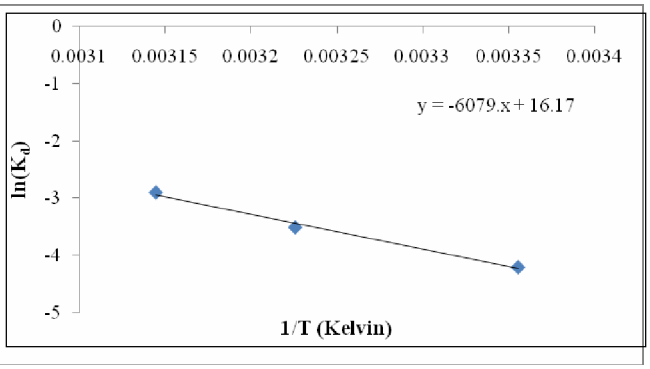

Fig. 3: Semi-logarithmic plot of K_d at 25^0, 37^{0}C and 45^{0}C* *vs. 1/T*.

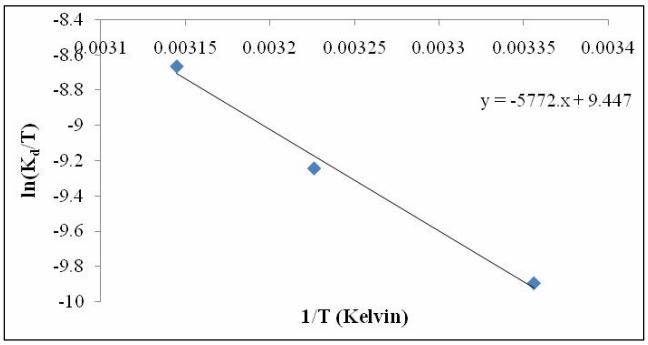

Fig. 4: *The unit of temperature was converted from ^{0}C to Kelvin); Semi-logarithmic plot of K_d/T vs. *1/T*.

calculated using the deactivation rate constants as given in eq-1. Hence the half-lives obtained for the enzyme at 25^{0}C, 37^{0}C and 45^{0}C were 46 hrs, 23 hrs and 13 hrs respectively.

The deactivation energy (E_d) determined from the slope of ln (K_d) against *1/T* (fig.3) was found to be 50.54 kJ/Mol.

Determination of thermodynamic parameters:

The thermodynamic parameters such as change in enthalpy (ΔH^*), entropy (ΔS^*) were determined from the slope of calculated from the slope and intercept of the plot of *ln* (K_d/T) vs. *1/T*, respectively, and Gibbs free energy of inactivation (ΔG^*) was estimated from Eq. 5 as mentioned in the Materials and Methods section. From

the fig.4, ΔH^*, ΔS^* and ΔG^* were determined to be -47.98 kJ/Mol, 0.078 kJ/Mol.K and -71.22-72.78 kJ/Mol respectively.

Discussion

Studies on thermostabilities of an enzyme are one of the important aspects regarding its structural and functional informations. It can also provide a good knowledge about the deactivation kinetics of the enzyme. The deactivation of the enzyme is a process in which secondary, tertiary or quaternary structure of a protein changes without breaking any covalent bonds. The thermostability studies revealed quite a higher stability of asparaginase at 25^0C when compared to other higher temperatures such as at 37^0C and 45^0C. This also leads to a higher half-life of 46 hrs at room temperature which implies that the enzyme is most stable at room temperature, thus making it suitable for long term storage at room temperature for experiments relating to acrylamide reduction in food products. Also, it's quite significant stability at 37^0C makes it more suitable for the treatment of cancer. However, this result is somewhat different to that reported by Kishore *et al.*, (2015) who reported the half life of cloned ASNase from *Pseudomonas fluorescens* to be 35 hrs at 28^0C and 45.5 hrs at 37^0C *i.e.* the enzyme showed more stability at higher temperature.

To develop an economic production design of L-asparaginase and enhance the feasibility of its therapeutic use, role of deactivation of the enzyme is needed to be explored. The deactivation energy (E_d) of 50.54 kJ/mol is quite lower as compared to the thermal deactivation energy of ASNase from *Pectobacterium carotovorum* MTCC 1428 at different pH values (Kumar *et al.*, 2011). Braga *et al.*, (2013) also reported quite higher deactivation energy of â-galactosidase enzyme in the range of 300-400 kJ/mol. Similarly, Pal *et al.*, (2013) reported the deactivation energy of almond β-galactosidase enzyme to be 124.53 kJ/mol.

An estimation of the thermodynamic parameters helps to understand the structure-stability relationships of their enzymes as well as the probable denaturation mechanism. The changes in enthalpy (ΔH^*) and entropy (ΔS^*) occurring during the thermal inactivation of ASNase were calculated using the transition state theory (Naidu and Panda, 2003). From fig.4, the larger activation enthalpy value of -47.98 kJ/mol was the characteristic for protein denaturation (Brown and Yada, 1991) and the smaller ΔS^* value which is close to zero, showed that thermal deactivation did not imply any relevant variation in the tertiary structure of the enzyme (Ustok *et al.*, 2010). The entropy of inactivation (ΔS^*) showed positive value, which indicates that there are no significant processes of aggregation, since had this happened, the values would have been negative (Marin *et al.*, 2003). The present studies coincide with that reported by Pal *et al.*, (2013) about almond β-galactosidase enzyme which showed the positive ΔS^* values at each temperature. Contradictory to the present report, there were earlier studies on xylanase purified from *Aspergillus niger* DFR-5 which undergoes significant processes of compaction/ aggregation with increase in the temperature (Pal and Khanum, 2010; Pal and Khanum, 2011; Kumar *et al.*, 2011). As the temperature increases the value of ΔG^* which are measures of the spontaneity of inactivation processes decreases and becomes more and more negative, which confirms that ASNase purified from *Pseudomonas fluorescens* has the potential to change the reaction from unfavorable condition to the favorable one.

Conclusion

L-Asparaginase enzyme from *Pseudomonas fluorescens* was purified and studied for its thermal stability at different temperatures including 25^0C, 37^0C and 45^0C as well as its thermodynamic parameters as it can provide information about the structure and function of the enzyme. It was observed that the enzyme showed the maximum stability at 25^0C as compared to the higher temperatures with a half-life of about 46 hrs. This could aid in the experiments relating to acrylamide reduction in food products and the enzyme could be stored for longer periods at room temperature. Moreover its quite significant half life at 37^0C can be helpful to be used against tumor cells. The values of change in enthalpy, entropy and Gibbs free energy of enzyme inactivation were found to be -47.98kJ/mol, 0.078kJ/mol. K and 71.22-72.78 kJ/mol respectively implying that there are no significant processes of aggregation as ΔS^* value was positive. Also the smaller value of ΔS^* indicated that thermal deactivation did not imply any relevant variation in the tertiary structure of the enzyme.

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