Purification, characterization and anticancer evaluation of L-methioninase from *Trichoderma harzianum*

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Abstract

The *Trichoderma harzianum* L-methioninase was purified 7.15-fold with a recovery of 47.9% and the specific activity of 74.4 U/mg of protein. The purified enzyme has an apparent molecular mass of 48 kDa on SDS-PAGE and exhibited maximum activity at pH 8 and 35 °C. The enzyme was catalytically stable below 50 °C and at a pH range of 6.0–8.5. The thermal inactivation of L-methioninase exhibited first-order kinetics with the k value between $5.71 \times 10^{-4} \text{ min}^{-1}$ and $1.83 \times 10^{-2} \text{ min}^{-1}$. The studies on thermodynamic parameters of L-methioninase indicated the compaction and aggregation of the enzyme molecule during denaturation. This is the first report of thermodynamic analysis of thermal inactivation in L-methioninase. The enzyme activity was enhanced by Li^+ and inhibited by Cu^{2+} , Co^{2+} , Fe^{2+} , Hydroxylamine and PMSF. The purified enzyme showed K_m , $V_{\max}$ and k_{cat} value of 1.19 mM, 21.27 U/mg/min and 16.11 s^{-1} , respectively. The L-methioninase inhibited the growth of human cell lines hepatocellular carcinoma (Hep-G2) and breast carcinoma (MCF-7) with IC_{50} values of 14.12 $\mu\text{g/ml}$ and 20.07 $\mu\text{g/ml}$, respectively. The in vivo antitumor activity of L-methioninase was evaluated against DAL cell lines bearing in Swiss albino mice. The enzyme effectively reduced tumor volume, packed cell volume, viable cell count and restored hematological parameters, serum enzyme and lipid profile to normal levels compared to DAL control mice. The present study has demonstrated the high efficacy of *Trichoderma harzianum* L-methioninase against cancer cell lines in vitro and in vivo conditions. The purified L-methioninase has significant thermal stability and better catalytic properties than the enzyme purified from other sources.

Keywords Antitumor · Cytotoxicity · Electrophoresis · Kinetic analysis · Sulforhodamine B assay · Thermodynamics

Introduction

L-methioninase is a potential therapeutic enzyme that catalyzes the degradation of L-methionine to methanethiol, α -ketobutyrate and ammonia. The enzyme requires pyridoxal-L-phosphate for γ elimination of L-methionine (Tanaka

et al. 1977), α , β elimination of L-cysteine and its analogs (Tanaka et al. 1985). Methionine is an essential amino acid in humans which performs a crucial role in mammalian metabolisms such as biosynthesis of protein, glutamine and polyamine (Wise and Thompson 2010; Halpern et al. 1974). The increased requirement of plasma methionine for protein synthesis and regulation of DNA expression was observed in several cancer cells (Cellarier et al. 2003). Under methionine deprivation, the cancer cells were arrested in the late S-G2 phase of cell cycle and undergo apoptosis (Guo et al. 1993). Restriction of cancer development using L-methioninase, for cancers that exhibit L-methionine dependence for their growth and proliferation, is a promising strategy for anticancer treatment (Cellarier et al. 2003).

L-Methioninase from bacterial origin has been purified and characterized from *Pseudomonas putida* (Ito et al. 1976), *Clostridium sporogenes* (Kries and Hession 1973), *Trichomonas vaginalis* (Lockwood and Coombs 1991), *Brevibacterium linens* BL2 (Dias and Weimer 1998) and

Accession number The rDNA sequence of *Trichoderma harzianum* was deposited to gene bank under accession number MH828332.1.

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Streptomyces variabilis (El Awady et al. 2017). In addition, the enzyme was purified from cultural filtrates of several fungi including *Aspergillus flavipes* (El-Sayed 2011), *Aspergillus ustus* (Abu-Tahon and Isaac 2016) and *Candida tropicalis* (Selim et al. 2015). L-methioninase is absent in mammals. Bacterial enzymes are reported to possess high immunogenicity, low substrate specificity, and hazardous effects on the kidney and liver (Sun et al. 2003). L-methioninase from fungal sources has fewer immunogenic properties and allergic reactions, with high substrate specificity, make it a potent source of enzyme for anticancer therapy.

The anticancer L-methioninase activity was extensively studied against numerous cancers cell lines, including breast, kidney, colon, lung, and prostate cell lines (Tan et al. 1998). *Aspergillus flavipes* L-methioninase displayed significant anticancer activity against prostate (PC3), liver (HEPG2) and breast (MCF7) cancers (El sayed et al. 2012). Recombinant L-methioninase was reported to possess broad selective efficacy towards various cancer cell lines in vitro conditions (Tan et al. 2010). Kawaguchi et al. (2018) reported the efficacy of Recombinant methioninase against a BRAF-V600E mutant melanoma patient-derived orthotopic xenograft (PDOX) nude mouse model. Sundar and Nellaiah (2013) reported that the treatment of Daltons Ascites Lymphoma cell lines-bearing mice with L-methioninase arrested the tumor growth and enhanced the lifespan of DAL-bearing mice.

Salim et al. (2019) isolated a highly active L-methioninase producing fungus from soil samples and was identified as *Trichoderma harzianum*. Response surface methodology and artificial neural network-linked genetic algorithm was employed to develop an optimized L-methioninase production medium. The production of enzyme was enhanced by 2.7-fold after optimization.

The present work describes the purification and characterization of L-methioninase from the fungus *Trichoderma harzianum* produced by submerged fermentation. Kinetic properties and thermodynamic parameters of purified enzyme were evaluated. The in vitro and in vivo anticancer properties of the enzyme were also investigated.

Materials and methods

Microorganism

The filamentous fungus used in this study was isolated from soil samples and was identified as *Trichoderma harzianum* (Accession no MH828332.1) by morphological and molecular methods (Salim et al. 2019). The culture was maintained on methionine glucose agar medium (Ruiz-Herrera and Starkey 1969).

Production of L-methioninase

L-methioninase production was carried out using liquid medium containing 13.9 g/l lactose, 11.37 g/l L-methionine, 1.58 g/l KH_2PO_4 , 3.98 g/l K_2HPO_4 , 0.5 g/l $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g/l $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.01 g/l of zinc chloride (Salim et al. 2019). The pH of the medium was adjusted to 7 with sodium hydroxide and the production was carried out for 5 days at 28 °C with constant shaking at 150 rpm. After incubation, mycelium was separated from the supernatant by centrifugation at 8000×g for 20 min at 4 °C and the crude supernatant was used as the source of enzyme.

Quantification of L-methioninase

The demethylating activity of L-Methioninase was quantified by 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) assay (Laakso and Nurmikko 1976). The reaction mixture was composed of 20 mM L-methionine in potassium phosphate buffer (pH 7.2), 0.1 mM pyridoxal-L-phosphate, and 0.25 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) in a final volume of 1.0 ml. The assay mixture was incubated for 30 min at 37 °C and the absorbance was measured at 420 nm. One unit (U) of L-methioninase activity is defined as the amount of enzyme that releases 1 μmol of free thiol per minute under optimal assay conditions.

The L-methioninase activity was also determined by estimating the α-ketobutyrate released from L-methionine degradation (Esaki and Soda 1987). One unit (U) of L-methioninase activity is defined as the amount of enzyme that released 1 μmol of α-ketobutyrate per minute at standard reaction conditions.

Purification of L-methioninase from *Trichoderma harzianum*

The culture supernatant containing crude protein was fractionated using ammonium sulfate precipitation in a step-wise manner at 0–30%, 30–70% and 70–90% of saturations. The protein precipitated was collected by centrifugation at 10,000×g for 20 min at 4 °C. The recovered protein was resuspended in a minimum amount of 25 mM potassium phosphate buffer (pH 7.2) and dialyzed against the same for 24 h with three changes of the buffer for desalting.

The dialyzed protein sample with L-methioninase activity was applied on to Diethylamino ethyl Sephadex anion exchange column (2×25 cm) pre-equilibrated with 25 mM potassium phosphate buffer (pH 7.2). The chromatographic column was washed with two-bed volumes of 25 mM potassium phosphate buffer (pH 7.2) to collect unbound protein fraction. The bound proteins were eluted in a stepwise

manner with a linear gradient of 0.1–1.0 M NaCl in the same buffer at a flow rate of 60 ml/h. The fractions were collected and assayed for protein at 280 nm. The protein concentration of samples was also determined by Lowry's (1951) method, using bovine serum albumin as the standard. Both bound and unbound proteins were assayed for L-methioninase activity. The active fractions were pooled and dialyzed and concentrated by ultra-filtration.

The concentrated protein sample was subjected to a gel filtration column (2 × 50 cm) with Sephacryl-S-300 HR as the matrix. The fractions were eluted using 25 mM potassium phosphate buffer (pH 7.2) at a flow rate of 30 ml/h with a sample size of 2 ml. The protein concentration of the fractions was estimated by measuring the absorbance at 280 nm and tested for L-methioninase activity. Active fractions were concentrated by freeze drying and stored at – 80 °C.

Characterization of the purified L-methioninase enzyme

Determination of molecular weight and homogeneity by electrophoresis

The purified enzyme was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the homogeneity and estimate the molecular weight of protein. SDS-PAGE was performed according to the method of Laemmli (1970), with a separating gel of 10% and stacking gel 5% containing 0.1% SDS. The gel was stained with Coomassie brilliant blue R-250 and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5. The molecular weight of L-methioninase was quantified in comparison with the mobility of protein in the standard marker.

Effect of pH on the activity and stability of purified L-methioninase

The effect of pH on L-methioninase activity was examined by performing the enzyme assay at different pH within the range of 4.0–12.0. The buffer systems used for this study include 50 mM concentrations of acetate buffer (pH 4.0–5.5), phosphate buffer (pH 6.0–7.5), Tris buffer (pH 8–9), glycine-sodium hydroxide buffer (pH 9.5–10.5) and phosphate-sodium hydroxide buffer (pH 11.0–12.0). For investigating the pH stability, the lyophilized enzyme was dissolved in various buffer systems mentioned above and was pre-incubated for 24 h before conducting the assay. All the assays were carried in triplicates with appropriate controls and the residual activity was estimated at standard assay conditions.

Effect of temperature on activity and stability of purified L-methioninase

The effect of temperature on L-methioninase activity was studied at different temperatures within the range of 20–70 °C in 50 mM potassium phosphate buffer (pH 8.0). The maximum enzyme was defined as 100% and the relative activity was determined as a fraction of this value. Thermostability of the purified enzyme was analyzed by pre-incubating the enzyme at temperatures in the range of 20–70 °C for different periods, 30, 60, 90, 120 and 150 min followed by estimation of enzyme activity at 37 °C. The residual activity was measured as the percentage of observed activity for the unheated enzyme.

The thermal inactivation of L-methioninase was studied in the range of 20–70 °C for different periods and the inactivation rate constant (k) was measured from a semi-log plot of residual activity against treatment time. Half-life ($t_{1/2}$) is the time taken to reduce the total enzyme activity to 50% and was determined using the following equation:

$$t_{1/2} = \ln(2)/k \quad (1)$$

The D value is the time required to reduce the enzyme activity to 10% of its original value. The D value is directly related to the inactivation rate constant k by the equation:

$$D = 2.303/k \quad (2)$$

Thermodynamics of L-methioninase denaturation

The activation energy for thermal deactivation (E_d) was determined from the slope of Arrhenius plot ($\ln k$ vs. $1/T$). The thermodynamic parameters, enthalpy of inactivation (ΔH^0), Gibbs free energy of inactivation (ΔG^0) and entropy of inactivation (ΔS^0) were calculated using the following equations:

$$\Delta H^0 = E_d - RT \quad (3)$$

$$\Delta G^0 = -RT \ln(kh/K_B T) \quad (4)$$

$$\Delta S^0 = \Delta H - \Delta G/\Delta T \quad (5)$$

where R is universal gas constant, K_B is the Boltzmann constant (1.38×10^{-23} J/K), h is the Planck constant (6.626×10^{-34} J s), T is the absolute temperature, and k (s^{-1}) is the inactivation rate constant at a particular temperature.

Effect of different metal ions on L-methioninase activity

The effect of various metal ions such as Ba^{2+} , Ca^{2+} , Cd^{2+} , Na^+ , K^+ , Mg^{2+} , Mn^{2+} , Cu^{2+} , Zn^{2+} , Li^+ , Co^{2+} and Fe^{2+} on

L-methioninase activity was analyzed. The purified enzyme was preincubated with the metal ions at a final concentration of 1 and 5 mM in 50 mM phosphate buffer, pH 8.0 for 30 min at 35 °C. The residual activity was quantified following standard assay method with L-methionine as substrate. The reaction mixture without any metal ion was considered as control with 100% activity.

Effect of inhibitors on the purified L-methioninase

The effect of activators and inhibitors was determined by pre-incubating the purified L-methioninase in 50 mM phosphate buffer at 35 °C for 30 min with the selected chemicals such as 2-mercaptoethanol, Dithiothreitol, sodium azide, Phenylmethylsulfonyl fluoride (PMSF), Hydroxylamine, Guanidine hydrochloride and Ethylenediaminetetraacetic acid (EDTA). The enzyme activity was measured under the standard assay conditions as described earlier. Residual activity was determined by taking the activity of the control sample without inhibitor as 100%.

Effect of organic solvents and detergents on L-methioninase activity

The stability of purified L-methioninase was evaluated against various organic solvents and detergents by pre-incubating the enzyme sample with the compound at 35 °C for 30 min. The organic solvents used for the present study are isopropanol, acetone, ethanol, methanol, dimethylsulfoxide (DMSO) and acetic acid. The detergents SDS, Tween 80, Tween 20 and Triton X 100 were used at a 1% concentration. The enzyme assay was performed on the pre-incubated enzyme and the residual activities were estimated. The activity recorded by the control sample with no additive was taken as 100%.

Substrate specificity of L-methioninase

The ability of the purified enzyme to catalyze the elimination reactions of various amino acids such as L-methionine, DL-homocysteine, L-cysteine, L-cystine, L-asparagine and DL-methionine was evaluated. The demethiolating activity of L-methioninase towards L-methionine, L-homocysteine, L-cystine and DL-methionine was evaluated using methanethiol release assay. For the evaluation of L-methioninase action on L-cysteine and L-asparagine, the enzyme's deaminating ability was assessed. The substrates were added to the assay mixture individually at a concentration of 20 mM. The relative activity was expressed as the percentage ratio of the L-methioninase activity determined against different substrates to enzyme activity with L-methionine.

Determination of kinetic parameters of L-methioninase

The kinetic parameters of purified L-methioninase towards selected substrates were analyzed in 50 mM potassium phosphate buffer (pH 8.0) at 35 °C using different concentrations of each (10–100 mM). The enzymatic kinetic parameters, Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were calculated from the Lineweaver–Burk plot. The k_{cat} value, the catalytic efficiency of enzyme was calculated using the following formula:

$$k_{cat} = V_{max}/E_t, \text{ where } E_t = \text{Total enzyme concentration.} \quad (6)$$

Evaluation of in vitro anticancer activity of L-methioninase on cancer cell lines

The breast cancer cell line (MCF-7) and hepatocellular carcinoma (Hep-G2) cell lines used in the experiment were maintained in Dulbecco's modified Eagles Medium (DMEM), supplemented with 10% Fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin-B (5 µg/ml) in a humidified atmosphere of 5% CO₂ at 37 °C. Sulforhodamine B (SRB) assay was performed to determine the anticancer activity of the purified L-methioninase against breast cancer (MCF7) and hepatocellular carcinoma (Hep-G2). Cells were seeded at a density of 0.5×10^5 cells/well in to 96-well microtiter plates containing DMEM medium. The microtiter plates were incubated at 37 °C in a humidified incubator with 5% CO₂ for 24 h. After incubation, enzyme samples at different concentrations were added to the wells and incubated for 48 h. The cells were fixed with 100 µl cold trichloroacetic acid (10%) for 1 h at 4 °C followed by staining with 100 µl SRB (0.4%) dissolved in 1% acetic acid for 30 min at room temperature. Subsequently, the cells were washed with 1% acetic acid to remove the unbound stain and air dried. The bound dye was solubilized with 100 µl of 10 mM Tris base (pH 10) per well. The absorbance was measured with an enzyme-linked immunosorbent assay (ELISA) microplate reader at 570 nm. The experiments were done in triplicates and the data are expressed as the mean percentage cell viability. The IC₅₀ of L-methioninase against cancer cell lines were calculated using sigmoidal dose–response curve-fitting models using GraphPad Prism Software version 7.

Evaluation of in vivo anticancer activity of purified L-methioninase

Experimental animals

Inbred female Swiss mice of 2-month old, weighing 20 ± 5 g, obtained from Sree Venkateshwara Enterprises, Pvt Ltd, Bangalore, India, were used for the study. They were housed at 22 °C under 12-h light/12-h dark cycle. Mice were fed with standard animal diet and water ad libitum freely throughout the study. The experimental protocols were performed after approval from the IAEC (Institutional animal ethical committee) (Reg No: NCP/IAEC/2018-19/29) and under the recommendations for the proper care and use of laboratory animals.

In vivo toxicity studies

The Swiss Albino mice were divided into five groups of three animals each. All the animals except group I were administered with a single dose of L-methioninase. The animals were injected intraperitoneally with 1 ml of different doses of L-methioninase (5, 10, 20, and 50 mg/kg). The untreated animals were kept as control (group 1). After administration of the enzyme, animals were observed for any toxic manifestation and mortality. At the end of 2 weeks, all the animals were killed and blood samples were collected from each mouse and used for the estimation of hematological parameters such as red blood cell count (RBC) and white blood cell count (WBC) and hemoglobin (Hb). The serum samples were used for the analysis of biochemical parameters such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

Antitumor study of L-methioninase on DAL-induced mice

Dalton's Ascites Lymphoma (DAL) cells were maintained in vivo in Swiss albino mice by intraperitoneal transplantation. Ascitic fluid was drawn out from DAL tumor-bearing mouse and further diluted to get $1 \times 10^6/0.1$ cells/ml. The animals were divided into five experimental groups each containing six animals: group 1 is normal control, group 2 is tumor control, group 3 is tumor-induced mice treated with standard drug 5 Flurouracil (20 mg/kg), and groups 4 and 5 are tumor-induced mice treated with 10 mg/kg and 20 mg/kg L-methioninase. All the groups were intraperitoneally injected with 0.1 ml of tumor cell suspension intraperitoneally except normal control.

All the treatments were given 24 h after the tumor inoculation, once daily for 14 days. After administering the last dose, the animals in each group were fasted overnight and three mice from each group were killed. The blood sample

was withdrawn from each mouse and used for the estimation of hematological parameters such as red blood cell count (RBC) and white blood cell count (WBC), and hemoglobin (Hb). The serum samples were used to analyze the biochemical parameters such as total cholesterol, triglycerides, AST, ALT, and ALP. The rest of the animals were provided with food and water ad libitum and observed to determine body weight, ascitic tumor volume, packed cell volume, tumor cell count, mean survival time (MST) and percentage increase in lifespan (%ILS).

Statistical analysis for in vivo studies

All the values are expressed as mean $\pm$ SEM. The data were statistically analyzed by one-way ANOVA, followed by Dunnett comparison using GraphPad Prism 7 software. The groups with $P < 0.05$ were considered significant.

Results and discussion

Purification of L-methioninase from *Trichoderma harzianum*

The extracellular L-methioninase of *Trichoderma harzianum* was purified by ammonium sulfate precipitation followed by ion exchange and gel filtration chromatography. Among the different fractions, 30–70% fraction of ammonium sulfate-precipitated protein showed 12.24 U/mg of L-methioninase activity with 1.17-fold purification. The protein was dialyzed, concentrated and the protein sample was further purified using DEAE Sephadex ion exchange column. The specific activity of the enzyme increased to 41.03 U/mg after anion exchange chromatography and the active fractions were pooled, dialyzed, concentrated and applied to gel filtration column. The enzyme was eluted from sephacryl-S-300 h column using phosphate buffer, concentrated by freeze drying and stored at -80 °C. The purification of protein using the Sephacryl 300HR gel filtration column increased the purity of the protein by 7.15-fold with a recovery of 47.9%. A summary of the purification steps is shown in Table 1.

Trichoderma harzianum L-methioninase have a higher specific activity of 74.4 U/mg of protein than those reported from other sources. L-methioninase from *Aspergillus flavipes* was purified by 12.1-fold with a 39.8% yield and had a specific activity of 14.6 U/mg (El-Sayed 2011). The purification of enzyme in *Candida tropicalis* was carried out in three steps with purification fold of 43.19 and 27.985% enzyme recovery (Selim et al. 2015). A purification fold of 270 was achieved with an overall yield of 8.4% for L-methioninase from *Pseudomonas putida* (Ito et al. 1976).

Table 1 Purification of L-methioninase from *Trichoderma harzianum*

Sample	Volume (ml)	Total enzyme (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Enzyme yield (%)
Crude	500	3334	320.5	10.4	1	100
Ammonium sulfate	80	3158	258.4	12.24	1.17	94.7
Ion exchange	42	2372	57.8	41.03	3.95	71.17
Gel filtration	18	1599	21.5	74.4	7.15	47.9

Characterization of purified L-methioninase

Determination of molecular weight and homogeneity by SDS-PAGE

The purity and homogeneity of L-methioninase were confirmed by SDS-PAGE. The molecular weight of the purified L-methioninase was analyzed using SDS–polyacrylamide electrophoresis which showed a single band with apparent molecular weight 48 kDa (Fig. 1). L-methioninase has been reported to be a multi-subunit enzyme (El-Sayed 2010). The tetrameric structure of L-methioninase was reported among various bacterial (Nikulin et al. 2008), fungal (El-Sayed et al. 2017), and protozoan (Lockwood and Coombs 1991) species. Ronda et al. (2011) elucidated the structure of *Citrobacter freundii* L-methioninase using X-ray crystallography and single-crystal spectroscopy studies. L-methioninase purified from different sources possessed molecular weight in the range of 43–48 kDa per subunit (Ito et al. 1976; El-Sayed 2010), which is in accordance with our result. A 47 kDa L-methioninase from *A. flavipes* (El-Sayed 2010), 46 kDa from *C. tropicalis* (Selim et al. 2015) and 43–45 kDa from *Streptomyces* spp. (Selim et al. 2016) have been previously reported. The molecular weight of the purified enzyme was within the range of L-methioninase isolated from different fungi.

Effect of pH on L-methioninase activity and stability

The L-methioninase from *Trichoderma harzianum* was active between pH 5 and 10 and exhibited maximum activity at pH 8. The enzyme activity was inhibited entirely below pH 4.0 and decreased sharply above pH 10.0. El Sayed (2011) has reported the optimum pH for L-methioninase from *A. flavipes* to be 8 which is in accordance with our result. Similar reports were also reported for L-methioninase from *Aeromonas* (Nakayama et al. 1984). The stability of an enzyme at physiological pH is desirable for antitumor properties. Figure 2a depicts the pH stability studies of L-methioninase in the range of 4–10. The enzyme was highly stable in the pH range 6.0–8.5 when incubated in buffers of various pH for 24 h. Almost 80% of enzyme activity was retained at this pH range. Lower pH stability at extreme acidic and alkaline conditions may be due to the dissociation of cofactor pyridoxal phosphate from the enzyme. Similar pH stability profile was also reported for L-methioninase from *Brevibacterium linens* (Pavani and Saradhi 2014), *Aspergillus ustus* (Abu-Tahon and Isaac 2016) and *Aspergillus flavipes* (El-Sayed 2011).

Fig. 1 SDS-PAGE image of L-methioninase from *Trichoderma harzianum*. Lane 1: marker, lane 2: ion exchange chromatography, lane 3: gel filtration chromatography, lane 4: ammonium sulfate-precipitated protein

Fig. 2 **a** Effect of pH on *Trichoderma harzianum* L-methioninase stability. **b** Thermal stability of *Trichoderma harzianum* L-methioninase

Effect of temperature on L-methioninase activity and stability

The effect of temperature on *Trichoderma harzianum* L-methioninase activity was examined over a range of 20–70 °C. The enzyme activity gradually increased with a rise in temperature up to 35 °C and declined thereafter. The optimum reaction temperature for L-methioninase was found to be 35 °C. The temperature optima reported for L-methioninase from *A. flavipes* (El-Sayed 2011), *Aspergillus ustus* (Abu-Tahon and Isaac 2016), *P. putida* (Esaki and Soda 1987) and *Aspergillus* spp. (Ruiz-Herrera and Starkey 1969) were also 35 °C. However, L-methioninase of *Brevibacterium linens* and *Streptomyces* spp. showed

optimum activity at 25 °C and 45 °C, respectively (Diaz and Weimer 1998; Selim et al. 2016).

The thermal stability of *Trichoderma harzianum* L-methioninase was tested by preincubating the enzyme in potassium phosphate buffer (pH 8) at temperatures ranging from 20 to 70 °C for different time periods. L-methioninase showed higher thermal stability below 50 °C (Fig. 2b) with 80% of the enzyme activity retained for 90 min. However, activity was significantly reduced above 60 °C and the enzyme activity was lost entirely after incubation at 70 °C for 2 h. L-methioninase from *Aspergillus flavipes* (El-Sayed 2011) and *Brevibacterium linens* (Diaz and Weimer 1998) had thermal stability below 40 °C. *Aspergillus ustus* L-methioninase (Abu-Tahon and Isaac 2016)

exhibited maximum activity at 35 °C followed by a gradual decrease until it retained only 55% of its activity at 50 °C. Whereas, L-methioninase purified from *C. tropicalis* (Selim et al. 2015) was thermostable at a higher temperature. The inactivation of the enzyme at a higher temperature may be due to the denaturation of the same.

To understand the inactivation kinetics of enzyme, the inactivation constant k was measured from semi-log plots of residual activity versus heat treatment time. Figure 3a shows that the inactivation followed first-order kinetic model. The increase in k value with a rise of temperature indicated faster inactivation of L-methioninase at a higher temperature (Table 2). The inactivation constant increased from 5.71×10^{-4} to 1.83×10^{-2} min with a rise in temperature from 20 °C to 70 °C. Half-lives ($t_{1/2}$) and decimal reduction time (D) of L-methioninase at different temperatures indicated enzyme stability to be dependent on temperature. The enzyme showed half-life of 20.23 h and 0.63 h at 20 and

70 °C, respectively (Table 2). The decimal reduction time (D) is the time required to reduce 90% of its initial activity and is also a reliable parameter in assessing enzyme's thermostability. The maximum D value reaches 67.22 h at 20 °C and a minimum of 2.09 h is observed at 70 °C. These results indicated lower stability of the enzyme at a temperature above 50 °C.

Evaluation of thermodynamic parameters of L-methioninase denaturation

An investigation of thermodynamics parameters is necessary for analyzing enzyme thermal denaturation process and the behavior of molecules in different conditions. The dependence of inactivation rate constant (k) on temperature was evaluated using the Arrhenius equation. The activation energy of denaturation (E_d) is the minimum energy required to initiate the thermal denaturation process. The activation

Fig. 3 **a** Thermal inactivation kinetics of L-methioninase at different temperature. **b** Arrhenius plot of deactivation rate constant of L-methioninase

Table 2 Kinetic and thermodynamic parameters for thermal inactivation of L-methioninase from *Trichoderma harzianum*

Temperature (°C)	k (min ⁻¹)	$t_{1/2}$ (h)	D (h)	ΔH^0 (kJ/mol)	ΔG^0 (kJ/mol)	ΔS^0 (J/mol K)
20	5.71×10^{-4}	20.23	67.22	55.64	88.11	-110.81
30	8.13×10^{-4}	14.20	47.21	55.56	92.11	-120.62
40	1.5×10^{-3}	7.70	25.58	55.47	93.71	-122.17
50	2.51×10^{-3}	5.25	17.44	55.39	95.41	-123.90
60	5.93×10^{-3}	1.95	6.50	55.31	96.08	-122.40
70	1.83×10^{-2}	0.63	2.09	55.22	95.80	-118.30

k inactivation rate constant, $t_{1/2}$ Half-life, D value time required to reduce the enzyme activity to 10%, ΔH^0 Enthalpy of inactivation, ΔG^0 Gibbs free energy of inactivation, ΔS^0 entropy of inactivation

energy for thermal inactivation of L-methioninase calculated using the Arrhenius plot (Fig. 3b) was 58.08 kJ/mol. The linearity of the plot indicated a unique temperature-dependent inactivation of the enzyme.

The enthalpy of inactivation (ΔH^0), Gibbs free energy of inactivation (ΔG^0) and entropy of inactivation (ΔS^0) of L-methioninase inactivation was measured and presented in Table 2. The average ΔH^0 for L-methioninase deactivation was 55.43 kJ/mol. The change in enthalpy with the rise in temperature indicates the transient changes in the enzyme conformation during thermal denaturation (Narwal et al. 2016). The positive ΔH^0 values indicate that the enzyme inactivation is an endothermic process. The decline of ΔH^0 values with an increase in temperature implies lower energy requirement for denaturing the enzyme at high temperature (Bhatti et al. 2006). The ΔG^0 and entropy for L-methioninase was found to be in the range of 88.11–95.80 kJ/mol. For purified L-methioninase from *Trichoderma harzianum*, entropy was found to be between -110.81 and -118.30 J/mol K.

The positive change in entropy indicates the enzyme and solvent disorder of the system. Usually, the entropy of the system is positive but negative values of change in entropy are observed in biological systems like proteins. The

negative entropy values could be due to increase in the order of the system, due to the compaction of the enzyme molecule, ordering of molecules of water in the vicinity of the hydrophobic residues and aggregation during denaturation (Gummadi 2003; Marin et al. 2003). Similar results were also observed in xylanase from *Aspergillus niger* (Pal and Khanum 2011) and *Trichoderma harzianum* chitinase (Kapat and Panda 1996). Thermal inactivation and thermodynamic studies indicate that with an increase in temperature, enzyme undergoes several transition phases leading to its aggregation. The thermodynamic analysis of L-methioninase has not been performed before from any sources. To our best knowledge, this is the first report on the thermodynamic analysis of L-methioninase.

Effect of metal ions

Metal ions play a prominent role in maintaining structural stability and protein integrity by binding to amino acids. The influence of different metal ions on L-methioninase activity was investigated using 1 mM and 5 mM concentration of the corresponding chloride forms at pH 8 and 35 °C. From Fig. 4, it is evident that L-methioninase did not require any specific metal ion for its catalytic activity. The

Fig. 4 Effect of metal ions on the activity of L-methioninase from *Trichoderma harzianum*

Li⁺ stimulated the enzyme activity with residual activity of 118.13% and 137.48% at 1 mM and 5 mM concentration. Ba²⁺, Na⁺ and K⁺ had no effect on enzyme activity. Cu²⁺, Co²⁺ and Fe²⁺ showed significant inhibition at 5 mM concentration and retained only 26.4, 49.2 and 19.68% of L-methioninase activity than control. Metal ions such as Ca²⁺, Mn²⁺, Mg²⁺, Zn²⁺ and Cd²⁺ showed minimal inhibition at 5 mM concentration. The inhibition of L-methioninase by Cu²⁺, Co²⁺ and Fe²⁺ was also reported by Selim et al. (2015). Inhibition of enzyme activity in the presence of Cu²⁺ might be indicative of presence of the sulfhydryl group in the active site of the enzyme (Selim et al. 2015).

Effect of inhibitors on the purified L-methioninase

Enzyme inhibitors are used as an indicator for determining the catalytic identity of the enzyme. The effect of inhibitors on L-methioninase activity is summarized in Table 3. The L-methioninase was undisturbed in the presence of EDTA and showed a relative activity of 96.46%. The insensitivity of EDTA towards L-methioninase indicated the non-metallic nature of the enzyme. The inhibitory effect of 2-mercaptoethanol and Dithiothreitol on L-methioninase, which resulted in the relative activity of 53.5% and 55.5%, suggests the presence of a disulfide bond for maintaining the molecular integrity of enzyme. Similar results were also observed for L-methioninase from *A. flavipes* (El-Sayed 2011) and *Streptomyces* spp. (Selim et al. 2016). PMSF and Hydroxylamine strongly inhibited the enzyme at 1 mM concentration with 20.3% and 3.5% residual activity, respectively. Apart

from being a serine protease, PMSF can also affect the thiol group of enzymes. The inhibitory effect of PMSF suggests the presence of cysteine for maintaining the molecular catalytic folding state of the enzyme (Kamran et al. 2015). The active site of *Aspergillus flavipes* L-methioninase was found to have conserved cysteine (El-Sayed et al. 2017). Hydroxylamine causes inactivation of the enzyme by dissociating pyridoxal phosphate from methioninase (Lockwood and Coombs 1991; Bertoldi et al. 2002). At 5 mM concentration, the enzyme was completely inhibited by these compounds. The enzyme was also susceptible to sodium azide and Guanidine HCl, reducing residual activity to 75.6 and 40.6%, respectively.

Effect of surfactants and solvents on the purified L-methioninase

Various solvents and surfactants were found to significantly influence the structure and function of the enzyme and its accessibility of the substrate. The effect of different organic solvents and surfactants on *Trichoderma harzianum* L-methioninase was evaluated and is summarized in Table 3. Most of the solvents, including methanol, ethanol, acetone and DMSO, reduced L-methioninase activity. Acetic acid and isopropanol significantly inhibited enzyme activity to $8.08 \pm 0.05\%$ and $23.56 \pm 0.8\%$. L-methioninase activity was moderately reduced by surfactants SDS, Tween 80, Tween 20 and Triton X 100. *Aspergillus flavipes* L-methioninase activity was also significantly reduced by Triton X 100 and Tween 20. The loss of enzyme activity may be due to the physical interference of the hydrophobic or hydrophilic amino acids (El Sayed 2011).

Substrate specificity of the L-methioninase

L-methioninase was able to catalyze the degradation of various sulfur containing amino acids and its derivatives. From Table 4, it is evident that highest affinity of L-methioninase is towards L-methionine, its natural substrate. Hence, the activity of L-methioninase towards L-methionine is considered as 100%. The order of affinity of L-methioninase

Table 3 Effect of inhibitors, detergents and solvents on L-methioninase from *Trichoderma harzianum*

Compound	Concentration	Residual activity (%)
2-Mercaptoethanol	5 mM	53.5 ± 1.7
Dithiothreitol	5 mM	55.5 ± 1.4
Sodium azide	5 mM	75.6 ± 2.1
PMSF	1 mM	20.3 ± 1.5
Hydroxylamine	1 mM	3.5 ± 0.5
Guanidine HCl	5 mM	40.6 ± 2.1
EDTA	5 mM	96.4 ± 1.6
SDS	1%	78.88 ± 2.3
Tween 20	1%	58.38 ± 2.1
Tween 80	1%	71.36 ± 3.2
Triton X 100	1%	75.15 ± 2.6
Methanol	1%	85.40 ± 2.4
Ethanol	1%	81.60 ± 1.9
Isopropanol	1%	23.56 ± 0.8
DMSO	1%	80.43 ± 1.7
Acetic acid	1%	8.08 ± 0.05
Acetone	1%	69.25 ± 2.1

Table 4 Substrate specificity of L-methioninase from *Trichoderma harzianum*

Substrate	Relative activity (%)
L-Methionine	100
L-Homocysteine	89.1 ± 1.26
L-Cystine	76.3 ± 1.32
L-Cysteine	85.8 ± 0.82
L-Asparagine	60.1 ± 0.92
DL-Methionine	83.6 ± 1.12

towards the tested substrates was L-methionine > L-Homocysteine > L-cysteine > DL-methionine > L-cystine > L-asparagine. L-methioninase is a multifunctional enzyme capable of hydrolyzing C–S and C–O bonds rather than C–C bonds (Manukhov et al. 2005). Most of the purified bacterial L-methioninase has a higher affinity towards L-ethionine and L-cysteine than to L-methionine (Lockwood and Coombs 1991; Kries and Hession 1973). L-methioninase from *Brevibacterium linens* (Dias and Weimer 1998) had higher specificity for DL-homocysteine than L-methionine. In addition, the enzyme from *T. vaginalis* (Lockwood and Coombs 1991) showed a greater affinity towards ethionine ($144 \pm 32\%$), homocysteine ($903 \pm 114\%$) and L-cystine ($108 \pm 14\%$) than L-methionine. However, in the current study, *Trichoderma harzianum* L-methioninase showed higher specificity for L-methionine than any other substrate. EL Sayed (2011) and Selim et al. (2015) have also reported a higher specificity of L-methioninase towards L-methionine.

Determination of kinetic parameters of the purified L-methioninase

Kinetic parameters of the purified L-methioninase from *Trichoderma harzianum* were evaluated by incubating the enzyme in varying concentrations of different substrates. Enzyme activities were measured under standard assay conditions and the catalytic properties, K_m , V_{max} and k_{cat} of purified enzyme were evaluated using Lineweaver–Burk plot. Table 5 depicts the kinetic

Table 5 Kinetic parameters of L-methioninase for different substrates

Substrate	K_m (mM)	V_{max} (U/mg/min)	k_{cat} (s^{-1})
L-Methionine	1.19	21.27	16.11
L-Homocysteine	1.99	17.85	13.47
L-Cysteine	3.35	16.67	12.62
L-Cystine	4.35	12.19	9.23

K_m Michaelis constant, V_{max} maximum rate of enzyme-catalyzed reaction, k_{cat} turnover number

parameters of L-methioninase for different substrates. L-methioninase from *T. harzianum* showed a higher affinity towards L-methionine with K_m and V_{max} of 1.19 mM and 21.27 U/mg/min, respectively. The enzyme also has higher catalytic efficiency towards L-methionine ($16.11 s^{-1}$). The low K_m value (1.19 mM) of *T. harzianum* L-methioninase towards L-methionine suggests high substrate specificity and its effectiveness against tumor cells. L-methioninase from *Pseudomonas putida* has been reported with a K_m of 1 mM (Esaki and Soda 1987) and the purified enzyme from *Aspergillus flavipes* (El-Sayed 2011) exhibited a K_m value of 6.5 mM. *Aspergillus flavipes* L-methioninase has a k_{cat} of $14.1 s^{-1}$ (El-Sayed 2011) and *C. freundii* (Manukhov et al. 2005) L-methioninase has a k_{cat} of $6.2 s^{-1}$. L-methioninase from *Trichoderma harzianum* showed higher catalytic efficiency when compared to L-methioninase from the above sources.

Evaluation of in vitro anticancer activity of purified L-methioninase

For evaluation of the in vitro cytotoxic activity of the purified *Trichoderma harzianum* L-methioninase, human breast carcinoma (MCF7) and human hepatocarcinoma (Hep-G2) cell lines were incubated with different doses of the enzyme. After 24 h of incubation, cell viability was determined by the SRB assay. The enzyme induced cytotoxicity in a dose-dependent manner, as illustrated in Fig. 5. The IC_{50} values of the enzyme required for inhibition of tumor cell growth were calculated using sigmoidal dose–response curve-fitting models. The purified L-methioninase exhibited an antiproliferative activity against Hep-G2 and MCF-7 cells with IC_{50} values of $14.12 \mu g/ml$ and $20.07 \mu g/ml$, respectively. The enzyme had highest activity against Hep-G2 cell lines followed by MCF-7 cell lines. One unit of *Trichoderma harzianum* L-methioninase inhibited the growth of Hep-G2 and MCF-7 by 47.62 and 33.84%, respectively. In vitro anticancer efficiency of L-methioninase was also reported by El sayed et al. (2012), which showed significant activity against numerous cancer cell lines. L-methioninase from

Fig. 5 In vitro cytotoxic activity of L-methioninase: **a** HEP-G2 and **b** MCF-7

Streptomyces spp. (Selim et al. 2016) was reported to possess remarkable L-methioninase activity against liver and breast cancer cell lines.

Studies on in vivo anticancer activity of purified L-methioninase on Dalton's ascites lymphoma-induced cancer in mice

In vivo toxicity studies of L-methioninase

In this experimental study, all the animals survived throughout the period without exhibiting any abnormalities. The L-methioninase treated animals did not show any toxic symptoms such as fatigue, weight loss or loss of appetite. The hemoglobin level, RBC cell count and total WBC count were found to be in the normal range. The liver enzymes AST, ALT and ALP were not significantly altered from the normal level of control animals. Hence, L-methioninase was found to be safe in the tested dose levels. The dose level of 10 mg/kg (low dose) and 20 mg/kg (high dose) was selected for anti-tumor studies.

Anti-tumor study of L-methioninase on DAL-induced mice

Effect of L-methioninase on tumor growth

A regular and rapid increase of ascitic tumor volume was found in DAL tumor control group. Ascitic fluid is the nutritional source of tumor cells and its increase is related to the nutritional requirement of tumors (Prasad and Giri 1994). Treatment with L-methioninase for 14 days in DAL tumor-bearing mice has led to a significant reduction in tumor volume, packed cell volume, and cancer cell count compared to tumor control (Table 6). The tumor volume of control group was 8.57 ± 0.49 ml which was reduced to 4.65 ± 0.7 ml and

3.47 ± 0.37 ml in the group treated with 10 and 20 mg/kg of L-methioninase, respectively.

A significant increase in body weight of the animals was observed in DAL control mice due to the rapid and progressive accumulation of ascites tumor cells. The bodyweight of the tumor control group was 33.86 ± 0.84 g and the treatment with L-methioninase (20 mg/kg) showed a marked reduction in the increased body weight of tumor-bearing mice 26.51 ± 1.37 g when compared to DAL control group. These results indicate the cytotoxic effect of L-methioninase on tumor cells. Sundar and Nellaiah (2013) have also reported the cytotoxic effects of L-methioninase on DAL cell lines.

Effect of L-methioninase on mean survival time and increase in life span

The major criterion considered for a potential antitumor drug is its efficacy of prolongation of lifespan. In the DAL tumor control group, the mean survival time was 20.6 days, while it increased to 37.1 days for L-methioninase (20 mg/kg) treated group. In DAL tumor control group, the average lifespan is 48.9% as compared to normal control. Whereas, an increase in life span of 58.06% and 79.83% was observed when treated with 10 and 20 mg/kg of L-methioninase, respectively (Table 6). For groups treated with 5-Fluorouracil, MST and % ILS were 39.5 days and 91.13% respectively.

Effect of L-methioninase on hematological parameters, serum enzymes and lipid profile

Myelosuppression and anemia are the most common problems encountered during cancer therapy (Hogland 1982; Price and Greenfield 1958). Anemia found in cancer patients is mainly due to the reduced RBC and hemoglobin production (Fenninger and Mider 1954). The inoculation of DAL cell lines in mice caused a significant increase in total WBC

Table 6 Effect of L-methioninase on body weight, cancer cell count, tumor volume, packed cell volume and lifespan of Daltons Ascites Lymphoma cell-induced mice

Parameters	Control	DAL control	DAL + STD (5-FU 20 mg/kg)	DAL + LD (L-met 10 mg/kg)	DAL + HD (L-met 20 mg/kg)
Body weight (g)	24.16 ± 1.16	33.86 ± 0.84^a	25.31 ± 1.68^b	28.6 ± 1.30^b	26.51 ± 1.37^b
Cancer cell count ($\text{ml} \times 10^6$)	0	7.60 ± 0.64^a	1.61 ± 0.33^b	4.06 ± 0.53^b	3.47 ± 0.37^b
Tumor volume (ml)	0	8.57 ± 0.49^a	3.23 ± 0.32^b	6.2 ± 0.59^b	4.65 ± 0.7^b
Packed cell volume (ml)	0	2.6 ± 0.2^a	0.45 ± 0.10^b	1.86 ± 0.22^b	0.76 ± 0.12^b
Mean survival time (days)	40.5	20.6	39.5	32.6	37.1
%ILS (percentage increase in lifespan)	—	—	91.13	58.06	79.83

All the values are expressed as mean $\pm$ SEM for 3 animals in each group

STD standard, 5-FU 5-Fluorouracil, LD low dose, HD high dose, L-MET L-methioninase

^aValues are significantly different from normal control at $P < 0.05$

^bValues are significantly different from cancer control at $P < 0.05$

Table 7 Effect of L-methioninase on hematological parameters, serum enzymes and lipid profile of Daltons Ascites Lymphoma cell-induced mice

Parameters	Control	DAL control	DAL + STD (5-FU 20 mg/kg)	DAL + LD (L-met 10 mg/kg)	DAL + HD (L-met 20 mg/kg)
Hemoglobin (gm/dl)	13.5 ± 0.36	8.32 ± 0.99 ^a	12.26 ± 0.77 ^b	10.23 ± 0.90 ^b	11.53 ± 1.59 ^b
RBC (cells/ml × 10 ⁶)	4.86 ± 0.64	3.04 ± 0.17 ^a	4.28 ± 0.47 ^b	4.02 ± 0.20 ^b	4.22 ± 0.34 ^b
WBC (cells/ml × 10 ⁶)	10.1 ± 0.79	16.5 ± 0.65 ^a	11.03 ± 1.20 ^b	13.8 ± 0.88 ^b	12.2 ± 0.6 ^b
AST(U/ml)	104.5 ± 2.06	140.9 ± 2.38 ^a	116.86 ± 1.45 ^b	128.76 ± 1.65 ^b	127.43 ± 0.96 ^b
ALT (U/ml)	30.83 ± 1.81	47.43 ± 2.60 ^a	39.93 ± 1.84 ^b	43.43 ± 1.95 ^b	41.43 ± 2.05 ^b
ALP (U/ml)	128.03 ± 2.04	237.50 ± 2.98 ^a	159.03 ± 2.54 ^b	167.53 ± 2.90 ^b	163.20 ± 1.21 ^b
Total cholesterol (mg/dl)	126.70 ± 2.3	184.9 ± 3.1 ^a	143 ± 2.13 ^b	156 ± 1.82 ^b	150 ± 1.92 ^b

All the values are expressed as mean ± SEM for three animals in each group

STD standard, 5-FU 5-Fluorouracil, LD low dose, HD high dose, L-MET L-methioninase, RBC red blood cells, WBC white blood cells, AST aspartate aminotransferase, ALT alanine amino transferase, ALP alkaline phosphatase

count and reduction of RBC cell count and hemoglobin compared to the normal control (Table 7). The treatment with L-methioninase (20 mg/kg) has significantly reduced WBC count to $12.2 \pm 0.6 \times 10^6$ cells/ml and increased RBC and hemoglobin to $4.22 \pm 0.34 \times 10^6$ cells/ml and 11.53 ± 1.59 g/dl, respectively, towards the normal level. These results indicate that the enzyme has a protective effect on the hematopoietic system without inducing myelotoxicity.

As shown in Table 7, the liver enzymes AST, ALT and ALP were significantly increased in DAL control mice. All three enzyme levels were restored to the normal range by treatment with 5-FU and L-methioninase. The elevated liver enzyme levels in the blood of DAL control may be due to liver damage and loss of functional integrity of the cell membrane. The inoculation of DAL cell lines has increased the levels of total cholesterol and triglycerides. The treatment with the standard drug and L-methioninase has reduced these altered parameters towards the normal levels. These results indicate the hepatoprotective nature of *Trichoderma harzianum* L-methioninase.

Conclusion

The present study reports the purification of novel L-methioninase from the fungus *Trichoderma harzianum*. The purified enzyme is a homo-tetramer with high specific activity. The enzyme was significantly stable over a broad range of pH and temperature. *Trichoderma harzianum* L-methioninase is found to be thermostable than enzyme purified from other sources. The thermal inactivation of L-methioninase exhibited first-order kinetics and the activation energy was found to be 58.08 kJ/mol using the Arrhenius model. Thermodynamic studies suggest that thermal inactivation of the enzyme is based on molecular structural changes. The purified L-methioninase showed a higher affinity towards L-methionine than other substrates,

which enhances its anticancer potential. L-methioninase also showed antiproliferative activity towards MCF-7 and HEP-G2 cell lines. The enzyme was effective in the reduction of tumor growth in DAL ascitic tumor model. This work reports the effectiveness of L-methioninase for inducing cytotoxic effect in tumor cells and inhibiting tumor progression in vitro and in vivo conditions. Therefore, the results of the present study indicate the potential of *Trichoderma harzianum* L-methioninase as an agent for cancer therapy.

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Author contributions AS conceived the original idea and designed the study. NS performed the experiment and wrote the paper. KJ provided help in performing the experiment.

Compliance with ethical standards

Conflict of interest The author declares that they have no conflict of interest. All the authors consent to the submission of this manuscript to the journal.

Ethical approval All the procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted and ethical approval was obtained from Committee for the purpose of control and supervision of experiments on animals (CPCSEA). (Reg No: 688/PO/Re/S/02/CPCSEA.), Approval No: NCP/IAE/2018-19/29.

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