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Original Article

PRODUCTION OF THERAPEUTIC METHOTREXATE DEGRADING ENZYME AND STUDIES ON ITS NANOCOMPLEXES WITH HUMAN SERUM ALBUMIN

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ABSTRACT

Objective: To improve the parent strain of *Variovorax paradoxus* for the production of methotrexate (MTX) degrading enzyme and to study the serum stability, release kinetics and functionality of the nano complexes of the enzyme with human serum albumin (HSA)

Methods: The activity of the enzyme was quantified by using the extinction coefficient of 8300 for the substrate, MTX. The mutant strain of *V. paradoxus* was isolated by exposing the cells to the UV light (302 nm) so that 50 % of the cells were killed. The enzyme was purified on DEAE-cellulose, and sephadex-G-100 columns and the purity of the enzyme was checked on 10 % SDS-polyacrylamide gel. The enzyme-HSA nano complexes were prepared by adopting desolvation-crosslinking method and their size was determined by using transmission electron microscope.

Results: MTX degrading enzymes are required to avoid the toxicity of the MTX during the treatment of cancer. The enzyme from *V. paradoxus* converts the MTX into non-toxic glutamate and 4-amino-N [10]-methylpteroate, and the culture utilizes the derived glutamate. Improving the production of this enzyme will be beneficial due to its therapeutic application. Different carbon and nitrogen sources didn't improve the production of this enzyme from the parent strain of *M. verrucaria*. The strain improvement was carried out by using UV radiation to improve the yields of the enzyme. The mutant strain produced around 6 times higher levels of the enzyme compared to the parent suggesting its advantage for the industrial production of the enzyme. Since this enzyme is of microbial origin, it was complexed to the safe carrier, HSA and these complexes showed their size in the nano-range. The nano complexes showed longer stability compared to the native enzyme in the serum, and the enzyme was readily released from the complex suggesting the protective role of the carrier, HSA. The nano complexes showed the higher degradation of MTX in the serum compared to the native enzyme suggesting their better functionality compared to the native enzyme.

Conclusion: Usage of mutant strain will be advantageous for the industrial production of the enzyme since it produces higher levels of enzyme compared to the parent strain. Enzyme-HSA nanocomputers will be a better choice for the therapeutic applications since they show better serum stability and functionality compared to the native enzyme.

Keywords: Methotrexate, *Variovorax paradoxus,* Glutamate, 4-amino-N[10]-methylpteroate, Strain improvement, UV radiation, Human serum albumin, Nanocomplexes, Native enzyme

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INTRODUCTION

MTX (4-amino-N [10]-methylpteroyl-L-glutamate) is an antagonist of folic acid and it has been used widely as a chemotherapeutic agent. Choriocarcinoma and Burkitt's lymphoma were treated by using MTX [1]. Usage of a high dose of MTX for the treatment of cancers will harm normal cells and leads to the toxicity, and it kills the cells by inhibiting the synthesis of DNA. This life-threatening toxicity is not avoidable with the available reagent leucovorin if there is a kidney failure in the patient. The enzymes that degrade MTX into the inactive components will be beneficial in this context. Enzymes that degrade MTX were reported from few microbial species [1-5]. However, these enzymes were not explored for therapeutic applications except carboxypeptidase G_2 [6, 7]. In the present study, the carboxypeptidase G that was reported long back [2] was explored since this enzyme was not completely characterized and explored for the therapeutic applications. The organism that produces this enzyme was not thoroughly exploited for the production of the enzyme. Hence, the studies that improve the production of this enzyme will benefit the pharma and biotech industries. In this study, different carbon and nitrogen sources were evaluated for the economic production of carboxypeptidase G in addition to carrying out the strain improvement of *V. paradoxus*. Since this enzyme is from the microbial source, it causes immunotoxicity if delivered directly into the system. Hence, in the present investigation, studies were carried out for complexing this enzyme with the safe nanocarrier, HSA and the nano complexes were evaluated *in vitro* for their functionality.

MATERIALS AND METHODS

Microbial cultures and reagents

The parent microbial culture was obtained from American type culture collection, USA and maintained on the agar slants containing folic acid (0.1%), KH_2PO_4 (0.05%), $K_2HPO_4.3H_2O$ (0.12%) and solutions of salt A ($MgSO_4.7H_2O$, 1%; $CaCl_2.2H_2O$, 0.1%; $FeSO_4.7H_2O$, 0.1%) and salt B ($MnSO_4$, 0.1%; Na_2MoO_4, 0.1%). This culture was initially deposited as *Pseudomonas* sp (2, 3) and subsequently renamed as *V. paradoxus* (ATCC 25301). The submerged culture was grown in the folic acid medium containing the above components for the production of the enzyme. MTX was procured from Sisco Research Laboratories, Mumbai, India and human serum was obtained from Sigma-Aldrich.

Enzyme assays

Enzyme activity on the MTX was assayed by using 100 µl of appropriately diluted cell lysate or purified enzyme. The reaction was started by adding the enzyme to the mixture containing MTX (300 µmol) & $ZnSO_4$ (0.2 mmol) at 37 °C and decrease in the absorbance was measured at 320 nm by using UV-Vis double beam spectrophotometer (Shimadzu, 1800).

The total reaction volume was 3.5 ml and it was buffered with Tris-HCl (0.1 mol, pH 7.4). The enzyme activity was quantified by using an extinction coefficient of 8300 for MTX. One unit corresponds to the amount of enzyme required to hydrolyze the 1 µmol of MTX per min.

To find out the stability of the enzyme either native enzyme or its HSA nano complexes were incubated with human serum at 37 °C and 50 rpm. Then samples were withdrawn at different points of time and assayed for the enzyme activity as described above. The release of enzyme from the enzyme-HSA nanocomplexes was studied *in vitro* by incubating the nanocomplexes with human serum at 37 °C and 50 rpm. At different time intervals, the serum was withdrawn, and nanocomplexes were pelleted by centrifuging at 10,000 rpm and 4 °C for 20 min and the released enzyme present in the supernatant was assayed on the MTX. The functionality of the nanocomplexes or native enzyme was studied *in vitro* by incubating either one of them with MTX (300 µM) containing human serum at 37 °C and 50 rpm. At different periods of time, the MTX present in the serum was monitored by measuring the optical density at 320 nm.

Isolation of the mutant

The parent strain of *V. paradoxus* was grown to the OD of 0.5 (at 578 nm) in the folic acid (0.1%) medium containing different salts. Then bacterial cells were diluted 1:10 and plated on the folic acid agar plates and exposed to the UV light (302 nm) to get 50% killing. Later the plates were incubated overnight at 4 °C in the dark and thereafter at 26 °C for 24h. Survived colonies were picked onto the folic acid agar plates coated with MTX (0.1 mM) and incubated at 26 °C for 24h. Emerged colonies were grown in the folic acid medium, and bacterial cell lysate was used for assaying the MTX degrading enzyme activity as described in the section 'enzyme assays'. The mutants producing higher enzyme activity compared to the parent were repeatedly subcultured and the mutant that stably overproduced the enzyme was selected.

Purification of the enzyme

The mutant culture of *V. paradoxus* was grown on the folic acid medium at 26-30 °C and 150 rpm for 24 h. The grown culture was centrifuged at 5000 rpm and 4 °C for 20 min and the cell pellet was washed twice with Tris-HCl buffer (0.05 mol, pH 7.4, 0.2 mmol $ZnSO_4$) and re-suspended in the same buffer containing 0.5 mmol EDTA and 0.2 mmol PMSF and sonicated (250 W, 30 KHz) on the ice. The cell suspension was centrifuged at 5000 rpm and 4 °C for 20 min and supernatant was precipitated with 80% ammonium sulphate at 4 °C. The precipitated protein was dissolved into Tris-HCl buffer (0.1 mol, pH 7.4) and dialyzed against the Tris-Acetate buffer (0.02 mol, pH 9.0) containing 0.2 mmol $ZnSO_4$ to remove the ammonium sulphate. Then the sample was loaded on the DEAE-cellulose column equilibrated with Tris-Acetate buffer (0.02 mol, pH 9.0) containing 0.2 mmol $ZnSO_4$ and the bound enzyme was eluted with 0.2-1.0 mol NaCl prepared in the same buffer. Fractions showing the enzyme activity were mixed and concentrated by using lyophilizer after the dialysis. The sample was loaded on the Sephadex-G-100 column equilibrated with Tris-HCl buffer (0.05 mole, pH 7.4, 0.2 mM $ZnSO_4$) and eluted with the same buffer. The fractions having the major enzyme activity were mixed and the purity of the enzyme was checked on the 10% SDS-polyacrylamide gel. The protein concentration was estimated by Bradford assay.

Preparation of nano-complexes

The enzyme-HSA nano-complexes were prepared by the desolvation-crosslinking method as described earlier [8]. Briefly, the enzyme was mixed with HSA in the ratio of 1:25 in the 0.1 mol Tris-HCl (pH 7.3, 0.2 mmol $ZnSO_4$) buffer containing 300 mM NaCl. The final protein concentration was 6 mg/ml. Four ml of acetone was added to the 1 ml of enzyme and HSA mixture (on the ice with mild stirring) drop by drop at the rate of 1 ml/min. Then 4 µl of glutaraldehyde (2.5 % in water) was added and continued the stirring for 20 min. The mixture was centrifuged at 1000 rpm and 4 °C for 5 min and the supernatant was removed. The pellet was suspended in the required amount of buffer and sonicated for 1 sec (20 cycles, bath Sonicator, 100 W, 33 KHz) on ice. The sample was assayed for the activity of the enzyme on MTX as described above.

Transmission electron microscopy

Suspended enzyme-HSA nano-complexes were drop casted on the carbon coated copper grid by placing a drop of the sample and allowing it to air dry at room temperature for 2 h. The image was captured at the resolution of 500 nm by using FEI Technai Transmission Electron Microscope. Average size and shape of the nano-complexes were studied.

RESULTS AND DISCUSSION

The parent culture that produces MTX degrading enzyme was isolated from Rock Creek mud by the enrichment culture [2]. This organism can use MTX as a sole carbon and nitrogen source [2]. This enzyme showed peptidase activity, and it appears to have considerable specificity to the carboxy terminal glutamate or glutamine [3], accordingly this MTX degrading enzyme was named as carboxypeptidase G [3]. This enzyme converts MTX into non-toxic glutamate and 4-amino-N [10]-methylpteroate, and the parent culture utilizes the derived glutamate [2]. This parent culture didn't show any growth on the carbon sources i.e. glucose, lactose, sucrose, maltose, glycerol and soluble starch and thereby there was no MTX degrading enzyme activity on these sources (table 1). However, parent strain could be able to grow on the folic acid, MTX and sodium glutamate and produced highest MTX degrading enzyme activity on the folic acid followed by sodium glutamate and MTX (table 1). When different nitrogen sources were added to the folic acid medium, the parent strain didn't show any improvement in the production of the enzyme (table 2). Hence, folic acid itself is sufficient for the organism to produce the MTX degrading enzyme.

Table 1: Effect of carbon sources on the production of MTX degrading enzyme by the parent strain of *V. paradoxus*

Carbon source	Activity (Units)
Glucose	0
Lactose	0
Sucrose	0
Maltose	0
Glycerol	0
Soluble starch	0
Folic acid	0.168
Methotrexate	0.067
Sodium glutamate	0.086

Table 2: Effect of addition of nitrogen sources to the folic acid medium on the production of MTX degrading enzyme by the parent strain of *V. paradoxus*

Nitrogen source	Activity (Units)
Potato extract	0.170
Corn steep liquor	0.198
Peptone	0.151
Soyapeptone	0.212
Diammonium hydrogen phosphate	0.100
Ammonium dihydrogen phosphate	0.156
Ammonium nitrate	0.142
Ammonium sulfate	0.179
Ammonium chloride	0.103
Tryptone	0.224
Folic acid alone	0.229

Physiological mutations have credible importance in the strain improvement for various fermentation processes. Hence, the strain improvement through the induced mutation is a regular practice in all industrial processes that use microbes. In all these studies, mutations were induced with ultraviolet rays, ionizing radiations and chemicals like lithium chloride, camphor, nitrous oxide, etc. Microbes were improved for the enzyme production by subjecting the spores to UV rays [9, 10]. However, microbes producing the therapeutic MTX degrading enzymes were not explored for the strain improvement. The mutations were induced into the parent strain of *V. paradoxus* by using UV radiation and the mutant (no. 1068) that overproduces the MTX degrading enzyme was isolated as described in the materials and methods section. The mutant (1068)

produced around 6 times higher levels of MTX degrading enzyme compared to the parent in the folic acid medium after 20 h of fermentation (fig. 1) suggesting its advantage for the industrial production of the enzyme. When time course studies were conducted the parent showed the production of the enzyme at 12 h and it reached maximum at 18 h and almost no change thereafter (fig. 2). Mutant showed higher production of the enzyme compare to the parent at all time points (fig. 2). However, the mutant showed the highest production at 24 h and slight decrease afterwards (fig. 2). Overproduction of the MTX degrading enzyme by the mutant at the same fermentation period will benefit the pharma industry.

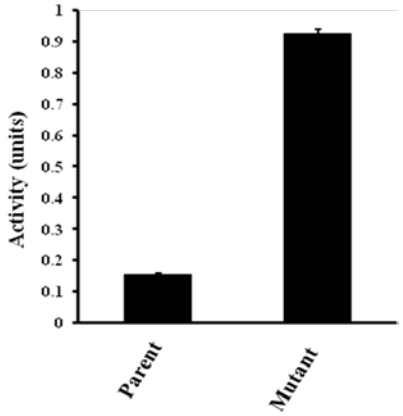

Fig. 1: Bar diagram showing the MTX degrading enzyme activities produced by the parent and mutant strains of *V. paradoxus*

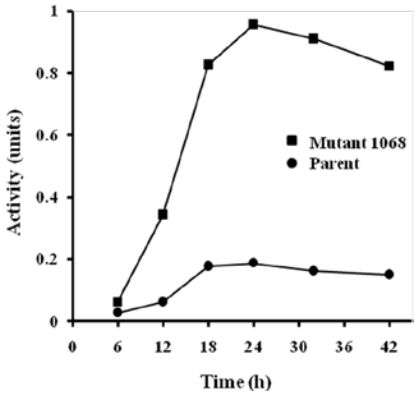

Fig. 2: Graph showing the time course studies on the production of MTX degrading enzyme by the parent and mutant strains of *V. paradoxus*

Though the microbial enzymes are helpful during the therapy, they are foreign to the human body and raise an immune response when injected, which will limit their repeated administration. Another problem is their instability in the system. Hence, one has to evolve with safe and effective carriers for systemic administration of enzymes for therapeutic purposes. Non-viral vectors are advantageous since they will minimise the host immune response. Number of non-viral carriers, especially nanoparticle systems were developed using polymers like poly (D,L-lactic-co-glycolic acid) (PLGA) [11], chitosan [12], poly-L-lysine [13]; dendrimers like polypropylene imine [14] and lipids like ethyl dimyristoyl phosphatidylcholine [15], 1,2-distearyl oxy-N,N-dimethyl-3-aminopropane [16], 1,2-dioleoyl-sn-glycero-3-trimethyl ammonium propane [17]. These delivery systems achieved moderate success due to the safety issues of the carrier materials. Among the above-mentioned carriers, PLGA, the only material approved for the human application, was criticized for incomplete delivery [18], restricting the complete utilisation of therapeutic dose. Hence, new carrier systems have to be explored to overcome such problems and to achieve safe and efficient delivery.

HSA remains a non-explored carrier for the delivery of the therapeutic enzymes even though it was approved for the parenteral use in humans. It is a 66 KDa protein and bind to different endogenous and exogenous molecules either covalently or reversibly with high affinity allowing it to serve as a transporter for various compounds [19]. Binding to HSA often results in the increased plasma solubility, reduced toxicity and increased the half-life of the bound molecule *in vivo* [20]. Another study revealed that HSA nanoparticles sustained the *in vitro* release of superoxide dismutase gene over 6 d, stabilized it against degradation and caused no cytotoxicity in ARPE 19 cells [21]. Its endogenous nature renders HSA highly efficient and safe as a carrier for therapeutic enzyme delivery. Hence, in the current study, HSA was used as a carrier material in the nanoparticles for the delivery of the therapeutic MTX degrading enzyme from the mutant strain of *V. paradoxus.*

The purified enzyme was used for the synthesis of enzyme-HSA nanocomplexes and they were prepared by adopting desolvation-cross linking method as described in the 'materials and methods' section. The complexes were in a rectangular shape with the length and breadth around 400 and 500 nm (fig. 3) respectively suggesting their nano size. When enzyme-HSA nanocomplexes or native enzyme was incubated with human serum at 37 °C *in vitro* with mild shaking the native enzyme lost most of the activity after 2 h and complete activity after 4 h (fig. 4) suggesting its short stability in the serum, whereas, enzyme-HSA nanocomplexes retained their complete activity up to 8 h and most of their activity up to 20 h of incubation (fig. 4). This suggests the better stability of the enzyme-HSA nanocomplexes in the serum compared to the native enzyme. This stability could be due to the protective effect of HSA since proteases present in the serum will degrade the enzyme. However, enzyme-HSA nanocomplexes lost their major activity after 24 h (fig. 4).

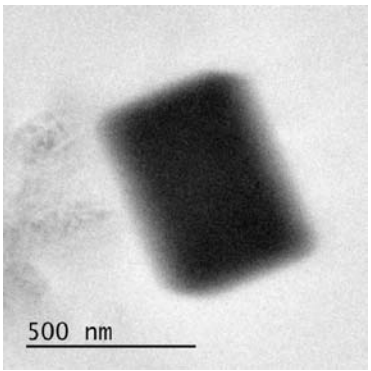

Fig. 3: Transmission electron microscopic image of enzyme-HSA nanocomplex

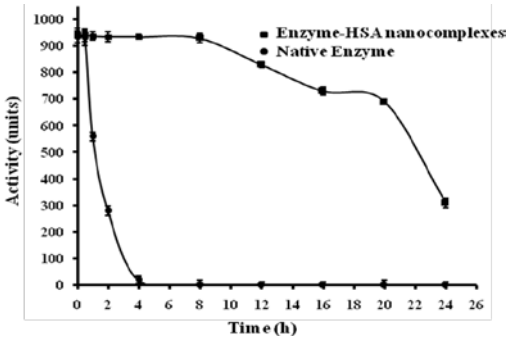

Fig. 4: Graph showing the activity of the native enzyme and its HSA nanocomplexes when incubated with the human serum at 37 °C for different periods of time

When the release of the enzyme from the enzyme-HSA nanocomplexes was studied *in vitro* by incubating the nanocomplexes with human serum around 80 % of the enzyme was released after 10 min and there was a complete release of the enzyme after 60 min (fig. 5) suggesting the loose binding of the majority of the enzyme to the HSA. The native enzyme could able to show significant degradation of the MTX-only after 30 min and there was no degradation after that (fig. 6, black bars) and this could be due to the degradation of the native enzyme by the proteases present in the serum. However, the enzyme-HSA nanocomplexes showed the significant degradation of the MTX just after the 5 min itself, and they degraded the MTX better than the native enzyme at all the points (fig. 6, grey bars) suggesting the protecting effect of the HSA on the enzyme in the nanocomplexes.

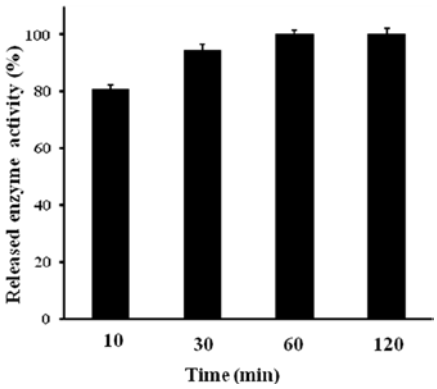

Fig. 5: Bar diagram showing the % release of the enzyme from the enzyme-HSA nanocomplexes when incubated with the human serum at 37 °C & 50 rpm for different periods of time

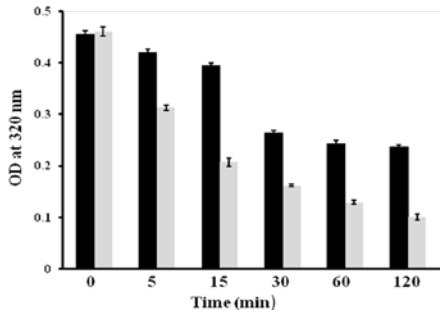

Fig. 6: Bar diagram showing the effect of native enzyme (black bars) or its HSA nanocomplexes (grey bars) on the MTX when they were incubated for different periods of time with the human serum containing MTX at 37 °C & 50 rpm

CONCLUSION

Due to the potential ability of the MTX degrading enzymes to overcome MTX toxicity increasing their production will definitely benefit the pharma sector. Strain improvement of the *M. verrucaria* was carried out to increase the production of MTX degrading enzyme since different carbon and nitrogen sources didn't improve the production from the parent strain. Mutant produced significantly higher levels of enzyme compared to the parent, and hence, usage of mutant will be advantageous for the industrial production of the enzyme. Enzyme-HSA nanocomplxes showed better serum stability and functionality compared to the native enzyme suggesting their better suitability for the therapeutic applications.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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