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Purification and Characterization of L-Arginine deiminase from Vibrio alginolyticus 1374

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ABSTRACT:

Aim: The aim of our present study was to purify and characterize l-arginine deiminase isolated from marine Vibrio alginolyticus1374.

Methodology: Arginine deiminase (AD), an arginine-degrading enzyme, has been used in the treatment of tumours, sensitive to arginine deprivation, such as malignant melanoma and hepatocellular carcinoma. Proteins of microbial origin are always associated with mild to severe hypersensitivities. Moreover, proteins from terrestrial sources were found to exhibit stability problems at physiological environment. Hence considering the fact that the characteristics of the enzyme depends upon sources from which it has been isolated, we have use marine organism for the present study expecting to yield therapeutically and physiologically stable enzyme. ADI production was carried out under optimal conditions by shake flask method. The enzyme thus obtained was purified by ammonium sulphate fractionation, ion exchange and gel permeation chromatography. The purity of the enzyme was further confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and it was characterized to know its properties.

Results: It was active at physiological pH, showed high substrate specificity towards l-arginine. It also exhibited high salt and temperature tolerance indicating good scope for its industrial and therapeutic applications.

Conclusions: ADI obtained from Vibrio alginolyticus 1374 can be a good candidate for the treatment of human cancers.

Keywords: *L-Arginine deiminase (ADI), Vibrio alginolyticus 1374; GU726873, ion exchange chromatography, gel permeation chromatography.*

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INTRODUCTION:

Vibrio alginolyticusis a halophilic microorganism generally found in marine waters[1, 2]. It is recognized as an important human pathogen causing mild superficial wound and ear infections [3]. Vibrio alginolyticus and various other microorganisms use ADS for energy production and for survival under extreme acidic conditions[4,5]. Arginine metabolism via ADS (Arginine Deaminase System) has been shown to generate sufficient energy to maintain growth of several bacteria such as Enterococcus faecalis[6-10]. Next to the natural role of energy production, arginine deiminases were found to be effective in treatment of arginino succinate synthetase (ASS) negative tumors[11, 12]. These tumors are auxotrophic in nature and they face nutrient starvation in the presence of arginine degrading enzymes such as ADI or l-arginase [13-18]. Hence these enzymes can be used for arresting the growth of several cancer cells. Beneficial effects of application of ADI have been also shown in treatment of certain cancers[19]. Even though the

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presence of ADI is found in many micro-organisms, the yield obtained from them is very less. Hence in our preliminary studies we have isolated a potential ADI producer from marine sources and optimized various nutritional as well as physical parameters for optimal production of the enzyme [20]. More over to use ADI as an effective candidate in the treatment of human cancers, it should be stable at biological pH and temperature. Hence in the present study we have purified and characterized the enzyme to know its properties. Immunogenicity and short plasma t ½ time associated with bacterial proteins such as ADI in the human body can be prevented by linking with polymers such as polyethylene glycol, dextran etc without loss of enzymatic activity.

MATERIALS AND METHODS:

Chemicals:L-Arginine,Nutrient agar used in the present study was procured from Hi-Media Laboratories, Bombay. Ammoniumsulphate, DEAE Sephacel, Sephacryl S-200 column, Sodium dodecyl sulphate polyacrylamide gel and remaining chemicals were purchased from Sigma Aldrich, Bengaluru, India.

Inoculum preparation : Marine Vibrio alginolyticus 1374 isolated from coastal areas of Chirala beach was maintained in fresh cultures of nutrient agar slants. 5ml of sterile distilled water was added to the nutrient agar slants, mixed well and inoculated into sterile nutrient broth medium and incubated at 37 ^{0} C. The 2% of the resulting suspension was used as an inoculum.

Production: Production media containing maltose – 2g, soya bean meal -2g and l-arginine – 2g in sea water based mineral arginine medium of pH 8 was found optimal for the production of ADI in our previous studies. A 2% of the inoculum was added into the production medium and incubated at 37°C for 120 h[21].

Purification: Cells from the medium were harvested by centrifugation at 10,000 rpm for 30 min. The supernatant thus obtained was subjected to purification by ammonium sulphate fractionation followed by ion-exchange column (1.5X30 cm of DEAE-Sephacel) and gelfiltration chromatography (SephacrylS-200 column (2.5x55cm). The purity of the enzyme was further confirmed by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) as described by Laemmli[22].

Analytical techniques: Citrulline concentration was used to measure enzyme activity colorimetrically using a modified version of the method described by Oginsky[23].
Enzyme protein: Protein content in the sample was determined by Lowry's method and the values were expressed in mg[24].

Enzyme characteristics: The purified ADI was characterized for its various properties. The characters analyzed included effect of pH (4 -10) and temperature (30 -80^{0}C) on enzyme activity and stability, effect of NaCl (0-20%) on enzyme activity, effect of substrate concentration (L - arginine; 0.01 - 1.0 M) on enzyme activity, effect of L-arginine (0.01M) and NaCl (10%) on temperature stability of ADI and the substrate specificity the enzyme.

RESULTS:

Enzyme purification:

ADI was purified to homogeneity after successive steps of purification. The crude extract contained 360 mg of protein and showed a total ADI activity of 192U units with a specific activity of 0.53 IU/mg protein. After final step of purification process the extract showed the specific activity of 280.6 IU/mg with yield of 43.5% (Table 1).

Determination of molecular weight

SDS-polyacrylamide gel electrophoresis of the purified l-arginine deiminase together with several proteins with known molecular weight indicated that the enzyme had a molecular mass of approximately 48 kDa (not shown).

Table 1: Purification of L-arginine deiminase

Step	TotalEnzyme Activity(IU)	TotalProtein (mg)	Specific Activity (IU/mg)	%Recovery	Purification Fold
Crudeextract	192	360	0.53	100	1
(NH4)2SO4 fractionation fractionation	169	265	0.637	88	1.20
Ion-exchange chromatography	112	0.87	128.7	58.3	242.8
Gelfiltration chromatography	87	0.31	280.6	45.3	529.43

Enzyme characteristics:The enzyme was active over a range of pH 4-10 with optimum at pH 8.0 (Fig.1). The activity considerably decreased at both low pH (5.0) and high pH (10.0).

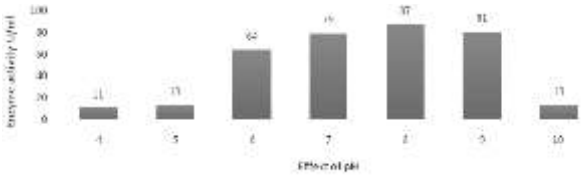

Fig 1: Effect of pH on the enzyme activity

The enzyme was active over wide temperature rangeof 25 and 50^{0}C (Fig. 2). The enzyme had highest activity at 37 ^{0}C.

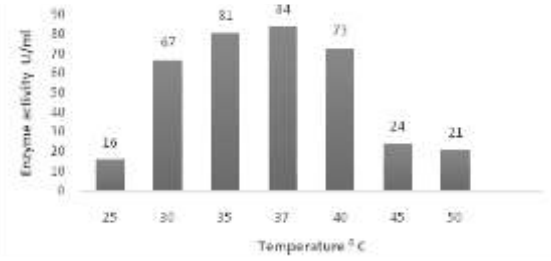

Fig 2: Effect of temperature on the enzyme activity

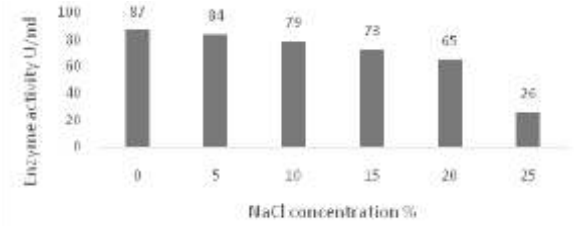

Fig.3: Effect of NaCl concentration on enzyme activity.

It retained its maximum activity between 0 to 15% NaCl concentration which indicated its salt tolerant nature of ADI fromVibrio alginolyticus (fig 3).

Effects of metal ions and some compounds:As shown in fig. 4, cobalt ions were important forthe activity of the enzyme. Other metal ions such as manganese, calcium and irontested hardly affected the enzyme activity.

Arginine deiminase was inhibited by sulfhydryl inhibitors like p-chloromercuribenzoate, Hg^{2+} and mersaryl (fig.5). As shown in fig. 6 ADI is not allosterically regulated by the intermediates and metabolic products of arginine degradation. No significant changes in ADI activity were observed in the presence of ATP, citrulline, and carbamoyl phosphate, respectively. The enzyme shows high substrate specificity towards l-arginine (fig.7).

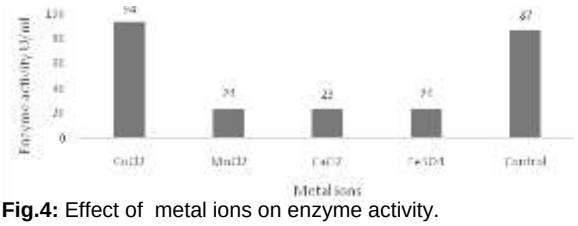

Fig.4: Effect of metal ions on enzyme activity.

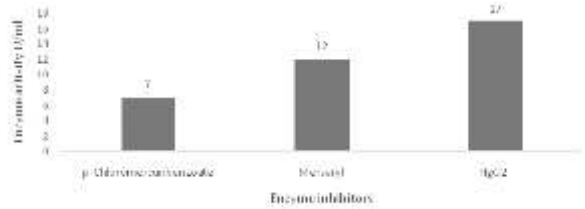

Fig.5: Effect of enzyme inhibitors on enzyme activity.

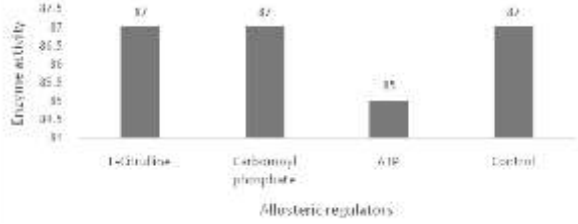

Fig.6: Effect of allosteric regulators on enzyme activity.

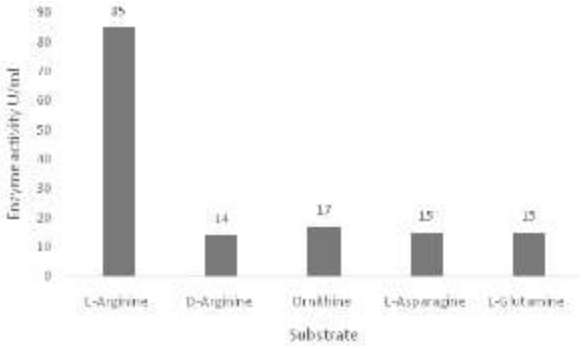

Fig. 7: Effect of substrates on enzyme activity.

DISCUSSIONS :

The impurities present in crude extract were removed by step by step process of purification. The specific activity of crude extract before purification was 0.53 IU/mg. Upon precipitation with ammonium sulphate, the specific activity was found to be increased by 1.2 fold and total protein content was found to be decreased by 1.35 fold. This indicates that the fractionation range ammonium sulphate used for precipitation was effective enough in removing proteins which are contaminants, with subsequent loss of total activity. With ion exchange chromatography, the specific activity was found to increase by 242.8 fold with decrease in protein concentration to about 413.7 times which indicates an efficient removal of contaminants. GFC was performed as final step of purification. The specific activity after GFC was found to be increased by 529.43 fold, with total protein content decreasing by 1161.2 fold. Three bands were observed in SDS-PAGE after IEC. After GFC, only one band was observed. From the bands observed, it can be claimed that the enzyme was purified successfully and its molecular mass was found to be approximately 48 kDa. Where as in case of Mycoplasma arthritidis and Mycoplasma arginini it was found to be 49 and 48 kDa respectively on SDS PAGE[25,26]. The specific activity of the purified enzyme from Vibrio alginolyticus1376 was found to be 280.6 IU/mg which is more when compared to Enterococcus (5.1IU/mg), Pseudomonas putida (76 ± 0.03 IU/mg) and recombinant E. coli (30-34 U/mg) [27-29].

The enzyme was active over a range of pH 4-10 with optimum at pH 8.0. The activity considerably decreased at both low pH (5.0) and high pH (10.0). ADI isolated from various sources were shown to have slightly acidic to neutral pH. ADI isolated from Streptococcus pyogenes M4959 was having optimal pH at 6.5 whereas that of Lactobacillus Buchneri NCDO110, Pseudomonas putida, Escherichia coli was at pH 6[30-32]. The enzyme had highest activity at 37 ^{0}C. The fact that ADI from Vibrio alginolyticus 1374 obviously works best at 37^{0}C and pH 7.4 makes it a suitable candidate for the application in treatment of ASS-negative tumors. Immunogenicity of bacterial proteins such as ADI in the human body can be circumvented by formulation with polyethylene glycol without loss of enzymatic activity.

It retained its maximum activity between 0 to 15% NaCl concentration which indicated its salt tolerant nature of ADI from Vibrio alginolyticus. Metal ions acts as co factors for several enzymes. Of various ions tested cobalt ion was important for the activity of the enzyme. Other metal ionshardly affected the enzyme activity. Arginine deiminase was inhibited by sulfhydryl inhibitors like p-chloromercuribenzoate, Hg2 +, and mersaryl. ADI isolated from Pseudomonas has also been reported to be inhibited by sulfhydryl inhibitors[33].

As shown in fig. 8 ADI of Vibrio alginolyticusis not allosterically regulated by the metabolites of arginine metabolism. No significant changes in ADI activity were observed in the presence of ATP, citrulline, and carbamoyl phosphate, respectively. While citrulline and carbamoyl phosphate had no effect on the activity of ADI of other bacteria either, for L. buchneri an inhibitory effect of ATP on ADI activity has been shown [34].

The kinetic constants of arginine deiminase:

Michaelis–Menten constant (Km) and maximum velocity (Vmax) of Vibrio alginolyticus 1374 (ADI reaction) were calculated according to Line weaver- Burk plot. Results showed that the average of Km and Vmax of l-arginine deiminase were, 1.51 ± 0.23 mM and 120.48 ± 3.24 U/ml//min respectively. The small value of Km indicates high substrate specificity of ADI towards arginine. ADI isolated form E. faecalis has Km and Vmax values of 3.2686 mM and 2.44 M/min [35]. The Km of ADI from Mycoplasma arginini was 0.2 mM and the Vmax was 50 U/mg[36]. The Km and Vmax values for arginine deiminase of Lactococcus lactis were 8.67 mM and 344.83 mol/min, respectively[37]. The purpose of use Michaelis–Menten constant is to estimate the approximate value of l-arginine levels in the cells for using it in a comparative study between enzymes isolated from different microorganisms when Km is low or small, the affinity of enzymes to substrate will be high [38].

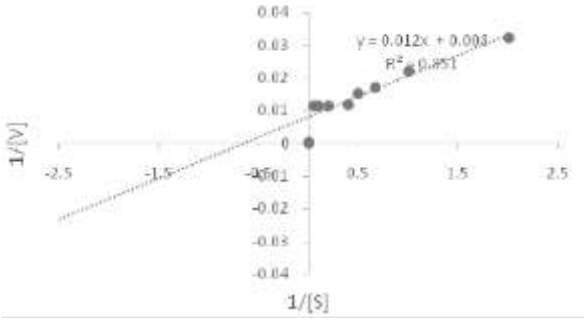

Fig. 8: The Line weaver –Burk plot to determine the Km and Vmax for ADI

CONCLUSIONS:

The ADI produced by the organism has several beneficial properties needed for atherapeutic and industrial enzyme. It has wide range of activity and stability at various temperatures and p H and is highly salt-tolerant. The substrate specificity towards l-arginine is also very high which means that it could be used in low amounts to achieve the desired effect. Preliminary studies showed that the purified ADI was an effective therapeutic agent in the treatment of cancer (unpublished data). Based on the results obtained in the present study, we conclude that the marine Vibrio alginolyticus 1374 has immense potential for large scale production of ADI having several beneficial properties for its use as an anticancer agent and in the food industry.

LIST OF ABBREVIATIONS

ADS= Arginine deiminase system
ADI = L-Arginine deiminase
ASS = argininosuccinate synthetase
IEC = Ion exchange chromatography,
GFC = Gel filtration chromatography,
SDS- PAGE = Sodium dodecyl sulphate polyacrylamide gel electrophoresis

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