Purification and Characterization of Adenosine Deaminase from *Penicillium politans*

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Abstract: Adenosine deaminase (ADA) an enzyme involved in purine metabolism, which catalyzes the hydrolytic deamination of adenosine to inosine and ammonia, was identified in extracts of *Penicillium politans*. The enzyme was partially purified and separated from cytidine deaminase using cold ethanol and acetone precipitation methods. A purification fold of about 20 and 186 were obtained for adenosine deaminase using Sephadex-G100 and DEAE-Sephadex A-25 chromatographic columns respectively. The enzyme exhibited optimum pH and temperature at 6.0 and 55°C respectively. Exposure of purified adenosine deaminase alone to 55°C, for 5 minutes resulted to a complete loss of activity. FeSO₄, CuSO₄, MnCl₂ and HgCl₂ at a concentration of 1mM and 20 mM caused a complete inhibition of adenosine deaminase activity, whereas calcium chloride and sodium molybdate slightly inhibit enzyme activity. On the other hand ZnSO₄ caused about 30% activation at a final concentration of 20 mM.

Key words: adenosine deaminase, cytidine deaminase, *Penicillium politans*, purification, enzyme properties

INTRODUCTION

Adenosine deaminase-ADA (E.C.3.5.4.) is an essential zinc- metalloenzyme of purine recycling or salvage pathways[2]. It act on RNA (ADARs) catalyzed adenosine to inosine conversion in RNA that is largely double stranded[18]. RNA editing by adenosine deaminase is catalyzed by members of an enzyme family known as adenosine deaminases that act on RNA[3]. In humans, adenosine deaminase activity occurs mainly in two kinetically distinct isoenzymes[10]. In patients with liver diseases such as chronic hepatitis and liver cirrhosis, increased serum adenosine deaminase activities have been reported[8,13]. Iwaki-Egawa and Watanabe[11], purified this enzyme from human and chicken liver. The purified enzyme had a molecular weight of approximately 42000 Da on SDS-PAGE. Adenosine deaminase has a wide spread distribution in animal tissues[19] and in all species the duodenum and spleen are the richest sources[4]. Bacterial tRNA adenosine deaminases (TadAs) catalyzed the hydrolytic deamination of adenosine to inosine at the wobble position of codons in mRNA. The genes encoding these deaminases are essential in bacteria and yeast, demonstrating the importance of their biological activity[16]. A number of these enzymes have been purified[5]. This enzyme is of special interest for its role in regulation of cellular growth and differentiation[9]. In particular, clinical and in vitro studies strongly suggest a casual relationship between genetically determined absence of this enzyme activity and the combined immunodeficiency disease, which is characterized by severe defects in cellular and humoral immunity[1]. In meat technology, this enzyme may play an important role in the post mortem nucleotide metabolism; nucleotide breakdown in the muscle produces inosine and hypoxanthine, and some of these nucleotides contribute to the typical flavor of meat[23,24]. Adenosine deaminase was purified (780 fold) from skeletal muscle of camel (*Camelus dromedarius*) to homogeneity level by using DEAE- Sephadex A-25 chromatography. The pH and temperature optima for enzyme activity were 7-7.5 and 25°C, respectively[2]. In 1974, Pichard *et al.*, studied the uptake and metabolism of adenosine using isolated membrane vesicles from *Micrococcus sodonensis*. Later, Pichard[21] described the purification and some properties of the soluble membrane-bound adenosine deaminase in the same organism. Purification and crystallization of adenosine deaminase from *Pseudomonas iodium* was made by Sakai and Jun[22]. Wolfenden *et al.*[26] purified takadiastase adenosine deaminase from *Aspergillus oryzae* and found that the enzyme has the ability to catalyze the deamination of free adenine, adenine nucleotides and...
oligonucleotides. The present paper reports on the purification and some biochemical properties of the purified adenosine deaminase from a filamentous fungus, namely *Penicillium politans*.

**MATERIALS AND METHODS**

**Organism:** A strain of *Penicillium politans* was obtained from the culture collection of the Department of Microbial Chemistry, Division of Genetic Engineering and Biotechnology, National research centre of Egypt.

**Media:** The organism was cultivated and kept on slants of modified Czapek-Dox's agar medium [Difco Manual, 1953] (g/100 ml) glucose, 3.0; NaNO₃, 0.2; KH₂PO₄, 0.1; KCl, 0.05; MgSO₄.7H₂O, 0.05 and Agar, 2.0. Media were sterilized by autoclaving (1.5 atm, 20 min [solid media 30 min]). The liquid medium used for growing the organism was the same without agar. Fifty ml portions of sterile medium each in 250 ml Erlenmeyer flask were inoculated with 1 ml of spore suspension and incubated at 30°C at static conditions for four days. The mycelia were harvested by filtration.

**Preparation of Cell-free Extracts:** The mats were ground with twice its weight of washed cold sand in a cold mortar and then extracted using distilled water. The slurry so obtained was centrifuged at 5500 rpm for 5 minutes. The supernatant was then used as the crude enzyme preparation for the process of enzyme purification.

**Chemical Methods:** Determination of ammonia was carried out by using Nesslers reagent[24]. Protein was determined according to the method of Lowry et al.,[17]. The protein content of the purified enzyme fractions was determined by the UV absorbance technique of Warburg and Christian [25], (1942).

**Enzyme Assay:** The activity of adenosine deaminase or cytidine deaminase was assayed by measuring ammonia that appeared, when the enzyme preparation was incubated with the ribonucleoside adenosine or cytidine. Units of deaminase activity can be defined as μmoles of the formed inosine or uridine / ml under specified conditions. Specific activity was calculated as μmoles / mg protein. The data given represent the mean value of three repetitions.

**Enzyme Purification:**

**Fractionation by Cold Acetone or Ethanol:** Cold acetone or ethanol (-15°C) was added to the crude extract at concentrations of 0-33%, 30-60%, 60-75% and 75-83.5% respectively. The precipitated protein was separated by centrifugation and dissolved in 6ml potassium phosphate buffer, pH 6.0 (0.02M).

**DEAE-Sephadex A-25 Chromatography:** The DEAE-Sephadex A-25 column (1.5x50cm) was equilibrated with 0.02M potassium phosphate buffer at pH6.0 and fraction N°2 (30-60%) from ethanol treatment was placed on the column. Elution was carried out by batchwise addition of 30 ml portions of increasing molarities of NaCl solution (0.0-0.3M) in 0.02M potassium phosphate buffer pH 6.0. Fractions of 5ml were collected at room temperature (25°C) at a flow rate of 30ml/hr. and the enzyme activity was then assayed in the eluent fractions.

**Gel Chromatography:** 6ml of Fraction N°2 which produced from (30-60%) ethanol precipitation was applied to Sephadex G-100 column (1.5cm x 50 cm) which had been equilibrated with 0.02M potassium phosphate buffer pH6.0. The enzyme was eluted from the column using the same buffer at a flow rate of 0.5ml / min. Each 5ml fraction of the eluent was collected separately at room temperature (25°C) and the activity of deaminase in each fraction was assayed.

**Effect of pH and Temperature:** Citrate-phosphate (pH 3.0-7.0) and potassium phosphate (pH 6.0-8.0) buffers were added separately to the reaction mixtures on an equimolar basis to study the effect of pH values on adenosine deaminase activity. To study the effect of temperature, a series of reaction mixtures were made, each was incubated at different temperature ranging from 20°C-80°C and after a fixed time interval (30minutes), an aliquot was withdrawn from each reaction mixture and analyzed for adenosine deaminase activity.

**Thermal stability behavior:** The effect of high degrees of temperature on adenosine deaminase activity was studied when pure enzyme were subjected to 55°C, 60°C, 65°C and 70°C (in the absence of substrate) for different time intervals (5, 10, 15, 20 and 30 minutes), an aliquot was withdrawn from each reaction mixture and analyzed for adenosine deaminase activity.

**RESULTS AND DISCUSSIONS**

**Results:**

**Purification of adenosine deaminase:** Different trials were made to purify the crude adenosine deaminase of *Penicillium politans*. Fractionation by cold ethanol
Table I: Partial purification of adenosine and cytidine deaminases from *Penicillium politans* by ethanol fractionation.

<table>
<thead>
<tr>
<th>Ethanol conc. (%)</th>
<th>Protein (mg)</th>
<th>Total Deaminase Activity (μ moles)</th>
<th>Specific Activity (μ moles/mg protein)</th>
<th>Activity (%)</th>
<th>Recovery (%)</th>
<th>Purification Fold</th>
<th>Adenosine</th>
<th>Cytidine</th>
<th>Adenosine</th>
<th>Cytidine</th>
<th>Adenosine</th>
<th>Cytidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>31.8</td>
<td>25.5 21.0</td>
<td>0.8 0.66</td>
<td>100 100</td>
<td>1.0 1.0</td>
<td></td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>F, 0 – 30</td>
<td>14.94</td>
<td>0.0 0.0</td>
<td>0.0 0.0</td>
<td></td>
<td></td>
<td></td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>F, 30 - 60</td>
<td>7.56</td>
<td>27.0 3.3</td>
<td>3.57 0.44</td>
<td>105.8</td>
<td>4.5</td>
<td>0.66</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>F, 60 - 75</td>
<td>3.69</td>
<td>6.6 20.4</td>
<td>1.78 5.53</td>
<td>25.8</td>
<td>2.2</td>
<td>8.38</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>F, 75 - 83.5</td>
<td>3.51</td>
<td>0.0 0.0</td>
<td>0.0 0.0</td>
<td></td>
<td></td>
<td></td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
</tbody>
</table>

Table II: Partial purification of adenosine and cytidine deaminases from *Penicillium politans* by acetone fractionation.

<table>
<thead>
<tr>
<th>Acetone conc. (%)</th>
<th>Protein (mg)</th>
<th>Total Deaminase Activity (μ moles)</th>
<th>Specific Activity (μ moles/mg protein)</th>
<th>Recovery (%)</th>
<th>Purification Fold</th>
<th>Adenosine</th>
<th>Cytidine</th>
<th>Adenosine</th>
<th>Cytidine</th>
<th>Adenosine</th>
<th>Cytidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>31.8</td>
<td>25.5 21.0</td>
<td>0.8 0.66</td>
<td>100 100</td>
<td>1.0 1.0</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>F, 0 – 30</td>
<td>12.15</td>
<td>0.0 0.0</td>
<td>0.0 0.0</td>
<td></td>
<td></td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>F, 30 - 60</td>
<td>8.64</td>
<td>22.5 20.6</td>
<td>2.6 2.36</td>
<td>88.2</td>
<td>3.25</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>F, 60 - 75</td>
<td>5.49</td>
<td>0.0 0.0</td>
<td>0.0 0.0</td>
<td></td>
<td></td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>F, 75 - 83.5</td>
<td>3.51</td>
<td>0.0 0.0</td>
<td>0.0 0.0</td>
<td></td>
<td></td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
<td>Adenosine</td>
<td>Cytidine</td>
</tr>
</tbody>
</table>

Fig. 1: Purification of adenosine deaminase in cell-free extracts of *Penicillium politans* by Sephadex-G100 column.

Fig. 2: Elution diagram of adenosine deaminase in cell-free extracts of *Penicillium politans* by DEAE-Sephadex A-25 column.

was carried out and four precipitate fractions were obtained (Table I). The results showed that fraction N°2 (30-60% ethanol) contains higher percentage of adenosine deaminase activity than cytidine deaminase by more than 8 times, however fraction N°3 (60-75% ethanol) contains higher percentage of cytidine.
Fig. 3: Time curve of purified adenosine deaminase from Penicillium politans. Reaction mixture contained: Adenosine, 12.5 μmoles, potassium phosphate buffer, pH 6.0, 250 μmoles, extract protein, 0.165 mg, total volume, 2.5 ml, temperature, 40°C and reaction time as indicated.

Fig. 4: Purified adenosine deaminase activity as a function of pH value in Penicillium politans. Reaction mixture contained: Adenosine, 2.5 μmoles, buffer, 50 μmoles, extract protein, 0.033 mg, total volume, 0.5 ml, temperature, 40°C and reaction time, 30 minutes.

deaminase activity as compared with adenosine deaminase by more than three times. Four acetone precipitate fractions were obtained when the crude extract was fractionated by cold acetone. Adenosine deaminase activity and cytidine deaminase activity were found only in acetone fraction N°2 (30-60%), (Table II). The fraction which was obtained by (30-60%) ethanol treatment was applied to Sephadex G-100 chromatographic column (Fig.1) to separate the two deaminases namely adenosine deaminase and cytidine deaminase and to obtain the highest purification fold of adenosine deaminase. From the results obtained, a purification fold of 20.5 was obtained for adenosine deaminase with fraction N°7 without any contamination of cytidine deaminase. Another trial to obtain a high purification fold of adenosine deaminase was also made, thus fraction N°2 which obtained with (30-60%) ethanol was also applied to DEAE-Sephadex A-25 chromatographic column. From the results obtained in Fig.(2), it clear that adenosine deaminase from Penicillium politans was efficiently purified using DEAE - Sephadex A-25, purification fold of about 186 was obtained with fraction N°47, also the same fraction showed an optimum percentage recovery of about 69% (Fig.2). The resulting high percentage recovery of enzyme can be interpreted by the release of some inhibitors that may effect the enzyme activity in the crude extracts.

Properties of Purified Adenosine Deaminase Of: Penicillium politans:

Time of Reaction: A reaction mixture was set up which contained adenosine, 12.5 μmoles, potassium phosphate buffer, pH 6.0 (250 μmoles) and 1 ml pure enzyme all in a total volume of 2.5 ml. Control reaction mixture without substrate was also made. Reaction mixtures were then incubated at 40°C for 2 hours. At different time intervals aliquots were removed for ammonia determination. From Fig. (3) it can be seen that 80% of adenosine has been converted into product (inosine) by the end of the incubation period (120 minutes).

Effect of pH and Temperature on Enzyme Activity:
The effect of pH on the purified enzyme activity was examined using two buffers (0.2M) at different pH values, namely citrate-phosphate (pH 3.0-7.0) and potassium-phosphate buffer (pH 6.0-8.0) (Fig.4). The enzyme exhibited maximum activity at pH 6.0. At pH 3.0 and 8.0 about 9.1% and 63.6% of adenosine deaminase activity is still present respectively. These results indicate that the deaminase activity has a broad range of pH values. On the other hand different reaction mixtures containing adenosine as substrate were incubated at different degrees of temperature ranged from 20-80°C for 30 minutes. Results obtained in Fig.(5) indicate that optimum temperature was obtained at 55°C and a considerable high amount of enzyme activity was recorded at 50°C (89%) and 60°C.
(83%) as compared with that obtained at 55°C. Increasing the temperature above 55°C resulted to a gradual decrease.

### Table III: Activity of purified adenosine deaminase of *Penicillium politans* in different buffer systems.

<table>
<thead>
<tr>
<th>Type of buffer (pH 6.0)</th>
<th>µmoles NH</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate-phosphate</td>
<td>0.75</td>
<td>68.2</td>
</tr>
<tr>
<td>Potassium-phosphate</td>
<td>1.1</td>
<td>100</td>
</tr>
<tr>
<td>Tris-acetate</td>
<td>1.1</td>
<td>100</td>
</tr>
</tbody>
</table>

Reaction mixture contained: Adenosine, 2.5 µmoles; buffers (pH 6.0), 50 µmoles as indicated; extract protein, 0.033 mg; total volume, 0.5 ml; temperature, 40°C and reaction time, 30 minutes.

### Table IV: Effect of some activators or inhibitors on purified adenosine deaminase by extracts of *Penicillium politans*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>20mM Relative activity (%)</th>
<th>1mM Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>130.4</td>
<td>91.3</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>95.7</td>
<td>91.3</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>60.9</td>
<td>43.5</td>
</tr>
<tr>
<td>Na molybdate</td>
<td>95.7</td>
<td>69.6</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Reaction mixture contained: Adenosine, 2.5 µmoles; potassium-phosphate buffer pH 6.0, 50 µmoles; extract protein, 0.033 mg; compound (2x10⁻² M and 10⁻³ M); total volume, 0.5 ml; temperature, as indicated and reaction time, 30 minutes.

Results obtained show that a complete loss of enzyme activity was noticed when the enzyme was incubated at 55°C (in absence of substrate) for 5 minutes indicating that adenosine plays a major role in the protection of catalytic site of enzyme at high degrees of temperature and that adenosine deaminase is less stable towards elevated degrees of temperature.

### Effect of Some Metal Ions:

The tested compounds investigated were zinc sulphate, ferrous sulphate, copper sulphate, calcium chloride, manganese chloride, magnesium chloride, sodium molybdate and mercuric chloride. They were added to each reaction mixture at a final concentration of 2x10⁻² M and 10⁻³ M. Table IV shows that FeSO₄, CuSO₄, MnCl₂ and HgCl₂ caused complete inhibition of enzyme activity at both concentrations, whereas CaCl₂ and sodium molybdate slightly decrease the enzyme activity. On other hand ZnSO₄ caused activation of enzyme activity at a final concentration of 2x10⁻² M.

### Discussion:

In this work we reported the presence of adenosine deaminase in extracts of *Penicillium politans*. The enzyme was partially purified and separated from cytidine deaminase using cold ethanol and acetone precipitation methods. Adenosine deaminase of
\textit{Pseudomonas iodonum} IFO 3558 was found to be unstable and ethyl alcohol was found to protect the enzyme almost completely from inactivation\cite{22} in agreement with our results that were obtained in extracts of \textit{Penicillium politans}. Concerning the effect of metals, adenosine deaminase from \textit{Micrococcus sodonensis} is unstable, particularly at low-protein concentration, it requires Mg\textsuperscript{2+} or Mn\textsuperscript{2+} ions, for stabilization\cite{20}. However, stabilization effects of neither Mg\textsuperscript{2+} nor Mn\textsuperscript{2+} were observed in case of adenosine deaminase from \textit{Pseudomonase iodonum} IFO 3558\cite{22}. In contrast to this result MnCl\textsubscript{2} caused a complete inhibition of adenosine deaminase in extracts of \textit{P. politans}. Ling et al.\cite{15} purified adenosine deaminase approximately 22-fold with a 25% activity yield, and found that optimal activity was obtained at pH 8.0 and 37°C in an extract of \textit{Klebsiella sp}. LF1202 when the organism was cultured in a medium containing adenosine as the sole source of carbon and nitrogen, whereas in our study adenosine deaminase was found in cell-free extracts of \textit{P. politans} when cultured on modified Czapek Dox’s medium, in which glucose (3%) was amended as the only carbon source. These results indicate that adenosine deaminase is a constitutive enzyme in this organism. The scheme of purification of adenosine deaminase in \textit{P. politans} under study is relatively simple, since, it required only DEAE-Sephadex A-25 column. By this procedure it was possible to separate adenosine deaminase from cytidine deaminase of the same organism and purify adenosine deaminase with about 186 purification fold whereas adenosine deaminase from camel skeletal muscle\cite{2] was purified using gel filtration and ion exchange column chromatography using FPLC and the enzyme was purified 778 fold, the results presented in this paper show that \textit{P. politans} adenosine deaminase is similar to that of \textit{Camelus dormedarius} in that is an essential zinc-metalloenzyme. On the other hand, Jun et al.\cite{12} purified extracellular adenosine deaminase from the culture filtrate of \textit{Streptomyces sp.} J-845S by ammonium sulphate precipitation and column chromatography on DEAE-Sephadex A-50, Sephadex G-100, and CM-cellulose. These authors reported that the enzyme was stable at pH 3.5-5.5, the optimum pH for the reaction was 5.0-6.0, and the optimum temperature was 55°C in agreement with our results.

\section*{REFERENCES}


