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Purification and characterization of intestinal adenosine deaminase from mice

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Abstract

Adenosine deaminase (ADA) was isolated from small intestine of mice and purified to utmost homogeneity. SDS-PAGE of purified ADA gave a molecular weight of 41 kDa. Western blot analyses gave a single reactive band at 41 kDa and the other band was an associated ADA binding protein. The purified enzyme was more stable in the alkaline pH. The optimum pH and the pI values were about 7.0 and 4.96, respectively. Km values of the small intestinal ADA for adenosine and 2′-deoxyadenosine were 23 and 16 μM, respectively. Purine riboside was a competitive inhibitor with Ki of 5 μM, whereas 2′-3′-o-isopropylidene adenosine acted as an uncompetitive inhibitor (Ki 66 μM). Activity of ADA was inhibited by the presence of theophylline (-40%), caffeine (-30%), and L-cysteine (-50%). Significantly, Hg^{2+} (100 μM) inhibited 98% of the initial ADA activity. In addition, various purine analogs such as inosine, purine, α-adenosine and adenine showed variable inhibitions on the activity of ADA. Relative ADA activity towards 3′-deoxyadenosine and 6-chloropurine riboside was lower by 30% and 40%, respectively. However, the activity towards 2′-o-methyl adenosine was higher (30%) compared to the activity obtained using adenosine. (Mol Cell Biochem **204:** 127–134, 2000)

Key words: intestinal adenosine deaminase, mice, purification, physicochemical and kinetic characterization

Introduction

Adenosine deaminase (ADA; EC 3.5.4.4) catalyzes the irreversible hydrolytic deamination of adenosine and 2′-deoxyadenosine to inosine and 2'-deoxyinosine, respectively [1]. The physiological function of ADA is crucial in regulating the steady state concentration of adenosine and 2′-deoxy-adenosine in a variety of systems, specially immunology, neurological and cardiovascular [2]. Genetic defect of ADA gene has been correlated with the lack of ADA enzyme giving rise to the severe combined immunodeficiency (SCID) disorder [3,4], where both T- and B-lymphocytes are unable to proliferate and mount the antigenic challenges. Changes in the activity of ADA have also been observed in many human diseases, like acquired immune deficiency syndrome (AIDS), various lymphomas, leukemias, anemia, short-limbed dwarfism, hepatitis, and hepato-cellular jaundice [5,6]. ADA is phylogenetically ubiquitous and widely distributed. Mammalian tissues, where the highest level of the enzyme activity is found, are thymus, spleen, gastrointestinal tract and placenta while muscle, lung and kidney show low level of ADA. In mice, the highest level of ADA is found in thymus and alimentary canal where the enzyme accounts for as much as 20% of the total protein [7]. High level of this enzyme in thymus and intestine presumably reflects the critical role of ADA in T-cell maturation [8,9]. Adenosine, a natural substrate of ADA, is believed to stimulate Cl^- secretion in intestinal epithelium which results in movement of isotonic fluid into the lumen, and help hydrate the mucosal surface [10].

Catalytically active ADA is a monomeric zinc metallo-enzyme having 1 mole of Zn^{2+} per mole of protein [11]. The monomeric catalytic moiety, however, may interact with other noncatalytic proteins termed as ADA-binding protein (ADA-BP) [12,13]. As a result, ADA exists in different molecular and electrophoretic forms and their distribution is tissue-specific. High molecular forms of ADA (230–440 kDa) are predominantly present in the tissues exhibiting low specific activity and the smaller forms (30–47 kDa) predominate in high ADA expressing

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tissues [14]. Intermediate molecular forms of ADA are also found in some tissues [15,16]. Recently, 2 ADA-binding proteins that co-precipitated with ADA have been identified, namely CD26 and A1 adenosine receptor [17-19]. ADA catalyzes the hydrolysis of several 6-substituted purine derivatives including a number of clinically important anti-metabolites. Many important drugs including anticancer, antibiotics, analgesics, coronary vasodilators are found to be inhibitors of ADA activity [20]. We have earlier reported an age-specific expression of intestinal ADA activity during postnatal development of mice [21]. Our findings also entailed corticosterone as an inhibitory and dibutyryl-cAMP as a stimulatory signal for intestinal ADA activity. Keeping in view the importance of ADA and its substrate analogs in the physiology of the gastrointestinal tract and also in various immuno disorders and unrelated diseases, we herein purified and characterized ADA from the small intestine of mice to get insight into the mode of regulation of this enzyme by various hitherto unstudied modulators including its related substrates.

Materials and methods

Materials

Small intestines from female Swiss albino mice (120 day old) were collected and processed immediately. Adenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 2′-o-methyladenosine, inosine, α-adenosine, purine, adenine, purine riboside, 6-chloropurine riboside, caffeine, theophylline, 2-mercapto-ethanol, dithiothreitol, L-cysteine, molecular weight markers for gel filtration, agarose, sodium azide, EDTA, Sephadex G-200, DEAE-cellulose were purchased from Sigma Chemical Co., USA. HRP-conjugated anti-rabbit antibody and substrate for HRP were from Bio-Rad, USA. A programmable electrophoresis unit, precasted electrophoresis polyacrylamide gels, agarose buffer stripes and coomassie brilliant blue R 350 strain tablets, and nitrocellulose papers were supplied by Pharmacia Biotech, Sweden.

Isolation and purification of ADA

ADA was isolated and purified from small intestine of mice by the procedure of Jaroszewicz and Kowalczyk [16] with certain modifications of our own. Small intestinal tubes were washed with chilled normal saline, minced and blotted dry. A 10% (w/v) homogenate of pooled small intestine (30 g) from 15–20 mice was made in ice-cold 20 mM imidazole buffer, pH 6.8 containing 0.25 M sucrose using a glass homogenizer fitted with a teflon pestle. All the steps were carried out at 4°C unless mentioned otherwise. The crude homogenate was centrifuged at 27,000 × g for 60 min. The supernatant thus obtained was brought to 40% ammonium sulfate saturation through gradual addition of solid $(NH_4)_2SO_4$ and was centrifuged at 15,000 × g for 15 min. The supernatant was brought to 70% ammonium sulfate saturation, pH adjusted to 6.5 and centrifuged at 20,000 × g for 30 min. The precipitate thus obtained was dissolved in 3–4 ml of 20 mM imidazole buffer, pH 6.8 and dialyzed for 36 h against similar buffer. Dialysate was then centrifuged at 20,000 × g for 30 min and pellet was discarded. The supernatant was loaded onto a Sephadex G-200 column (1.6 × 75 cm), which was pre-equilibrated with 20 mM imidazole buffer, pH 6.8, containing 20 μM sodium azide. Fractions of 3 ml were collected at a flow rate of 14 ml/h. Enzymatically active fractions were pooled, dialyzed against 10 mM imidazole buffer, pH 6.8 containing 10 μM dithiothreitol and 20 μM sodium azide. The dialysate obtained was applied onto a column (2.5 × 14 cm) of DEAE-cellulose, which was pre-equilibrated with 10 mM imidazole buffer containing 20% glycerol, 5 μM Zn^{2+}, 10 μM dithiothreitol and 20 μM sodium azide, pH 6.8 at a flow rate of 30 ml/h. The column was extensively washed with the equilibrating buffer. A linear gradient of 0-0.4 M NaCl in the same buffer was applied to elute the bound fractions. The active peak fractions were collected, pooled and dialyzed against 100 mM sodium citrate buffer, pH 6.0 before further use.

Enzyme assay and protein estimation

The activity of ADA was measured spectrophotometrically in a Hitachi Model U-2000 spectrophotometer by the method of Kalchar [22] and Yoshida and Aikawa [23] with certain modifications [21]. The standard assay was carried out at 25°C in 3 ml of 100 mM sodium citrate buffer, pH 6.0 with 100 μM adenosine and 50 μl of suitably diluted enzyme preparation that gave a linear decrease in absorbance at 265 nm. Protein concentration of enzyme preparation was determined by the method of Bradford [24] using BSA as standard. The activity of ADA was expressed as units (μmol adenosine deaminated per min) per mg protein.

Electrophoretic studies

Native- and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoreses were performed on a programmable pharmacia phastSystem, using precasted gradient gels (continuous 4–15%) according to the protocols described in the user's manual [25]. Similarly isoelectric focusing was performed on a precasted polyacrylamide gel of pH range 3–9. Sample was run in duplicate together with spleen ADA from Sigma. After focusing run is over, the gel was divided in 2 halves. One half was processed for general protein staining

using coomassie brilliant blue, while the other half was washed with 100 mM sodium citrate buffer, pH 6.0 and processed for zymogram analysis [27]. The area along the lane of the sample on the isoelectric focusing gel was sliced into 1 mm sections after removing the plastic gel backing. Each slice was placed into individual eppendorf tubes containing 100 mM sodium citrate buffer, pH 6.0 and crushed gently with a glass rod and left for 3 h at 4°C. Each tube was then centrifuged at 20,000 × g for 30 min. Supernatant thus obtained was assayed for ADA activity.

Molecular weight of purified ADA was determined by SDS-PAGE using standard markers as phosphorylase b (94 kDa), BSA (64 kDa), ovalbumine (43 kDa) carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactoalbumine (14 kDa). A linear calibration curve of R_m versus log molecular weight was plotted and the value obtained is presented in table 2.

Immunological studies

An adult rabbit was immunized by administering 0.2 ml (1 mg/1 ml) of purified intestinal ADA with equal volume of Freund's complete adjuvant subcutaneously. This was followed by two booster doses (0.2 mg) of intestinal ADA with incomplete Freund's adjuvant after an interval of 30 days. Blood was collected after 4 days of second booster dose and the serum was prepared as a source of ADA antibody for further analysis. The cross-reactivity of the antibody with purified ADA was checked by Ouchterlony's immunodiffusion method in 1.5% (w/v) agarose gel at pH 6.8, using BSA, purified malate dehydrogenase and inorganic pyrophosphatase as controls.

Western blotting

Western blotting was carried out for analyzing electrophoretic patterns of ADA protein using the polyclonal ADA antibodies and rabbit HRP-conjugate antibodies as primary and secondary antibodies, respectively. Electrophoretic blotting was performed on pharmacia PhastSystem using semi-dry transfer kit (Phast Transfer) following the instructions given in the user's manual. Immuno-detection of ADA was carried out according to protocol described in the Bio-Rad instruction manual with certain modifications.

Kinetic studies

The kinetic studies for intestinal ADA were carried out using the purified enzyme. Km values for intestinal ADA were determined by Michaelis-Menten and Lineweaver-Burk plots using various concentrations (0–100 μM) of adenosine and 2′-deoxyadenosine as substrates. Ki values were determined by Dixon's plot at two fixed concentrations (40 and 100 μM) of adenosine using varying concentrations of purine riboside and 2′-3′-o-isopropylidene adenosine as inhibitors. ADA activities were measured, using adenosine as substrate in the presence of (100 μM) inosine, purine, α adenosine and adenine. Activities of ADA for various related substrates (100 μM), 3′-deoxyadenosine, 2′-o-methyladenosine and 6-chloropurine riboside were determined using the activity for adenosine as standard control. ADA activities for adenosine in the presence of 100 μM of theophylline, caffeine and Hg^{2+} and 1 mM L-cysteine were also studied.

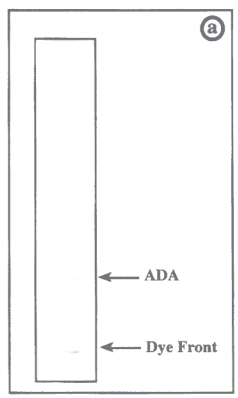
Results

Purification and physicochemical properties of ADA

Using the procedures outlined, the degree of purification of ADA from the small intestine of mice was obtained at 1500-fold with a yield of 6.5% (Table 1). The fold of purification was significantly increased at DEAE-cellulose ion exchange step wherein ADA got eluted as a single peak at an ionic strength of 0.17 M NaCl. The native-PAGE pattern of purified ADA from intestine of mice showed a single band indicating that the ADA has been purified till utmost homogeneity (Fig. 1a). In contrast, SDS-PAGE resolved the purified preparation into 2 bands of molecular weight 31 and 41 kDa (Fig. 1b, left). In order to identify the bands obtained from the purified ADA, Western blotting was performed and only the band having molecular weight of 41 kDa was detected in the Western blotting for ADA (Fig. 1b, right; Table 2). Specificity of the ADA antibody raised in rabbit was examined by double immunodiffusion technique. A single precipitin line for purified intestinal ADA was observed and no precipitin line could be seen with any of the control proteins (Fig. 2). Similar to the SDS-PAGE pattern, in the isoelectric focusing, 2 bands of protein were observed, whereas the marker spleen ADA has been focused at 3 locations as expected (Fig. 3, left). Taking the locations of 3 isoelectric forms of marker spleen ADA as reference, 1 band of purified ADA was located at about pH 4.96 and the other at pH higher than 6. To identify the 2 proteins (bands) of different isoelectric pH, Western blotting and band activity for ADA were carried out. Interestingly, Western blotting detected only the band of isoelectric pH 4.96 (Fig. 3, right). This finding was further strengthened by band activity assay, where the ADA activity was detected in the protein band having isoelectric pH 4.96 but not in the other protein band.

Table 1. Purification chart for intestinal adenosine deaminase of mice

STEPS	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (U)	SPECIFIC ACTIVITY (U/.mg protein)	PURIFICATION FOLD	YIELD (%)
CRUDE EXTRACT	8370.0	167.4	0.02	–	100.00
27,000 × g SUPERNATANT	3653.0	147.7	0.04	2	88.23
$(NH_4)_2SO_4$ (40%–70%)	1023.0	68.4	0.07	3	40.86
G-200 SEPHADEX	10.4	19.0	1.82	91	11.35
DEAE-CELLULOSE	0.4	10.9	30.18	1509	6.51



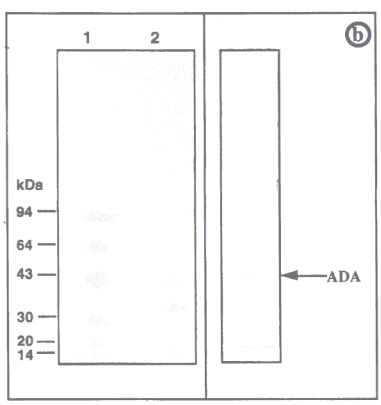


Fig. 1. (a). Native polyacrylamide gel electrophoresis of purified ADA from intestine of mice. 3-5 µg of purified ADA was electrophoresed as described in methods section. Arrows indicate the respective location of ADA and dye front. (b). SDS-polyacrylamide gel electrophoresis (left) of purified ADA (lane 2) using standard low molecular weight markers (lane 1) and Western blotting (right) pattern of SDS-polyacrylamide gel electrophoresis. Arrow indicates the position of ADA reactive band. Similar amount of purified preparation was used for these experiments also.

Kinetic analysis of ADA

The computed Km values of the small intestinal ADA for adenosine and 2′-deoxyadenosine were 23 and 16 µM, respectively (Table 2). Purine riboside inhibited the ADA activity competitively with a Ki of 5 µM (Fig. 4a). In contrast, 2′-3′-o-isopropylidene adenosine acted as an uncompetitive inhibitor with Ki of 66 µM (Fig. 4b).

The pH dependence for the reaction catalyzed by intestinal ADA of mice showed a bell shaped curve, dropping about 50% of the activity below pH 5 on acidic side and above pH 9 on the basic side. Whereas pH stability profile showed a hyperbolic curve with a plateau from pH 6.5–9 (Data not shown). About 95% of the activity of enzyme was retained after preincubating at pH 9 for 45 min whereas, at pH 5 about 45% of the activity was lost. Activity of purified intestinal ADA from mice was inhibited 40%, 30% and 50% in presence of theophylline, caffeine, and L-cysteine, respectively. In the presence of 100 µM Hg^{2+}, 98% of the original ADA activity of mice was lost (Fig. 5a). Activity of purified ADA was also inhibited in presence of purine to an extent of 40–50%. Furthermore, α-adenosine inhibited the activity by 20%, whereas, inosine and adenine gave an inhibition of 10%

Table 2. Values of various physicochemical and kinetic parameters of purified intestinal ADA from mice. Astericks indicate the values obtained by computing the data using Sigma enzfitter programe. ¶ represents the value obtained from linear calibration curves of Rm vs log MW on SDS-PAGE.

Parameters	Values
Molecular weight	41 kDa¶
Km for adenosine	23 µM*
Km for 2′-deoxyadenosine	16 µM*
Ki for purine riboside	5 µM*
Ki for 2′-3′-o-isopropylidene adenosine	66 µM*
pI	4.96

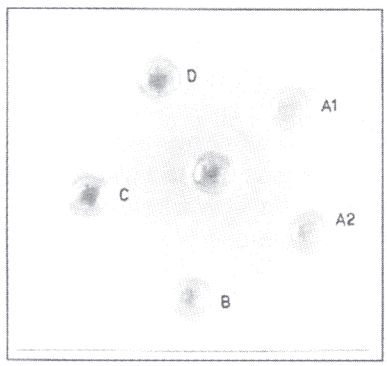


Fig. 2. Ouchterlony double immunodiffusion of mice intestinal ADA antiserum (central well) against purified ADA in duplicate (well-A1, A2), inorganic pyrophosphatase (well-B), BSA (well-C), and malate dehydrogenase (well-D). 40–50 µg of purified ADA with similar amounts of other control proteins were used for the immunodiffusion as detailed in the Materials and methods section.

(Fig. 5b). This indicated inosine, adenine and α-adenosine as weak inhibitors, while purine as a strong inhibitor of ADA. Relative activity of ADA towards 3′-deoxyadenosine, 2′-o-methyladenosine and 6-chloropurine riboside as substrates showed that 3′-deoxyadenosine and 6-chloropurine riboside gave rise to lower activity by 30% and 40% respectively, than it's deamination activity of adenosine. In contrast, ADA activity towards 2′-o-methyl adenosine was higher by 30% compared to the activity towards adenosine (Fig. 5c).

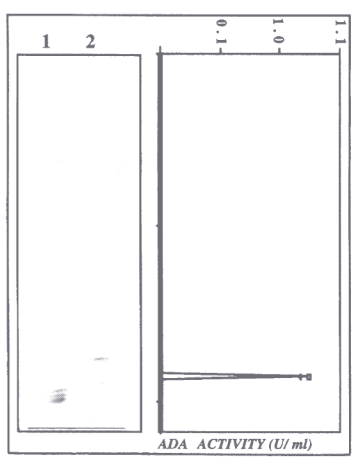
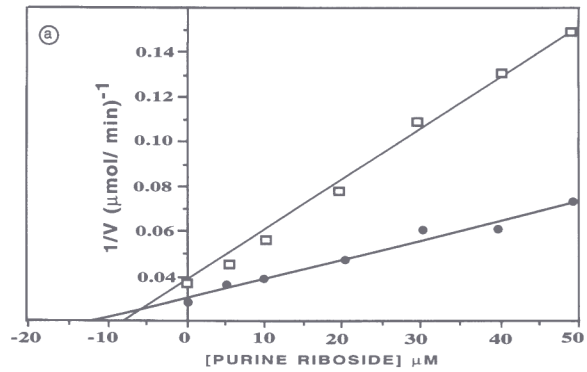


Fig. 3. Isoelectric focusing electrophoresis (left) of purified intestinal ADA (lane 2) using spleen ADA (lane 1) and ADA activity assay of the IFE gel (right). 3–5 µg of purified intestinal ADA and the spleen ADA were used for electrophoresis. The details of experimental procedures are described in the Materials and methods section.



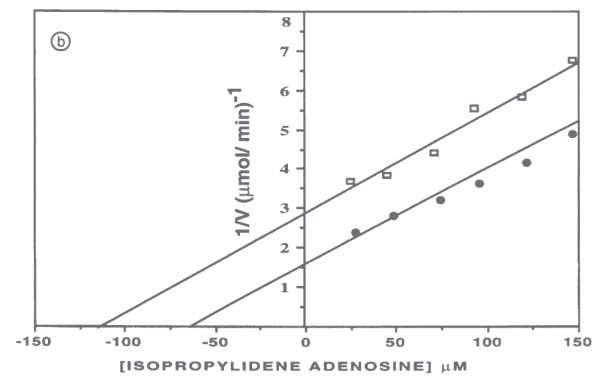


Fig. 4. Dixon's plots for purine riboside (a) and 2′-3′-o-isopropylidene adenosine (b) inhibition of mice intestinal ADA. -□- and -●- are at 40 and 100 µM of adenosine, respectively. 50 µl of purified ADA (0.1 mg/ml) was used for the inhibition studies as given in the Materials and methods section. The plots were drawn using Macintosh Cricket graph v 1.3.

Discussion

Adenosine deaminase has generated considerable importance as its genetic deficiency leads to severe combined immuno deficiency (SCID) disorder where the patient is unable to mount the antigenic challenges due to impairment of both T- and B-cell mediated immune responses. In order to have an enzyme replacement therapy for such patients, the physico-chemical and kinetic characterization of adenosine deaminase from purified preparation may provide the insight into the modulatory properties of the enzyme. We have attempted to purify ADA to utmost homogeniety and characterize its physicochemical and kinetic properties. In previous purification procedures [16,26] the loss of ADA activity was reported during ion-exchange chromatography. We have tried to improve it by addition of DTT, Zn^{2+} and 20% glycerol in the purification buffer during ion exchange separation where the lost of activity was always marked without these additions. This gave higher fold of ADA purification after DEAE-cellulose chromatography. A single band of small intestinal

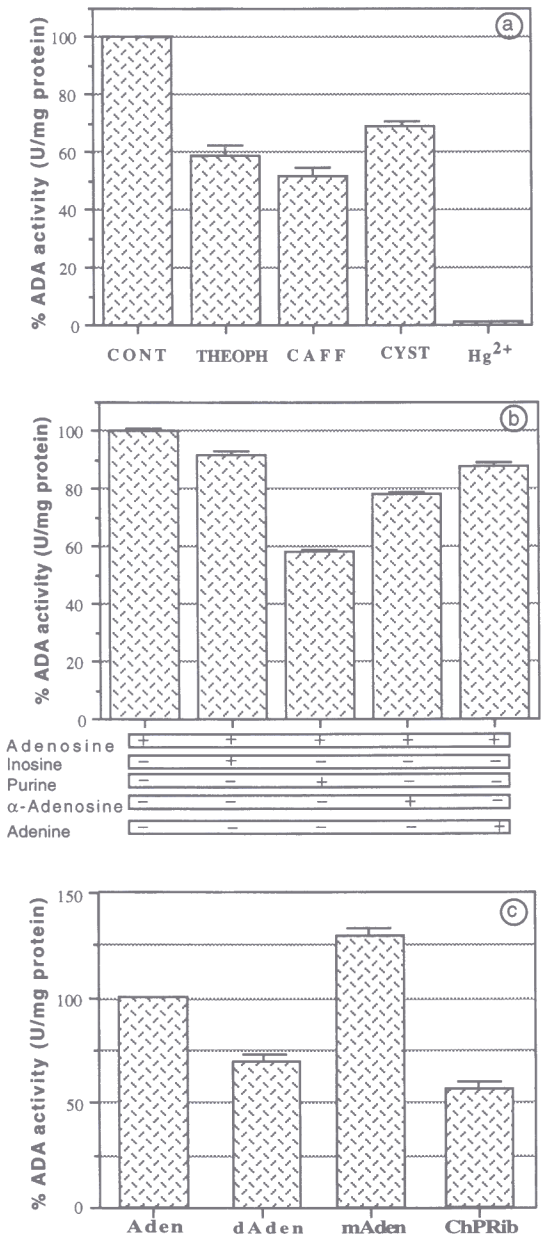


Fig. 5. (a). Effects of various modulators on the activity of purified intestinal ADA from mice. Values are mean of 3 separate experiments and bars represent standard deviation. CONT, THEOPH, CAFF, and CYST indicate control, theophylline, caffiene, and L-cysteine, respectively. 50 µl of purified ADA (0.1 mg/ml) was used in various modulator studies. Details of concentration of each modulators are given in the Materials and methods section. (b). Effects of various purine analogs on intestinal ADA activity of mice. + and - signs indicate the presence and absence, respectively of analogs. These analogs were used at a concentration of 100 µM each. (c). Relative activity of intestinal ADA towards 3′-deoxyadenosine (dAden), 2′-o-methyladenosine (mAden) and 6-chloropurine riboside (ChPRib). Values are presented as percentage taking the activity toward adenosine (Aden) as 100%. Bars indicate standard deviation. Details of experimental procedures are given in the Materials and methods section.

ADA on native-PAGE indicates that the enzyme was purified to homogeneity. Western blots after SDS-PAGE showed that the purified ADA is a single subunit with molecular weight of 41 kDa. This agrees with the presumption that lower molecular form of ADA predominates in the tissues exhibiting highest activity of ADA [14] and consistent with the report of human thyroid and erythrocytes ADA whose molecular weights are 42 kDa [16]. Though, the other band on the SDS-PAGE might be ADA complexing protein co-purified along with the ADA (13). Isoelectric focusing and band activity studies indicate that intestinal ADA has a single isoelectric form of pI ~ 4.9, which is similar to one of the isoelectric forms of spleen ADA. This was further confirmed by western blot analysis at the end of isoelectric focusing. Immunological comparison of the purified ADA from small intestine of mice has indicated that the anti-ADA antibodies raised in rabbit were specific for ADA and gave a single immunoreactive band during western blot analysis.

Our findings indicate that the intestinal ADA exist in 1 molecular form having acidic character which contrast to various molecular forms present in other tissues such as spleen [14]. Kinetic characterization of ADA from the small intestine of mice exhibited the estimated Km values for adenosine and 2′-deoxyadenosine as 23 µM and 16 µM, respectively. The Km value for adenosine is similar to the reported earlier in mice [26,27], however, that for 2′-deoxy-adenosine the values are rarely available in the literature. The lower km value of small intestinal ADA for 2′-deoxyadenosine might reflect faster deamination of this metabolite to help greater role in the physiology of gastrointestinal tract. This suggestion is well corroborated with the earlier report that 2′-deoxyadenosine contributes more to lymphotoxicity than adenosine [28]. Accumulation of deoxyadenosine causes imbalance of deoxyribonucleotide precursor for DNA synthesis through inhibition of ribonucleotide reductase activity [29].

Analyses of the data indicate that purine riboside is a strong competitive inhibitor of ADA with Ki of 5 µM. In contrast, 2′-3′-o-isopropylideneadenosine acts as an uncompetitive inhibitor to ADA with Ki of 66 µM that is about 13-fold higher than that of purine riboside, indicating differential mode of inhibition by these inhibitors. The observed values of Ki and the optimum pH are consistent with the earlier reports on cloned ADA from mice [26,27]. pH stability studies indicate that mice intestinal ADA is most stable in the neutral and basic region of pH range from 6.5 to 8.0, however, slight instability started at pH 8.5 upward. The pH dependence and stability characteristics observed in this study is well corroborated with the properties of small form of ADA. This might also parallel with the physiological milieu of gastrointestinal tract. Our findings indicate that caffeine, L-cysteine and Hg^{2+} inactivate ADA, albeit to a varying degree. It has earlier been reported that caffeine inhibits ADA activity in vivo as well [30]. Variable

inhibitions by purine analogs such as inosine, purine, α-adenosine and adenine, on the activity of ADA indicate that the presence or absence of ribose moiety has no significant impact on the inhibitory activity.

Relative activity studies of intestinal ADA of mice with respect to 3′-deoxyadenosine, 2′-o-methyladenosine and 6-chloropurine riboside show that the ADA is involved in deamination and dechloronation of 3′-deoxyadenosine and 6-chloropurine riboside, respectively, albeit to a lesser extent as compared to deamination of adenosine. Purified intestinal ADA show increased activity for 2′-o-methyladenosine compared to that for adenosine. This observation of greater mice intestinal ADA activity with 2′-o-methyladenosine differs from the ADA of marine bivalved mollusc. Methyl group of this substrate may be more suitable for substrate alignment leading to relatively higher catalytic activity. This indicates that 2′-o-methyladenosine might serve as a better analog for substrate binding site as well as catalytic site. In conclusion, our findings suggest that mice intestinal ADA shares many physicochemical and kinetic properties with the small molecular forms of ADA reported from other sources. It also reflects that ADA activity can be modulated by various modulators including substrate analogs which may be useful in controlling ADA activity levels in various kinds of ADA-related diseases.

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