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PURIFICATION AND CHARACTERIZATION OF ADENOSINE NUCLEOSIDASE FROM BARLEY LEAVES

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Summary

Adenosine nucleosidase (adenosine ribohydrolase, EC 3.2.2.7) has been purified to a nearly homogeneous state from barley leaves. The enzyme is soluble in concentrated salt solution while it aggregates and precipitates at low ionic strength, factors which enabled a simple purification procedure to be carried out. A molecular weight of 66 000 ± 3000 was estimated for the native enzyme by gel filtration. In sodium dodecyl sulphate polyacrylamide gel electrophoresis of the most purified fraction a single major band of polypeptide chains, with molecular weight of 33 000, was observed. Thus, the native enzyme seems to be dimer of α_2 type.

The pH optima are 4.7 and 5.4 for citrate and (N-morpholino)ethanesulphonic acid buffers, respectively. Adenine and adenosine protect the enzyme against heat inactivation. The enzyme is resistant to -SH reagents, dithiothreitol inhibits it. The K_m for adenosine varied from 0.8 to 2.3 μM depending on temperature and buffer system. The K_m for deoxyadenosine was 120 μM. Besides adenosine, of several nucleosides tested only adenosine N_1-oxide, deoxyadenosine and purine riboside acted as substrates. Adenine as well as its derivatives, including plant hormones (cytokinins), have an inhibitory effect on the enzyme. The K_i values of some modified nucleosides and free bases were determined. The physiological role of adenosine nucleosidase in plants is discussed.

Introduction

The biosynthetic pathway of 5'-adenylic acid seems to be common to all groups of organisms [1—3]. There are, however, differences in catabolic fates

Abbreviations: MES, (*N*-morpholino)ethanesulphonic acid; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

of the AMP adenine moiety in microorganism, animal and plant material. In animal tissues the adenylic acid deaminase (EC 3.5.4.6) converts AMP to IMP [4]. In *Azotobacter vinelandii* 5′-AMP is hydrolyzed to adenine and ribose 5′-phosphate by adenylic acid ribosidase (EC 3.2.2.4) [5,6]. 5′-AMP can be decomposed to adenosine by the action of 5′-nucleotidase (EC 3.1.3.5) [7,8] or nonspecific alkaline [9] and acidic [10,11] phosphatases in several organisms. At least the following three variants of further adenosine degradation are known:

1. Deamination catalyzed by adenosine deaminase (EC 3.5.4.4), the major route of adenosine metabolism in mammalian cells[4].

2. Adenosine can be a substrate for purine-nucleoside phosphorylase (EC 2.4.2.1) as in *Salmonella typhimurium* [12]. Adenosine phosphorylase activity was also found in some species of mycoplasmas [13].

3. In plants, adenosine is irreversibly hydrolyzed to adenine and ribose. This reaction is catalyzed by adenosine nucleosidase (adenosine ribohydrolase, EC 3.2.2.7). Its activity was demonstrated in preparations from leaves of soyabean [14], wheat [15], cabbage [16], Brussels sprout [17] and potato [18]. To date, partially purified preparations of adenosine nucleosidase from Brussels sprouts are the best described. Knowledge concerning the properties of the other adenosine nucleosidases is limited.

The paper presents a simple procedure enabling to purify the adenosine nucleosidase from barley leaves to a nearly homogeneous state in two days. Of the nucleoside hydrolases only uridine nucleosidase from bakers' yeast has been purified to homogeneity so far [19].

A new method has been worked out in this laboratory for estimation of adenosine nucleosidase activity [20] and since this method facilitated the very rapid determination of enzyme activity in sufficiently small quantities of purified fractions, the isolation and characterization of the adenosine nucleosidase from barley leaves were realizable.

Materials

[8-^{14}C]adenosine, [U-^{14}C]adenosine, [8-^{14}C]ATP, d[U-^{14}C]ATP and [U-^{14}C]-AMP were obtained from Amersham Radiochemical Centre, U.K. [U-^{14}C]-guanosine, [U-^{14}C]uridine, [U-^{14}C]cytidine and [U-^{14}C]thymidine were from the Institute for Research, Production and Application of Radioisotopes, Prague, Czechoslovakia. [U-^{14}C]deoxyadenosine was prepared from d[U-^{14}C]-ATP after incubation with bacterial alkaline phosphatase (Worthington, U.S.A.) and purified by thin layer chromatography. The common nucleic bases, common ribonucleosides, hypoxanthine, kinetin, zeatin, morpholinoethane sulfonic acid (MES), Aquacide, inosine and 2,6-diaminopurine were from Calbiochem, U.S.A. *p*-Hydroxymercuribenzoate, xanthine, 6-methylamino-purine riboside, cordycepin, 6-(γ,γ-dimethylallylamino)purine were purchased from Sigma, U.S.A. 6-Methyladenine, 6-dimethylaminopurine, dithiothreitol, *N*-ethylmaleimide, 2′,3′-AMP, Coomassie Brilliant Blue R-250 and the proteins of known molecular weight were from Serva, G.F.R. Iodoacetamine was from Schuchardt, G.F.R. PPO and POPOP were from Fluka, Switzerland.

D-Ribose and precoated plates for thin layer chromatography were obtained

from Merck, G.F.R. Acrylamide and TEMED were from B.D.H., U.K. SDS and bisacrylamide were from Koch-Light, U.K. Tris and 2-mercaptoethanol were from Loba-Chemie, Austria. NaF and $ZnCl_2$ were from Reachim, U.S.S.R. RbCl, CsCl and 6-benzylaminopurine were from Chemapol, Czechoslovakia. LiCl was obtained from UCB, Belgium. Adenine arabinoside was from I.C.N., U.S.A. 8-Aza-adenine was from Reanal, Hungary. Adenosine N_1-oxide, deoxyadenosine, purine riboside, kinetin riboside, 6-mercaptopurine riboside were from Waldhof, G.F.R. Deoxyguanosine, deoxyinosine, deoxyuridine and deoxycytidine were obtained from P-L Biochemicals, U.S.A. Formycin A was a gift of Professor D. Shugar. 2′-*O*-methyladenosine and 3′-*O*-methyladenosine were gifts of Dr. Z. Kazimierczuk. Cyclopentane analogue of adenosine, 9-(β-DL-2α,3α-dihydroxy-4β-(hydroxymethyl)cyclopentenyl)adenine, was obtained from Drs. Y.F. Sheely and J.D. Clyton [21]. L-adenosine was purchased from N.S.C., U.S.A. The cellulose nitrate filters were from Schleicher and Schüll, G.F.R. Sephadex G-100, Sephadex G-25 and Dextran Blue were from Pharmacia, Sweden. The chromatographic paper and cellulose powder CF 11 were obtained from Whatman, U.K. Other reagents were purchased from Polskie Odczynniki Chemiczne, Poland. The seeds of barley, *Hordeum vulgare* var. Ksenia, were obtained from Centrala Nasienna, Czarnków.

The following buffers were used routinely: buffer A: 50 mM Tris · HCl (pH 8.0); buffer B: 50 mM Tris · HCl (pH 8.0)/0.2 M KCl; buffer C: 50 mM Tris · HCl (pH 8.0)/1M KCl.

Methods

Preparation of the plant material

The barley seeds were washed several times with tap water and placed on humid cellulose wool to germinate. The seedlings were grown under natural light conditons at 20—22°C. After 7 days the green leaves were cut, weighed, chopped and pulverized with solid CO_2 in a coffee blendor. The powder thus obtained was stored at −20°C until enzyme extraction was undertaken.

Enzyme assay

Except for slight modifications, adenosine nucleosidase was assayed as previously described [20]. The standard incubation mixture of total volume 50 μl contained 50 mM MES/KOH buffer, pH 5.4, 2 mM [8-^{14}C]adenosine (32 000 cpm) and enzyme fraction (0.02—5 μg of protein). The samples were incubated at 35°C for 10 min and the reaction was stopped by addition of 30 μl of 20 mM unlabelled adenosine and heating at 80—85°C for 2 min. Aliquots of 60 μl were withdrawn and placed on cellulose nitrate discs for adsorption of the product (adenine) released. The adsorption procedure, washing and counting of the radioactivity retained on filters were carried out as described previously [20].

One unit of activity is the amount of enzyme which catalyzes the hydrolysis of 1 μmol of adenosine per min under standard incubation conditions.

The activity is also expressed in katals according to the recommendations of the Commission on Biochemical Nomenclature [22].

High capacity filters (24 mm in diameter, type BA 85, specially made for this purpose by Schleicher and Schüll) adsorbed adenine with a 70% efficiency.

Protein determination

Protein concentrations were determined by the turbidimetric tannin method of Mejbaum-Katzenelenbogen [23] using bovine serum albumin as standard. In column eluates only the absorbance at 280 nm was monitored.

Molecular weight determination

The molecular weight of adenosine nucleosidase was determined by gel filtration accordingly to the method of Andrews [24]. The 1.6 × 64 cm column of Sephadex G-100 equilibrated with 50 mM Tris · HCl buffer (pH 8.0) containing 0.5 M KCl was calibrated with the following standards of known molecular weight: cytochrome *c* from horse heart (12 400), chymotrypsinogen A (25 000), ovalbumin (43 000), and bovine serum albumin (67 000).

Subunit structure

The preparation of standards and adenosine nucleosidase samples, and sodium dodecyl sulphate polyacrylamide gel electrophoresis were as described by Weber et al. [25]. The gels were stained for proteins with Coomassie Brilliant Blue R-250 in water/methanol/acetic acid mixture. In order to check if the subunits were linked by disulphide bridges the reducing agent 2-mercaptoethanol was omitted in the denaturing mixture.

Substrate specificity

The adenylates, several common nucleosides, and rare adenosine derivatives were checked as substrates for adenosine nucleosidase. A given compound was incubated overnight with purified enzyme fraction. 10 μl of the mixture were then spotted on Whatman 3 MM sheet or on the plate precoated with the cellulose. The ascending paper (PC) or thin layer (TLC) chromatographies were carried out in the following solvents: I, *n*-butanol/acetic acid/water (12/3/5); II, isopropanol/HCl/water (130/33/37); III, distilled water. The chromatograms were scanned under ultraviolet light. The nucleosides from which the enzyme could release the free base were recognized as substrates. When labelled substrates were used for incubation (see Materials) the regions corresponding to possible products were cut from chromatograms and their radioactivity was counted.

Thermostability

The solution containing 20 μg of purified enzyme protein per ml was divided into several 50-μl portions. Each of them was kept in a water bath at a given temperature for 5 min and then placed on ice. The remaining adenosine nucleosidase activity was estimated in the standard enzyme assay. When the effect of adenine, adenosine or ribose on the thermostability was investigated these compounds were added at a final concentration of 1 mM to the enzyme fraction before it was submitted to heat inactivation. The activity found in the sample kept on ice was taken as 100% of the remaining activity.

Other details of particular experiments are given in legends to figures.

Results

Routine purification of the enzyme

The pulverized barley leaves (fresh weight 300 g) were extracted twice with double volume of buffer B. The slurry was passed through four layers of cheesecloth and the filtrate was discarded. Afterwards the leaf tissue was extracted twice with buffer C. The last filtrates were collected and centrifuged (20 min, 20 000 × *g*). The adenosine nucleosidase activity was found in the fraction salted out from the supernatant between 50 and 70% of ammonium sulphate saturation. This fraction was dissolved in a small volume of buffer C and dialyzed overnight against buffer A. The precipitate obtained was collected as a pellet after centrifugation at 10 000 × *g* for 15 min and the supernatant discarded. To solubilize the nucleosidase the pellet was suspended in small volume of buffer A containing 0.1 M KCl and after 15 min the suspension was centrifuged. The supernatant was discarded and the remaining pellet was extracted with buffer A containing increasing concentrations of KCl. All the operations were repeated four times with buffer A containing 0.2, 0.3, 0.4 and 1 M KCl. respectively. The activity of adenosine nucleosidase was found in the supernatants containing 0.2, 0.3 and 0.4 M KCl. These fractions were pooled and concentrated in a dialysis bag by means of Aquacide II. Gel filtration was the last step of the purification procedure. A single peak of the adenosine nucleosidase activity eluted at 1.43 void volume. The fraction of the highest specific activity was about 95% pure (see Discussion). The fractions with at least 70% of the maximum activity were frozen and kept on solid CO_2 for future characterization. All the operations were carried out at 0—4°C. The typical protocol for purification is given in Table I.

Comments on purification

Besides being found in leaves, adenosine nucleosidase activity was also found

TABLE I

PURIFICATION OF ADENOSINE NUCLEOSIDASE FROM BARLEY LEAVES

The purifcation procedure was carried out as described in the text. 300 g of fresh leaves were used.

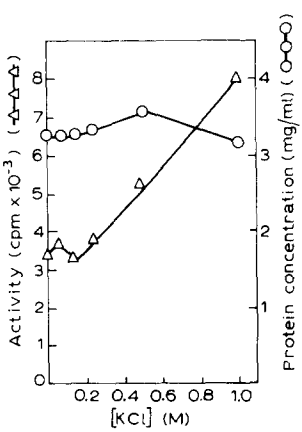
Step	Protein		Activity				Yield
	wt mg	vol. ml	Total		Specific		%
			units	nkat	units/mg	kat/kg	
Crude extracts							
with buffer B	1800	800	72	1200.2	0.04	0.0006	26.2
with buffer C	480	480	168	2800.5	0.35	0.0058	73.8
Resolubilization							
with buffer A containing 0.2-0.4 M KCl	4.64	6	4.17	69.5	0.90	0.015	1.74
Sephadex G-100							
pooled fractions	0.24	12	2.86	47.6	11.92	0.2	1.20
peak fraction	0.04	2	1.22	20.3	30.50	0.5	0.53

in barley seedling coleoptiles and roots. The leaves were preferred, however, since they are the most suitable for harvest and give the highest specific activity. This activity changed as seedlings developed. The specific activity reached its maximum in the leaves of 7—8-day old seedlings. It is then about 10-fold higher than in 3-day or 11-day old plants. Pulverization of the barley leaves with solid CO_2 appeared to be a very convenient technique of homogenization. It facilitated the efficient enzyme extraction. It had been shown that the higher the ionic strength of the buffer used for extraction, the higher the specific activity of adenosine nucleosidase that could be found in the extract (see Fig. 1). The extraction procedure with the use of two buffers, with low ionic strength and high ionic strength, applied in the first step of purification (Table I) allowed very efficient removal of the bulk of contaminating proteins, with however a 30% loss of total enzyme activity.

In the preliminary experiments the resolubilization of the enzyme from the precipitate obtained in low-salt buffer was carried out as presented in Fig. 2. The last step of purification, gel filtration on Sephadex G-100 (Fig. 3), in which enzyme already of high purity is used, yielded an almost homogeneous preparation.

Molecular weight and subunit structure

The molecular weight of 66 000 ± 3000 was determined for barley leaf adenosine nucleosidase by gel filtration (see Fig. 3). The SDS polyacrylamide gel electrophoresis of the purest fractions done in the presence or absence of mercaptoethanol revealed the main band with molecular weight of 33 000, Fig. 4.



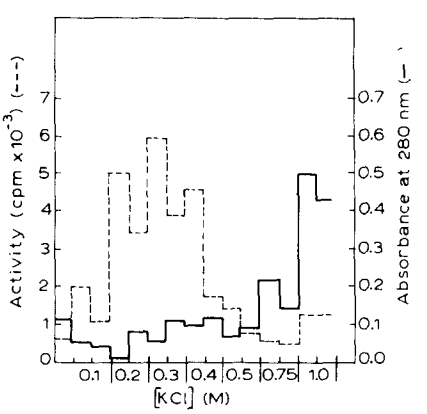


Fig. 1. Influence of buffer ionic strength on the efficiency of adenosine nucleosidase extraction. Equal portions of 7-day old leaves were extracted with buffer A containing indicated KCl concentration. After centrifugation the protein concentration and activity of the adenosine nucleosidase were estimated in the supernatants.

Fig. 2. Solubilization of adenosine nucleosidase from the protein precipitate resulted during dialysis against low-salt buffer. The precipitate was suspended in buffer A containing 0.1 M KCl and mixed with some cellulose powder CF 11. A smal (about 0.3 ml) column was formed. The enzyme was eluted by a stepwise gradient. The 0.5-ml fractions of the eluate, with KCl concentration indicated, were collected, the absorbance at 280 nm was measured and the activity of the enzyme estimated.

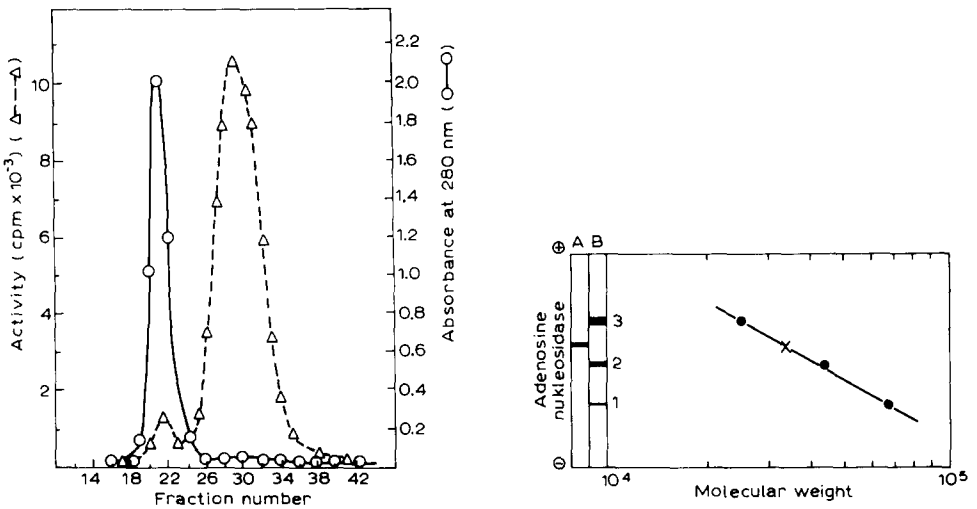


Fig. 3. Gel filtration of adenosine nucleosidase. The 1.5-ml sample containing 4.5 mg of the resolubilized protein was applied to a Sephadex G-100 column (1.6 X 64 cm) equilibrated with buffer A containing 0.5 M KCl. The flow rate was 16 ml per h. 2-ml fractions were collected, the absorbance at 280 nm was measured and the enzyme activity estimated. The fractions 27—32 were kept.

Fig. 4. SDS polyacrylamide gel electrophoresis of purified adenosine nucleosidase; molecular weight estimation of polypeptide chains. About 20 μg of SDS-denatured protein from the best-purified fraction was applied to each of the 7.5% polyacrylamide gel columns and electrophoresis was carried out as described in Methods. A: pattern of the gel on which the adenosine nucleosidase was run; B: pattern of the gel on which the standards were run: 1, bovine serum albumin; 2, ovalbumin; 3, chymotrypsinogen A.

The data presented indicate that the active adenosine nucleosidase occurs in 0.5 M KCl buffer solution as a dimer consisting, possibly of identical polypeptide chains which are not bound by a disulphide bridge.

Enzyme stability

The purified fractions of adenosine nucleosidase were frozen and thawed several times without any detectable loss of activity. The presence of salt, however, was essential. When the enzyme was frozen in diluted salt solution, it lost its activity completely. The enzyme was also more stable during prolonged standing at room temperature in 0.5 M KCl or 0.5 M potassium phosphate buffer than in the low-salt solution.

An attempt was made to dry the crude extract of the nucleosidase in the beads of Sephadex G-25, according to the procedure of Schneider et al. [26]. It was found that in the dried state the enzyme could be stored for several months. When necessary it could be eluted from the beads with water. The enzyme being stabilized within the Sephadex matrix facilitates mailing and it can withstand elevated temperatures.

Reaction products

It was demonstrated by thin layer chromatography that after prolonged incubation of the enzyme with uniformly labelled [^{14}C]adenosine, radioactivity could be found only in the spots of adenine and ribose.

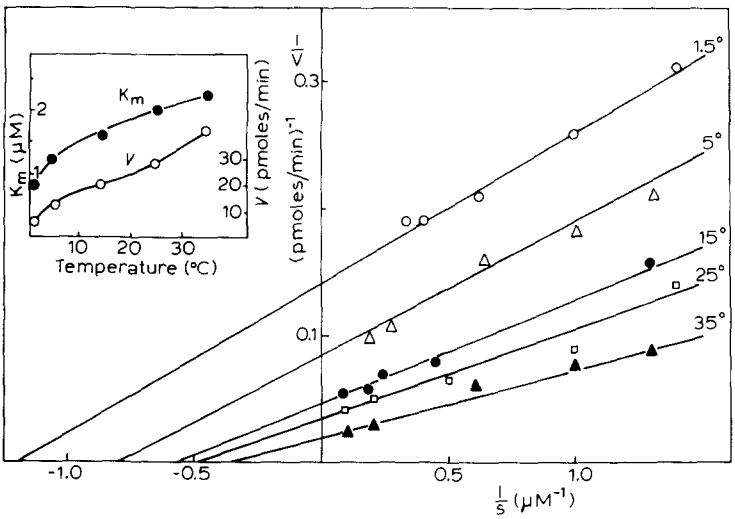


Fig. 5. Effect of temperature on K_m and V values of adenosine nucleosidase. The initial velocities of adenosine nucleosidase were determined at indicated temperatures by incubating 100-μl samples containing 0.02 μg of the enzyme in 0.5 M potassium phosphate buffer, pH 5.8, and at varied concentrations of [^{14}C]adenosine. Each point on the graph was obtained by extrapolation to zero time incubation of three tubes run at different times. Because of considerable activity of the enzyme in the ice bath and also during the first seconds of denaturing heating, the incubation time for each tube was carefully controlled. The incubation was started by addition of [^{14}C]adenosine preheated to the required temperature and stopped by addition of 50 μl 20 mM unlabelled adenosine, followed by heating at 95°C for 3 min. The product [^{14}C]adenine, was collected from 60-μl volumes by adsorption on cellulose nitrate filters as described in Methods. The data were corrected for adsorption of [^{14}C]adenosine by running control samples without enzyme. The K_m and V values were evaluated from the graph.

Kinetic studies and inhibitory effects

The Michaelis constant for adenosine was estimated in two buffer systems from the Lineweaver-Burk plot. With the use of potassium phosphate buffer the K_m was determined at different temperatures. As shown in Fig. 5 the values varied from 0.8 μM at 1.5°C to 2.3 μM at 35°C. The Arrhenius plot (not shown here) drawn from these data indicate a low-temperature coefficient (1.4) in the temperature range 5—35°C. Thus, the enzyme compared with other enzyme systems shows relatively high activity at temperatures close to 0°C. The potassium phosphate buffer was chosen for kinetic studies due to high reproducibility of the enzyme activity. The K_m value determined in MES buffer, pH 5.4 in the absence of phosphate ions was 1.2 μM at 35°C. The enzyme also shows activity with deoxyadenosine as substrate (Table II). The K_m for deoxyadenosine in MES buffer, pH 5.4 is much higher (120 μM) than for adenosine. The affinity of deoxyadenosine to the enzyme was also studied by using it as an inhibitor. As can be seen from Fig. 6 and Table III, the K_i obtained for this compound is 50 μM when using potassium phosphate buffer. Cordycepin (3'-deoxyadenosine) is not hydrolyzed by the adenosine nucleosidase. Cordycepin shows, however, inhibitory properties, though in terms of K_i value it is almost four times weaker than 2'-deoxyadenosine.

One of the products, adenine, is a strong competitive inhibitor, while ribose does not inhibit the enzyme at concentrations up to 10 mM nor does it enhance the inhibitory effect of adenine. Other derivatives of adenine,

TABLE II

SUBSTRATE SPECIFICITY OF ADENOSINE NUCLEOSIDASE

26 compounds were investigated as possible substrates for adenosine nucleosidase from barley leaves. R_F values obtained in different chromatographic systems for adenine nucleotides, nucleosides and respective free bases are included. Details of these experiments are described in the Methods. Asterisk indicates those compounds found to be substrates.

Compound	R_F value				
	TLC Solvent system		PC Solvent system		
	I	III	I	II	III
* Adenosine	0.48	0.45	0.44	—	0.50
* Deoxyadenosine	0.49	0.50	—	—	—
Cordycepin	—	0.52	—	—	—
Cyclopentane analogue of adenosine	0.41	0.45	—	—	—
2′-O-methyladenosine	0.58	—	—	—	—
3′-O-methyladenosine	0.60	—	—	—	—
Adenine arabinoside	0.38	0.51	—	—	—
Formycin A	0.36	0.44	—	—	—
5′-AMP	—	—	0.14	—	—
5′-ATP	—	—	0.03	—	—
2′,3′-AMP	—	—	0.16	—	—
Adenine	0.53	0.29	0.55	—	0.30
Guanosine	—	—	—	0.42	—
Deoxyguanosine	—	0.48	—	—	—
Guanine	—	—	—	0.27	—
Inosine	0.27	0.80	—	—	—
Deoxyinosine	—	0.68	—	—	—
Hypoxanthine	0.33	0.50	—	—	—
Xanthosine	0.28	0.88	—	—	—
Xanthine	0.34	0.32	—	—	—
*Purine riboside	0.50	0.76	—	—	—
Purine	0.61	0.58	—	—	—
*Adenosine N_1-oxide	—	0.70	—	—	—
Adenine N_1-oxide	—	0.58	—	—	—
6-Methyladenosine	—	0.54	—	—	—
6-Methyladenine	—	0.28	—	—	—
Kinetine riboside	—	0.65	—	—	—
Kinetine	—	0.32	—	—	—
6-Mercaptopurine riboside	0.30	0.60	—	—	—
Cytidine	—	—	0.33	—	0.77
Deoxycytidine	—	0.70	—	—	—
Cytosine	—	0.68	0.40	—	0.64
Uridine	—	—	0.38	—	—
Deoxyuridine	—	0.73	—	—	—
Uracil	—	0.65	0.49	—	—
Thymidine	—	—	0.57	—	—
Thymine	—	—	0.62	—	—

including some cytokinins, indicated in Table III, are also inhibitors, less strong, however, than adenine. Comparing the K_i values of various derivatives of adenine and adenosine conclusions can be drawn with regard to complementarity of structure and charge interactions of the enzyme with adenosine (see Discussion).

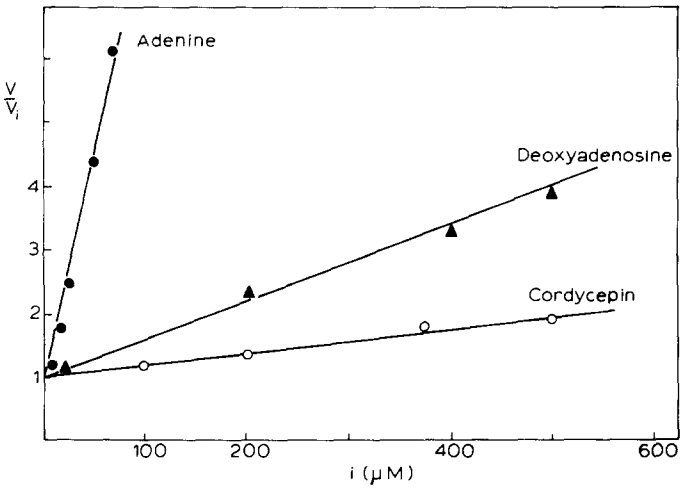


Fig. 6. The inhibitory effects of adenine, deoxyadenosine and cordycepin on adenosine nucleosidase. The incubation mixture contained in 100 μl: 0.02 μg of the enzyme in 0.5 M potassium phosphate buffer, pH 5.8, 5 μM [8-^{14}C]adenosine (20 000 cpm) and various concentrations of the indicated inhibitors. The incubations run at 25°C and the time of incubation was varied depending on the concentration of the inhibitor to obtain 7—10% substrate conversion. The product was estimated as described in the legend to Fig. 5 and in Methods. The data were not corrected for the inhibitory contribution of the product as this was considered to be negligible under these conditions.

TABLE III

EFFECTS OF PURINE DERIVATIVES ON ACTIVITY OF ADENOSINE NUCLEOSIDASE

The relative velocities of *N*-glycosidic bond cleavage of nucleosides were calculated from the data shown in Fig. 7. Substrate activity indicated by "0" was obtained after 24 h of incubation under conditions described in Fig. 7.

Compound	K_i * (μM)	Activity relative to adenosine (%)
Adenine	4	—
Purine	360	—
8-Aza-adenine	100	—
6-(γ,γ-dimethylallylamino)-purine	68	—
Zeatin	133	—
Kinetin	380	—
6-Benzylaminopurine	102	—
6-Methylaminopurine	90	—
6-Dimethylaminopurine	350	—
2,6-Diaminopurine	550	—
6-Mercaptopurine	500	—
Cytosine	1300	—
2'-Deoxyadenosine	50	58
L-adenosine	160	0
Adenosine N_1-oxide	130	89
Purine riboside	180	9
Cordycepin (3'-Deoxyadenosine)	250	0
N^6-methyladenosine	120	0
5'-Adenylic acid	approx. 500	0

* The K_i values were calculated from the slopes (see Fig. 6) according to the method of Dixon and Webb [27]. Slope = $K_m/K_i \cdot (1/K_m + S)$. The K_m value estimated at 25°C equals 2.0 μM (Fig. 5).

Substrate specificity

Among the naturally occurring nucleosides only adenosine and deoxyadenosine were hydrolyzed by adenosine nucleosidase from barley leaves. Of the other nucleosides tested only adenosine N_1-oxide and purine riboside (nebularine) were substrates (see Table II). The *N*-glycosidic bond in adenosine N_1-oxide and in deoxyadenosine was hydrolyzed at a similar rate to that in adenosine, but in purine riboside it was hydrolyzed at about 1/10 of this speed (see Fig. 7 and Table III).

Effect of pH

The optimal pH for enzyme activity varied with the buffer used and was found to be 4.7 in 40 mM citrate buffer and 5.4 in 50 mM MES/KOH buffer.

Effects of metal ions and other reagents

The chlorides of the following metal ions were tested at 1, 10 and 100 mM concentrations: Li^+, K^+, Na^+, Rb^+, Cs^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}. However no influence on the hydrolysis of adenosine was observed. No inhibition or stimulation was noticed either in the presence of 10 mM NaF, 1 mM EDTA, or any other naturally occurring nucleoside at 1 mM concentration.

Such -SH reagents as *p*-hydroxymercuribenzoate (0.125 mM), *N*-ethylmaleimide (1 mM) and iodoacetamide (1 mM) did not affect the nucleosidase activity during 20 min preincubation at room temperature. On the other hand

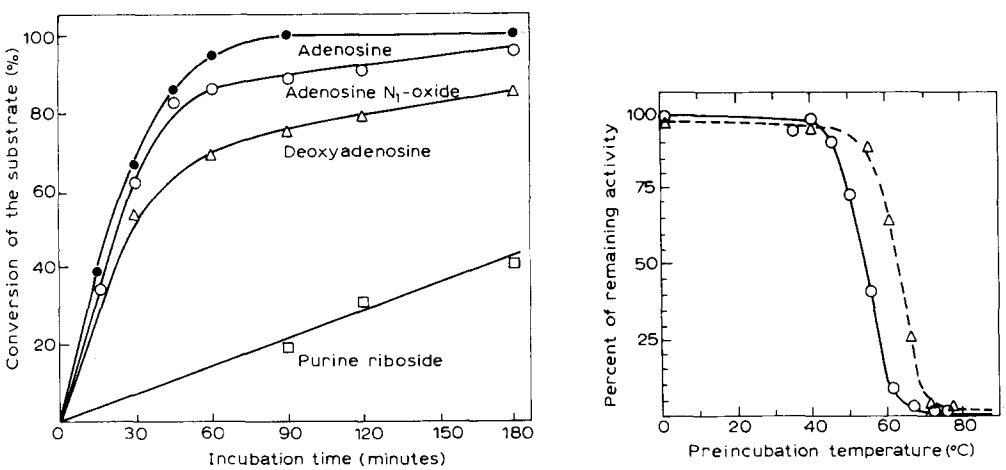


Fig. 7. The time-course of adenosine, deoxyadenosine, adenosine N_1-oxide and purine riboside hydrolysis. The hydrolysis of the nucleosides indicated was carried out at 35° C using the following reaction mixture: 50 mM MES/KOH, pH 5.4, 2 mM nucleoside, 0.1 M KCl and the purified enzyme fraction in a total volume of 0.2 ml. At intervals, 5-µl aliquots were withdrawn and pipetted on TLC plates subsequently developed with distilled water. The regions of the released base were measured densitometrically in Vitratron TLD-100 densitometer at filter 254 nm. Samples incubated for 12 h (after which for all substrates the hydrolysis is complete) were used as references for the evaluation of the substrate conversion at the times indicated.

Fig. 8. Thermostability of adenosine nucleosidase. The experiments were carried out as described in Methods. Enzyme denatured in the absence of adenine or adenosine (○———○); enzyme denatured in the presence of 1 mM adenine or adenosine (△- - - - -△).

up to 60% inhibition has been found when the enzyme was preincubated with 10 mM dithiothreitol for 20 min.

Effect of temperature and thermostability

Adenosine nucleosidase exhibited a very high temperature optimum, reaching 58°C. Such an unusually high value may in part be due to the protective effect of adenine and adenosine against heat inactivation. As demonstrated in Fig. 8, 1 mM adenine and/or adenosine shifts the enzyme denaturation curve by about ten degrees towards higher temperatures. The ribose molecule had no effect in this respect.

Discussion

In relation to total proteins extractable from barley leaves, 350-fold purification of adenosine nucleosidase was achieved. The property discovered of adenosine nucleosidase, to interconvert between the solubilized and precipitated states depending upon the salt concentration was essential for the purification of the enzyme. Very useful also was the convenient method of determination of adenosine nucleosidase activity.

On comparing the intensity of the main and two less mobile faint bands on SDS gels and taking into account the sample size on the one hand, and the sensitivity of the protein to staining with Coomassie Brilliant Blue [25] on the other, the purity of the best-purified fraction was assessed to be about 95%.

Besides the electrophoretic pattern also the correlation between the molecular weights of the native enzyme (estimated on Sephadex G-100) and its subunits (estimated on SDS gels) suggests high purity of the adenosine nucleosidase.

However, since the enzyme aggregates and precipitates in low-salt solutions the disc electrophoresis of the native molecule could not be carried out.

The adenosine nucleosidase purified from barley leaves was free of nucleotidases, nonspecific phosphatases and other nucleosidases and therefore it can be used as a convenient analytical tool. In conjunction with the analytical method of adsorption of labelled adenine on cellulose nitrate filters, the preparation of adenosine nucleosidase could be employed as a reagent in the assays of the following enzymes: adenosine kinase (EC 2.7.1.20), 5′nucleotidase (EC 3.1.3.5), 3′nucleotidase (EC 3.1.3.6), adenosine deaminase (EC 3.5.4.4), 3′,5′-cyclic AMP phosphodiesterase (EC 3.1.4.17) and adenosylhomocysteinase (EC. 3.3.1.1), (in preparation).

Kinetic studies of the enzyme at different temperatures revealed its low temperature coefficient. This property seems to have its physiological significance for seedlings exposed to sudden changes of temperature in springtime. The high level of the enzyme in 7-day old seedlings, which still rely mainly on the limited energy and mineral supply from their seeds, suggests that adenosine nucleosidase in plants, as purine phosphorylases in microorganisms and animals [28], is a member of the purine (adenine) salvage pathway. Besides adenosine nucleosidase, other enzymes of the adenine salvage pathway: adenine phosphoribosyltransferase (EC. 2.4.2.7) and adenosine kinase (EC 2.7.1.20) were demonstrated in plant materials [30—32]. Thus, adenine can be re-utilized, and

moreover, as in animal cells [33,34], the free adenine was reported to be an inhibitor of de novo purine biosynthesis in wheat seedlings [35].

The unusually high affintiy of the enzyme to the substrate found in two buffer systems ($K_m \approx 1$ μM) suggests very low concentrations of adenosine the enzyme has to deal with in the cell. The deoxyadenosine is also hydrolyzed but the K_m value for this compound is by about two orders of magnitude higher. Since the concentration of deoxyadenosine in plant tissues is expected to be much lower than adenosine, the significance of the enzyme for its metabolism is dubious. The strong inhibitory effect of the product (adenine) also found by others [17] indicates a simple feedback control system. Since as low as micromolar concentrations of adenine inhibit adenosine nucleosidase, it is expected that the product remains at low concentration in the cell and the enzymes metabolizing adenine, like adenine phosphoribosyltransferase or adenine deaminase (EC 3.5.4.2), if present, should have a high affinity for the substrate. The occurrence and concentration of free adenine in plants are not well established and there are contradictory reports on the matter [36,37] due to a lack of reliable extraction methods [38].

The differentiated binding of various adenine derivatives to adenosine nucleosidase represented by the inhibition constants in Table III permits the conclusion that the amino group at carbon 6 of the purine ring is responsible for strong binding of adenosine to the enzyme, however it is not essential for the enzyme activity. Substitution of one hydrogen at N-6 by a methyl group causes a dramatic decrease in affinity, but the substitution by groups larger than methyl does not change the affinity significantly. It seems that the N-6 amino group is not inserted in the enzyme "pocket", and consequently it is not complementary to the protein in terms of structure but only in terms of charge interaction. On the other hand, 2,6-diaminopurine shows a relatively high K_i (Table III) suggesting that there is a structural hindrance in its binding to the enzyme. Comparative kinetic studies on other purine derivatives would reveal whether this region of adenine is critical for structural complementarity. Though the ribose molecule has not been found to the inhibitory for adenosine nucleosidase, nor has it protected the enzyme against heat inactivation, the ribose moiety of the nucleosides seems to show complementarity with the enzyme (see K_i for purine and purine riboside in Table III).

The 3′-hydroxyl group of adenosine seems to be important for both binding to the enzyme and enzyme activity (see cordycepin in Fig. 6 and Table III). The lack of a 2′-hydroxyl group in 2′-deoxyadenosine does not interfere with enzyme activity, the binding of deoxyadenosine to the enzyme, however, is significantly decreased as compared with adenosine. In comparison with other enzymes metabolizing purines many of the derivatives studied (listed in Table III) are strong inhibitors for adenosine nucleosidase. Since they can be used only at low concentration due to their low solubility, the enzyme combined with the sensitive method of its estimation is a good model for further studies on purine-protein interactions.

Addendum

While a revised version of this paper was being prepared a paper on adenosine nucleosidase partially purified from leaves of *Beta vulgaris* was published: (1976) Poulton, J.E. and Butt, V.S., Planta 131, 179—185.

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