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PURIFICATION AND PROPERTIES OF *N*-RIBOSYLADENINE RIBOHYDROLASE FROM POTATO LEAVES

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Key Word Index—*Solanum tuberosum*; Solanaceae; *N*-ribosyladenine ribohydrolase.

Abstract—*N*-Ribosyladenine ribohydrolase, an enzyme that catalyzes the hydrolytic cleavage of adenosine in potato leaves, was extracted and purified 163-fold. Chromatography of the partially purified enzyme on DEAE-cellulose columns resolved the activity into several fractions. The existence of these distinct and separate activities was confirmed by electrophoresis in polyacrylamide gels. The MW of the purified enzyme was estimated as 62 400 daltons based on its sedimentation properties in sucrose density gradients. Kinetic studies indicated that the hydrolysis of adenosine proceeds irrreversibly at rates which can formally be described by Michaelis–Menten kinetics, but which are slightly more complex because of product inhibition by adenine. The enzyme was further characterized by a K_m of $2{\cdot}76 \times 10^{-4}$ M and by a product inhibition constant of $0{\cdot}57 \times 10^{-4}$ M. On the basis of its high substrate specificity for adenosine and deoxyadenosine and other properties, a physiological role for *N*-ribosyladenine ribohydrolase is considered.

INTRODUCTION

DESPITE the widespread occurrence of nucleosidases that catalyze the cleavage of purine and pyrimidine β-ribofuranosides,[1–12] relatively little is known about the role of these enzymes in cellular metabolism. A number of nucleosidases in bacteria and fungi have been thoroughly studied in terms of their kinetic properties, but few cases are known where the physiological role of these enzymes has been unequivocally established. The situation is even less satisfactory for the nucleosidases of higher plants. With one or two exceptions, interest here has centered mainly on whether nucleoside cleavage is hydrolytic or phosphorolytic or occurs by some other mechanism.[5,6,9,10]

On the other hand, definitive studies on the kinetic properties of several nucleosidases from higher plants have been made. Mazelis and Creveling[2] isolated and purified a purine nucleosidase from Brussel sprouts and showed that it catalyzes the hydrolytic cleavage of adenosine with relatively high specificity for this substrate. A similar study by Achar and

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Vaidyanathan[7] on uridine hydrolase from mung bean seedlings led these investigators to speculate that this enzyme could be important in regulating the metabolism of pyrimidines and therefore, to some extent, the synthesis of nucleic acids.

Pursuant to studies on the adenosine-splitting activity in extracts of potato leaves,[9] we decided to further investigate the properties of the enzyme in order to establish its role in both healthy and diseased plants. The present paper describes the purification and properties of *N*-ribosyladenine ribohydrolase and attempts to determine its function based on these properties.

RESULTS

Purification

The individual steps used in purification and their effect on purification are delineated in Table 1. Despite the low fold purification obtained from isoelectric precipitation, this step was necessary because, without it, the ensuing step using ethanol was ineffectual. Moreover acidification at an earlier stage of purification resulted in substantial losses in activity.

TABLE 1. PURIFICATION OF *N*-RIBOSYLADENINE RIBOHYDROLASE FROM POTATO LEAVES

Purification step	Total protein (mg)	Total units (mmol/min)	Activity Recovery (%)	Sp. act. (mU/mg)	Fold purification per sep
Leaf extract	14 678	954·1	100	65	—
Protaine sulfate precipitation	4488	673·2	70·6	150	2·3
$(NH_4)_2SO_4$ precipitation (75%)	2416	536·4	56·2	222	1·5
$(NH_4)_2SO_4$ fractionation (40–60%)	682	328·0	34·4	481	2·2
Isoelectric precipitation (pH 4·8)	454	267·0	28·0	588	1·2
Ethanol precipitation	28	140·5	14·7	5016	8·5
DEAE-cellulose chromatography	10·2	108·2	11·3	10 607	2·1
			Total fold purification		163·1

An attempt at further purification, following precipitation with ethanol, was made by chromatography on DEAE-cellulose columns. A typical elution profile is illustrated in Fig. 1 and shows that activity applied to the column was further resolved into several peaks. The fold purification of the major peak is that represented in Table 1 and this is the fraction to which all subsequent analyses refer. No attempt was made to further delineate the properties of the minor peaks shown in Fig. 1. However, in order to rule out the possibility that these peaks were artefacts of purification or of the column itself, eluates of the individual peaks were pooled, concentrated, and rechromatographed on separate columns. The relative positions of the peaks remained unchanged. Furthermore, when activity of the earlier steps in purification was chromatographed on DEAE-cellulose, the elution profiles were in all cases similar to those obtained with the purified material. In addition, electrophoresis of activity from the ammonium sulfate fractionation step in polyacrylamide gels resulted in three separate and distinct bands: a diffuse, slow-moving band which corresponded to the major peak observed in the eluate from DEAE-cellulose and two sharp, faster moving bands which corresponded to the minor peaks.

pH Optimum

The effect of changing pH on the rate of hydrolysis of adenosine is depicted in Fig. 2. Whether the extremely sharp optimum at pH 4·0 is a reflection of the charge state of the 6-amino group of adenosine ($pK\check{a} = 3{\cdot}3$) or of the dissociation of the functional groups in the active site of the enzyme itself is unknown. However, the relative increase in activity observed in phosphate buffer in the interval pH 6·0–7·0 was not due to contamination by nucleoside phosphorylase as might be anticipated. Evidence for this conclusion will be developed more fully in a later section.

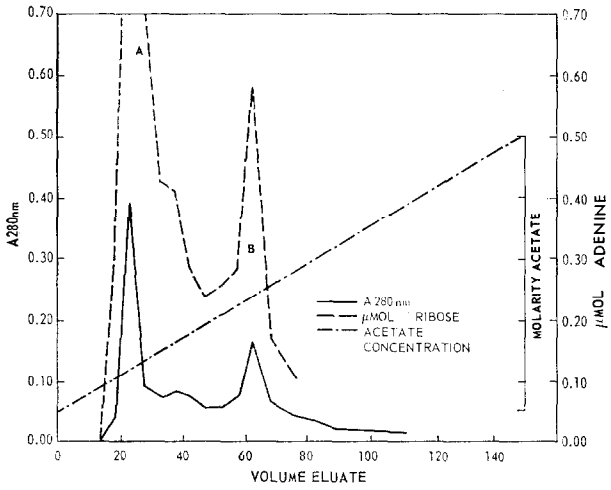

FIG. 1. ELUTION PROFILE OF THE PARTIALLY PURIFIED ENZYME ON DEAE-CELLULOSE.

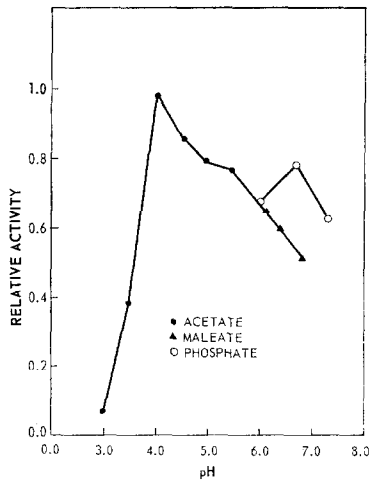

FIG. 2. EFFECT OF pH ON THE RATE OF HYDROLYSIS OF ADENOSINE.

For Fig. 1. The precipitate obtained by ethanol precipitation was dissolved in 0·01 M acetate buffer, pH 5·4 and applied to a DEAE-cellulose column, 1·5 × 25 cm. Elution was conducted with a linear gradient of acetate (0·05–0·50 M, pH 5·4). The flow rate was maintained at 15 ml/hr and 5·0 ml fractions were collected; these were monitored for absorbance at 280 nm and for enzyme activity.

Activation and Inhibition

Results from a number of experiments on *N*-ribosyladenine ribohydrolase are summarized in Table 2. Except for the marked inhibition by one of the products, adenine, the effect of the compounds listed was so slight as to rule out any significant effect under physiological conditions.

In connection with the activation of the enzyme, the possibility that activity was contained in a sub-cellular particle was also examined. However, when leaf extracts prepared in isotonic medium were subjected to sonication, a regime of freezing and thawing, or treated with deoxycholate and subsequently incubated with adenosine under isotonic conditions, there was no significant increase in activity as compared to controls. Moreover, even though fractionation of sub-cellular constituents by sedimentation in sucrose gradients did produce a band of particulate matter corresponding to spherosomes (density = 1·01 g/cm^3)[13] the activity of the hydrolase was not enriched in this fraction.

[13] P. H. MATILE, J. P. BALZ, E. SEMADENI and M. JOST, *Z. Naturforschg.* **20**, 693 (1965).

Specificity

In order to establish the substrate specificity of the partially purified enzyme, its activity was tested on a number of compounds that are structurally similar to adenosine. As shown in Table 3, the results demonstrate that although the enzyme catalyzed the cleavage of adenosine and deoxyadenosine almost equally, it failed to show measurable activity with the other compounds listed. This absence of activity coupled with the observed catalysis of deoxyadenosine indicated that specificity may depend primarily upon the nature of the group at the 6-position in the purine ring. Hence it was concluded that the nature of the attached pentose moiety in the nucleoside could be of secondary importance in determining specificity. If this were so, it might be possible to catalyze the cleavage of 6-aminonucleosides in which there is some substitution at the 2 and 3 positions of the pentose. Accordingly, an attempt was made to see if a mixture of the 2′- and 3′-isomers of adenylic acid could serve as substrates for the enzyme.

TABLE 2. EFFECT OF VARIOUS SUBSTANCES ON THE ACTIVITY OF *N*-RIBOSYLADENINE RIBOHYDROLASE

Substance	Final concn. (mM)	Relative activity
$Ca(NO_3)_2$	100	98
$Co(NO_3)_2$	100	95
$CuSO_4$	100	103
NaF	100	93
LiCl	100	100
$MgSO_4$	100	110
$MnCl_2$	100	110
$ZnCl_2$	100	98
Na_2HAsO_4	100	93
p-Chloromercuribenzoate	0·01	110
NaN_3	0·10	99
Ribose	8·0	103
Adenine	4·0	36
Control	8·0	100

TABLE 3. SPECIFICITY OF *N*-RIBOSYLADENINE RIBOHYDROLASE

Substrate	Concn. (mM)	Relative activity
Adenosine	8·0	100
Inosine	8·0	0
Xanthosine	8·0	0
DMAP riboside*	8·0	0
Cytidine	8·0	0
Deoxyadenosine	8·0	93
Deoxycytidine	8·0	0
Deoxyguanosine	8·0	0
Adenosine-3′-(2′) PO_4	8·0	0
Adenosine-*N*-oxide	4·0	0
Guanosine	4·0	0

* Dimethylaminopurine.

Incubation of the enzyme with these isomers in the presence of 0·1 M NaF (preliminary experiments had shown that the enzyme preparation contained small amounts of a non-specific acid phosphatase whose activity was completely inhibited by this concentration of NaF) failed to produce measurable hydrolysis as determined by tests for ribose. Examination of reaction mixtures by paper chromatography confirmed this observation.

A similar approach was taken in order to demonstrate the absence of contaminating nucleoside phosphorylase. Because incubation of the enzyme with adenosine in phosphate buffer with and without NaF resulted in the same amount of reducing sugar, it was concluded that increased activity in the presence of phosphate was due to direct activation. (In those cases where NaF was added to inhibit acid phosphatase, the product of nucleoside phosphorylase activity would be ribose-1-phosphate and this would not react as a reducing sugar under the conditions of the test).

Kinetics

Initial velocities at substrate concentrations ranging from 0·72 to 8·7 K_m were obtained by extrapolation to zero time in plots of product concentration vs. time. The corresponding estimates of initial velocity ranged from 0·44 to 0·92 V_m and were used to calculate K_m and V_m along with their respective standard errors. Analysis of these data by the procedure of Wilkinson[14] yielded values of $2{\cdot}76 \times 10^{-4}$ M $\pm 0{\cdot}32 \times 10^{-4}$ (11·6%) and 0·241 μm/min $\pm$ 0·007 (2·9%) for K_m and V_m respectively. In order to detect any obvious departures from Michaelis–Menten kinetics, values for the initial velocity, v_0 plotted against $v_0/(S)$[15] and this gave a straight line.

Time-course studies at several substrate concentrations were used to follow the extent of reaction as well as to estimate the product inhibition constant, K_p. Application of a least squares treatment to these curves yielded expressions formally equivalent to those produced by Michaelis–Menten kinetics and homeomorphic with competitive inhibition.[16] The value for K_p by this method was apparently about 5-fold higher than that expected based on a comparison of the extent of reaction at a given time in the presence and absence of product inhibition.[17] In addition, the rate at which the velocity falls off from zero to first order kinetics at substrate concentrations of the order 10 K_m also indicated that the affinity of the enzyme for the product, adenine, is greater than that predicted above. Application of the graphical procedure of Dixon[18] confirmed these conclusions and gave a value of $0{\cdot}57 \times 10^{-4}$ M for K_p.

Molecular Weight

The MW of *N*-ribosyladenine ribohydrolase was estimated from its relative rate of sedimentation in sucrose density gradients. A mean falue of $S_{20,w} = 5{\cdot}0 \pm 0{\cdot}16$ ($N = 5$) was calculated on the assumption that the error due differences in partial specific volume would be negligible.[19] Similarly, the corresponding value for the MW of 62 400 daltons was computed on the premise that the enzyme is essentially spherical. This estimate of MW was in accord with that predicted from the elution pattern of the enzyme from Sephadex G100.

DISCUSSION

One of the enigmas associated with the behaviour of *N*-ribosyladenine ribohydrolase is its apparent latency in the intact potato leaf. As originally shown by Page[20] and confirmed many times in subsequent studies, uptake of adenosine via the petiole is followed by a relatively rapid conversion to inosine and hypoxanthine with only traces of adenine found in leaf extracts. By contrast, when leaf tissue is macerated, subjected to freezing and thawing, or injured by fungal invasion, adenosine conversion occurs predominantly by way of direct hydrolysis to adenine and ribose. As was shown in the present work, the explanation for this differential degradation based upon the possibility that the enzyme exists in the cell as a lysosomal or spherosomal constituent appears unlikely.

Perhaps a better explanation for the cryticity of this enzyme under normal physiological

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conditions can be found in a consideration of the relative affinities of the enzymes which affect the substrate, adenosine. For example, a relatively higher affinity on the part of adenosine deaminase for adenosine (conversion to inosine) coupled with the product inhibition observed with *N*-ribosyladenine ribohydrolase could mean that under conditions of low substrate concentration, the predominant activity is deamination. In case of injury or stress, on the other hand, the leaf responds with increased activity of degradative enzymes which directly or indirectly lead to increased levels of adenosine. Presumably this is what happens when the leaf is invaded by the fungus, *Phytophthora infestans*:[9] the pronounced phosphatase activity of the fungal haustorium together with that indigenous to the plant markedly increases the levels of adenosine by hydrolysis of adenylic acid. A concomitant shift in pH of lower values would also be expected to favour hydrolysis over deamination. Moreover, failure to observe measurable amounts of deamination when extracts of healthy leaf tissue are incubated with adenosine must mean that some inactivation of the deaminase does occur under these conditions.

What then is the physiological role of *N*-ribosyladenine ribohydrolase? On the one hand, the extremely low pH optimum and the hydrolytic nature of the reaction point to an essentially catabolic role for this enzyme. However, on the other hand, the high substrate specificity and the low activity observed under normal conditions suggest otherwise. It seems to us that the enzyme may represent a mechanism for 'funnelling off' adenine in accordance with cellular demand for this compound. Some support for this conclusion can be drawn from our other work (to be published) where ^{14}C-labelled adenine was fed to potato leaves and underwent rapid incorporation into the ribonucleic acid fraction. Analysis of alkaline hydrolysates of this labelled nucleic acid showed that both adenylic and guanylic acids were labelled and approximately to the same extent. In keeping with the function for *N*-ribosyladenine ribohydrolase postulated above, we would therefore propose that the enzyme operates as part of a salvage pathway for the recovery of preformed purines which are reutilized as precursors in the synthesis of ribonucleic acid. Whether this is the exclusive function of the enzyme remains to be determined.

EXPERIMENTAL

Extraction and purification. Potato plants (*Solanum tuberosum*, L. var. Green Mountain) were grown in the greenhouse, harvested at the end of 7 weeks, and used as the source of enzyme.

Freshly excised leaflets (400 g) were washed with distilled H_2O and homogenized for 2 min at 5° in 600 ml of 0·05 M Tris-HCl buffer, pH 7·4 containing 0·001 M EDTA. The resulting slurry was strained through Miracloth and the residue squeezed by hand to remove occluded buffer.

This crude extract was transferred to a magnetic stirrer and neutralized aq. protamine sulfate (1%) was added dropwise at the rate of 12 ml/100 ml of extract. The resulting suspension was centrifuged at 20 000 *g* for 1 hr at 0°; the supernatant was decanted and adjusted to 75% saturation with $(NH_4)_2SO_4$. After sitting overnight at 5°, the precipitate was recovered by centrifugation and suspended in 150 ml of the extracting buffer solution.

Following overnight dialysis against 6 l. of the same buffer solution, the concentrated extract was fractionated by the stepwise addition of $(NH_4)_2SO_4$. The precipitate obtained between 40 and 60% saturation was dissolved in 20 ml of 0·01 M Tris-HCl buffer, pH 7·4 and this solution adjusted to pH 4·8 with HOAc. The resulting suspension was centrifuged, the precipitate discarded, and the supernatant solution titrated to pH 7·0 with dil. NaOH.

Further purification of the enzyme was achieved by fractionating the neutralized solution with absolute EtOH at 0°, and the precipitate obtained between the addition of 0·5 and 1·5 vol. EtOH was dissolved in 10 ml of 0·01 M acetate buffer, pH 5·4. This solution was dialyzed to remove traces of EtOH and used for further purification by chromatography on DEAE-cellulose columns.

Protein estimation. Protein concentration was estimated by Lowry's procedure[21] on samples prepared as

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follows: proteins were precipitated from 1·0 ml vol. by the addition of 0·2 ml of 2·4 M $HClO_4$. The acidified solution was centrifuged, the supernatant solution discarded, and the precipitate washed twice with acetone. The washed precipitate was dissolved in 0·1 N NaOH and protein estimated as described above using bovine serum albumin as standard.

Assay of enzyme activity. Two methods were used to determine the activity of the enzyme. In one case activity was followed by estimating the amount of ribose released according to the procedure of Nelson.[22] This method of determining ribose as reducing sugar was particularly well-suited to kinetic studies because of its high sensitivity. Other instances where this procedure was used are cited in the text. In order to facilitate the assay of a large number of samples—the situation when individual fractions in column eluates were monitored for activity—a procedure based on the quenching of adenosine in alkaline solution was used to follow activity.[9] The extent of reaction was calculated from the expression: $X = (12{\cdot}44–2{\cdot}06\,Y)/(3{\cdot}14\,Y + 6{\cdot}08)$ where X represents the degree of hydrolysis expressed as a fraction, and Y, the ratio of absorbances at 250 and 280 nm; i.e. $Y = A_{250\text{ nm}}/A_{280\text{ nm}}$. The application of this expression was verified by determining the amount of adenine in mixtures that contained known amounts of adenine and adenosine. Comparison of this method with that based on the estimation of ribose as reducing sugar showed that the results agreed to within 5%.

Initial velocities. Reaction mixtures for the determination of initial velocities were prepared by transferring 1·0 ml vol. of enzyme solution containing 125 mU of activity to 4·0 ml of the appropriate substrate concentration in 0·1 M acetate buffer, pH 4·0. The tubes containing the reaction mixture were covered with parafilm, inverted 3× in rapid succession, and incubated at 30°. After the appropriate time interval, 1·0 ml of the reaction mixture was transferred to Sunderman tubes that contained 0·2 ml N NaOH, and the amount of ribose was estimated. The number of μmol of ribose released was plotted against time and the initial velocities obtained by extrapolation to zero time.

Chromatography and electrophoresis. Columns of DEAE-cellulose (Whatman DE52) and Sephadex (Pharmacia, G100) were prepared and packed by standard procedures. Elution characteristics and packing of columns was checked by observing the elution behaviour of uridine (0·5 μm) in the case of DEAE-cellulose and high MW DNA in the case of Sephadex before samples were applied. Electrophoresis was conducted on 5% polyacrylamide gels and activity followed by two different procedures. In one instance, gels were sectioned into 1 mm slices and these were placed directly into solutions of the substrate. For the second procedure, the intact gels were incubated with substrate containing xanthine oxidase, DPN, phenazine methosulfate, and the dye, NB tetrazolium.[23] The basis for the selective staining in this procedure depended upon the observed reaction of adenine with xanthine oxidase to produce 2,8-dihydroxyadenine.[24]

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[23] G. J. Brewer, *An Introduction to Isozyme Techniques*, p. 126, Academic Press, New York (1970).
[24] H. Klenow, *Biochem. J.* **50**, 404 (1952).