Phytochemistry, Vol. 25, No. 1, pp. 33–37, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00

PURIFICATION AND CHARACTERIZATION OF A 5'-NUCLEOTIDASE FROM *ZEA MAYS* MICROSOMES

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(*Revised received* 28 *May* 1985)

Key Word Index—*Zea mays*; Gramineae; maize; nucleotide metabolism; 5'-nucleotidase.

Abstract—A 5'-nucleotidase (EC 3.1.3.5) from *Zea mays* seedling shoot microsomes has been purified 125-fold to apparent homogeneity. The enzyme is competitively inhibited by cAMP (K_i = 5.2 μM) and is also inhibited by adenosine in a noncompetitive manner ($K_i \leq 57$ μM). The inhibition by adenosine allowed us to distinguish the specific 5'-nucleotidase activity from that of nonspecific acid phosphatase activity in crude tissue homogenates. When an assay based on that observation is used, about half of the total 5'-nucleotidase activity in a crude homogenate is estimated to be associated with the microsomal membranes. The microsomal enzyme has been solubilized and purified. Estimates from gel filtration and from gel electrophoresis in sodium dodecylsulphate suggest that the enzyme is composed of two subunits of M_r 24 500 and 25 500. The purified enzyme is specific for nucleoside monophosphates, with the activity assayed with *p*-nitrophenyl phosphate, α- and β-glycerol phosphates and ribose-5-phosphate ranging from 0 to 3% of that with 5'-AMP. K_m values for purine nucleotides are somewhat lower than for pyrimidine nucleotides. Divalent cations had no effect on the enzyme activity. A possible role for the enzyme in nucleotide pool size regulation is discussed.

INTRODUCTION

5'-Nucleotidases from animal cells have been implicated in a number of important cellular functions such as transmembrane nucleotide transport [1–3] and nucleotide pool size regulation [4–7]. The description of 5'-nucleotidase activities from higher plant sources have been very few despite the possible importance of this group of enzymes. Polya described 5'-nucleotidase activities from potato [8] and wheat seedling leaves [9] and suggested an involvement with a cyclic nucleotide regulatory system. The regulation of cytokinin metabolism by a 5'-nucleotidase from wheat germ has been suggested by Chen and Kristopeit [10]. Eastwell and Stumpf have shown the presence of a 5'-nucleotidase in the chloroplasts of Swiss chard [11].

In this report, we describe the purification and characterization of a 5'-nucleotidase from corn coleoptile microsomes and suggest a possible role for the enzyme in the regulation of the adenine nucleotide pool size.

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RESULTS

Purification of 5'-nucleotidase

The 5'-nucleotidase activity in extracts of corn seedlings was first noticed as an activity that accompanied a microsomal K^+-ATPase until the final step of the ATPase preparation [12]. Consequently, it was possible to purify the nucleotidase to apparent homogeneity with minor modifications of the scheme used for the K^+-ATPase. Because of the large amounts of nonspecific phosphatase activity present in the seedling tissues, it was difficult to determine specific 5'-nucleotidase activity present before chromatography on a Sephacryl S-200 column, at which point the last of the phosphatase activity was resolved from the 5'-nucleotidase activity. In estimation of the amount of 5'-nucleotidase in the presence of nonspecific phosphatases, advantage was taken of the fact that 5'-

Table 1. Purification of 5'-nucleotidase

Isolation step	Total activity (μmol Pi/hr) −adenosine	+2 mM adenosine	5'-Nucleotidase activity (μmol Pi/hr)	Specific activity (μmol Pi/hr/mg)	Fold purification	% recovery
1465 *g* supernatant	1412	1234	178	0.35	—	100
12 000 *g* supernatant	1283	1047	236	0.57	1.6	131
Microsome suspension	418	308	110	0.77	2	61
Detergent extraction	251	102	149	2.5	7	84
Hexyl agarose	76	22	54	5.7	16	30
1st Sephacryl S-200 column	30	5	25	29	81	14
2nd Sephacryl S-200 column	22	5	17	44	125	10

Table 2. 5'-Nucleotidase substrate specificity

200 μM substrate	Relative activity	K_m values
5'-AMP	100	57.7 μM
5'-GMP	116	57.1 μM
5'-CMP	46	333.3 μM
5'-UMP	71	200.0 μM
5'-IMP	99	81.6 μM
5'-ATP	0	
3'-AMP	20	
cAMP	18	
p-Nitrophenyl phosphate	3	
Ribose-5-phosphate	0	
β-Glycerol phosphate	1	
α-Glycerol phosphate	3	
Zeatin riboside 5'-phosphate	17	

Table 3. Effect of inhibitors on 5'-nucleotidase activity

Inhibitor	% of control*
0.2 mM 6-chloropurine riboside	57
0.2 mM 6-mercaptopurine riboside	91
0.2 mM 6-γ-γ-dimethylallylpurine riboside	64
0.2 mM kinetin riboside	64
0.2 mM adenosine-5'-sulphate	79
0.2 mM cAMP	24
0.2 mM ATP	88
0.2 mM adenosine	53
2 mM adenosine	18
2 mM 8-bromoadenosine	4
2 mM 8-azidoadenosine	8

*The hydrolysis of 200 μM 5'-AMP in the absence of inhibitors has been used as the standard of 100% of control.

nucleotidase is inhibited by adenosine (Tables 1 and 3), whereas nonspecific phosphatase activity is not affected (not shown). The 5'-nucleotidase activities shown in Table 1 are calculated from the differences in AMPase activity in the presence and absence of 2 mM adenosine.

In the 1465 *g* supernatant, the hydrolysis of 5'-AMP is inhibited *ca* 12% by 2 mM adenosine. This is a measure of the hydrolysis by the 5'-nucleotidase. Presumably the remaining 88% of the 5'-AMP hydrolysis is due to acid phosphatase activity. However, since the assay was performed at pH 6.8 (far from the optimal pH for an acid phosphatase), this is probably a low estimate of the amount of acid phosphatase present in this sample relative to 5'-nucleotidase. This method of estimating 5'-nucleotidase activity is subject to large relative errors when the amount of phosphatase is much greater than the amount of 5'-nucleotidase, but it has allowed us to estimate the 5'-nucleotidase activity during the purification of the enzyme when other assay procedures were useless. Overall, 125-fold purification was achieved with 10% recovery of activity.

Characterization of the purified 5'-nucleotidase

From the position of elution of the 5'-nucleotidase from a calibrated Sephacryl S-200 column, the apparent M_r was calculated to be 49 000 ± 6000 (Fig. 1). However, when the purified enzyme was examined by SDS-PAGE, two bands appeared corresponding to M_rs of 24 500 and 25 500.

According to an estimate of the carbohydrate content of the enzyme by the periodate-Schiff method of Mantle and Allen [13], the enzyme contains 40% (w/w) carbohydrate.

The results of the SDS-PAGE of the 5'-nucleotidase preparation after a second treatment on a Sephacryl S-200 column show the presence of two bands. Although this indicates that there are two different proteins present in the denatured 5'-nucleotidase preparation, the sum of the estimated M_rs of the two bands is very close to the value of 49 000 ± 6000 for the native protein. This suggests that the

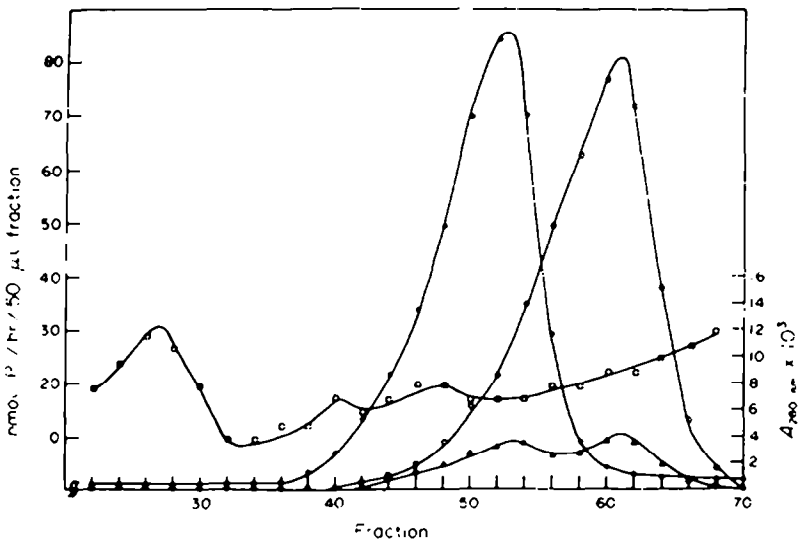

Fig. 1. Sephacryl S-200 column chromatography of 5'-nucleotidase and K^+-ATPase activities. A 6-ml sample of protein eluted from the hexyl agarose column was applied to the Sephacryl S-200 column (2.6 × 80 cm), eluted with buffer D (see Experimental) at a flow rate of 0.77 ml/min. 5'-Nucleotidase (●), K^+-ATPase (○), acid phosphatase (□), $A_{280\,nm}$ (△).

purified 5'-nucleotidase may be composed of two subunits and has been purified to apparent homogeneity.

This preparation of enzyme was used for the production of polyclonal antibodies. An Ouchterlony diffusion plate using the anti-5'-nucleotidase antiserum and the purified 5'-nucleotidase protein showed one precipitin line between the 5'-nucleotidase well and the 5'-nucleotidase antiserum well, while there were no precipitin lines present between the 5'-nucleotidase and the preimmune serum wells. The presence of one precipitin line gives further indication of the homogeneity of the 5'-nucleotidase preparation.

Once the 5'-nucleotidase has been purified by the Sephacryl S-200 chromatography, it may be stored at −20° without appreciable loss of enzymatic activity for a period of months. However, incubating the enzyme for variable lengths of time at 37° before substrate addition results in a decline in the activity of the enzyme. The presence of 0.01 % sucrose dilaurate prevented the inactivation of the enzyme (not shown) and resulted in a linear time course of product production. Similar results were obtained with bovine serum albumin or Zwittergent 3,12 added to the enzyme. The rate of the reaction is proportional to enzyme concentration over a 10-fold range of protein concentrations (0.05–0.5 μg/ml).

A pH profile encompassing the maximum and minimum physiological values found in the plant cell as established by ^{31}P NMR studies [14] is shown in Fig. 2. There is no clear optimal pH for the enzyme, although it is inhibited in the higher pH range. The remainder of the experiments reported here were done at pH 6.8.

Substrate specificity

K_m values for the enzyme indicate stronger affinity for the purine nucleoside monophosphates than for pyrimidine nucleotides (Table 2). The cytokinin derivative zeatin-5'-monophosphate is a relatively poor substrate, while the glycerol phosphates, ribose-5-phosphate and *p*-nitrophenylphosphate, are virtually inactive as substrates.

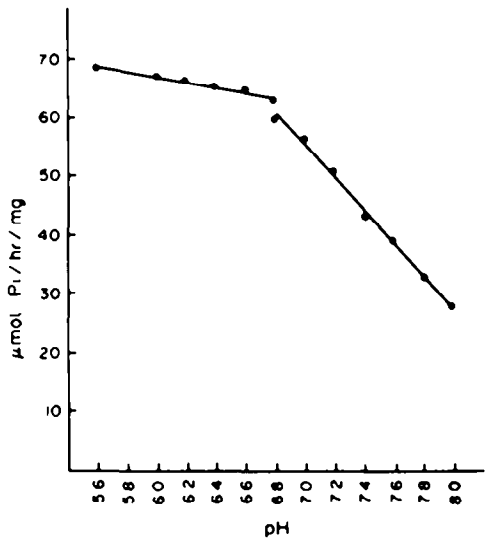

Fig. 2. The pH profile of the purified 5'-nucleotidase. Buffers used: pH 5.6, 10 mM MES–imidazole; pH 6.0–6.8, 10 mM bis-Tris HCl; pH 6.8–8.0, 10 mM HEPES–imidazole.

Effects of various inhibitors

A series of substrate and product analogs were tested with the 5'-nucleotidase for their ability to inhibit the activity of the enzyme (Table 3). The plant hormones zeatin and kinetin did not greatly affect the enzyme. Adenosine and cAMP were among the most effective inhibitors tested. cAMP acted as a competitive inhibitor with a K_i of 5.2 μM as determined by analysis of replots of intercepts and slopes from the Lineweaver–Burk plot [15]. Adenosine inhibited the 5'-nucleotidase in a non-competitive manner. A replot of the $1/v$ intercepts vs adenosine concentration yields a concave upward curve, characteristic of a steady-state random Uni-Bi mechanism [15] from which one cannot extract an exact value for the K_i; however, the Lineweaver–Burk plot indicates that the inhibition constant for adenosine is less than the K_m for the substrate used (5'-AMP, K_m = 57 μM).

A number of divalent cations were tested for their influence on the 5'-nucleotidase. The enzyme is not affected significantly by the presence of 100 μM $MgCl_2$, $MnCl_2$, $CoCl_2$, $CaCl_2$, $ZnSO_4$, $FeSO_4$ or $NiSO_4$.

Association of the 5'-nucleotidase with membranes

5'-Nucleotidase activity bound to the microsomes accounts for *ca* half of the total 5'-nucleotidase activity found in the corn shoot. This activity is not removed by washing the membranes with buffer A; however, upon treatment with detergent and KCl, the previously bound

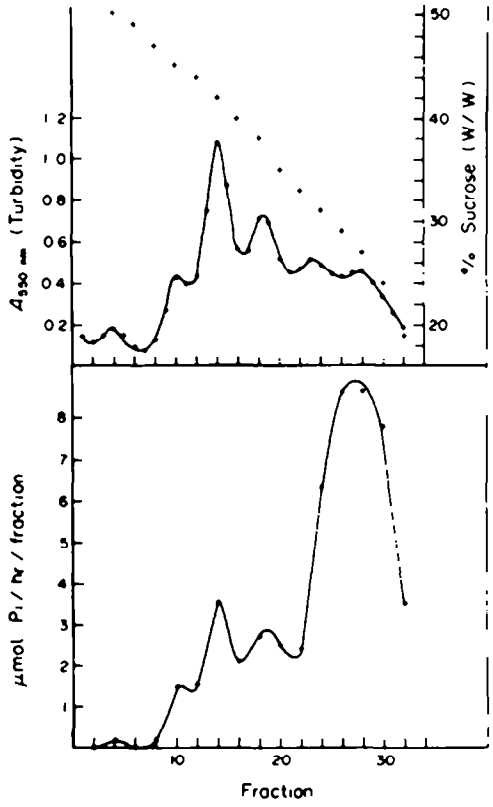

Fig. 3. Sucrose density gradient profile of the 80000 *g* microsome pellet. Top panel: (○) sucrose concentration; (●) $A_{550\ nm}$ turbidity. Bottom panel: (●) specific 5'-nucleotidase activity.

activity, along with the major portion of the total membrane protein, becomes solubilized. Subjecting washed microsomal membranes to continuous sucrose density gradient centrifugation (Fig. 3) shows that the specific 5'-nucleotidase activity is distributed among all of the resolved membrane fractions. However, the major fraction of the activity is located in the region of the gradient thought to be associated with the tonoplast and the endoplasmic reticulum membranes (1.08–1.13 g/cc[2]) [16–19].

DISCUSSION

The 5'-nucleotidase activity from corn coleoptile microsomes has been purified 125-fold from the crude extract. The low number associated with the purification of the 5'-nucleotidase to apparent homogeneity could be the result of two possibilities: (1) the activity of the crude homogenate material has been overestimated or (2) the activity of the purified material has been underestimated. A likely explanation for an underestimation of the purified enzyme activity is that an activating factor, present in the crude homogenate, has been removed from the 5'-nucleotidase during the purification scheme. It is unclear what the actual situation is; however, the combination of a very low turnover number for the 5'-nucleotidase (~ 5) and the unlikely prospect that the 5'-nucleotidase comprises 0.8% of the total protein in the cell indicates that the 'fold-purification' of the enzyme is suspect.

The 5'-nucleotidase activity described here is a membrane-bound glycoprotein, composed of two similar subunits, with a native M_r of 49 000 for the whole enzyme. The enzyme is inhibited by nucleosides and specifically hydrolyses nucleoside-5'-monophosphates as substrates. The physical characteristics of the corn microsomal 5'-nucleotidase are very similar to those described for the 5'-nucleotidase found in potato [8] and wheat seedling leaves [9], all having M_rs of *ca* 50 000 and being composed of two subunits. A comparison of the kinetics of the different enzymes shows that although there are similarities between them, the characteristics of the enzyme vary from source to source. All three enzymes are noncompetitively inhibited by adenosine, but the maize enzyme has at least an order of magnitude higher affinity for adenosine than do the other two enzymes. The substrate specificity of the three enzymes also shows that they are quite similar with the exception that the potato and the wheat seedling leaf 5'-nucleotidase preparations have very high *p*-nitrophenylphosphatase (*p*NPPase) activity while the purified maize microsomal 5'-nucleotidase is essentially without *p*NPPase activity. This probably is due to a more effective removal of phosphatase during purification of the maize enzyme, rather than difference in specificities of the enzymes from different plants. In regard to the subcellular location of the enzymes, the 5'-nucleotidase from maize is tightly associated with the microsomal membranes (Fig. 3), whereas the potato and wheat seedling leaf 5'-nucleotidases as well as the chloroplast enzyme [11] seem to be soluble enzymes, based on the procedures used to isolate them.

Polya [8, 9] has suggested that the 5'-nucleotidase from potato and wheat seedling leaves may be regulated by cyclic nucleotides. We also found a significant competitive inhibition of the maize 5'-nucleotidase by cAMP, with a K_i of 5.2 μM. The concentration of cAMP found in corn coleoptiles [20] is *ca* 10^{-7} M, therefore a physiologically relevant regulation by cyclic nucleotides is not excluded, although it is more likely that the inhibition is simply a case of interaction of the enzyme with a substrate analog.

A more obvious role for the enzyme is regulation of the level of nucleoside monophosphates in the cell. It may be a key enzyme in determining whether a nucleoside monophosphate is degraded to a nucleoside and subsequently to the respective base, or rephosphorylated to di- or triphosphate. As shown by Saglio and Pradet [21], the depletion of energy source (e.g. soluble sugars) and the consequent drop in the respiration rate of plant cells result in a drop in the adenine nucleotide pool size in the cell, but the ratio of the high energy nucleotides (ATP and ADP) to the total nucleotide pool (ATP, ADP, AMP) remains constant [22]. A similar effect of 2-deoxyglucose (2-DG) and ethionine on corn root adenine nucleotide levels was reported by Lin and Hanson [23]. The addition of 10 mM 2-DG resulted in a drop in ATP and ADP levels (47 and 24 nmol/g fresh weight, respectively) whereas the AMP levels increased by only 8 nmol per g fresh weight. A mechanism for these changes is to allow for the hydrolysis of the nucleoside monophosphates *via* 5'-nucleotidase action in coordination with the drop of the high energy nucleotides. This hypothesis might be tested by inhibiting the 5'-nucleotidase activity in intact tissues. Our attempts to do so using 8-azidoadenosine and 8-bromoadenosine were unsuccessful, but it may be possible with another inhibitor.

EXPERIMENTAL

Zea mays hybrid B73Ht × Mo17Ht was obtained from the M. Brayton Seed Co., Ames, IA and W64A seed was generously supplied by Dr. Charles Martinson, Department of Plant Pathology, Weed and Seed Sciences, Iowa State University, Ames, IA.

All the compounds tested as substrates for the enzyme were obtained from Sigma, with the exception of zeatin riboside-5'-phosphate which was the kind gift of Dr. C.-M. Chen, University of Wisconsin-Parkside, Kenosha, WI. All the inhibitors tested were also from Sigma with the exception of adenosine which was obtained from Calbiochem as was the Zwittergent 3,12 detergent. Hexyl agarose was obtained from Miles and the Sephacryl S-200 was from Pharmacia.

Preparation of microsomes. Corn seeds were germinated at 28° in the dark. After 3 days, the etiolated shoots were removed from the seedlings, rinsed with deionized H_2O, weighed and cut into 1-cm lengths. Typically, between 50 and 150 g of shoot segments were added to buffer A (0.25 M sucrose, 1.25 mM $MgCl_2$, 1.25 mM EDTA, pH 7.2 with Tris) at a ratio of 4 ml/g shoots. All subsequent steps were performed at 4°.

The suspension was homogenized in a Polytron homogenizer (Brinkman) for 30 sec at setting 4 followed by 60 sec at setting 6. Polyvinylpyrrolidone (0.25 g/g fr. wt of shoots) was added and mixed thoroughly. The mixture was then filtered through four layers of cheesecloth to remove the bulk of the fibrous material before centrifugation.

The filtrate was centrifuged at 1465 *g* for 10 min, the pellet discarded, and the supernatant centrifuged at 12 000 *g* for 20 min with the resulting pellet again being discarded. The 12 000 *g* supernatant was centrifuged at 80 000 *g* for 90 min to pellet the microsomes. This pellet was resuspended in buffer A (0.1 ml/g shoots) and then repelleted at 80 000 *g* for 90 min. The washed pellet was resuspended in 1 ml buffer A per 7 g shoots.

Enzyme purification. Zwittergent 3,12 and KCl were added to

give final concns of 1% and 1 M, resp. After 10 min at 20°, the suspension was centrifuged at 80000 *g* for 90 min and the supernatant saved. The Zwittergent extract was dialysed 6 hr against buffer A, then overnight against buffer B (0.25 M $NaClO_4$, 1.25 mM $MgCl_2$, 1.25 mM EDTA pH 8 with Tris). The dialysis retentate was applied to a hexyl agarose column (1.6 × 30 cm) equilibrated previously with buffer B. The column was then eluted with 50 ml buffer B. The 5'-nucleotidase was retained on the column during this elution but was eluted at a higher salt concn (80 ml of buffer C: 2 M $NaClO_4$, 1.25 mM $MgCl_2$, 1.25 mM EDTA pH 8 with Tris).

The 5'-nucleotidase peak from the hexyl agarose column was concd to *ca* 4 ml by using Aquacide II-A (Calbiochem. The enzyme concentrate was dialysed against buffer D (1 M NaCl, 1.25 mM $MgCl_2$, 1.25 mM EDTA pH 8 with Tris), then applied to a Sephacryl S-200 column (2.6 × 80 cm) equilibrated with buffer D.

The peak of 5'-nucleotidase activity eluted between the activity peaks of an acid phosphatase and a K^+-ATPase, so that the pooled 5'-nucleotidase fractions were contaminated by the other two enzymes. The pooled 5'-nucleotidase fractions were reconcentrated with Aquacide II-A and reapplied to the Sephacryl S-200 column. The 5'-nucleotidase pool obtained from the second Sephacryl column treatment was essentially free of K^+-ATPase and acid phosphatase activity.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A portion of the 5'-nucleotidase after the second Sephacryl S-200 column was prepared for SDS-PAGE by precipitation with 5% trichloroacetic acid (TCA) and washed with cold EtOH. Slab gels of acrylamide (3% stacking, 10% separating) were prepared according to the method described in ref. [24].

Analytical methods. Protein was determined by using the TCA precipitation modification of refs [25] or [13]. The amount of carbohydrate associated with the 5'-nucleotidase was estimated by using the periodic acid/Schiff reaction described in ref. [26]. The phosphate assay [27] was used to measure the activity of the 5'-nucleotidase in the purification scheme up to the hexyl agarose step. After chromatography on the hexyl agarose column, the enzyme was assayed by a modification of the phosphate assay of ref. [28], developed in this laboratory [29]. This method is about 10-fold more sensitive than the Fiske–SubbaRow method.

Enzyme assay conditions. The assay for the 5'-nucleotidase contained 10 mM bis-Tris adjusted to pH 6.8 with HCl, 0.01% sucrose dilaurate, 0.05–0.20 μg enzyme protein and 200 μM 5'-AMP unless otherwise stated. All assays were incubated for periods of 1 hr or less at 37° in a total vol. of 1 ml.

The assay for the K^+-ATPase contained 10 mM HEPES adjusted to pH 7.5 with imidazole, 20 μg BSA, ± 250 mM KCl and 1 mM 5'-ATP. The enzyme was assayed at 37° for 1 hr or less in a total vol. of 1 ml. The activity reported is the increment due to the addition of KCl. The assay for the acid phosphatases contained 10 mM MES adjusted to pH 5.5 with Tris, and 200 μM *p*-nitrophenylphosphate. The enzyme was assayed at 37° for 30 min or less in a total vol. of 1 ml.

Sucrose density gradient centrifugation. The 80000 *g* microsomal pellet was resuspended in 2 ml of 0.25 M sucrose, 1 mM Tris, pH 7.2 with MES. This suspension was floated on a linear (20–50%, w/w) sucrose gradient. This gradient was centrifuged in a SW27 rotor at 112840 *g* for 2 hr under a slow acceleration mode in a Beckman L-8 ultracentrifuge. The gradient was fractionated by puncturing the bottom of the tube and collecting drops.

The kinetic parameters for the 5'-nucleotidase were determined by using the OMNITAB computer program of ref. [30].

Polyclonal antibodies were produced against the purified 5'-nucleotidase in New Zealand white rabbits according to the following schedule. Four hundred micrograms of purified 5'-nucleotidase was injected as an emulsion with Freunds' complete adjuvant, subcutaneously in the back of the rabbit. After one month, a booster injection of 200 μg of 5'-nucleotidase (in 0.9% NaCl, 0.1 M HEPES adjusted to pH 7.4 with imidazole) was injected in the ear vein. Nine days following the booster injection, the rabbit was bled and tested for the presence of 5'-nucleotidase antibodies. The specificity of the rabbit antiserum was determined using an Ouchterlony double diffusion test. The purified 5'-nucleotidase was tested against the immune and the preimmune rabbit serum.

Acknowledgements—Journal Paper No. J-11374 of the Iowa Agriculture and Home Economics Experiment Station. Project No. 2560.

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