Nucleic Acid Degrading Enzymes of Barley Malt. III. Adenosine Nucleosidase from Malted Barley[1,2]

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ABSTRACT

Adenosine nucleosidase, an enzyme that catalyzes the hydrolytic cleavage of adenosine to adenine and ribose, was examined in malted barley. The enzyme was purified by precipitation with ammonium sulfate, and by column chromatography with diethylaminoethyl (DEAE)-Sephadex, Sephadex G-200, and hydroxylapatite. The enzyme was specific for adenosine and 2'-deoxyadenosine and had no discernable effect on other naturally occurring nucleosides. The molecular weight of the enzyme was approximately 120,000. The optimum pH was about 5.0 and the K_m value for adenosine was 0.27 m*M*. Adenosine nucleosidase showed no metal ion requirement for optimum activity and was inhibited by Zn^{2+}, Cu^{2+}, and Hg^{2+} ions. The enzyme in solution was completely inactivated after 15 min at 60° C.

Purine and pyrimidine bases, the products of the enzymatic breakdown of malt nucleic acids, are of interest to brewers in terms of yeast growth and fermentation (11) and, in some cases, as possible flavor enhancers (6,30). It is necessary to have adequate levels of free purine and pyrimidine bases available in wort if a long lag phase in fermentation is to be avoided. The rate of protein and enzyme synthesis by yeast depends to a large degree not only upon the level of amino acids in the wort but also upon the amount of nucleic acid precursors. Slow fermentations have been shown to be caused by a deficiency in the level of free nucleic acid bases in wort that restricts the rate of yeast growth (27).

Adenine and adenosine are also of interest because they are components of adenosine triphosphate and adenosine diphosphate, both of which are involved in energy transfers within the cell (3). Other compounds, such as flavin adenine dinucleotide, coenzyme A, and nicotinamide adenine dinucleotide, contain adenine and play familiar roles in cell metabolism.

Thompson et al (27) reported that slowly fermenting worts contained less than 10 mg of adenine per liter, compared with approximately 40 mg/L in normally fermenting worts. They also reported that the rate of fermentation could be stimulated by the addition of free bases. The free bases could not be replaced by nucleosides or nucleotides, which brewing yeasts are unable to utilize to any significant extent (27). A 10-fold decrease in adenine occurs during fermentation, which implies the uptake of this compound by yeast (6,21).

Ribonucleic acid and DNA are hydrolyzed by nucleases and diesterases first to oligonucleotides and eventually to mononucleotides. The latter are converted to nucleosides by the nucleotidases, and the glycosidic linkages between the purine or pyrimidine bases and the sugar moieties are cleaved by nucleosidases to yield the free purine and pyrimidine bases (1).

Two types of nucleosidases have been described (18): one catalyzes a reversible phosphorolytic cleavage of the glycosidic bond, and the second catalyzes an irreversible hydrolysis. Heppel and Hilmoe (8) have demonstrated both a hydrolytic system and a phosphorolytic system in bakers' yeast. Nucleoside phosphorylase from yeast splits inosine and guanosine, but only in the presence of phosphate or arsenate. The requirement for inorganic phosphate is absolute. On the other hand, the nucleoside hydrolase of yeast splits inosine, adenosine, guanosine, xanthosine, and a number of synthetic nucleosides in the absence of phosphate and arsenate.

Adenosine phosphorylase from extracts of *Escherichia coli* (13), uridine phosphorylase from rat liver (29), pyrimidine nucleoside phosphorylase from *Haemophilus influenzae* (23), thymidine phosphorylase from *Salmonella typhimurium* (9), and purine nucloside phosphorylase from *S. typhimurium* and rat liver (10) are reported in the literature.

Nucleoside hydrolase purified from various sources shows either relatively broad specificity with respect to the sugar and base involved or very narrow specificity. Riboside hydrolase from fish muscle (25) and microbial nucleosidases from *Lactobacillus delbruckii* (24), *L. pentosus* (28), *Pseudomonas fluorescens* (26), and yeast (8) are reported to catalyze the hydrolytic cleavage of several nucleosides to free bases and ribose. On the other hand, uridine nucleosidase from yeast (5,16,17) and adenosine hydrolase from brussels sprouts (18) have been reported to hydrolyze only one substrate among the naturally occurring purine and pyrimidine nucleosides. No studies of any purified nucleoside hydrolase from barley malt have been reported. However, a previous study in this laboratory revealed the presence of nucleoside hydrolase activity in crude malt extracts (14). This activity, with adenosine as substrate, was low in sound, mature barley or steeped barley. Enzyme activity increased more than 10-fold during germination, and results using an ASBC mash (2) indicated that the enzyme may be expected to be active during a substantial portion of a typical mash schedule.

Much of the earlier literature on nucleosidases from a variety of sources is based on data obtained using either unspecific spectrophotometric assays that involve changes in extinction or reducing sugar methods that can be rendered inaccurate by sugars from other sources. In the present paper, we describe an extensive purification of adenosine nucleosidase from malt to yield a preparation that will hydrolyze only adenosine, and some of the properties of this enzyme that were examined using a rapid, quantitative high-performance liquid chromatography (HPLC) assay procedure.

EXPERIMENTAL

Preparation of Malted Barley

The cultivar Robust, chosen for its high level of adenosine nucleosidase activity, was grown at Fargo, ND, in 1983. Cleaned barley was steeped at 16° C to 45% moisture and was germinated at 16° C and 100% relative humidity for five days. After germination, the sample was kilned for 24 hr at 45° C, conditions under which almost no activity loss occurred (14). The resulting malt was milled with a Udy cyclone mill (Udy Corp., Boulder, CO).

Purification of Adenosine Nucleosidase

Adenosine nucleosidase was purified by the method of Yamada (29) with some modifications. All steps described were performed at 0–4° C. One kilogram of ground malt was stirred for 2 hr at 4° C with 4.0 L of 0.1 *M* phosphate buffer, pH 5.0. The suspension was centrifuged at 10,000 × *g* at 4° C for 30 min. The supernatant was designated as the crude extract.

Preliminary studies indicated that most of the adenosine nucleosidase activity was present in the protein fraction

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precipitated between 50 and 70% saturation with ammonium sulfate. The crude extract was adjusted to 50% ammonium sulfate saturation by the slow addition of solid ammonium sulfate near pH 7.0. After each addition of ammonium sulfate, the solution was stirred for 30 min and centrifuged at 10,000 × *g* for 30 min. The supernatant was then brought to 70% saturation by the further addition of ammonium sulfate and was stirred for 30 min. After centrifugation at 10,000 × *g* at 4° C for 30 min, the precipitate (50–70% ammonium sulfate fraction) was resuspended in 0.02*M* potassium phosphate buffer (pH 8.0) containing 10 m*M* β-mercaptoethanol and was dialyzed overnight against the same buffer.

Half of the dialyzed fraction (110 ml) was placed on a column (4.5 × 60 cm) of diethylaminoethyl (DEAE)-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.02*M* phosphate buffer (pH 8.0), and the column was washed with 500 ml of the same buffer. Elution with a linear gradient of KCl was begun by allowing 0.4*M* KCl in 1.0 L of the 0.02*M* phosphate buffer to flow into a mixing chamber containing 0.5 L of the phosphate buffer and then onto the column. Fractions of 15 ml were collected at a flow rate of 40 ml/hr. Fractions containing enzyme activity from two consecutive runs were pooled. The enzyme was precipitated with ammonium sulfate (85% saturation) and centrifuged as described above. The precipitate was resuspended in 0.02*M* phosphate buffer (pH 8.0) and dialyzed overnight against the same buffer.

The dialyzed fraction was placed on a column (2.5 × 45 cm) of Sephadex G-200 (Pharmacia) equilibrated with phosphate buffer (0.02*M*, pH 8.0), and elution was performed at a constant flow rate of 20 ml/hr. The active fractions (5.0 ml each) were pooled and treated with ammonium sulfate to 85% saturation. After centrifugation, the pellet was dissolved in 3.0 ml of 0.02*M* phosphate buffer (pH 7.2) and dialyzed overnight against the same buffer.

A column (0.9 × 60 cm) of hydroxylapatite (Bio-Rad, Richmond, CA) was equilibrated with 0.02*M* phosphate buffer (pH 7.2). The enzyme was applied to the column and was eluted with a linear gradient of phosphate buffer by allowing 300 ml of 0.5*M* phosphate buffer (pH 7.2) to flow into a mixing chamber containing 300 ml of 0.02*M* phosphate buffer (pH 7.2). Fractions (3 ml) were collected, and highly active fractions were pooled.

Enzyme Assay

Adenosine (Sigma Chemical Co., St. Louis, MO) was dissolved at a level of 0.3 m*M* in 0.1*M* phosphate buffer (pH 5.0). The reaction mixture consisted of 4.0 ml of substrate solution and 50 μl of enzyme solution. The reaction mixture was incubated at 37° C for 30 min, after which it was heated for 5 min at 100° C to inactivate the enzyme. The amount of adenine released was determined by the HPLC method described by Qureshi et al (20).

HPLC analyses were performed with an Altex model 110A solvent metering pump and a variable-wavelength ultraviolet detector (Shoeffel Instrument Corp., model SF 770) at 254 nm. Peak areas were quantified by electronic integration (Hewlett Packard, model 3380A). The column was a μBondapak C_{18} high-efficiency column (300 × 3.9 mm, 10 μm particle size, Waters Associates, Milford, MA). Adenine was eluted isocratically with a solution of H_2O, acetic acid, and tetrabutylammonium phosphate (98.35:1.50:0.15). Activity was expressed as micromoles of adenine produced per minute under the conditions described above. Determination of activity with other nucleosides was performed under the conditions of incubation given above, and HPLC analyses of products utilized isocratic systems described previously (14).

Protein Determination

Protein was estimated by the method of Bradford (4).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed on 6% (w/v) gels with sodium lactate buffer (pH 3.1) according to the procedure of Khan et al (12). Methyl green was used as the tracking dye, and 50 μg of freeze-dried protein in 50 μl of 0.01*M* sodium lactate buffer (pH 3.1) was applied to each slot. The proteins were located with Coomassie Blue R-200.

To assay the enzyme activity of the protein fractions so separated, another sample of the active enzyme solution was run in another slot of the gel under the same conditions. Each of the 2-mm sections cut from the gel was extracted in 2.0 ml of 0.1*M* Tris-acetate buffer (pH 5.0) for 18 hr at 4° C. The reaction mixture was composed of one of the solutions thus prepared (0.5 ml) and 4.0 ml of 0.3 m*M* adenosine in 0.1*M* phosphate buffer (pH 5.0) or 0.1*M* Tris-acetate buffer (pH 5.0). The aforementioned assay procedure was used.

Determination of Molecular Weight

The molecular weight of the enzyme was determined by gel filtration of enzyme and standard proteins in 0.05*M* potassium phosphate buffer (pH 7.4) containing 0.1*M* NaCl, on a 2.5 × 45 cm column of Sephadex G-200 equilibrated with the same buffer. The standard proteins used (kit for molecular weights, Sigma Chemical Co., St. Louis, MO) were β-amylase, alcohol dehydrogenase, albumin, and carbonic anhydrase with molecular weights of 200,000, 150,000, 66,000 and 29,000, respectively. The molecular weight of the enzyme was determined by reference to a standard curve of log molecular weight versus K_{av} of the standard proteins, where

$$K_{av} = (V_e - V_o)/(V_t - V_o),$$

and V_o = void volume (ml), V_t = total volume (ml), and V_e = elution volume (ml) (19).

Evidence for Hydrolytic Cleavage

The enzyme used was dialyzed against Tris-acetate buffer (0.1*M*, pH 5.0) for 72 hr. After dialysis, the inorganic phosphate in the enzyme preparation was determined using the colorimetric method of Chen (7). The formation of adenine was measured by HPLC according to the standard assay procedure except that phosphate buffer was replaced by Tris-acetate buffer (0.1*M*, pH 5.0). The assay mixture consisted of 4.0 ml of 0.3 m*M* adenosine and 0.2 ml of dialyzed enzyme.

The formation of adenosine from adenine and ribose-1-phosphate (reverse reaction) was measured by HPLC as described above. The incubation mixture contained 2.0 ml of 0.3 m*M* adenine, 2.0 ml of 0.3 m*M* ribose-1-phosphate, and 0.2 ml of dialyzed enzyme in Tris-acetate buffer (0.1*M*, pH 5.0).

pH Optimum

Adenosine was prepared at a concentration of 0.3 m*M* in 0.1*M* phosphate buffer adjusted to pH values between 3.0 and 9.0 in 0.5 pH unit increments. Activities were determined in duplicate with 0.05 ml of enzyme as described above.

Optimum Temperature

Adenosine was incubated with 0.05 ml of enzyme solution for 30 min at 30, 37, 50, 60, and 70° C and assayed as described above.

Thermal Stability

Aliquots (0.05 ml) of purified enzyme in 3.6 ml of 0.1*M* phosphate buffer, pH 5.0 or pH 8.0, were held at 40, 50, 60, and 70° C for 15 min (3,26). After cooling, 0.4 ml of 3.0 m*M* adenosine was added and residual activities were determined as described above.

Determination of K_m

The K_m values were determined by plotting the reciprocal of the reaction velocity against the reciprocal of the substrate concentration. Substrate concentrations were 0, 0.017, 0.020,

TABLE I
Purification of Malt Adenosine Nucleosidase

Purification Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
I. Crude extract	14,430	40.3	0.0028	···	100.0
II. Ammonium sulfate	2,450	22.9	0.0093	3.3	56.8
III. DEAE-Sephadex	160	10.83	0.0677	24.0	26.9
IV. Sephadex G-200	22.0	7.09	0.322	115	17.6
V. Hydroxylapatite	1.8	5.59	3.16	1,130	13.9

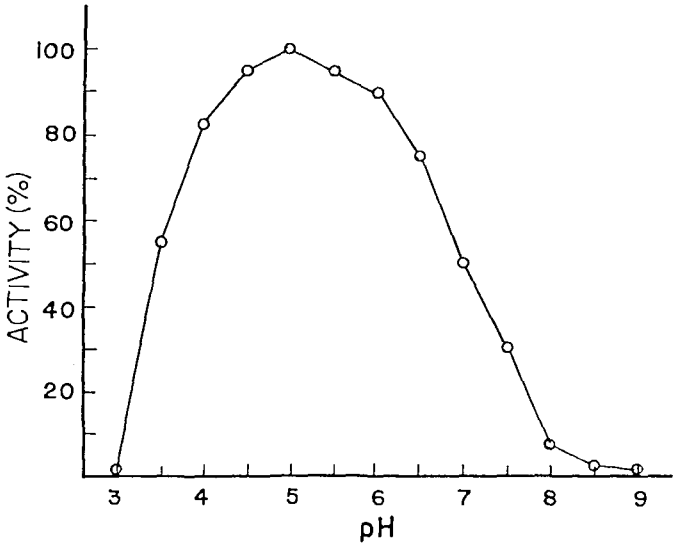

Fig. 1. Effect of pH on adenosine nucleosidase activity.

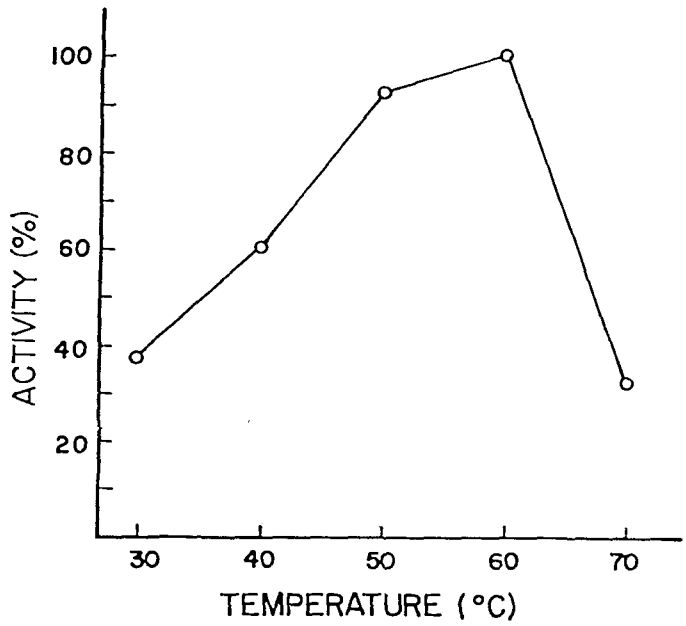

Fig. 2. Optimum temperature of adenosine nucleosidase.

).025, 0.033, 0.05, and 0.1 m*M*. The reaction mixture consisted of 1.0 ml of substrate in 0.1 *M* phosphate buffer (pH 5.0) and 0.05 ml of enzyme. The reaction mixture was incubated for 10 min at 37° C and was assayed as described above.

RESULTS AND DISCUSSION

Purification of Adenosine Nucleosidase

Adenosine nucleosidase was purified over 1,000-fold in specific activity from an extract of barley malt (Table I). This is five times greater than the extent of purification of the partially purified adenosine nucleosidase isolated by Mazelis and Creveling (18) from brussels sprouts. A single peak of adenosine nucleosidase activity was observed during the course of purification. The specific activity of the purified enzyme was 3.16 units/mg protein. The purified enzyme was free of detectable levels of adenosine deaminase, adenine deaminase, and other nucleosidases.

The adenosine nucleosidase from malt was found to be quite labile during the course of purification. The use of β-mercaptoethanol was necessary to maintain activity during the various purification steps.

When adenosine nucleosidase was stored at 4° C in 20 m*M* phosphate buffer (pH 7.2) containing 10 m*M* β-mercaptoethanol it was stable for up to twelve weeks, retaining 70% of its original activity at 4° C. However, more than 50% of the original activity was lost in less than one week at −20° C.

The purified malt enzyme preparation was also subjected to analytical polyacrylamide gel electrophoresis and stained with Coomassie Blue. One major band and three minor bands were observed. Extracts of the gel segments containing each of the bands revealed that all adenosine nucleosidase activity was associated with the major protein component.

Substrate Specificity

The action of malt adenosine nucleosidase on various natural nucleosides was determined at a substrate concentration of 0.3 m*M* at pH 5.0 for 30 min. The enzyme was active against only adenine-containing compounds. The deoxyribonucleoside, 2′-deoxyadenosine, was hydrolyzed at 96% of the rate of adenosine. None of the following nucleosides served as substrates: inosine, uridine, cytidine, guanosine, thymidine, xanthosine, 2′-deoxyinosine, 2′-deoxyguanosine, 2′-deoxycytidine, or 2′-deoxyuridine. Thus, the malt enzyme has a very narrow specificity. The partially purified adenosine hydrolase from brussels sprouts has also been shown to be a highly specific enzyme (18).

Molecular Weight

Elution volumes of the standard proteins and purified adenosine nucleosidase during gel filtration (column volume, 220 ml) were as follows: β-amylase, 121.5 ml; alcohol dehydrogenase, 138.0 ml; bovine serum albumin, 148.5 ml; carbonic anhydrase, 185.0 ml; enzyme (duplicate), 137.5 and 137.5 ml; and blue dextran (void volume), 91.5 ml. The molecular weight of adenosine nucleosidase was thus estimated to be about 120,000. This indicates that malt adenosine nucleosidase is larger than other nucleosidases studied so far. For example, the uridine nucleosidase purified from yeast has a molecular weight of 32,000 (18).

pH Optimum

The pH activity curve for the adenosine nucleosidase of malt is shown in Figure 1. The optimum pH, using adenosine as the substrate, was around 5.0 in potassium phosphate buffer. Adenosine nucleosidase activity in 0.1 *M* phosphate buffer (pH 5.0) was approximately the same as that in Tris-acetate buffer (0.1 *M*, pH 5.0), which suggests that this enzyme does not require inorganic phosphate ion.

Various values have been reported in the literature for the optimum pH of adenosine hydrolase preparations from other sources. Mazelis and Creveling (18) reported an optimum pH for adenosine hydrolase from brussels sprouts between pH 3.5 and 4.5, whereas Terada et al (26) reported a pH optimum for nucleoside hydrolase from *Pseudomonas fluorescens* of 8.5 using adenosine as the substrate.

Optimum Temperature and Thermal Stability

The effect of temperature on adenosine nucleosidase activity is shown in Figure 2. The activity of the enzyme increased between 30 and 60° C, the apparent optimum temperature for enzyme activity under the standard assay conditions. The activity dropped off sharply as the temperature rose above 60° C. The energy of activation, E_a, of this enzyme-catalyzed reaction over the temperature range of 30–50° C was calculated from the Arrhenius equation (22). A value of 10,600 cal/mol (44.5 kJ/mol) was obtained.

Heating the enzyme in the absence of substrate at 60° C for 15 min at pH 5.0 or pH 8.0 in 0.1 *M* phosphate buffer completely inactivated the enzyme (Fig. 3). These results are in good agreement with the mashing study, which showed a rapid decline in activity as the temperature rose to 70° C (14).

Evidence for Hydrolytic Cleavage Mechanism

Nucleoside phosphorylase is reported to be widely distributed in various organisms, and the phosphorolytic cleavage of nucleosides seems to be the ubiquitous pathway of nucleoside metabolism (18). Therefore, it was necessary to determine whether nucleoside cleavage by the enzyme from malt was hydrolytic (adenosine + H_2O → adenine + ribose) or phosphorolytic (adenosine + P_i → adenine + ribose-1-P). The following evidence, however, excluded the possibility of phosphorolytic cleavage.

First, careful elimination of inorganic phosphate from the enzyme preparation by dialysis for 72 hr and from the other components of the assay mixture did not cause any decrease in the reaction rate, and the addition of phosphate to such a system failed to enhance the activity (Table II). Second, an attempt to detect phosphorolytic activity by its reverse reaction gave only negative results; when the enzyme was incubated with adenine and ribose-1-phosphate, no adenosine was formed (Table II). This evidence strongly suggests that the mechanism of enzymatic cleavage of the riboside linkage in adenosine by the malt enzyme involves a hydrolytic rather than a phosphorolytic reaction.

TABLE II
Evidence Against Phosphorolytic Cleavage

Incubation Time (min)	Forward Reaction (μmol adenine formed) P_i (m*M*)[a] 0	0.3	3	Reverse Reaction[b] (μmol adenosine formed)
20	0.31	0.31	0.31	<0.02
40	0.60	0.58	0.60	<0.02
60	0.82	0.82	0.83	<0.02
80	1.07	1.02	0.98	<0.02

[a] Phosphate in the enzyme preparation was undetectable using the colorimetric method of Chen (7).
[b] Ribose-1-phosphate (2.0 ml, 0.3 m*M*) and adenine (2.0 ml, 0.3 m*M*) were incubated at 37° C. No adenosine was detected. The values listed indicate the limit of detection.

Effect of Substrate Concentration

The effect of varying the concentration of adenosine on the initial rate of hydrolysis is shown in Figure 4. Linearity of the plot indicated that the enzyme was not subject to substrate inhibition at high concentrations of adenosine. The V_{max} value for adenosine,

TABLE III
Effect of Common Inhibitors on Adenosine Nucleosidase

Inhibitor	Activity Remaining (%)
Mercuric chloride	13
Cupric sulfate	49
Zinc chloride	57
p-Chloromercuribenzoate	80
Calcium chloride	94
EDTA[a]	98
Magnesium chloride	99
Potassium cyanide	100
Iodoacetic acid	100
Glutathione	100

[a] EDTA, ethylenediaminetetraacetic acid.

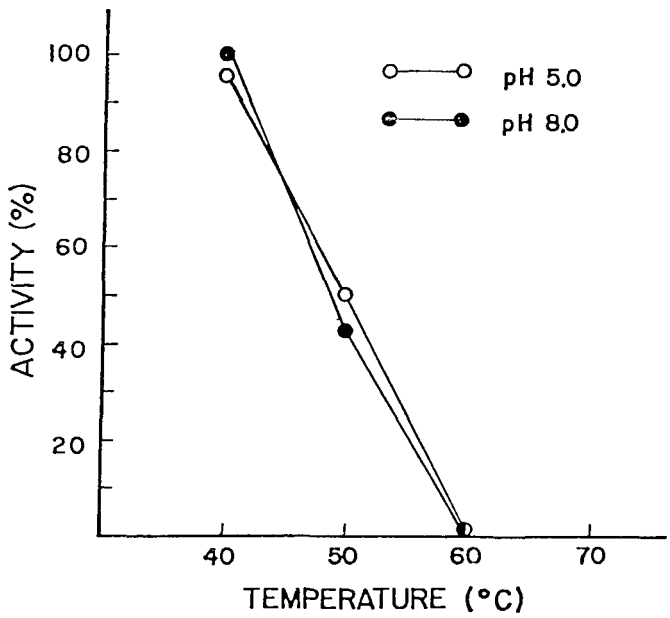

Fig. 3. Heat inactivation of adenosine nucleosidase at pH 5.0 and 8.0.

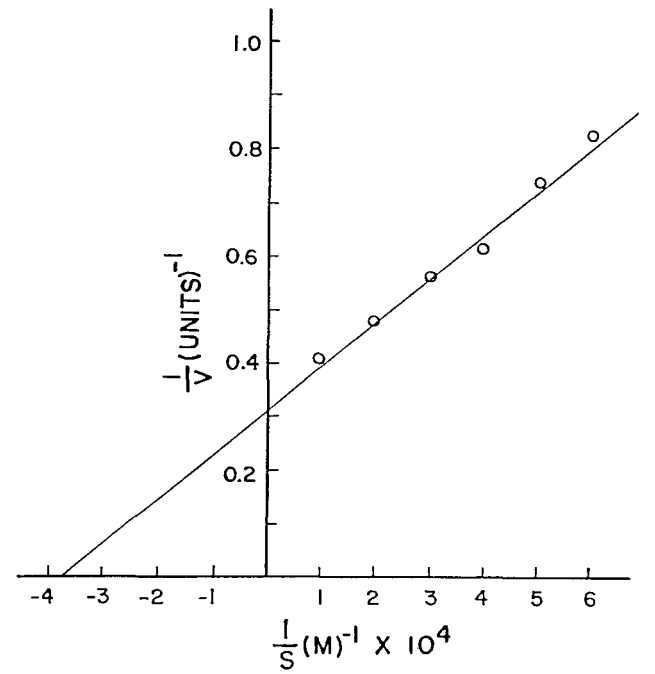

Fig. 4. Effect of substrate concentration on adenosine nucleosidase activity. The initial rate, V, is expressed as (μmol/min)/mg protein and the substrate concentration, S, as m*M*.

calculated from the double reciprocal plot (15), was 3.23 μmol/min per milligram of protein, and the K_m value was 0.27 m*M*. This value for the K_m of the malt enzyme, measured at its pH optimum (pH 5.0), is much lower than that of the adenosine hydrolase from brussels spouts measured at its pH optimum. The enzyme from brussels sprouts has a K_m of 2.4 m*M* at pH 4.0 (18).

Effect of Inhibitors and Activators

The effects of some common enzyme inhibitors and activators on purified adenosine nucleosidase are summarized in Table III. For this study, the reagent was added at a concentration of 1 m*M* before assaying for adenosine nucleosidase activity in the usual manner. The enzyme did not require the addition of any metal ions for full activity, and various ions were inhibitory when assayed under the standard assay conditions. *p*-Chloromercuribenzoate, zinc chloride, copper sulfate, and mercuric chloride, each at a final concentration of I m*M*, caused considerable inhibition. In contrast, ethylenediaminetetraacetic acid (EDTA), and magnesium and calcium chlorides were without effect.

CONCLUSIONS

Adenine, an important constituent of wort, is produced by malt adenosine nucleosidase. An over 1,000-fold purification of the enzyme was effected by means of ammonium sulfate precipitation and DEAE-Sephadex, Sephadex G-200, and hydroxylapatite chromatography. The purified enzyme has a molecular weight of 120,000, a pH optimum of 5.2, and a temperature optimum of about 60° C. The K_m of the enzyme for adenosine is 0.27 m*M*. It was inhibited by mercuric chloride, cupric sulfate, zinc chloride, and *p*-chloromercuribenzoate, but was not affected by several other common enzyme inhibitors.

The results of this study show that this nucleosidase, which is specific for adenine compounds, catalyzes a hydrolytic rather than a phosphorolytic cleavage of the substrate to produce adenine and ribose or deoxyribose.

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