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PURINE NUCLEOSIDE PHOSPHORYLASE OF CHICKEN LIVER

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SUMMARY

Purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) has been purified 125-fold from the homogenate of chicken livers and some of the properties of the purified enzyme have been studied. This enzyme had a pH optimum at around 6.0. At high substrate levels of inosine the reaction rate was increased, suggesting that substrate activation of the enzyme had occurred. The enzyme activity was completely lost after 48 h at −20°. The inhibition by nucleotides and SO_4^{2-} is a characteristic of this enzyme and has not been previously reported.

INTRODUCTION

The earlier investigation concerning purine nucleoside phosphorylase has been thoroughly reviewed by FRIEDKIN AND KALCKAR[1] at 1961. More recently, studies have been attempted to elucidate the reaction mechanism of the enzyme obtained from human erythrocytes[2–7]. We have reported the developmental pattern of purine nucleoside phosphorylase in chicken liver[8]. Enzyme activity was low prior to hatching and then increased rapidly after hatching. However, the enzyme found after hatching, unlike the embryonic enzyme, was extremely unstable, suggesting that different molecular forms of this enzyme exist. To obtain crucial evidence of a molecular difference between the embryonic and chick enzyme, the partial purification and characterization of purine nucleoside phosphorylase of chicken liver have been investigated.

MATERIALS AND METHODS

Materials

The following chemicals were used in this study:guanosine, xanthosine, adenosine, nucleotides, Tris and crystalline bovine serum albumin from Sigma Chemicals Co.; inosine, cytidine and uridine from Kohjin Co. Ltd.; protamine sulfate and hypo-

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xanthine from Nutritional Biochemicals Corp.; DEAE-cellulose, Sephadex gel and Whatman chromatography cellulose powder from Brown, Pharmacia and W & R Balston. All other reagents were of analytical grade. Calcium phosphate gel was prepared according to the method of TSUBOI AND HUDSON[9]. Milk xanthine oxidase was purified up to the $(NH_4)_2SO_4$ step by the method of BALL[10].

Methods

The assay methods and the definition of the enzyme unit of specific activity were described previously[8]. In this study, the standard reaction mixture contained 200 μmoles of Tris–acetate buffer (pH 6.0), 27 μmoles of inosine, 60 μmoles of phosphate buffer (pH 6.0) and the enzyme preparation in a total volume of 2.7 ml. When nucleosides other than inosine were used as substrate, the enzyme activity was determined by measuring the rate of production of ribose-1-*P*. The reaction was stopped by the addition of $HClO_4$ to a final concentration of 0.3 M and heating for 15 min at 50° to hydrolyze ribose-1-*P*. After centrifugation, an aliquot of the supernatant was neutralized by the addition of concentrated $KHCO_3$ solution. An aliquot of the neutralized solution was assayed for the reduction of sugar by the method of SOMOGYI–NELSON[11,12]. Protein was determined by the method of LOWRY *et al.*[13]. 2-Mercaptoethanol and SO_4^{2-} were removed from the enzyme solution by gel filtration on Sephadex G-25, since the former interfered with protein determination and the latter was a potent inhibitor of purine nucleoside phosphorylase of chicken liver.

RESULTS

Purification of purine nucleoside phosphorylase of chicken liver

The latter few steps of the purification were adopted according to the method of KIM *et al.*[2].

Homogenate and extract

Fresh chicken livers were obtained from the local meat market. All subsequent operations were conducted at 4° unless otherwise specified. 500 g of tissue were homogenized in a Waring blendor with 4 vol. of 0.05 M acetate buffer (pH 6.0) containing 0.01 M 2-mercaptoethanol. 2-Mercaptoethanol was maintained at this concentration in the following operations since purine nucleoside phosphorylase activity disappeared rapidly in its absence. The homogenate was centrifuged at 10 000 × *g* for 20 min.

Protamine treatment

1% protamine solution in 0.05 M acetate buffer (pH 6.0) was added to the supernatant so as to give a final concentration of 0.12%. The mixture was stirred for 60 min, and the clear dark red supernatant was obtained after centrifugation at 10 000 × *g* for 20 min.

$(NH_4)_2SO_4$ fractionation

The supernatant was brought to 0.3 saturation by adding solid $(NH_4)_2SO_4$ (22.8 g/100 ml). After stirring for 30 min, the precipitate was removed by centrifugation at 10 000 × *g* for 20 min. To the supernatant was added solid $(NH_4)_2SO_4$

to 0.65 saturation (24 g/100 ml). The precipitate was separated by centrifugation at 10 000 × *g* for 20 min and redissolved in about 200 ml of 0.05 M acetate buffer (pH 6.0).

Calcium phosphate gel adsorption

The enzyme solution was dialyzed for 3 h against 0.05 M acetate buffer (pH 6.0) which was changed once during the dialysis. To 295 ml of the enzyme solution, calcium phosphate gel in an amount of 0.3 mg (dry wt.) per mg protein was added and the mixture was stirred for 60 min. The gel was collected by centrifugation at 5000 × *g* for 10 min and washed twice with ice-cold 0.01 M 2-mercaptoethanol. The enzyme was eluted from the gel by successive treatments with 150, 150 and 75 ml of 0.15 M phosphate buffer (pH 7.5).

$(NH_4)_2SO_4$ fractionation

The enzyme solution was refractionated with $(NH_4)_2SO_4$. The precipitate, obtained between 0.3 and 0.6 saturation of $(NH_4)_2SO_4$, was dissolved in about 20 ml of 0.05 M Tris–HCl buffer (pH 7.5).

DEAE-cellulose column chromatography

$(NH_4)_2SO_4$ was removed from the above enzyme fraction through gel filtration on a Sephadex G-25 column (5 cm × 41 cm) which had been equilibrated with 0.05 M Tris–HCl buffer (pH 7.5). Red-colored fractions were collected as they contained the enzyme. The desalted enzyme solution was placed on the DEAE-cellulose column (2 cm × 25 cm) which had been equilibrated with 0.05 M Tris–HCl buffer (pH 7.5). The column was then washed with 600 ml of the same buffer and eluted stepwise with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.1 M, 0.2 M and 0.4 M NaCl, respectively. The enzyme activity was found in the fractions of Tris–HCl buffer containing 0.2 M and 0.4 M NaCl.

Calcium phosphate gel–cellulose column chromatography

The concentration of NaCl in the pooled enzyme solution was diluted to about 0.05 M with 0.05 M Tris–HCl buffer (pH 7.5), and the diluted enzyme solution was loaded on the DEAE-cellulose column (2 cm × 11 cm) which had been conditioned as described above. About 22 ml of concentrated enzyme solution was eluted with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.5 M NaCl. The buffer was changed to 1 mM phosphate buffer (pH 6.0) by gel filtration on Sephadex G-25 which also removed the salt. The desalted enzyme solution was loaded on a calcium phosphate gel–cellulose column (3 cm × 21 cm) which had been equilibrated with 1 mM phosphate buffer (pH 6.0). The ratio of the cellulose to the gel was 20:1 (dry wt.). The column was washed with 0.05 M phosphate buffer (pH 7.5) until no protein was found at 280 nm. The enzyme was eluted with the linear gradient of 0.05 M to 0.2 M phosphate buffer (pH 7.5) in a total volume of 600 ml. 7-ml fractions were collected. The enzyme peak emerged between Tubes 52 and 77, corresponding to the phosphate concentration of about 0.12 M to 0.16 M.

Sephadex G-100 column chromatography

The enzyme solution was diluted to about 0.05 M phosphate, followed by

TABLE I

PURIFICATION OF PURINE NUCLEOSIDE PHOSPHORYLASE FROM CHICKEN LIVER

The standard assay method was described in the text.

Fraction	*Total vol. (ml)*	*Total activity (units)*	*Protein (mg/ml)*	*Specific activity (units/mg)*
(1) Homogenate	2410	8200	36.1	0.094
(2) Supernatant at 10 000 × *g* for 20 min	1780	6060	14.9	0.226
(3) Protamine sulfate	1985	4450	11.8	0.190
(4) $(NH_4)_2SO_4$ (0.3–0.65 satn.)	295	3930	57.0	0.234
(5) Calcium phosphate gel	363	2790	7.97	0.965
(6) $(NH_4)_2SO_4$ (0.3–0.6 satn.)	34.0	2290	65.2	1.03
(7) DEAE-cellulose column	22.2	779	7.65	4.58
(8) Calcium phosphate gel–cellulose column	131.0	376	0.27	10.6
(9) Sephadex G-100	35.1	255	0.62	11.7

concentration with DEAE-cellulose as described above. The concentrated enzyme solution was applied to the Sephadex G-100 column (2 cm × 41 cm) previously equilibrated with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.2 M NaCl. The elution was carried out with the same buffer and 5-ml fractions were collected. The enzyme emerged as a single peak between tubes 6 and 15.

The specific activity of purine nucleoside phosphorylase was 11.7 after this purification step and represented a 125-fold increase over the original homogenate. The results of a typical purification are summarized in Table I.

Some properties of purine nucleoside phosphorylase

The following results were obtained with the enzyme preparation which had been purified up to the Sephadex G-100 gel filtration step.

pH optimum

The optimal pH of the purified enzyme preparation was found to be around 6.0 (Fig. 1).

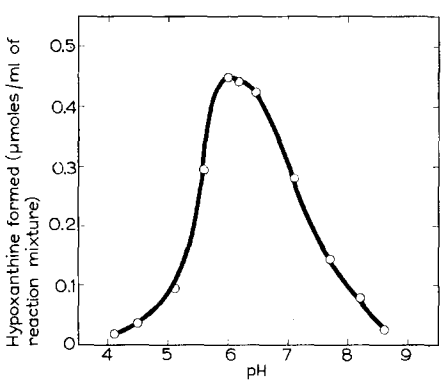


Fig. 1. Effect of pH on the activity of purine nucleoside phosphorylase. The enzyme activity was determined by the standard assay method. 0.2 M acetate and Tris–acetate buffer were used in order to cover the pH range, 4.0 to 5.5, and 5.5 to 9.5, respectively.

TABLE II

SPECIFICITY OF PURINE NUCLEOSIDE PHOSPHORYLASE OF CHICKEN LIVER FOR PHOSPHOROLYSIS

The reaction mixture contained 3 μmoles of nucleosides, 36 μmoles of phosphate buffer (pH 6.0), 320 μmoles of acetate buffer (pH 6.0) and the enzyme preparation in a total volume of 1.5 ml. The reaction mixture was incubated at 37° for 10 min. The succeeding procedures were as described in the text.

Substrate	*Relative activity (%)*
Inosine	100
Guanosine	61.0
Xanthosine	37.0
Adenosine, cytidine, uridine, AMP, IMP	0

Substrate specificity

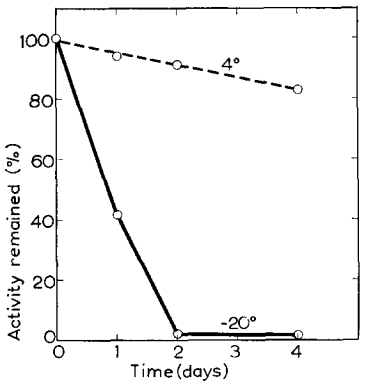
The enzyme catalyzed phosphorolysis of inosine, guanosine and xanthosine and maximum activity was observed with inosine as a substrate (Table II).

Stability

The activity of the crude enzyme decreased gradually at −20° as noted previously[8]. The purified enzyme lost its activity completely at −20° after 48 h, whereas at 4° only 10% of the activity was lost after 48 h as shown in Fig. 2.

Inhibition

Nucleotides, especially ADP, and Na_2SO_4 markedly inhibited phosphorolysis



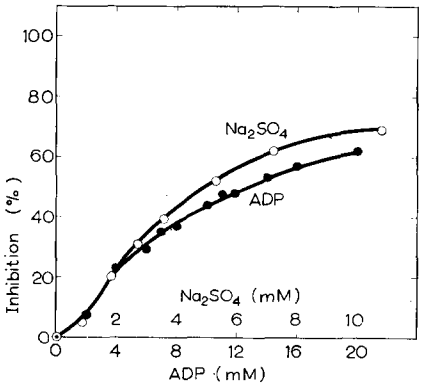


Fig. 2. Stability of purified purine nucleoside phosphorylase. The purified enzyme, which was eluted through calcium phosphate gel-cellulose column with phosphate buffer (pH 7.5), was allowed to stand at 4° or −20°. The enzyme activity was determined at indicated time by the standard assay method.

Fig. 3. Inhibition by Na_2SO_4 or ADP. The enzyme activity was determined shortly after the last procedure of purification had been over. The reaction mixture contained 5.4 μmoles of inosine, 360 μmoles of acetate buffer (pH 6.0) and the enzyme preparation in a total volume of 1.5 ml. ADP or Na_2SO_4 was added in the concentrations indicated. The reaction was stopped by adding 1.5 ml of 0.6 M $HClO_4$. The precipitate was removed by centrifugation and an aliquot of the supernatant was neutralized by adding concentrated $KHCO_3$. Hypoxanthine of neutralized solution was determined as described previously[8].

(Fig. 3). Na_2SO_3 was less potent, whereas carbonate and nitrate did not show inhibition. This inhibition appears to be a characteristic of this enzyme and has not been reported previously. This enzyme was also inhibited by *p*-chloromercuribenzoate. The inhibition can be slightly prevented by the prior addition of 2-mercaptoethanol[14–18].

Substrate activation at high concentration of inosine

When the concentration of inosine was varied at the constant level of phosphate (22.2 mM), the Lineweaver–Burk plots yielded a straight line at low concentrations but curved downward at high concentration of the substrate (Fig. 4).

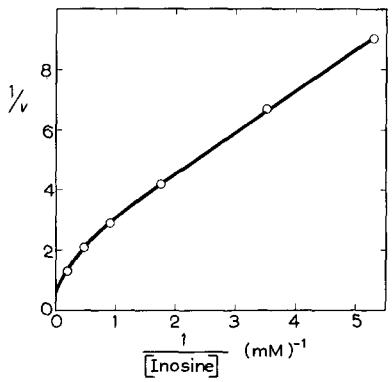


Fig. 4. The effect of concentration of inosine on the velocity of phosphorolysis. Double-reciprocal plots of initial velocity against inosine concentration. The reaction mixture, in a total volume of 2.7 ml, contained various amount of inosine, 200 μmoles of Tris–acetate buffer (pH 6.0), 60 μmoles of phosphate buffer (pH 6.0) and the enzyme preparation. The reaction mixture was incubated at 37° for 10 min. Hypoxanthine formed in the reaction mixture was determined as described previously[8].

DISCUSSION

pH optimum of this enzyme at around 6.0 with a single sharp peak appears to be a characteristic of this enzyme, as other workers have reported that purine nucleoside phosphorylase from other sources had a pH optimum at 7.0–8.0. The liver enzyme appears similar to the enzyme of fish muscle[19] which has a pH optimum at 6.0 with a broad single peak, as well as the enzyme from human erythrocytes which has a pH optimum at 6.0 for arsenolysis[18].

As described previously[8], crude chick enzyme is unstable in comparison with embryonic enzyme as it gradually loses activity on storage at −20°. The purified enzyme also was inactivated markedly at −20°, whether it contained 2-mercaptoethanol or not. This is the first time that purine nucleoside phosphorylase has been found to be labile when stored at −20°.

The phosphorolysis of inosine at a constant level of phosphate increased with high concentration of inosine. This observation might support the hypothesis of PARKS *et al.*[2–5] that the purine nucleoside phosphorylase is multivalent with cooperative interaction between active sites. The inhibition of purine nucleoside phos-

phorylase by nucleotides or SO_4^{2-} has not been reported by previous workers. Freshly purified enzyme was not inhibited to the same extent by ADP or Na_2SO_4, however the degree of inhibition increased with time or storage at 4°. It remains unexplained why the unpurified enzyme was inhibited more effectively than the fresh purified enzyme. The answer to this question might hold the key to the structure and function of purine nucleoside phosphorylase from chicken liver. Attempts to retain the sensitivity to inhibition of freshly purified enzyme, such as the addition of heat-treated chicken liver homogenate (100°, 15 min) or the addition of enzyme-free eluate from DEAE-cellulose column to fresh purified enzyme solution, however, were unsuccessful.

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