Biochimica et Biophysica Acta, 438 (1976) 159—168

BBA 67822

CYTOSOL 5'-NUCLEOTIDASE FROM CHICKEN LIVER

PURIFICATION AND SOME PROPERTIES

YOSHITSUGU NAITO * AND KEIZO TSUSHIMA

Department of Biochemistry, Yokohama City University School of Medicine, Yokohama (Japan)

(Received November 26th, 1975)

Summary

1. 5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) from the cytosol of chicken liver has been purified 1860-fold with an overall yield of 20% by a combination of precipitation at pH 5.3, $(NH_4)_2SO_4$ fractionation, calcium phosphate gel adsorption, phosphocellulose chromatography and gel filtration with Sephadex G-200. The enzyme has been shown to be highly purified, as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. This is the first time it has been possible to obtain a purified 5'-nucleotidase from the cytosol of animal tissue.

2. An $S_{20,w}$ of 9.7 S for 5'-nucleotidase was obtained by the use of sucrose density gradient centrifugation and a Stokes radius of 5.1 nm was estimated by gel filtration techniques. From these values and the assumed partial specific volume of 0.725 cm^3/g, the molecular weight of the enzyme was calculated to be 205 000. One major band, corresponding to a molecular weight of 51 000, was detected after sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicating that the native enzyme was composed of four identical subunits.

3. Some properties of the purified enzyme, including pH optimum, Mg^{2+} dependency and substrate specificity, resembled closely those of the partially purified enzyme from chicken liver acetone powder as reported by Itoh, R., Mitsui, A. and Tsushima, K. (1967) Biochim. Biophys. Acta 146, 151—159.

Introduction

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) which preferentially hydrolyzes IMP and GMP was reported originally by Itoh et al.

* To whom correspondence should be sent.

[1,2] and this enzyme was found to be located in the supernatant fraction of chicken and rat liver [3,4]. The activity of this enzyme in chicken liver was much higher than in rat liver [4], suggesting the importance of this enzyme in nitrogen metabolism in uricotelic animals [5,6]. We reported the partial purification of this enzyme from the acetone powder of animal livers [1,2]. Fritzson [3] also partially purified this enzyme from rat liver cytosol. The other kinds of cytosol 5′-nucleotidase have been partially purified from sheep brain [7], rat and guinea pig skeletal muscle [8], bovine liver [9] and pigeon heart [10], but the best purification were only 20—80-fold. This paper describes the purification of the cytosol 5′-nucleotidase from chicken liver to approximately homogeneity and some of its properties.

Materials

All of the nucleotides obtained were sodium salts. 5′-IMP and 5′-AMP were purchased from Kohjin, 2′,(3′)-GMP from Boehringer Mannheim and the other nucleotides from Sigma. Disodium phenylphosphate and sodium β-glycerophosphate were obtained from E. Merk; ribose 5-phosphate and ovalbumin from Sigma; Tris, rabbit muscle pyruvate kinase, bovine liver catalase and yeast alcohol dehydrogenase from Boehringer Mannheim; bovine serum albumin from Nutritional Biochemicals Corp.; acrylamide and *N,N′*-methylenebisacrylamide from Tokyo Kasei and *N,N,N′,N′*-tetramethylethylenediamine from Wako Pure Chemical. All other chemicals were of reagent grade or of the highest quality available. Phosphocellulose was purchased from Brown and Sephadex G-200 from Pharmacia. Calcium phosphate gel was prepared according to the method of Tsuboi and Hudson [11].

Methods

Assay of enzyme activity. The 5′-nucleotidase was assayed routinely in the following incubation mixture: 100 mM Tris/maleate buffer (pH 6.5), 10 mM $MgCl_2$, 3 mM 5′-IMP, 1 mg of bovine serum albumin and the enzyme preparation in a total volume of 1.0 ml. After 15 min of incubation at 37°C the reaction was terminated by the addition of 1.0 ml of 10% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation and inorganic phosphate (P_i) released was determined by the method of Chen et al. [12]. One unit of activity corresponds to the release of 1 μmol of P_i per min and specific activity is defined as the units per mg of protein. Acid phosphatase was assayed according to the method previously described [1]. Alkaline phosphatase was assayed under the condition described by Naito and Tsushima [13].

Protein determination. Protein was determined according to the method of Lowry et al. [14]. 2-Mercaptoethanol which interferes with protein determination by this method was removed from the enzyme solution by gel filtration on a column of Sephadex G-25 equilibrated with 10 mM sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl. Crystalline bovine serum albumin was used as the standard.

Gel filtration experiments. The molecular weight and the Stokes radius were determined by gel filtration [15,16]. A column (1.5 × 68 cm) of Sephadex

G-200, equilibrated with 0.05 M Tris · HCl buffer (pH 7.4) containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 0.2 M NaCl, was prepared. The column was calibrated by chromatographing 3—6 mg of the following standard proteins: ovalbumin, bovine serum albumin, yeast alcohol dehydrogenase, catalase and pyruvate kinase. Fractions of 0.93 ml were collected at a flow rate of 6 ml/h. The elution volume (V_e) of the protein was determined by monitoring the absorbance of 280 nm or the enzyme activity, while blue dextran which absorbs at 625 nm was used to determine the void volume of the column (V_o). The total volume (V_t) was determined by the elution volume of 5′-AMP.

Sucrose density gradient centrifugation. The sedimentation coefficient was determined by sucrose density gradient centrifugation according to the method of Martin and Ames [17]. The linear gradient used was 5—20% (w/v) sucrose in 0.05 M Tris · HCl buffer (pH 7.4) containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 0.2 M NaCl. Centrifugation was performed at 5°C for 14 h at 34 000 rev./min in a Hitachi 65P ultracentrifuge equipped with an RPS 40 rotor. After centrifugation the gradients were divided into 5-drop fractions which were assayed for enzyme activity. The sedimentation coefficient of the standard, catalase, was that given by Martin and Ames [17].

Gel electrophoresis. Analytical disc gel electrophoresis was performed by the procedure of Davis [18] using 5% polyacrylamide gel. For histochemical analysis of 5′-nucleotidase activity in the gels, the 5′-nucleotidase reaction mixture, with bovine serum albumin omitted, was supplemented with 2 mM Pb $(NO_3)_2$. The gels were rinsed briefly in distilled water and immersed in reaction mixture at 37° C until visible lines of white lead phosphate precipitate appeared. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn [19] using 7.5% polyacrylamide gel. Chymotrypsinogen A, ovalbumin, glutamate dehydrogenase and bovine serum albumin were used as standards.

Results

Purification of the enzyme

All of the steps in the enzyme purification were performed at 0—4°C. All buffers used for enzyme purification steps consisted of 0.05 M Tris · HCl, pH 7.4, 1 mM EDTA and 10 mM 2-mercaptoethanol, unless otherwise specified.

Step 1: Preparation of 9000 × g supernatant. 800 g of fresh chicken livers obtained from a local meat market was coarsely minced and homogenized in 3200 ml of the buffer for 5 min using a Waring blendor. The homogenate was centrifuged at 9000 × *g* for 20 min and the precipitate was discarded.

Step 2: Precipitation at pH 5.3. The pH of the supernatant fluid was adjusted to 5.3 with 2 M acetic acid. After 30 min, the precipitate formed was collected by centrifugation at 9000 × *g* for 20 min and suspended in 800 ml of the buffer. The suspension was adjusted to pH 7.4 with 1 M Tris.

Step 3: $(NH_4)_2SO_4$ fractionation. The suspension was brought to 20% saturation by slow addition of solid $(NH_4)_2SO_4$. After 30 min the precipitate was removed by centrifugation and solid $(NH_4)_2SO_4$ was again added to the supernatant to 40% saturation. The precipitate was collected, resuspended in approx. 400 ml of the buffer and dialyzed for 18 h against 5 l of the buffer with two

changes. The dialyzed suspension was centrifuged for 60 min at 130 000 × *g* to remove most of the insoluble materials.

Step 4: Calcium phosphate gel adsorption. To the supernatant solution, calcium phosphate gel (2 mg dry weight per mg protein) was added and the mixture stirred for 30 min. The gel was collected by centrifugation and washed once with 700 ml of the buffer containing 0.05 M $(NH_4)_2SO_4$. The enzyme was eluted from the gel three times by successive treatment with 300 ml of the buffer containing 0.2 M $(NH_4)_2SO_4$. The enzyme was precipitated with $(NH_4)_2SO_4$ (40% saturation) and dissolved in 150 ml of the buffer.

Step 5: Phosphocellulose column chromatography. The enzyme preparation from Step 4 was applied to a phosphocellulose column (1.5 × 20 cm) equilibrated with the buffer containing 0.2 M NaCl. The column was washed with the same buffer and the enzyme was then eluted with a linear gradient of 0.2—0.6 M NaCl in the buffer. Fractions of 4 ml were collected at a flow rate of 40 ml/h. The enzyme was found to be eluted at approx. 0.4 M NaCl. A typical elution profile is shown in Fig. 1. Active fractions were pooled, precipitated with $(NH_4)_2SO_4$ as before and dissolved in 1.5 ml of the buffer.

Step 6: Sephadex G-200 gel filtration. The active fraction from the previous step was purified further by gel filtration on a column (2.5 × 40 cm) of Sephadex G-200 equilibrated with the buffer containing 0.2 M NaCl. The enzyme was eluted at a flow rate of 14 ml/h and fractions of 2 ml were collected as shown in Fig. 2. The elution profiles of the protein and the enzyme activity showed a coincident shoulder ahead of the main peak. A rechromatography on a Sephadex G-200 column of the concentrated peak fractions showed a similar elution profile and the enzyme was not purified further. The shoulder was assumed to be due to aggregates of the enzyme proteins which were produced during the concentration process. The pooled, concentrated active fractions were dissolved in 3 ml of the buffer. This enzyme solution was used as the source of enzyme for the following experiments.

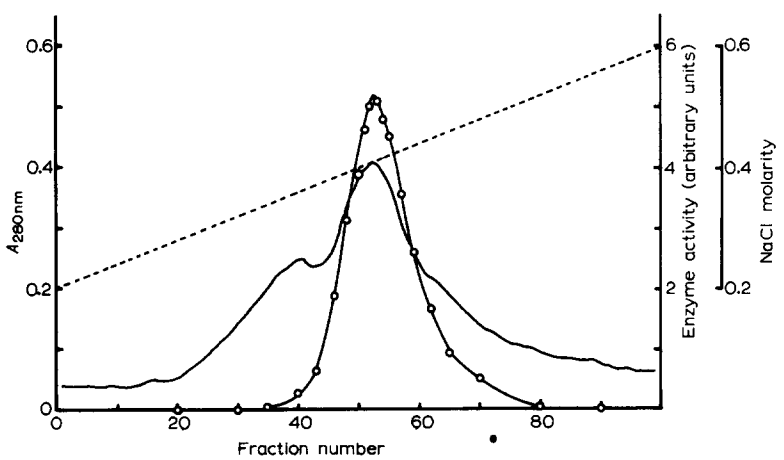

Fig. 1. Phosphocellulose column chromatography of 5′-nucleotidase from chicken liver. The enzyme was eluted with a linear gradient of 0.2—0.6 M NaCl in the buffer (with 200 ml in each reservoir). ———, protein concentration as $A_{280\ nm}$; o———o, activity of 5′-nucleotidase; - - - - - -, NaCl molarity. Other details were described in the text.

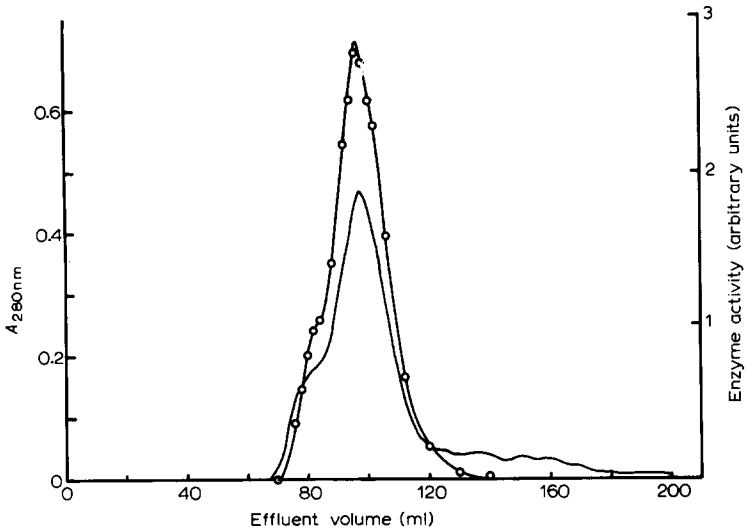

Fig. 2. Sephadex G-200 gel filtration of 5′-nucleotidase. ———, protein concentration as A_{280} nm; o———o, activity of 5′-nucleotidase. Other details were described in the text.

The purification procedure is summarized in Table I. The specific activity of the enzyme purified 1860-fold was 37 untis/mg, the highest values of 5′-nucleotidase ever obtained from animal tissues. The overall recovery was 20%.

Stability of the enzyme

The activity of the crude enzyme decreased 5—10% a day at 4°C, whereas the purified enzyme lost less than 7% of its original activity when stored over a period of 2 months at 4°C in the buffer. A significant loss of activity was observed when the purified enzyme solution was diluted down to 10 μg per ml with the buffer. For the assay of the 5′-nucleotidase in an appropriate activity we always diluted the enzyme with the buffer which contained 10 mg per ml of bovine serum albumin.

Purity of the enzyme

Acid and alkaline phosphatase activities were not detected in the purified

TABLE I

PURIFICATION OF CYTOSOL 5′-NUCLEOTIDASE FROM CHICKEN LIVER

5′-Nucleotidase activity was determined with 5′-IMP as substrate under the conditions described in the text.

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification ratio
1. 9000 × *g* supernatant	72 600	1480	0.020	100	1
2. pH 5.30 precipitate	27 000	1420	0.053	96	2.7
3. $(NH_4)_2SO_4$ 20—40% saturation	9 600	952	0.099	64	5
4. Calcium phosphate gel	2 320	748	0.322	51	16
5. Phosphocellulose	14.5	438	30.2	30	1510
6. Sephadex G-200	7.9	293	37.2	20	1860

enzyme preparation under the assay conditions described in Methods. The purified preparation gave three protein-staining bands, an intermediate major band and two faint minor bands, on polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulphate (data not shown). All of these bands corresponded to 5′-nucleotidase activity detected using 5′-IMP as substrate and the intermediate band gave the most dense deposit. No other protein bands were present. On sodium dodecyl sulphate/polyacrylamide gel electrophoresis, as shown in Fig. 3a, one major and a faint minor protein-staining band were detected. The number of bands was reduced as compared with that on the gel electrophoresis in the absence of sodium dodecyl sulphate. Since the major and minor bands corresponded to the molecular weights of 51 000 and 100 000, respectively, which were calculated as described below, the minor band appeared to correspond to a probable dimeric form of the subunit.

Molecular weight of the native enzyme and subunit composition

The molecular weight and the Stokes radius of the 5′-nucleotidase were investigated by gel filtration on a column of Sephadex G-200. Under the conditions used the molecular weight and the Stokes radius were estimated to be 180 000 and 5.1 nm, respectively (Figs. 4a and 4b). Further investigation was performed to determine the molecular weight by employing sucrose density

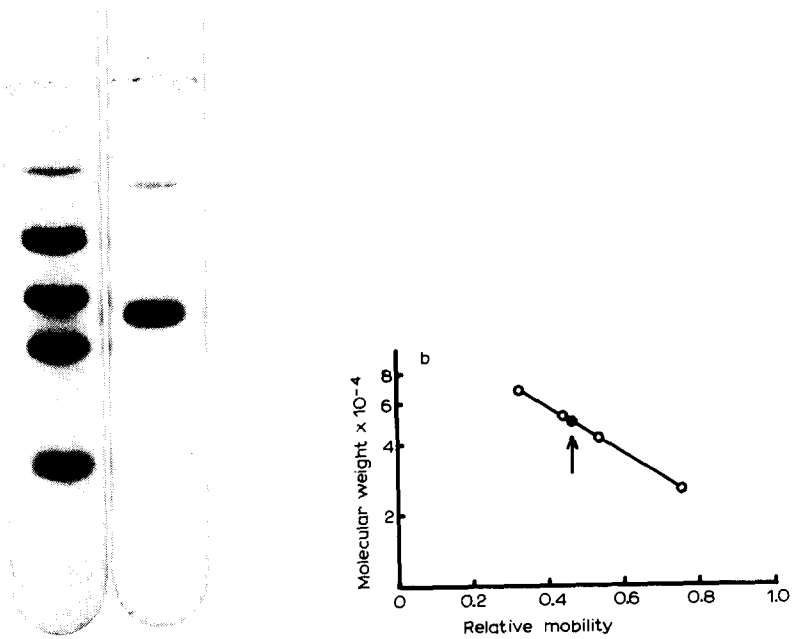

Fig. 3. (a) Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of the purified 5′-nucleotidase. Left, electrophoresis of standard proteins. Right, electrophoresis of the purified enzyme. Details of the electrophoresis are given under Methods. (b) Subunits molecular weight of 5′-nulceotidase as determined by sodium dodecyl sulphate gel electrophoresis. Relative mobility of the enzyme and four standard proteins (chymotrypsinogen A, 25 700; ovalbumin, 43 000; glutamate dehydrogenase, 53 000; and bovine serum albumin, 68 000) were plotted against the molecular weight. An arrow indicates the position of the 5′-nucleotidase.

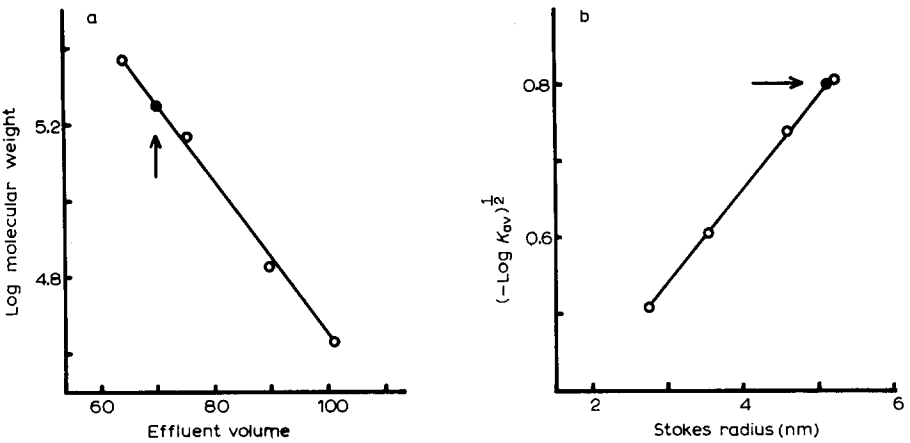

Fig. 4. The molecular weight and the Stokes radius of the purified enzyme estimated by Sephadex G-200 gel filtration. (a) Determination of molecular weight. The standards used were ovalbumin, 43 000; bovine serum albumin, 68 000; yeast alcohol dehydrogenase, 148 000 and pyruvate kinase, 237 000. An arrow indicates the position of the 5'-nucleotidase. (b) Determination of Stokes radius. The standards used were ovalbumin, 2.75 nm; bovine serum albumin, 3.5 nm; yeast alcohol dehydrogenase, 4.6 nm and catalase, 5.2 nm. Details of the procedure are given under Methods. An arrow indicates the position of the 5'-nucleotidase.

gradient centrifugation. The sedimentation coefficient of the enzyme was found to be 9.7 S and the molecular weight was calculated to be 199 000 from this value. We also used a procedure described by Siegel and Monty [16] to confirm the molecular weight obtained. From the sedimentation coefficient of 9.7 S, the Stokes radius of 5.1 nm and the assumed partial specific volume of 0.725 cm^3/g, a molecular weight of the native enzyme was calculated to be 205 000 by use of the equation: $M = 6\pi\eta Nas/(1 - \bar{\nu}\rho)$, where M = molecular weight, N = Avogadro's number, a = Stokes radius, s = sedimentation coefficient, $\bar{\nu}$ = partial specific volume, ρ = density of medium and η = viscosity of medium.

Polyacrylamide gel electrophoresis of the purified 5'-nucleotidase in the presence of sodium dodecyl sulphate revealed one major protein band (Fig. 3a). The molecular weight of this polypeptide was determined by calibrating sodium dodecyl sulphate polyacrylamide gels with standard proteins of known subunits molecular weight (Fig. 3b). The molecular weight of the polypeptide was 51 000, indicating that the native enzyme was composed of four identical subunits. The same molecular weight was obtained for the subunit using 5 and 10% polyacrylamide gels.

pH optimum and substrate specificity

The 5'-nucleotidase has a pH optimum around 6.5 when assayed under the standard condition with IMP or AMP as substrate (Fig. 5). 5'-Nucleotidase activity was not detected without Mg^{2+} in this pH range. The relative activities of the enzyme towards a number of nucleoside monophosphates and other phosphate esters are shown in Table II. The enzyme was found to have a high specificity for 5'-nucleotides. IMP, GMP, dGMP and xanthosine 5'-monophosphate were dephosphorylated more rapidly than any other substrates tested.

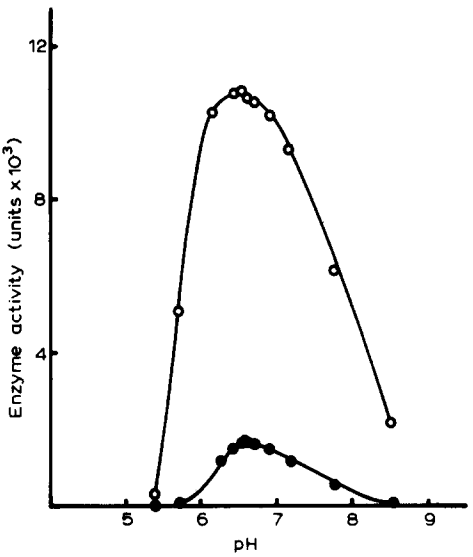

Fig. 5. Effect of pH on reaction velocity. Standard assay conditions were used except the pH of the buffer was varied. The pH was measured at 20°C in the complete reaction mixture. ○———○, 5′-IMP as substrate; ●———●, 5′-AMP as substrate.

The apparent K_m values were estimated as shown in Table II from Lineweaver-Burk plots of the reaction velocities at various concentrations of the 5′-nucleotides. The activities with all of the nucleotides tested followed Michaelis-

TABLE II

SUBSTRATE SPECIFICITY OF 5′-NUCLEOTIDASE

The reaction mixture of 1.0 ml contained 100 μmol of Tris/maleate buffer (pH 6.5), 10 μmol of $MgCl_2$, 3 μmol of substrate, 1 mg of bovine serum albumin and the enzyme preparation. All rates are referred to as relative to the hydrolysis of 5′-IMP which is taken as 100. The K_m values were determined from Lineweaver-Burk plots of the reaction velocities at varied substrate concentrations. The substrate concentration ranges used were 0.1—4 mM for 5′-IMP; 0.5—4 mM for 5′-GMP and 5′-dGMP; 5—40 mM for 5′-UMP and 10—80 mM for 5′-AMP.

Substrate (3 mM)	Relative activity (%)	K_m (mM)
5′-IMP	100	0.31
5′-GMP	96	0.53
Xanthosine 5′-monophosphate	43	
5′-AMP	13	18
5′-UMP	10	23
5′-CMP	7	
5′-dGMP	75	1.4
5′-dAMP	5	
5′-dUMP	3	
5′-dTMP	2	
5′-dCMP	0.6	
2′, (3′)-GMP *	4	
2′, (3′)-AMP *	0	
Ribose 5-phosphate	2	
Phenylphosphate	0	
β-Glycerophosphate	0	

* Mixed isomers.

Menten type kinetics in the concentration range used. The K_m values for AMP and UMP are about 50 times higher than for IMP and GMP.

Discussion

5′-Nucleotidase from the supernatant fraction of chicken liver homogenate was highly purified. The purified enzyme was relatively stable as compared with the crude one.

The enzyme was eluted as a single symmetrical peak on phosphocellulose column chromatography and the enzyme from the final purification step was not purified further by rechromatography on a Sephadex G-200 column. The gel electrophoresis in the presence of sodium dodecyl sulphate showed a decrease in the number of protein bands as compared with the gel electrophoresis in the absence of the detergent. And the faint minor band observed on the gel in the presence of sodium dodecyl sulphate was considered to be a probable dimeric form of the subunits. These results suggested that electrophoretic heterogeneity of the purified enzyme in the absence of sodium dodecyl sulphate was an artifact. The purified enzyme was therefore considered to be approximately homogeneous.

The molecular weight determined by Sephadex G-200 gel filtration was 180 000. This value was somewhat lower than the molecular weight of 199 000 obtained by sucrose density gradient centrifugation and deviated from the sum of the molecular weight of the subunit determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. It has been described that the gel filtration elution volumes of proteins were more accurately correlated with the Stokes radii than with the molecular weights [16,20,21]. We therefore used a procedure developed by Siegel and Monty [16] to determine the molecular weight of this 5′-nucleotidase. The molecular weight of the native enzyme was calculated to be 205 000, whereas that of the dissociated subunit was 51 000. The 5′-nucleotidase thus appears to be a tetramer, being composed of four identical subunits. The value of 205 000 is close to that of 190 000 determined by gel filtration for 5′-nucleotidase from membrane fraction of bovine cerebral cortex [22], higher than those for 5′-nucleotidase from sheep brain, 140 000 [7], mouse liver plasma membrane, 140 000—150 000 [23] and Ehrlich ascites tumor cells, 125 000 [24] and lower than that for bovine pituitary gland 5′-nucleotidase, 237 000 [25]. The 5′-nucleotidase from mouse liver plasma membrane has been reported to be composed of two identical subunits, each with a molecular weight of 70 000—75 000 [23].

The catalytic properties of the purified 5′-nucleotidase, including pH optimum, Mg^{2+} requirement and substrate specificity resembled closely those of partially purified enzyme from chicken liver acetone powder [1]. However, the purified enzyme was completely devoid of the activity towards phenylphosphate and β-glycerophosphate. The apparent K_m values for IMP and GMP were about one-third of those of the partially purified enzyme. The apparent K_m values for UMP and AMP were much higher than the values, 2.2 and 6.8 mM, respectively, reported by Fritzson [3] for the 5′-nucleotidase from rat liver cytosol. Further investigations on the molecular and catalytic properties of this enzyme are now in progress.

Acknowledgement

This work was supported in part by a Scientific Research Grant from the Ministry of Education of Japan.

References

1 Itoh, R., Mitsui, A. and Tsushima, K. (1967) Biochim. Biophys. Acta 146, 151—159
2 Itoh, R., Mitsui, A. and Tsushima, K. (1968) J. Biochem. Tokyo 63, 165—169
3 Fritzson, P. (1969) Biochim. Biophys. Acta 178, 534—541
4 Naito, Y., Itoh, R. and Tsushima, K. (1974) Int. J. Biochem. 5, 807—810
5 Itoh, R. and Tsushima, K. (1972) Biochim. Biophys. Acta 273, 229—235
6 Itoh, R. and Tsushima, K. (1974) J. Biochem. Tokyo 75, 715—721
7 Ipata, P.L. (1968) Biochemistry 7, 507—515
8 Cozzani, I., Ipata, P.L. and Ranieri, M. (1969) FEBS Lett. 2, 189—192
9 Itoh, R. and Tsushima, K. (1971) Int. J. Biochem. 2, 651—656
10 Gibson, W.B. and Drummond, G.I. (1972) Biochemistry 11, 223—229
11 Tsuboi, K.K. and Hudson, P.B. (1957) J. Biol. Chem. 224, 879—887
12 Chen, Jr., P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756—1758
13 Naito, Y. and Tsushima, K. (1973) Biochim. Biophys. Acta 327, 398—404
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Andrews, P. (1965) Biochem. J. 96, 595—606
16 Siegel, L.M. and Monty, K.J. (1966) Biochim. Biophys. Acta 112, 346—362
17 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
18 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 401—427
19 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
20 Ackers, G.K. (1964) Biochemistry 3, 723—730
21 Ackers, G.K. (1967) J. Biol. Chem. 242, 3237—3238
22 Tanaka, R., Morita, H. and Teruya, A. (1973) Biochim. Biophys. Acta 298, 842—849
23 Evans, W.H. and Gurd, J.W. (1973) Biochem. J. 133, 189—199
24 Murray, A.W. and Friedrichs, B. (1969) Biochem. J. 111, 83—89
25 Lisowski, J. (1966) Biochim. Biophys. Acta 113, 321—331