COPURIFICATION OF CHICKEN LIVER SOLUBLE THIAMINE MONOPHOSPHATASE AND LOW MOLECULAR WEIGHT ACID PHOSPHATASE

I. K. KOLAS, A. F. MAKARCHIKOV

Grodno State Agrarian University;
Institute of Biochemistry of Biologically Active Compounds,
National Academy of Sciences of Belarus;
e-mail: a_makarchikov@yahoo.com

Thiamine monophosphatase (ThMPase) is an enzyme of thiamine metabolism in animals whose molecular nature has still to be elucidated. In this study we have achieved a 714-fold purification of a soluble enzyme possessing ThMPase activity from a chicken liver extract. In addition to ThMPase, acid phosphatase activity was traced during purification. Both activities proved to have coincident elution profiles at all chromatographic steps implying the same enzyme involved. The molecular weight of the enzyme was 18 kDa as estimated by gel filtration. Along with ThMP and p-nitrophenyl phosphate, the purified enzyme was capable of hydrolyzing flavin mononucleotide as well as phosphotyrosine. Subcellular distribution of ThMPase activity was also explored indicating its cytosolic localization. The results of the present work imply the involvement of low molecular weight acid phosphatase in thiamine metabolism in the chicken liver.

K e y w o r d s: thiamine monophosphatase, low molecular weight acid phosphatase, HPLC, chicken liver.

Thiamine (vitamin B_1) fulfills vital biochemical functions in all living organisms studied to date. In animals, thiamine diphosphate (ThDP) is well known to serve as a coenzyme for at least five enzyme systems involved in energy producing and metabolism of carbohydrates, branched-chain amino acids as well as 3-methyl-branched fatty acids [1]. Usually, ThDP accounts for about 80% of the total thiamine content in the cell. Apart from its coenzyme form, several other phosphorylated derivatives of vitamin B_1 are present in biological objects, namely thiamine monophosphate (ThMP), thiamine triphosphate (ThTP) and thiamine adenosine triphosphate (AThTP). Quantitatively, the amount of these compounds, with some exceptions in the case of ThTP, varies from less than 1% for AThTP and ThTP to 2-15% for ThMP in terms of tissue total thiamine content [2-7]. While evidence exists indicating both ThMP and AThTP are likely to be involved in mechanisms of short-term biochemical adaptation [8, 9], a possible biological role of ThMP has not been recognized yet. In bacteria, yeast and plants this compound was found to be an intermediate on thiamine biosynthesis pathway, the enzyme thiamine phosphate synthase (EC 2.5.1.3) joining 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate and 4-methyl-5-β-hydroxyethylthiazole monophosphate to produce ThMP. Some bacterial species, like *Escherichia coli* and *S. typhimurium*, are also capable of direct phosphorylation of thiamine to ThMP by the enzyme thiamine kinase (EC 2.7.1.89) [10]. In animal cells, ThMP is believed to be produced exclusively by the hydrolysis of ThDP. In addition, ThMP can be transported into the cell from the blood plasma, where it constitutes up to 80% of total thiamine, through reduced folate carrier, RFC1 [11]. The hydrolysis to thiamine is then a necessary step for ThMP to serve as a substrate in coenzyme synthesis by thiamine pyrophosphokinase (EC 2.7.6.2) in eukaryotic organisms [1, 12]. Little knowledge is available from the literature concerning the nature of ThMP hydrolyzing enzymes in animal tissues. It is only known that this compound could be a substrate for

alkaline phosphatase (ALKP, EC 3.1.3.1) [13, 14] and fluoride-resistant (prostatic) acid phosphatase (PAP, EC 3.1.3.2) [15, 16], both enzymes being membrane-bound proteins. Quite recently, we have revealed a soluble ThMPase activity in chicken liver and hypothesized that this activity might belong to a low-molecular-weight acid phosphatase (LMW-AP), also known as a low-molecular-weight protein phosphotyrosine phosphatase (LMW-PTP) [17]. The present work is to study ThMPase subcellular distribution as well as to purify and characterize its basic properties in chicken liver.

Materials and Methods

Fresh livers of broiler chicken ROSS 308 were obtained from local poultry farm, placed on ice and frozen during about 1 h after the animals were killed. The tissues were stored at –20 °C before use. Sephadex G-50, Sephadex G-75, SP Sephadex C-50 and Sephacryl S-200 were purchased from Pharmacia (Uppsala, Sweden), Servacel P-23, ThMP, phenylmethylsulfonyl fluoride (PMSF), pyridoxal-5-phosphate and protein molecular weight markers were from Serva (Heidelberg, Germany), dithiothreitol (DTT), p-nitrophenyl phosphate (p-NPP), flavin mononucleotide (FMN), phosphotyrosine, β-glycerophosphate, glucose-6-phosphate, fructose-1,6-diphosphate, inosine 5′-monophosphate, adenosine 5′-monophosphate, 2-morpholinoethanesulfonic acid (MES) and trichloroacetic acid (TCA) were from Sigma (St. Louis, Mo., USA). All other chemicals were of analytical grade.

All operations on preparing subcellular fractions were performed at 0-4 °C (in an ice bath). A sample of fresh liver tissue was forced through a 1-mm pore press and homogenized by 10 strokes in a teflon/glass Potter-Elvehjem device at 600 rpm in 10 volumes of 10 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 0.5 mM EDTA and 0.1 mM $MgCl_2$. The homogenate was passed through four layers of wet gauze and centrifuged at 600 g for 10 min. The pellet was washed by suspending in 10 volumes of the initial buffer and centrifuging (600 g, 10 min) to give a crude nuclear fraction. To prepare mitochondria the post-nuclear supernatants were pooled and subjected to further centrifugation at 12 000 g for 10 min. The particles were washed once with the same medium yielding a crude mitochondrial fraction. Post-mitochondrial supernatants were then combined and adjusted to pH 7.5 with 0.5 M Tris-HCl, pH 8.0, followed by the addition of $CaCl_2$

to a final concentration of 15 mM. In two hours, the microsomal fraction was obtained as a precipitate (14 000 g, 10 min), while the remaining supernatant represented the fraction of cytosol. The particular fractions were suspended in 50 mM Tris-HCl buffer, pH 7.3, containing 0.15 M KCl, 0.2 mM EDTA and frozen at –80 °C along with the cytosol for storage.

Lactate dehydrogenase (LDH, EC 1.1.1.27), succinate dehydrogenase (EC 1.3.5.1) and glucose-6-phosphatase (EC 3.1.3.9) activities, as well as DNA content, were assayed in subcellular fractions by conventional methods [18-21].

The standard mixture for ThMPase activity determination contained 25 mM Tris-maleate buffer, pH 6.0, 1 mM ThMP, 20 µg of bovine serum albumin (BSA) and an aliquot of enzyme solution in a total volume of 0.2 ml. The reaction was carried out for 20-60 min at 37 °C and stopped by adding 0.1 ml of 10% TCA. Then the mixture was centrifuged (10 min, 2500 rpm), and the amount of inorganic phosphate (P_i) liberated during the reaction was determined in accordance to the method of Lanzetta P. A.et al. [22]. Kinetic experiments required some alterations in the assay with respect to pH, substrate concentration or metal composition; it is indicated as necessary. During ion-exchange chromatography, when the enzyme elution was carried out with phosphate, ThMPase activity was monitored by HPLC. In this case, TCA was extracted prior to analysis with 9 volumes of diethyl ether. Then a 50 µl aliquot was oxidized with 30 µl of 4.3 mM potassium ferricyanide in 12.5% NaOH, diluted with 0.9 ml of 50 mM Na-phosphate buffer, pH 7.2, and adjusted to pH ~8.0 adding 35 µl of 1 M HCl. A 10-µl sample was injected into the chromatographic system (Agilent 1200) and the separation was performed at 25 °C at a flow rate of 1 ml/min on a Zorbax SB-C18 column (3×100 mm) in 50 mM NaH_2PO_4, pH 8.0, containing 2.5 % tetrahydrofuran. Thiochrome derivatives of thiamine and ThMP were quantified using a fluorometric detector (λ_{ex} = 365 nm, λ_{em} = 433 nm).

Acid phosphatase (AP) activity was assayed by incubating an aliquot of enzyme solution for 5-10 min in a total volume of 0.1 ml of 25 mM Tris-maleate buffer, pH 6.0, containing 10 µg of BSA and 0.5 mM p-NPP. The reaction was terminated by addition of 1.0 ml of 0.1 M NaOH, and the absorbance of the solution was measured at 405 nm after centrifugation (10 min, 2500 rpm). The amount of p-nitrophenol formed was calculated from ε_{405} = 18 300 $M^{-1}cm^{-1}$.

When studying the enzyme substrate specificity, the amounts of Pi liberated were measured under conditions of ThMPase assay.

A unit of activity (U) was defined as the amount catalyzing the formation of 1 μmole of P_i per min. Specific activity was expressed in units per mg of protein.

Molecular mass of the purified enzyme was estimated by gel filtration on a Sephacryl S-200 column (1.0×27 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, and calibrated with standard size proteins: cytochrome c (12.4 kDa), α-chymotrypsinogen (25.7 kDa), ovalbumin (45 kDa), BSA (67 kDa) and LDH (135 kDa). The sample of 0.5 ml was applied to the column and eluted at a flow rate of 5 cm/h. Enzyme activity was located in the eluate (V_e) by both ThMPase and acid phosphatase assays; the void volume (V_o) of the column was determined using blue dextran 2000. The value of molecular mass was calculated from the plot of log M_r *versus* the log of V_e/V_o ratio.

Protein was determined from the absorbance at 280 nm or according to the method of Bradford [23] with BSA as a standard.

Statistical calculations were done with Graph-Pad Prism 5.0. The means ± standard errors are presented for the data.

Results and Discussion

Though the presence of ThMP in animal tissues was shown more than 50 years ago, not much data concerning its enzymology have appeared since then. Keesling seems to become the first who studied ThMPase activity in rat tissues in 1960 [24], observing two pH optima at 6.0 and 9.0 in the acetone powders of the kidney and brain, but only the acid one in the liver. However, it was not until 1982, when Ogawa K. et al. [14] based on experiments with levamisole inhibition of ThMPase activity in mouse tissues at pH 8.5 concluded that the enzyme appears to be ALKP. It was also the time when ThMPase stained in small-diameter dorsal root ganglia neurons was recognized as a fluoride-resistant AP, well-known histochemical marker [15]. More recently, Zylka M. J. et al. [16] have found this fluoride-resistant TMPase to be identical to the transmembrane isoform of PAP.

The above non-specific enzymes would be expected to participate in ThMP metabolism in animals, but it is evident that neither ALKP nor PAP both being membrane-bound ecto-phosphatases

concern ThMP cleavage inside the cell. So the molecular nature of TMPase is currently unknown. In the search for an enzyme which would be a constituent of the intracellular thiamine metabolizing system in birds, we started studying some properties of ThMPase activity in chicken liver [17]. Two phosphatases were revealed to contribute to ThMP hydrolysis in liver homogenate. One of them possessing a pH-optimum of 9.0 was a membrane-bound enzyme inhibited strongly by levamisole in an uncompetitive manner with K_i of 53 μM, indicating the involvement of ALKP. On the other hand, at acidic pH (5.0-6.0) the activity proved to be due to the presence of a soluble phosphatase. The last one had a molecular mass of 17.8 kDa, as estimated by gel filtration of liver extract, and seemed to show activity toward *p*-NPP and FMN along with ThMP. Analysing the literature led us to hypothesize that the soluble ThMPase might be identical to LMW-AP which is widely expressed in eukaryotic organisms, including chicken liver [25]. In an attempt to identify this ThMPase we have conducted some further research. At first, the localization of acid ThMPase activity was examined in subcellular fractions isolated from chicken liver according to a standard protocol of differential centrifugation. As can be seen in Fig. 1, about 69% of total ThMPase activity was recovered in the cytosol. This fraction also contained more than 80% of LDH, while only a few percents of total DNA, succinate dehydrogenase and glucose-6-phosphatase were measurable as contaminants (data not shown). The mitochondrial and microsomal fractions contained up to 18% of ThMPase activity altogether. A relatively high amount (13%) of TMPase activity measured in the nuclear fraction was probably due to unbroken and damaged cells, which were pelleted with nuclei. It is also possible that PAP, which was shown to be expressed in low amounts in human liver [26], contributes to ThMPase activity in the particular fractions. Thus, based on the overlapping of ThMPase activity profile with the distribution of LDH, one may conclude that soluble acid ThMPase of chicken liver is localized in the cytosol like LMW-AP, which has been known for few decades to be a cytosolic enzyme [27].

Further, we carried out the purification of soluble ThMPase from chicken liver tracing both ThMPase and p-NPPase activity during chromatographic steps. The purification procedure included 8 steps.
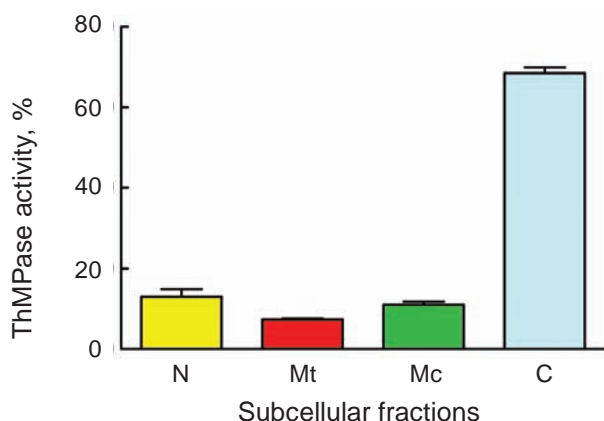
Fig. 1. Subcellular distribution of ThMPase activity in chicken liver. N – nuclear fraction, Mt – crude mitochondrial fraction, Mc – microsomal fraction, C – cytosol fraction

Step 1. Extract preparation. Chicken livers (100 g) stored at –20 °C were thawed, cut into pieces and homogenized with 250 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M KCl, 0.2 mM EDTA, 5 mM DTT and 0.1 mM PMSF, in an MWP-1 blender at a maximal speed for 2 min. The homogenate was centrifuged at 10 000 g for 60 min. The supernatant was passed through 16 layers of gauze and used as a crude extract.

Step 2. Acid treatment. Acetic acid (1 M) was slowly added to the extract under constant stirring to lower pH to 5.0. In 30 min protein aggregates were precipitated by centrifugation (10 000 g, 30 min) and discarded. Cold 1 M Tris-HCl, pH 8.9, was then added to the supernatant to give a pH value of 7.5.

Step 3. Ammonium sulfate fractionation. The solution from the previous step was brought to 35% saturation by adding solid $(NH_4)_2SO_4$. After 30 min of gentle stirring, the precipitate was removed by centrifugation at 10 000 g for 30 min. Then the second portion of ammonium sulfate was added to 60% saturation, the suspension was stirred for 30 min and the precipitate was collected by centrifugation (10 000 g, 30 min).

Step 4. Gel filtration on Sephadex G-75. The precipitate was dissolved in MES buffer (20 mM MES, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, pH 6.0) to give approximately 40 ml volume, and chromatographed on a Sephadex G-75 column (5×75 cm) in the same buffer at a flow rate of 5 cm/h. Fractions of 6.5 ml were collected and analyzed for ThMPase and p-NPPase activities as well as protein content. The elution profiles for both activities proved to be coincident (data not shown). The frac-

tions enhanced in specific activity as compared with the previous step were pooled for further processing.

Step 5. Ion exchange chromatography. The combined fractions were applied to an SP Sephadex C-50 column (1.6×25 cm) equilibrated with MES buffer. The column was washed with the same buffer and elution was carried out with a 300 ml linear gradient of increasing phosphate concentration from 0 to 100 mM at a flow rate of 15 cm/h. Like in step 4, both ThMPase and p-NPPase emerged from the column in the same fractions (data were not shown). The fractions possessing a high specific activity were combined for the next step.

Step 6. Ammonium sulfate precipitation. To prepare the enzyme for further purification, the solution was salted out with solid $(NH_4)_2SO_4$ at 80% saturation and the aggregated proteins were collected by centrifugation for 30 min at 10 000 g.

Step 7. Gel filtration on Sephadex G-50. The precipitate from the previous step was dissolved in MES buffer to give a final volume of 5 ml and loaded on a Sephadex G-50 column (2.5×100 cm) equilibrated with the same buffer. The chromatography was carried out at a flow rate of 5 cm/h and fractions of 6 ml were collected. As one might expect, ThMPase and p-NPPase elution profiles matched again (Fig. 2), implying the involvement of the same enzyme. The active fractions were pooled and subjected to further purification.

Step 8. Servacel P-23 chromatography. The fractions from the previous step were applied on a Servacel P-23 column (0.7×3 cm) equilibrated with MES buffer and the column was washed with 10 ml of the same buffer. The enzyme did not bind to the resin emerging from the column in the effluent, which was then concentrated with Corning Spin-X UF Concentrators and used for analysis. The resulting preparation had a specific ThMPase activity of 2.5 U/mg exceeding 714-fold the activity of the extract. Summary of the purification procedure is shown in Table 1.

Thus, during purification ThMPase and *p*-NPPase activity coeluted on each chromatographic step, indicating they reside on the same protein.

As a comment on the purification procedure, it is to be noted the unexpectedly high effectiveness of ThMPase precipitation from SP Sephadex C-50 effluent. This manipulation gave a 3.4-fold increase in specific activity with respect to the previous step. Evidently, this was due to much lower ThMPase solubility as compared to contaminating proteins.
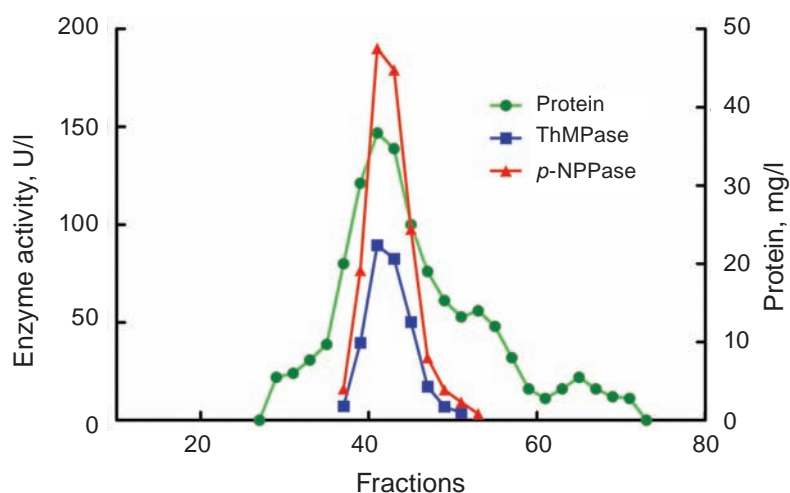
Fig. 2. Elution profiles of protein, ThMPase and low molecular weight acid phosphatase (reduced twofold) from a Sephadex G-50 column. The volume of each fraction is 6 ml

After the final ThMPase solution was concentrated, an apparent molecular mass of the enzyme was estimated by gel filtration on a calibrated Sephacryl S-200 column (1.0×27 cm). ThMPase was found to emerge from the column as a single activity peak. The standard proteins gave a linear plot of log Mr versus log V_e/V_o, from which the apparent molecular mass of ThMPase was calculated to be 18 kDa (data not shown). This value matches the molecular mass of LMW-AP from various sources which is known being a small protein of 14-21 kDa as determined by similar techniques [25, 28-32].

Kinetic experiments revealed that the purified enzyme exhibits a bell-like dependence of ThMPase activity on the acidity of the reaction medium in the range of pH values from 4.5 to 7.5, with pH-optimum being observed around pH 5.5 (data not shown). None of the divalent cations tested, such as Mg^{2+}, Mn^{2+} or Ca^{2+}, each at 5 mM concentration, affected the rate of ThMP hydrolysis in the presence of the enzyme (data not shown).

The studying of the effect of ThMP concentrations on an initial reaction velocity at pH 5.5 in the absence of metal ions revealed the enzyme to obey a classical Michaelis-Menten kinetics giving a hyperbolic saturation curve (Fig. 3), with K_m of 0.90 ± 0.07 mM and V_{max} of 8.6 ± 0.3 μmol/min/mg as estimated by nonlinear regression fit. The same values of kinetic parameters (K_m = of 0.91 mM, V_{max} = 8.1 μmol/min/mg) were also computed from the Hanes plot. It is to be noted, that the K_m of the enzyme for ThMP in the chicken liver extract was evaluated before to be 0.73 ± 0.02 mM [17]. Thus, in general, the kinetic behavior of purified ThMPase

Table 1. Purification of thiamine monophosphatase from chicken liver

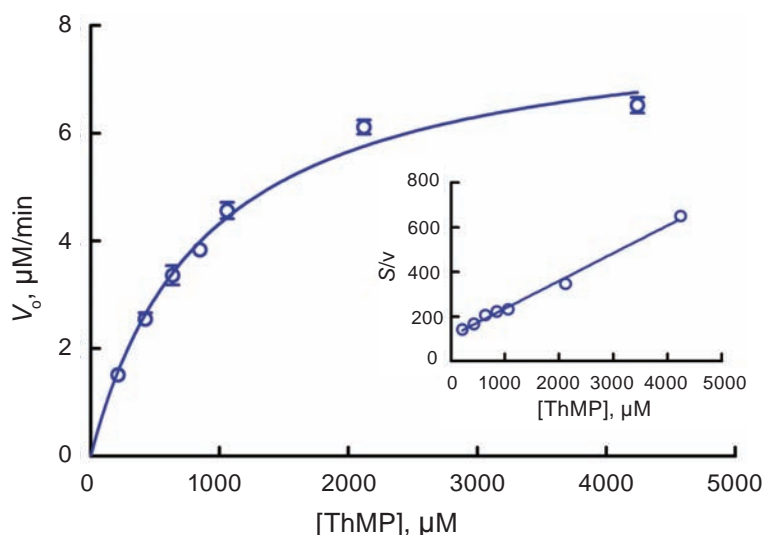| Step	Volume, ml	Total protein, mg	Total activity, U	Specific activity, U/mg	Yield, %	Purification, fold
Crude extract	273	7644	26.7	3.5×10^{-3}	100	–
Acid treatment	253	5262	23.7	4.5×10^{-3}	88.8	1.3
Ammonium sulfate, 30–60 S	40	1360	8.2	6.0×10^{-3}	30.7	1.7
Sephadex G-75	261	157	6.7	4.3×10^{-2}	25.1	12
SP Sephadex C-50	96	15.4	4.9	3.2×10^{-1}	18.4	91
Ammonium sulfate, 80 S	5	3.5	3.9	1.1	14.6	314
Sephadex G-50	50	1.4	2.4	1.7	9.0	486
Servacel P-23	60	0.88	2.2	2.5	8.2	714

Fig. 3. Effect of varying ThMP concentrations on the initial reaction velocity. Inset: The Hanes plot of the data. The reaction was carried out for 30 min at 37 °C in the mixture consisted of 25 mM Tris-maleate, pH 5.5, 20 µg of BSA and 0.19 µg of the purified enzyme preparation in a volume of 0.2 ml

is very close to the kinetics observed in the crude extracts [17].

Table 2 shows the hydrolysis rates of various phosphate monoesters by the chicken liver enzyme. Among compounds tested only p-NPP, FMN, phosphotyrosine and ThMP were efficiently hydrolyzed. No hydrolysis of other substances, including β-glycerophosphate, glucose-6-phosphate, fructose-1,6-diphosphate, IMP, AMP, NADP and pyridoxal-5-phosphate, occurred at appreciable rates. Essentially, the same restricted substrate specificity towards p-NPP, FMN and phosphotyrosine is characteristic of LMW-AP [28, 32-35].

After the ability of LMW-AP to hydrolyze phosphotyrosine was discovered [36, 37], the enzyme was postulated to function as a protein phosphotyrosine phosphatase. Indeed, LMW-AP was shown to be capable of hydrolyzing a variety of phosphorylated protein substrates, including platelet-derived growth factor, ephrin and insulin receptors [38]. However, the physiological meaning of such activity was challenged and the involvement of the enzyme in riboflavin metabolism was proposed instead [39, 40]. In any way, the results of the present work strongly suggest that ThMP is a natural substrate for LMW-AP (LMW-PTP) which thereby could be related to thiamine metabolism.

T a b l e 2. Substrate specificity of ThMPase from chicken liver (M ± SEM, n = 3)

Substrate	P_i released, nmol	%
p-Nitrophenyl phosphate	26.3 ± 1.1	100
Flavin mononucleotide	19.1 ± 2.6	73
Phosphotyrosine	8.4 ± 0.5	32
Thiamine monophosphate	4.6 ± 0.5	18
Pyridoxal-5-phosphate	< 0.5	< 1
β-Glycerophosphate	< 0.5	< 1
Glucose-6-phosphate	< 0.5	< 1
Fructose-1,6-diphosphate	< 0.5	< 1
Inosine 5′-monophosphate	< 0.5	< 1
Adenosine 5′-monophosphate	< 0.5	< 1

The amount of P_i released was determined after the reaction was carried out for 20 min (the case of *p*-NPP, FMN, phosphotyrosine and ThMP) or 60 min (for the other compounds) at 37 °C in the mixture consisted of 25 mM Tris-maleate, pH 5.5, 1 mM substrate, 20 µg of BSA and 0.19 µg of the purified enzyme preparation in a volume of 0.2 ml

РОЗЧИННА ТІАМІНМОНОФОСФАТАЗА І НИЗЬКОМОЛЕКУЛЯРНА КИСЛА ФОСФАТАЗА З ПЕЧІНКИ КУРЧАТИ НЕРОЗДІЛЬНІ В ПРОЦЕСІ ОЧИСТКИ

І. К. Колос, О. Ф. Макарчиков

Гродненський державний аграрний університет;
Інститут біохімії біологічно активних
сполук НАН Білорусі;
e-mail: a_makarchikov@yahoo.com

Тіамінмонофосфатаза (ТМРаза) є ензимом обміну тіаміну в тварин, молекулярна природа якого на сьогодні невідома. Мета роботи полягала у вивченні субклітинного розподілу ТМРази, одержаної та очищеної з печінки курчат. Розчинний ензим, який виявляв ТМРазну активність, було очищено в 714 разів. Разом із ТМРазною активністю вимірювали також і активність кислої фосфатази. На всіх стадіях колоночної хроматографії профілі елюції ТМРази і кислої фосфатази збігалися, що свідчило про те, що обидві реакції каталізуються одним й тим самим ензимом. Встановлено, що очищений ензим мав молекулярну масу 18 кДа і, крім ТМР та *п*-нітрофенілфосфату, каталізував гідроліз флавінмононуклеотиду і фосфотирозину. Показано, що ТМРаза локалізована в цитозольній фракції клітин печінки курчат, а низькомолекулярна кисла фосфатаза бере участь у метаболізмі тіаміну.

К л ю ч о в і   с л о в а: тіамінмонофосфатаза, низькомолекулярна кисла фосфатаза, HPLC, печінка курчат.

РАСТВОРИМАЯ ТИАМИНМОНОФОСФАТАЗА И НИЗКОМОЛЕКУЛЯРНАЯ КИСЛАЯ ФОСФАТАЗА ИЗ ПЕЧЕНИ ЦЫПЛЕНКА НЕРАЗДЕЛИМЫ В ПРОЦЕССЕ ОЧИСТКИ

И. К. Колос, А. Ф. Макарчиков

Гродненский государственный
аграрный университет;
Институт биохимии биологически
активных соединений НАН Беларуси;
e-mail: a_makarchikov@yahoo.com

Тиаминмонофосфатаза (ТМРаза) является энзимом обмена тиамина у животных, молекулярная природа которого в настоящее время неизвестна. Цель работы заключалась в изучении субклеточного распределения ТМРазы, полученной и очищенной из печени цыплят. Растворимый энзим, обладающий ТМРазной активностью был очищен в 714 раз. Вместе с ТМРазной активностью измеряли также и активность кислой фосфатазы. На всех стадиях колоночной хроматографии профили элюции ТМРазы и кислой фосфатазы совпадали, что свидетельствовало о том, что обе реакции катализируются одним и тем же энзимом. Установлено, что очищенный энзим имел молекулярную массу 18 кДа и помимо ТМР и *п*-нитрофенилфосфата катализировал гидролиз флавинмононуклеотида и фосфотирозина. Показано, что ТМРаза локализована в цитозольной фракции клеток печени цыплят, а низкомолекулярная кислая фосфатаза участвует в метаболизме тиамина.

К л ю ч е в ы е   с л о в а: тиаминмонофосфатаза, низкомолекулярная кислая фосфатаза, HPLC, печень цыплят.

References

1. Makarchikov AF. Vitamin B1: metabolism and functions. *Biochem (Moscow). Suppl. Ser. B: Biomedical Chemistry.* 2009; 3(2): 116-128.
2. Makarchikov AF, Lakaye B, Gulyai IE, Czerniecki J, Coumans B, Wins P, Grisar T, Bettendorff L. Thiamine triphosphate and thiamine triphosphatase activities: from bacteria to mammals. *Cell Mol Life Sci.* 2003; 60(7): 1477-1488.
3. Bettendorff L, Wirtzfeld B, Makarchikov AF, Mazzucchelli G, Frédérich M, Gigliobianco T, Gangolf M, De Pauw E, Angenot L, Wins P. Discovery of a natural thiamine adenine nucleotide. *Nat Chem Biol.* 2007; 3(4): 211-212.
4. Gangolf M, Czerniecki J, Radermecker M, Detry O, Nisolle M, Jouan C, Martin D, Chantraine F, Lakaye B, Wins P, Grisar T, Bettendorff L. Thiamine status in humans and content of phosphorylated thiamine derivatives in biopsies and cultured cells. *PLoS One.* 2010; 5(10): e13616.
5. Egi Y, Koyama S, Shikata H, Yamada K, Kawasaki T. Content of thiamin phosphate esters in mammalian tissues – an extremely high concentration of thiamin triphosphate in pig skeletal muscle. *Biochem Int.* 1986; 12(3): 385-390.
6. Iwata H, Matsuda T, Tonomura H. Improved high-performance liquid chromatographic determination of thiamine and its phosphate esters in animal tissues. *J Chromatogr.* 1988; 450(3): 317-323.
7. Miyoshi K, Egi Y, Shioda T, Kawasaki T. Evidence for *in vivo* synthesis of thiamin triphosphate by cytosolic adenylate kinase in chicken skeletal muscle. *J Biochem.* 1990; 108(2): 267-270.
8. Lakaye B, Wirtzfeld B, Wins P, Grisar T, Bettendorff L. Thiamine triphosphate, a new signal required for optimal growth of *Escherichia coli* during amino acid starvation. *J Biol Chem.* 2004; 279(17): 17142-17147.
9. Gigliobianco T, Lakaye B, Wins P, El Moualij B, Zorzi W, Bettendorff L. Adenosine thiamine triphosphate accumulates in *Escherichia coli* cells in response to specific conditions of metabolic stress. *BMC Microbiol.* 2010; 10: 148.
10. Jurgenson CT, Begley TP, Ealick SE. The structural and biochemical foundations of thiamin biosynthesis. *Annu Rev Biochem.* 2009; 78: 569-603.
11. Zhao R, Gao F, Goldman ID. Reduced folate carrier transports thiamine monophosphate: an alternative route for thiamine delivery into mammalian cells. *Am J Physiol Cell Physiol.* 2002; 282(6): C1512-C1517.
12. Goyer A. Thiamine in plants: aspects of its metabolism and functions. *Phytochemistry.* 2010; 71(14-15): 1615-1624.
13. Vovk AI, Babiy LV, Muravyova IV. Relative reactivity of thiamine monophosphate and thiamine diphosphate upon interaction with alkaline phosphatase. *Ukr Biokhim Zhurn.* 2002; 74(1): 93-96. (In Russian).
14. Ogawa K, Sakai M, Inomata K. Recent findings on ultracytochemistry of thiamin phosphatases. *Ann N Y Acad Sci.* 1982; 378: 188-214.
15. Knyihár-Csillik E, Bezzegh A, Böti S, Csillik B. Thiamine monophosphatase: a genuine marker for transganglionic regulation of primary sensory neurons. *J Histochem Cytochem.* 1986; 34(3): 363-371.
16. Zylka MJ, Sowa NA, Taylor-Blake B, Twomey MA, Herrala A, Voikar V, Vihko P. Prostatic acid phosphatase is an ectonucleotidase and suppresses pain by generating adenosine. *Neuron.* 2008; 60(1): 111-122.
17. Kolas IK, Makarchikov AF. Identification of thiamine monophosphate hydrolyzing enzymes in chicken liver. *Ukr Biochem J.* 2014; 86(6): 39-49. (In Russian).
18. King TE. Preparation of succinate dehydrogenase and reconstitution of succinate oxidase. *Methods Enzymol.* 1967; 10: 322-331.
19. Nordlie RC, Arion WJ. Glucose-6-phosphatase. *Methods Enzymol.* 1966; 9: 619-625.
20. Vassault A. Lactate dehydrogenase. UV method with pyruvate and NADH⁺. *Meth Enzym Anal.* 1983; 3: 118-126.
21. Burton K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J.* 1956; 62(2): 315-323.
22. Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. An improved assay for nanomole amounts of inorganic phosphate. *Anal Biochem.* 1979; 100(1): 95-97.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72: 248-254.

24. Kiessling KH. Thiamine phosphatases in liver, kidney, and brain of the rat. *Acta Chem. Scand.* 1960; 14: 1669-1670.

25. Haider S, Naz R, Khan AR, Saeed A. Acid phosphatases in chicken's liver. *J Chem Soc Pak.* 1993; 15(1): 64-71.

26. Graddis TJ, McMahan CJ, Tamman J, Page KJ, Trager JB. Prostatic acid phosphatase expression in human tissues. *Int J Clin Exp Pathol.* 2011; 4(3): 295-306.

27. Panara F, Mileti A. Subcellular localization of high- and low-molecular weight acid phosphatases from chicken liver. *Int J Biochem.* 1986; 18(11): 1057-1059.

28. Siddiqua A, Rehmat M, Saeed A, Amin S, Naz R, Sherazi M, Khan GM, Saeed A. Acid phosphatases from the liver of Labeo rohita: purification and characterization. *Biol Pharm Bull.* 2008; 31(5): 802-808.

29. Panara F. Isolation and partial characterization of high and low molecular weight acid phosphatases from chicken liver. *Int J Biochem.* 1985; 17(11): 1213-1217.

30. Heinrikson RL. Purification and characterization of a low molecular weight acid phosphatase from bovine liver. *J Biol Chem.* 1969; 244(2): 299-307.

31. Okada M, Owada K, Nakagawa H. [Phospho-tyrosine]protein phosphatase in rat brain. A major [phosphotyrosine]protein phosphatase is a 23 kDa protein distinct from acid phosphatase. *Biochem J.* 1986; 239(1): 155-162.

32. Fujimoto S, Murakami K, Ishikawa A, Himi K, Ohara A. Two distinct low-molecular-weight acid phosphatases from rat liver. *Chem Pharm Bull (Tokyo).* 1988; 36(8): 3020-3026.

33. Zhang ZY, Van Etten RL. Purification and characterization of a low-molecular-weight acid phosphatase – a phosphotyrosyl-protein phosphatase from bovine heart. *Arch Biochem Biophys.* 1990; 282(1): 39-49.

34. Khan AR, Naz R, Hassan M, Saeed A. The low and high molecular weight acid phosphatases in sheep liver. *J Chem Soc Pak.* 1997; 19: 60-69.

35. Granjeiro JM, Taga EM, Aoyama H. Purification and characterization of a low-molecular-weight bovine kidney acid phosphatase. *An Acad Bras Cienc.* 1997; 69(4): 451-460.

36. Chernoff J, Li HC. A major phosphotyrosyl-protein phosphatase from bovine heart is associated with a low-molecular-weight acid phosphatase. *Arch Biochem Biophys.* 1985; 240(1): 135-145.

37. Boivin P, Galand C. The human red cell acid phosphatase is a phosphotyrosine protein phosphatase which dephosphorylates the membrane protein band 3. *Biochem Biophys Res Commun.* 1986; 134(2): 557-564.

38. He RJ, Yu ZH, Zhang RY, Zhang ZY. Protein tyrosine phosphatases as potential therapeutic targets. *Acta Pharmacol Sin.* 2014; 35(10): 1227-1246.

39. Fuchs KR, Shekels LL, Bernlohr DA. Analysis of the ACP1 gene product: classification as an FMN phosphatase. *Biochem Biophys Res Commun.* 1992; 189(3): 1598-1605.

40. Granjeiro JM, Ferreira CV, Jucá MB, Taga EM, Aoyama H. Bovine kidney low molecular weight acid phosphatase: FMN-dependent kinetics. *Biochem Mol Biol Int.* 1997; 41(6): 1201-1208.