EXPERIMENTAL ARTICLES

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NON-COENZYME PROPERTIES OF THIAMINE: EVALUATION OF BINDING AFFINITY TO MALATE DEHYDROGENASE ISOFORMS

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The aim of this work was evaluation of binding affinity of thiamine to malate dehydrogenase (MDH) isoforms. The methods of affinity chromatography, SDS PAGE electrophoresis, and MALDI-TOF mass spectroscopy, as well as molecular modeling *in silico* were used in the study. Affinity sorbent (T-AS) contained C2-conjugated thiamine fragment as an anchor which was bound to activated Sepharose 4B via a spacer composed of *N*-4-azobenzoylcaproic acid hydrazide. A commercial preparation of MDH from the porcine heart was chosen for the experimental study. Analysis of the protein content in fractions performed by the Bradford method showed that three separate protein peaks with MDH activity were obtained after elution with a thiamine solution. The results of one-dimensional electrophoresis of the initial MDH preparation and pooled fraction of proteins which were eluted from the affinity sorbent with a thiamine solution demonstrated that almost all protein fractions detected in the commercial MDH preparation were also present in eluates obtained by T-AS affinity chromatography. Four isoforms of MDH, including cytoplasmic malate dehydrogenase (MDH1), mitochondrial malate dehydrogenase (MDH2) and its isoform, and malate dehydrogenase 1B (MDH1B) were specifically bound to the affinity sorbent. According to the molecular docking results, the most preferred for both monomeric and homodimeric MDH1 and MDH2 could be ligand position at the NAD (NADH) binding site. Additional binding site could be located between two subunits of the homodimeric form of enzyme. Our results confirm the previously obtained data and expand an idea of ability of MDH isoforms to interact with the thiamine molecule *in vivo*. These data can also be useful for identification of thiamine binding protein (ThBP) which was previously isolated from rat brain, taking into account the possible partial homology of this protein with proteins that show MDH activity.

Key words: thiamine, affinity chromatography, malate dehydrogenase, protein affinity for thiamine, molecular docking, thiamine binding protein.

During the years of research in the field of biochemistry and physiology of vitamin B_1 (thiamine), numerous experimental evidences have been accumulated that show a wide range of metabolic changes occurring in case of deficiency or excess of thiamine. Some of these alterations, even with the involvement of other metabolic interaction, cannot be explained only by known coenzyme functions of ThDP (thiamine diphosphate) [1–3].

The presence of two substituted heterocyclic fragments in thiamine molecule provides reactivity of this natural compound in the enzymatic and chemical non-enzymatic transformations as well as ability to interact with other proteins and cellular structures [4]. One of the effective approaches for elucidation of the non-coenzyme mechanisms of participation of thiamine in the processes of cell activity [5–7] may be the identification

of functionally active proteins that exhibit affinity for thiamine or its derivatives.

The protein that was named "thiamine binding protein" (ThBP) was isolated from the rat brain synaptosomes [8, 9] as possible protein target for thiamine. It turned out that in addition to thiamine-binding activity (TBA), this protein has the ability to specifically hydrolyze phosphoric esters of thiamine, namely, thiamine monophosphate, thiamine diphosphate and thiamine triphosphate (ThTP, ThDP, and ThMP, respectively) [9]. The ThBP was isolated using an affinity sorbent that contained thiamine fragment as an anchor bound to the matrix via a spacer (T-AS) and subsequent gel filtration on Sephadex G-150 [8]. The eluate fractions having the ThDPase- and thiamine-binding activities were selected and combined. The proteins contained in these fractions were separated by SDS-PAGE electrophoresis and examined by MALDI-TOF mass spectrometry (MS). It turned out unexpectedly that preliminary MS analysis showed a very high probability of malate dehydrogenase (MDH) being present in these fractions (unpublished data). The presence of proteins with MDH activity in the eluates with T-AS, as well as some other proteins, including dehydrogenase enzymes, was confirmed by MALDI-TOF mass spectrometry analysis by specialists from Germany and Belgium [6]. The role of thiamine fragment in the MDH binding to T-AS was confirmed by using two sorbents: T-AS and AS without thiamine as an anchor [10–11]. Since several MDH isoforms exist in animal cells and they are isolated together during purification of this enzyme from tissues, we set out to test whether the high affinity for thiamine is inherent in any MDH isoform or is characteristic of only some of them.

For this purpose, using T-AS, we subjected a commercial preparation of MDH from the porcine heart to affinity chromatography and analyzed proteins in eluates. This paper describes the results of the studies, including evaluation of binding affinity of thiamine to MDH isoforms.

Materials and Methods

Sample preparation. A commercial preparation of MDH from a porcine heart (Reanal, Hungary) with an initial specific activity of 100 units/mg protein (one unit converts 1.0 μmole of oxalacetate and β-NADH to L-malate and β-NAD per min at pH 7.5 at 25 °C) was maintain as ammonium sulfate at — 20 °C. The specific activity of MDH preparation was 92.5 units/mg protein at the time of the experiment. 40.8 mg of the preparation was dissolved in 24 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) and applied to a column with T-AS.

Affinity chromatography. The affinity sorbent (T-AS) was synthesized by reaction of thiamine with Sepharose 4B which was activated with bromocyan followed by covalent conjugation of hydrazide of *N*-4-azobenzoylcaproic acid according to the method of Klyashchitsky with some modification [8, 12].

A solution of the commercial preparation MDH was applied to an affinity sorbent column, previously equilibrated with Krebs-Ringer bicarbonate buffer (pH 7.4), at a flow rate of 0.2 ml/min. Unbound or weakly associated proteins were washed out until absorbance of the eluate at 280 nm approached background level. The proteins bound to T-AS were eluted in several stages. At the first stage, elution was carried out with a 10 mM thiamine chloride solution (pH 7.4). Next, stepwise elution was continued as previously described for TBP [8]: 1 M NaCl in 10 mM Tris-HCl buffer (pH 7.4); 2 M urea in 10 mM Tris-HCl buffer (pH 7.4). The elution rate was 0.4 ml/min; fractions with a volume of 2 ml were collected.

In each fraction, MDH activity and protein concentration were analyzed. The fractions obtained by elution with each type of element were combined and concentrated on "Amicon Ultra-15" ultrafilters, 3 kDa Centrifugal Ultrafilters with replacing the buffer twice with 10 mM Tris-HCl buffer (pH 7.4). The sorbent was regenerated with 8 M urea solution and then washed with 4 volumes of distilled water. The sorbent was stored in a 0.02% sodium azide solution.

Enzymatic Activity Measurement. The MDH activity was estimated by measuring the rate of oxidation of NADH in the presence of oxaloacetate as a substrate. The decrease in the absorbance at 340 nm was determined in reaction mixture (200 μl, pH 7.2) of the following composition: oxaloacetic acid (0.3 mM) in 20 mM potassium phosphate buffer and NADH (0.14 mM) at 25 °C [13].

Measurement of protein concentration. The protein concentration in the solution was calculated based on spectrophotometric monitoring at 280 nm, as well as using the Lowry technique. The Bradford method was used [14] in cases when other ways do not provide a possibility to do this.

SDS PAGE electrophoresis. One-dimensional electrophoresis under denaturing conditions was run in 10% polyacrylamide gel (PAGE) with 0.1% SDS (sodium dodecyl sulfate) according to the Laemmli method [15] at the starting constant current of 20 mA and voltage of 80 V and maintaining 20 mA and 140 V during separation.

2D-SDS PAGE. The 2D-electrophoresis procedure was performed using a Protean IEF Cell protein isofocus device (Bio_Rad, USA) and a Mini-PROTEAN 3 cell SDS PAGE electrophoresis device (Bio_Rad, USA) according to the manufacturer's instructions. Separation in the first direction was carried out by isoelectric focusing on IPG strips pH 3–10 (Bio_Rad). The strips were passively rehydrated for 16 hours in a solution containing a protein fraction obtained by specific elution with thiamine (170 μg of protein per sample) and rehydration buffer (8 M urea, 2% CHAPS (M/V), 50 mM dithiothreitol, 0.2% ampholines pH 3–10 (M/V) Bio-Lyte® ("Bio_Rad"). The isoelectric focusing procedure was carried out at a voltage of 250 V for 20 min, then for 2 h with a linear rise in voltage to 10,000 V and for 5 hours at 10,000 V until a total of 14,000 Wh is achieved. After isoelectric focusing, the strips were incubated for 10 minutes in a balancing buffer I (0.375 M Tris-HCl buffer (pH 8.8) containing 6 M urea, 20% glycerol, 2% SDS and 2% (w/v) dithiothreitol), and then in equilibration buffer II (0.375 M Tris-HCl buffer (pH 8.8), containing 6 M urea, 20% glycerol, 2% SDS and 2.5% iodoacetamide (Fluka®)). For the separation of proteins in the second direction, 8–16% vertical gradient gels prepared according to the standard protocol were used. Gels were stained with silver using a kit ProteoSilver™ Silver Stain Kit (Sigma®) in accordance with the procedures described in the kit manual. The size of the gels was 0.15×7.2×8.6 cm.

MALDI-TOF mass spectroscopy. Stained protein spots (Fig. 3) were excised from the gel, washed from stain and treated with bovine trypsin (Applied Biosystems, USA). 0.5 μl of the peptide mixture was mixed on the target with 1.1 μl of a 10 mg/ml solution of α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma, USA) in 50% acetonitrile with 0.1% trifluoroacetic acid and dried in air. Mass spectra were obtained on a Voyager DE PRO MALDI-TOF mass spectrometer serial № 6393 (Applied Biosystems, USA) in the mode of positive ions in the mass range from 500 to 5 000 Da, voltage 20 kV, reflex mode. The obtained mass spectra were calibrated using external peptides (Sequazyme kit standarts, Applied Biosystems) and internal peaks (matrix MH^+ 666.0293 and peaks of trypsin autolysis, MH^+ 2163.0566 and 2273.1599). The list of peptide peaks was formed using the Data Explorer 4.1 service program. Proteins were identified by sets of peptide mass values after trypsinolysis using the Peptide Fingerprint option of the Mascot online resource (Matrix Science, USA) (http://www.matrixscience.com/search_form_select.html). The following search parameters were set: accuracy of determining the mass of 0.5 Da, a database of primary protein sequences of NCBI and SwissProt, Mammalian taxon, one missed cleavage, the possibility of various modifications of cysteine with acrylamide and the oxidation of methionine.

Molecular docking. The molecular docking was carried out by program Autodock Vina [16] using monomeric and dimeric crystal structures of cytoplasmic porcine MDH1 and mitochondrial human MDH2 (PDB server https://www.rcsb.org). The unnecessary chains, water molecules and ligands were deleted from the crystal structures before docking simulation. MGLTools 1.5.6 program was used for preparing the docking files. The structure of thiamine was drawing in MarvinSketch program [17] and optimized in program MOPAC with using AM1 semi-empirical quantum mechanical method [18]. The analysis of obtained complexes of MDH with thiamine was performed by Discovery Studio.

Bioinformatics Resources. The following bioinformatics tools were used in the course of the work: Jalview (http://www.jalview.org); The Basic Local Alignment Search Tool (BLAST), Align two or more protein sequences with the Clustal Omega program and Peptide search service (https://www.uniprot.org); ExPASy Bioinformatics Resource Portal from SIB (https://www.expasy.org); Sequence & Structure Alignment from PDB (http://www.rcsb.org). We used also Phobius which is a combined transmembrane topology and signal peptide predictor (http://phobius.sbc.su.se) and Protein isoelectric point calculator (http://isoelectric.org).

Results and Discussion

Affinity chromatography of the commercial MDH preparation was carried out mainly according to the protocol developed for ThBP [8], however, as the first step, elution with a 10 mM thiamine solution

in 10 mM Tris-HCl buffer (pH 7.4) was additionally introduced. The yield of protein fractions with T-AS was summarized in Fig. 1. It should be noted that usually during chromatography, the appearance of protein fractions in eluate is monitored by measuring the extinction at 280 nm. We also used this protocol, however, at the some wavelengths, an intensive absorption of the thiamine was observed (Fig. 1, *A*). Therefore, we duplicated the determination of protein content in each fraction using the Bradford method, for which the presence of thiamine is not an obstacle.

Analysis of the protein content in fractions using the Bradford method showed that three separate protein peaks with MDH activity were obtained after elution with a thiamine solution (Fig. 1, *B*). Table 1 shows the balance of proteins from the commercial MDH preparation after the chromatographic procedure.

Data shown in Fig. 1 and in Table 1 indicate that more than half of the proteins with MDH activity of the sample used did not interact with the sorbent. At the same time, almost all proteins with MDH activity that binds to T-AS were eluted from the column with a thiamine solution. According to the data presented in Table 1, about 45% of MDH activity was found in thiamine eluates from T-AS. Moreover, after passing through a column with T-AS, the specific MDH activity of proteins decreases. In the next two stages of elution, namely the elution with NaCl and urea solutions, only trace amounts of proteins with MDH activity were removed. These results confirm the high affinity of proteins with MDH activity to thiamine.

We pooled all protein fractions with MDH activity, which were eluted with a thiamine solution, to remove thiamine on an Amicon ultrafilter, thus also increasing relative protein concentration. Lyophilic dried proteins of the selected fractions were studied using the methods of one-dimensional and two-dimensional electrophoresis and mass spectrometric analysis.

One-dimensional protein electrophoresis. One-dimensional denaturing electrophoresis of lyophilized proteins was carry out according to Laemmli, and the staining of protein bands was carried out using two methods: traditional staining with the Coomassie solution and more sensitive silver staining.

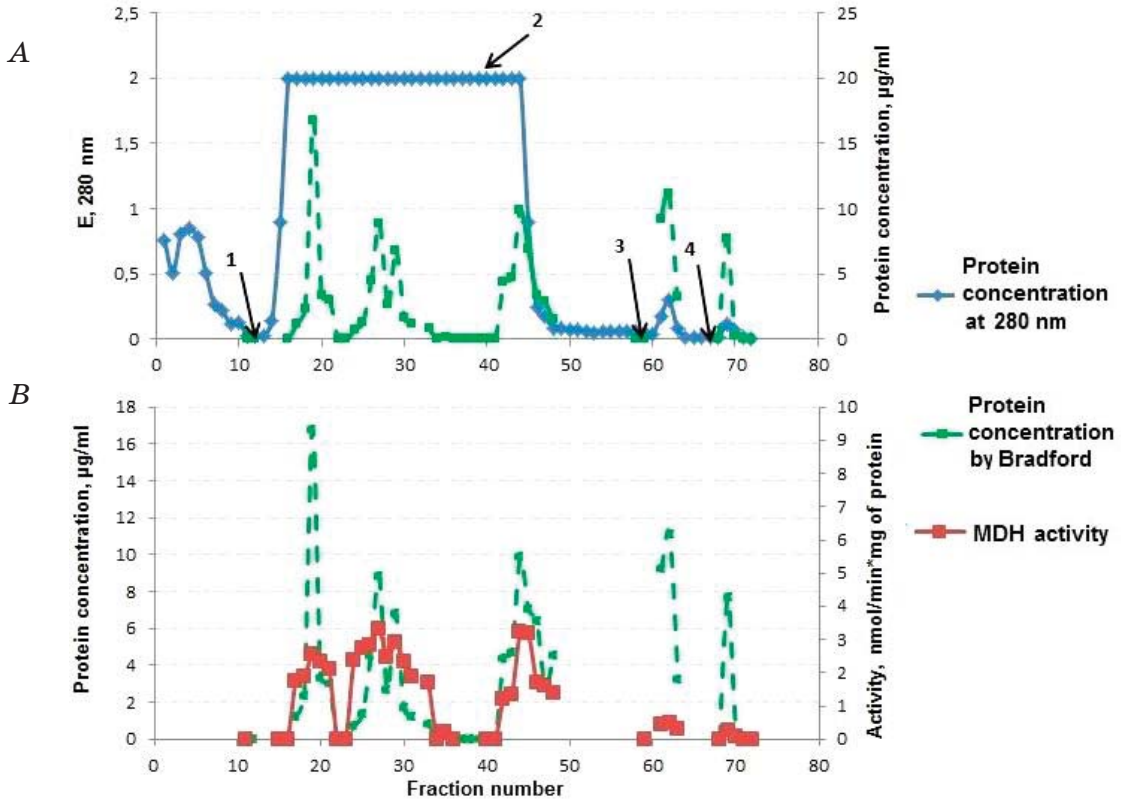


Fig. 1. **Affinity chromatography on T-AS of an MDH preparation from a porcine heart:** *A* — protein yield, controlled using two different methods: extinction at 280 nm; Bradford protein analysis (registration at a wavelength of 595 nm); the arrows indicate the change in the eluting solution: 1–10 mM thiamine; 2–10 mM Tris-HCl buffer (pH 7.4); 3–1 M NaCl; 4–2 M urea; *B* — activity of MDH in fractions

Table 1. **The yield of proteins after affine chromatography of MDH preparation on T-AS column**

Sample/fraction	MDH specific activity, μmoles/min·mg protein	Protein concentration, mg/ml	V, ml	Total activity, μmoles/min	Recovery of MDH activity, %
Applied to T-AS	92.50	1.70	24	3774.0	100
Unbound proteins	54.85	1.46	25.4	2034.1	53.9
10 мМ thiamine pH 7.4 eluate	62.1	0.28	98.5	1712.7	45.4
1 M NaCl eluate	2.49	0.15	13	4.9	0.13
2 M urea eluate	0.75	0.13	21.5	2.1	0.06

The typical results of one-dimensional denaturing Laemmli electrophoresis after silver staining are presented in Fig. 2. The results demonstrate that the preparation loaded on the column contains heterogeneous mix of proteins. The most intense protein bands are observed in the region of 52 and 26 kDa.

The bands migrated to Mr 70–72 kDa and the corresponding subunit (35–36 kDa) are less intense. Of interest there is the 140 kDa band both on the track on which the initial commercially available MDH preparation was applied (3), and on the tracks on which thiamine eluates with T-AS were separated (4 and 5). This observation may indicate that the tetrameric form of MDH binds to T-AS, but the possibility of aggregation of the MDH protein during concentration of eluates with AS on Amicon filters cannot be ruled out. The bands corresponding to 140, 72, and 36 kDa in lanes 4 and 5 are developed more clearly than that in the lane with the initial preparation. This is probably due to partial purification of the proteins on an affinity sorbent.

The results of one-dimensional electrophoresis of the initial MDH preparation and pooled fraction of proteins, which were removed from the affinity sorbent with a thiamine solution, demonstrated that almost all protein fractions detected in the commercial MDH preparation are also present in eluates with T-AS. However, some differences can be noted. Firstly, after affinity chromatography, the ratio of the intensity of the protein bands changes; secondly, a new band with Mr 42 kDa is clearly visible. The observed changes can be explained by different affinities of individual isomers to thiamine, which leads to the concentration of some of them on T-AS.

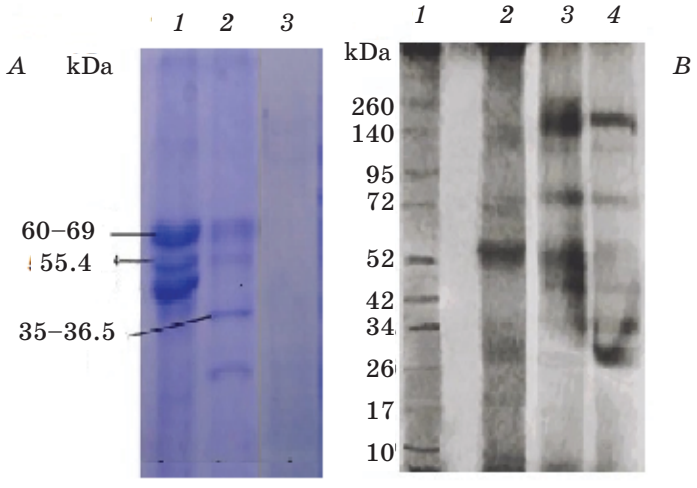


Fig. 2. **Electrophoregrams of the initial commercial MDH preparation and MDH eluted from T-AS:**
A — Protein staining with Coomassie solution. Samples loaded onto tracks: *1* — a mixture of marker proteins: bovine serum albumin (Mm = 68–69 kDa), glutamate dehydrogenase (55.4 kDa), lipoamide dehydrogenase (35–36.5 kDa); *2* — commercial MDH from a porcine heart; *3* — proteins eluted from t-AS with thiamine solution, pH 7.4; *B* — Protein staining with silver reagent (ProteoSilver™ Silver Stain Kit (Sigma®)). Samples loaded onto tracks: *1* — Spectra™ Multicolor Broad Range Protein Ladder from Thermo Fisher Scientific™; *2* — commercial MDH from a porcine heart; *3* and *4* — proteins eluted from t-AS with thiamine solution, pH 7.4 (various experiments)

2D electrophoresis and MALDI TOF MS analysis. Known MDH isoforms of various cellular localization and from various sources differ not only in molecular weight, but also in isoelectric point. To separate the proteins eluted from T-AS taking into account these parameters was possible using 2D electrophoresis. Therefore, the combined and concentrated fractions of proteins eluted with a thiamine solution were then subjected to two-dimensional electrophoresis for more accurate identification.

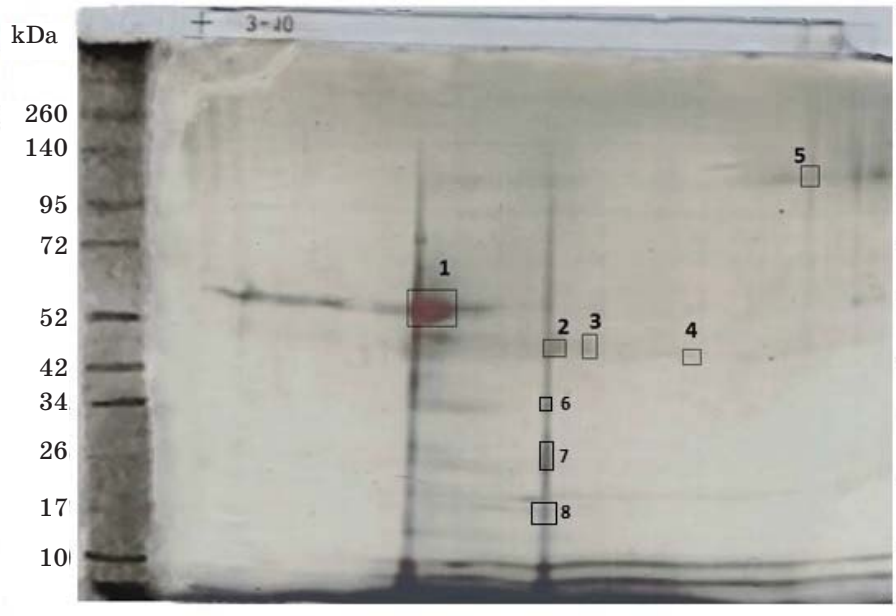
Proteins focused on spots 1–8, which were detected on a 2D electrophoregram (Fig. 3), were subjected to mass spectrometric studies. The most convincing results of this analysis are summarized in Table 2.

Data in Table 2 demonstrate that four isoforms of MDH are bound with an affinity sorbent. Three isozymes (products of different genes) of MDH are known, namely, MDH, cytoplasmic (MDH1 or simply MDH); malate dehydrogenase, mitochondrial (MDH2 or Mor1); malate dehydrogenase 1B (MDH1B or rCG_22254). Each isozyme has a large number of isoforms, splice variants, and variants with different post-translational modifications (references in Table 2). Protein spot No. 1 in Fig. 3 has the largest area, on the basis of which it is very likely to conclude that this protein is the basic constituent of the studied MDH preparation. According to MS analysis, this protein corresponds to MDH1B (Table 2), which is confirmed by the alignment of the found Mascot peptides against the primary structure of porcine MDH1B (Fig. 4).

Current information on porcine MDH1B, as well as other similar isozymes from other organisms, is limited. These isoforms have molecular weights in the range of 52–58 kDa, and their subunits — 26–29 kDa. Using the BLAST tool from the UniProt database, it was found that putative malate dehydrogenase 1B from Sus scrofa is 83.5% homologous to the human isoform with (3) putative malate dehydrogenase (MDH1B) from Homo sapiens (Human). According to UniProt, the highest expression levels are characteristic of the following tissues: pituitary, cortex, testes, epithelial cells of the bronchi and lungs. This protein can be either cytoplasmic or membrane-bound or secreted as shown by the Phobius resource.

All other protein spots found on the 2D electrophoregram are very poorly expressed and correspond to traces of proteins that are shared with MDH1B when MDH was isolated from the porcine heart. Besides MDH1V, three different isoforms of MDH were identified on electrophoregram (Fig. 3), namely in spots No. 4, No. 6 and No. 7. The fact that these proteins bound to T-AS were eluted with thiamine may indicate the presence of thiamine affinity sites on their surface.

The presence of a lingual lipase among proteins that interact with T-AS and eluted



No. of spot on the electrophoregram	pI	Mr, kDa
1	5.41–5.78	50–56
2	6.86–6.98	43–46
3	7.22	43–46
4	8.31–8.43	42
5	9.39–9.64	136–140
6	6.74–6.86	34–36
7	6.74–6.86	21–26
8	6.74–6.86	17–19

Fig. 3. Scanned image of a 2D electrophoregram of MDH protein from a porcine heart, eluted from T-AS with 10 mM thiamine (pH 7.4) and spots parameters
Rectangles indicate protein stains that were removed for analysis using the MALDI TOF MS method

Table 2. **Enzymes with MDH activity identified after 2D electrophoresis in the thiamine eluate obtained from T-AS**

№ of protein	Name of the protein	Mass, Da	Score	Matches (number of unique peptides)	Calculated pI a verage (from-up to different counting systems) (pI calculated- result of Mascot)	Protein sequence coverage	№ spot in which the protein is identified	References
Spot No.1								
1	Putative malate dehydrogenase 1B	55984	82	16	5.59 (4.03–6.16) (6.3)	24%	1	https://www.uniprot.org/uniprot/A0A287AW19, https://www.uniprot.org/uniprot/F1SHD
Spot No.2								
2	Gastric triacylglycerol lipase precursor	44559	54	11	5.83 (3.598–6.616) (6.12)	27%	2	https://www.uniprot.org/uniprot/P04634, https://rgd.mcw.edu/rgdweb/report/gene/main.html?id=708441
	Alternative name(s): Lingual lipase, lipase F, gastric type, RNLIP; Rnlp	45238	42	10	6.55 (3.757–7.374) (6.84)	26%	4	
3	ubiquitin-conjugating enzyme E2 variant 3 isoform X1	52128	73	11	6.11 (4.285–6.882) (6.80)	31%	2	https://www.uniprot.org/uniprot/A0A481B758
	ubiquitin-conjugating enzyme E2 variant 3 isoform X3	37054	55	9	5.68 (4.297–6.291) (5.91)	32%	2	https://www.uniprot.org/uniprot/A0A480K0S2
	ubiquitin-conjugating enzyme E2 variant 3 isoform X2	41068	52	9	6.18 (4.338–6.971) (6.46)	29%	2	https://www.ncbi.nlm.nih.gov/protein/XP_020938920.1?report=genbank&log$=protalign&blast_rank=1&RID=JWCCSSCK014
Spot No.4								
4	PREDICTED: malate dehydrogenase, mitochondrial isoform X1	42173	80	6	7.12 (4.484–8.603) (8.23)	22%	4	https://www.uniprot.org/uniprot/W5PW05
5	Gastric triacylglycerol lipase (precursor)	44559	54	11	5.83 (3.598–6.616) (6.12)	27%	2	https://www.uniprot.org/uniprot/P04634,
	Alternative name(s): Lingual lipase, lipase F, gastric type, RNLIP; Rnlp	45238	42	10	6.55 (3.757–7.374) (6.84)	26%	4	https://rgd.mcw.edu/rgdweb/report/gene/main.html?id=708441
6	Lysosomal acid lipase/cholesteryl ester hydrolase precursor	40362	42	6	6.55 (3.757–7.374) (6.84)	28%	4	https://www.uniprot.org/uniprot/A0A286ZVK3, https://www.uniprot.org/uniprot/A0A287ABD8, https://www.ebi.ac.uk/QuickGO/term/GO:0016788, https://www.ebi.ac.uk/QuickGO/annotations?geneProductId=A0A286ZVK3

Table 2. End

1	2	3	4	5	6	7	8	9
	Spot No.5							
7	Succinate dehydrogenase cytochrome b560 subunit, mitochondrial	18506	23	3	9.34 (9.343–10.39) (9.87)	45%	5	[Singer et al., 2006], https://www.uniprot.org/uniprot/D0VWV4
	Spot No.6							
8	Malate dehydrogenase, mitochondrial	35557	97	6	7.88 (4.803–9.244)(8.93)	39%	6	https://www.uniprot.org/uniprot/P00346
9	ubiquitin-conjugating enzyme E2 variant 3 isoform X3	37054	45	7	5.68 (4.297–6.291) (5.91)	26%	6	https://www.uniprot.org/uniprot/A0A480K0S2
10	L-lactate dehydrogenase A chain	36595	20	2	7.31 (4.616–8.458) (8.18)	11%	6	https://www.uniprot.org/uniprot/P00339
	Spot No.7							
11	Mitochondrial malate dehydrogenase 2, NAD, partial	28416	57	4	6.53 (4.262–7.492) (7.12)	31%	7	https://www.uniprot.org/uniprot/F6TYW4
	Spot No.8							
12	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9	21775	24	2	7.99 (4.455–8.951) (8.82)	21%	8	https://www.uniprot.org/uniprot/Q02369

with a thiamine solution (spot 2 in Fig. 3 on the 2D electrophoregram) is of interest for TBP identification. The MDH activity [19], as well as the phosphoesterase activity [20, 21] was proposed for proteins of this class.

Molecular docking. The blind docking approach, which provided scanning all surface of target protein molecule, was used for identification of possible binding sites for thiamine and interaction with MDH1 and MDH2. The calculations were carried by Autodock Vina using structures of porcine MDH1 and MDH2 (PDB code 4MDH [22] and 1MLD [23] respectively) and human MDH2 (PDB codes 4WLE, 4WLF, 4WLN, 4WLO, 4WLU, and 4WLV). The human recombinant MDH2 applied to the docking system were taken from the crystal structures which were obtained at different co-crystallization conditions with coenzyme and (or) different substrates. The calculations were performed using monomeric (chain A) and homodimeric (chains A and B) forms of the enzyme.

According to the obtained results, the monomers of MDH2 may have three sites for thiamine binding (Fig. 5). Chain A of MDH2 (PDB codes: 4WLE, 4WLO, and 4WLU) as well as chain A of MDH1 (PDB code 4MDH) showed binding affinity for thiamine in site 1, responsible for binding of fragment of nicotinamide adenine dinucleotide (NAD/NADH coenzyme system). The monomeric structures with PDB codes 4WLF, 4WLN and 4WLV demonstrated also possible binding of thiamine to site 2 outside of catalytic region. Only monomer of MDH2 from porcine heart exhibits some affinity to thiamine with involving amino acid residues near the substrate binding center (site 3).

Blind docking calculations was also performed with catalytically active homodimeric forms of MDH consisted of chain A and chain B. As a result, two additional binding sites 4 and 5 for thiamine were found on surface of homodimer (Fig. 5), additionally to the sites 1–3 described above. Both of the sites 4 and 5 are located between two enzyme subunits. It should be noted that only porcine MDH2 (PDB code 1MLD) is able to bind thiamine molecule at the site 5. The binding modes of thiamine at the site 1 are the same both for monomeric and dimeric MDH structures.

The site-oriented docking was carried out for detailed analysis of the binding sites 1–5. As seen from Table 3, the binding of thiamine molecule to all MDH structures is energetically more preferred in case of its location at site

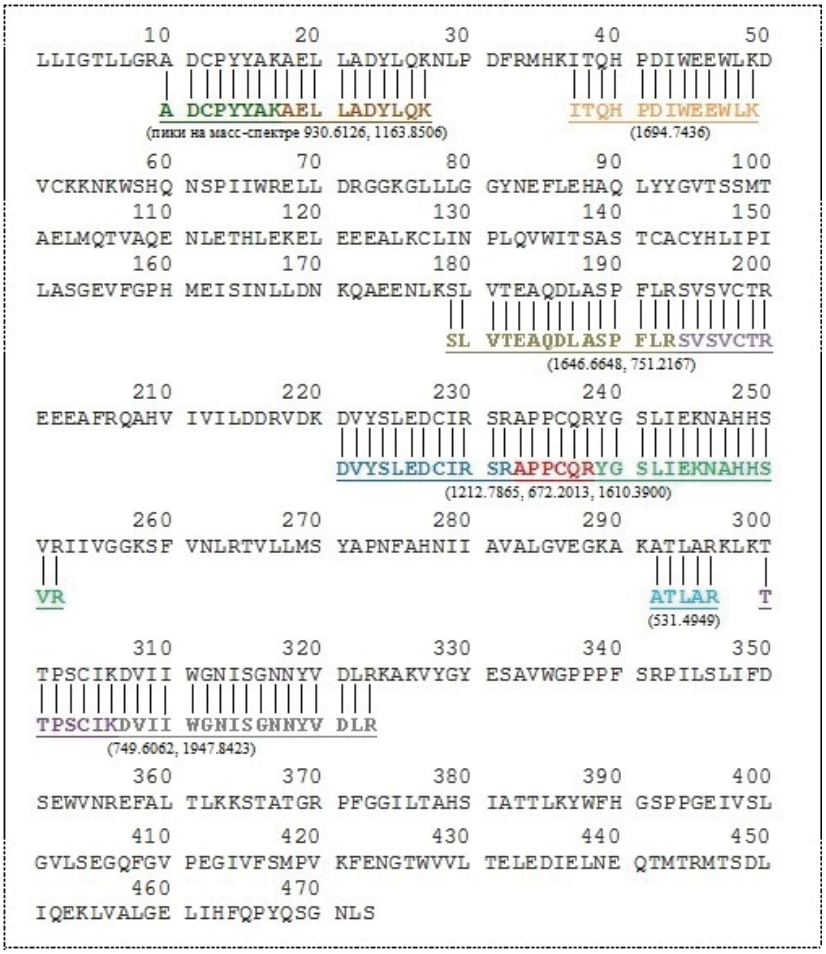


Fig. 4. **Alignment of peptides detected by mass spectrometry and identified using Mascot against porcine MDH1B (UniProtKB-F1SHD0)**
(in spot No. 1 of Fig. 3)

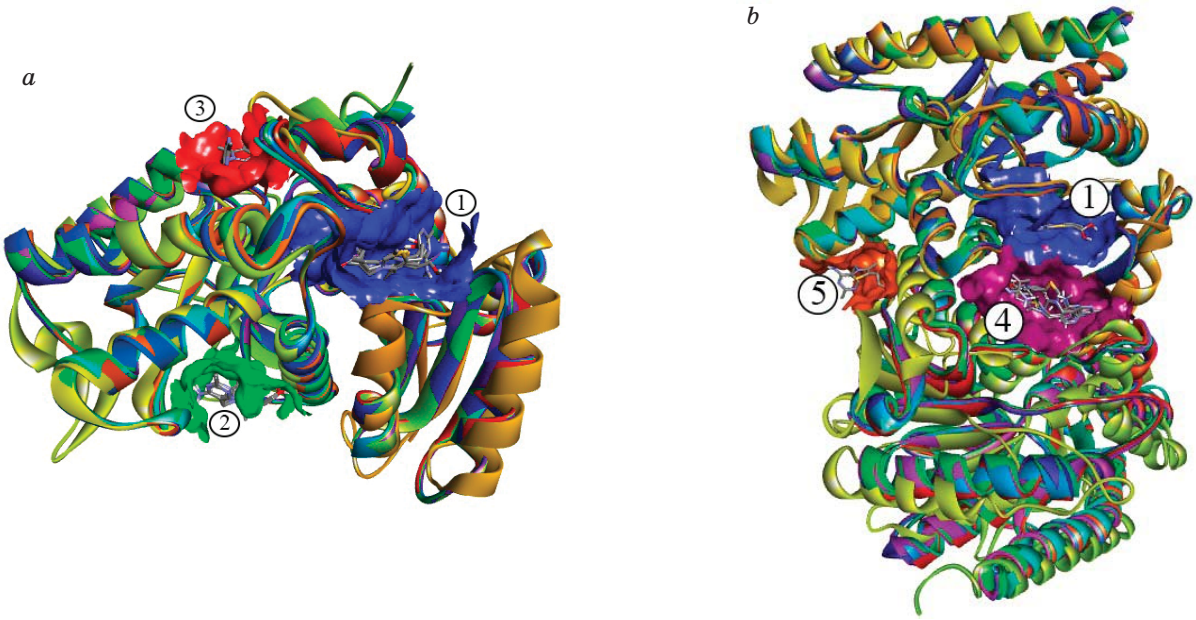


Fig. 5. **Possible binding sites of thiamine on surface of MDH1 and MDH2 monomers and additional sites** for their homodimeric structures:
a — monomeric forms; *b* — homodimeric structures

Table 3. **The predicted binding affinity of thiamine to the possible binding sites of the monomeric structures of MDH**

PDB code	Affinity, kcal/mol				
	Site 1	Site 2	Site 3	Site 4	Site 5
1MLD	−5.8	−5.2	−5.4	−5.9	−6.2
4MDH	−6.9	−4.9	−6.5	−6.1	−6.2
4WLE	−6.1	−5.6	−5.1	−5.9	−5.1
4WLF	−6.1	−5.6	−5.2	−6.0	−5.5
4WLN	−6.1	−5.6	−5.1	−5.8	−5.2
4WLO	−6.7	−5.5	−5.7	−6.1	−5.4
4WLU	−6.8	−5.7	−5.4	−5.9	−5.5
4WLV	−6.7	−5.7	−5.0	−5.9	−5.4

1 and site 4 as compared to the other sites. However, the affinity of vitamin B_1 to site 4 is still less than that to site 1.

The superposition of thiamine bound to site 1 of human MDH2 (PDB codes 4WLF, 4WLO, 4WLU, and 4WLV) is showed in Fig. 6, *a*. According to such position, the pyrimidine part of ligand forms hydrogen bonds with amino acid residues of Ser246 and Ala247, whereas OH-group of 5-(2-hydroxyethyl) substituent is located near Asp57. The hydrophobic and van der Waals contacts between thiamine and residues of Gly35, Ile36, Pro99, Gly101, and Pro103 contribute in stabilization of the enzyme-ligand complex (Fig. 6, *b*). It is noteworthy that C2-H of the thiazolium cycle is oriented outward from the binding site, allowing to expect similar binding pose of conjugated thiamine fragment of affinity adsorbent.

The positions of thiamine molecule at site 4 of homodimeric porcine MDH2 (PDB code 1MLD) and human MDH2 (PDB codes 4WLF, 4WLN, 4WLU, and 4WLV) is presented in Fig. 7, *a*. The most preferred model with human MDH2 (PDB code 4WLF) demonstrates hydrogen bonds between ligand and amino acid residues Thr248 of chain A as well as Gly35 and Gln38 of chain B. The position of thiamine provides hydrophobic and van der Waals interactions with residues of Gln38 and His60 of chain A, and residues of Lys241 and Ala244 of chain B (Fig. 7, *b*).

The data obtained suggest a possible regulatory role of thiamine in MDH2 functioning. It should be mentioned that overexpression of MDH2 may be the cause of docetaxel and doxorubicin resistance in cancer cells [24, 25]. In this connection, inhibitors of MDH2 may be beneficial for cancer treatment.

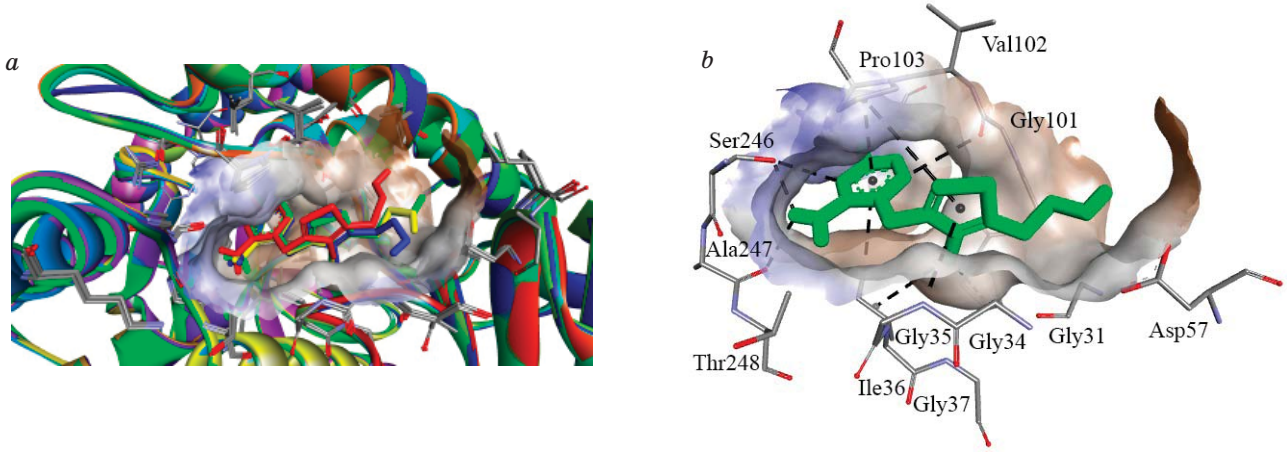


Fig. 6. **Superposition of thiamine molecules at site 1 of chains a of human MDH2:**
a — PDB codes 4WLF (ligand red), 4WLO (ligand blue), 4WLU (ligand green), and 4WLV (ligand yellow);
b — binding mode of thiamine at site 1 of human MDH2 (PDB code 4WLU)

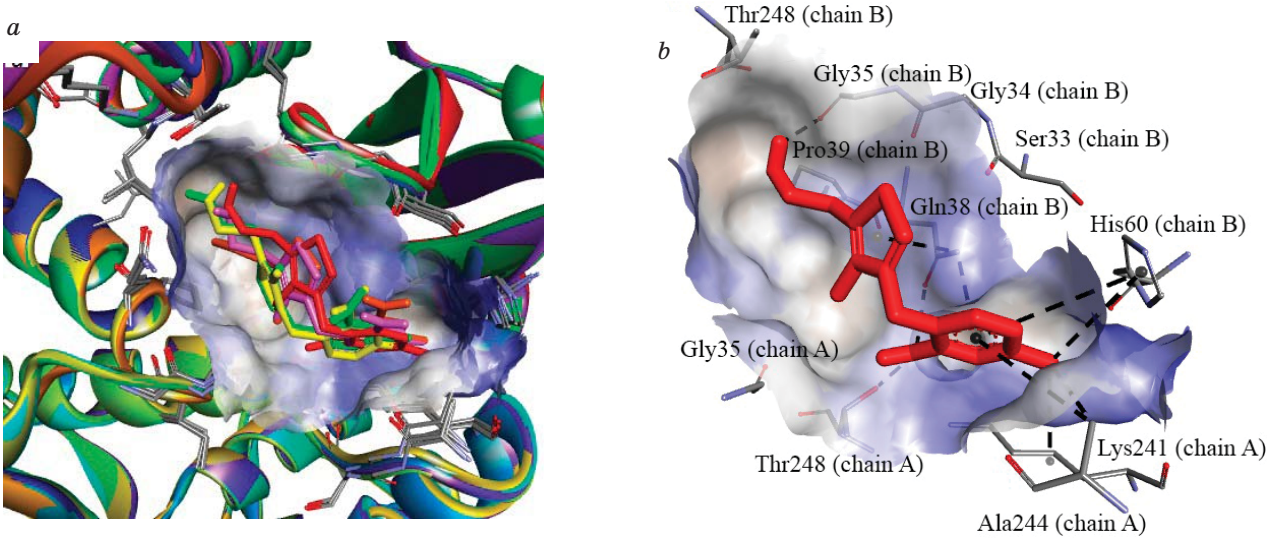


Fig. 7. **Superposition of thiamine molecule at site 4 of MDH:**
a — at site 4 of homodimer porcine MDH2 (PDB code 1MLD, ligand orange), human MDH2 (PDB codes 4WLF (ligand red), 4WLN (ligand pink), 4WLU (ligand green), and 4WLV (ligand yellow);
b — binding mode of thiamine at site 4 of homodimer human MDH2 (PDB codes 4WLF

For example, synthetic MDH2 inhibitors were reported to suppress hypoxia-inducible factor-1 by regulating mitochondrial respiration and integrating metabolism into anti-cancer efficacy [26]. However, given the observed and predicted affinity to MDH2, the possible regulatory role of thiamine may be related to the inherent factors of MDH2 functioning but not to the catalytic activity of the enzyme.

In conclusion, using T-AS, we have demonstrated experimental data concerning possible binding affinity of thiamine to MDH isoforms such as putative MDH1B, cytoplasmic MDH1, and mitochondrial MDH2. According to molecular docking results, the most preferred can be the binding position at the NAD (NADH) binding site in case of both monomeric and homodimeric MDH1 and MDH2. In case of the homodimer, additional binding site can be located between two subunits. Our results confirm the previously obtained data [6] and expand an idea of ability of MDH isoforms to interact with the thiamine molecule *in vivo*. These data can also be useful for identification of ThBP which was previously isolated from rat brain, taking into account the possible partial homology of this protein with proteins that show MDH activity.

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НЕКОЕНЗИМНІ ВЛАСТИВОСТІ ТІАМІНУ: ОЦІНЮВАННЯ АФІННОГО ЗВ'ЯЗУВАННЯ З ІЗОФОРМАМИ МАЛАТДЕГІДРОГЕНАЗИ

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Метою роботи було оцінювання афінності зв'язування тіаміну з ізоформами малатдегідрогенази (MDH). Дослідження включало використання методів афінної хроматографії, SDS PAGE електрофорезу і MALDI-TOF мас-спектроскопії, а також молекулярного моделювання *in silico*. Афінний сорбент (т-АС) містив С2-кон'югований тіаміновий фрагмент як якір, що був зв'язаний з активованою сефарозою 4В через спейсер, який складається з гідразиду N-4-азобензоїлкапронової кислоти. Для експериментального дослідження було обрано комерційний препарат MDH із серця свині. Аналіз вмісту протеїну у фракціях з використанням методу Бредфорда показав, що після елюювання розчином тіаміну було отримано три основних окремих піки протеїнів з малатдегідрогеназною активністю. Результати одновимірного електрофорезу вихідного препарату MDH і об'єднаної фракції протеїнів, які було знято з афінного сорбенту розчином тіаміну, свідчать, що майже всі фракції протеїну, виявлені в комерційному препараті MDH, також присутні в елюатах з т- АС. Встановлено, що чотири ізоформи MDH зв'язалися з афінним сорбентом, зокрема цитоплазматична малатдегідрогеназа (MDH1), мітохондрійна малатдегідрогеназа (MDH2) та її ізформа і малатдегідрогеназа 1В (MDH1B). Згідно з результатами молекулярного докінгу найкращим як для мономерної, так і гомодимерної MDH1 і MDH2 може бути положення ліганду в сайті зв'язування NAD (NADH). У разі гомодимера додатковий сайт зв'язування може бути розташований між двома субодиницями ензиму. Наші результати підтверджують раніше отримані дані й розширюють уявлення щодо здатності ізоформ MDH взаємодіяти з молекулою тіаміну *in vivo*. Ці дані також можуть бути корисні для ідентифікації тіамінзв'язувального протеїну (ThBP), виділеного раніше з мозку щура, беручи до уваги можливу часткову гомологію цього протеїну з протеїнами, які виявляють малатдегідрогеназну активність.

Ключові слова: тіамін, афінна хроматографія, малатдегідрогеназа, афінність протеїнів до тіаміну, молекулярний докінг, тіамінзв'язувальний протеїн.

НЕКОЭНЗИМНЫЕ СВОЙСТВА ТИАМИНА: ОЦЕНКА АФФИННОГО СВЯЗЫВАНИЯ С ИЗОФОРМАМИ МАЛАТДЕГИДРОГЕНАЗЫ

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Целью работы была оценка аффинности связывания тиамина с изоформами малатдегидрогеназы (MDH). Исследование включало использование методов аффинной хроматографии, SDS PAGE электрофореза и MALDI-TOF масс-спектроскопии, а также молекулярного моделирования *in silico*. Аффинный сорбент (Т-АС) содержал С2-конъюгированный тиаминовый фрагмент в качестве якоря, который был связан с активированной сефарозой 4В через спейсер, состоящий из гидразида N-4-азобензоилкапроновой кислоты. Для экспериментального исследования был выбран коммерческий препарат MDH из сердца свиньи. Анализ содержания протеина во фракциях с использованием метода Брэдфорда показал, что три основных отдельных пика протеина с малатдегидрогеназной активностью были получены после элюирования раствором тиамина. Результаты одномерного электрофореза исходного препарата MDH и объединенной фракции протеинов, которые были сняты из аффинного сорбента раствором тиамина, показали, что почти все фракции протеина, обнаруженные в коммерческом препарате MDH, также присутствуют в элюатах с Т- АС. Четыре изоформы MDH были связаны с аффинным сорбентом, а именно цитоплазматическая малатдегидрогеназа (MDH1), митохондриальная малатдегидрогеназа (MDH2) и ее изформа и малатдегидрогеназа 1В (MDH1B). Согласно результатам молекулярного докинга наиболее предпочтительным как для мономерной, так и гомодимерной MDH1 и MDH2 может быть положение лиганда в сайте связывания NAD (NADH). В случае гомодимера дополнительный сайт связывания может быть расположен между двумя субъединицами. Наши результаты подтверждают ранее полученные данные и расширяют представление о способности изоформ MDH взаимодействовать с молекулой тиамина *in vivo*. Эти данные также могут быть полезны для идентификации тиаминсвязывающего протеина (ThBP), который был ранее выделен из мозга крысы, принимая во внимание возможную частичную гомологию этого протеина с протеинами, которые проявляют активность MDH.

Ключевые слова: тиамин, аффинная хроматография, малатдегидрогеназа, аффинность протеинов к тиамину, молекулярный докинг, тиаминсвязывающий протеин.