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Monoclonal antibodies to Coenzyme A.

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Aim. To generate monoclonal antibodies to Coenzyme A (CoA) with antigenic epitope specificity different to previously developed anti-CoA mAb (1F10) by introducing alternative approach for antigen preparation animal immunization. **Methods.** Cross-linking of CoA to Keyhole Limpet Hemocyanin (KLH) and to Bovine serum albumin (BSA) using reversible chemical crosslinker SPDP. Hybridoma technique. Western blot analysis. Immunoprecipitation. ELISA. **Results.** Monoclonal antibody (A11) that specifically recognizes CoA in various immunoassays and has the same antigenic epitope specificity as existing anti-CoA mAb (1F10) has been generated. **Conclusions.** Application of different ways for CoA conjugation to the carrier proteins to create an antigen for immunization does not affect the antigenic epitope specificity of generated anti-CoA antibodies most probably due to the existence of only one immunogenic epitope in CoA molecule.

K e y w o r d s: CoA, hybridoma technique, monoclonal antibody, SPDP crosslinking.

Introduction

Coenzyme A is an essential cofactor in all living cells with diverse cellular functions [1–5]. CoA and its thioesters apart from participating in critical anabolic and catabolic pathways can also directly regulate the activity of proteins by allosteric mechanisms and gene expression by protein acetylation [6]. There are numerous data regarding implication of CoA and its derivatives in the pathogenesis of some diseases and conditions, such as diabetes, cancer, cardiac hypertrophy [7–14] as well as neurodegeneration [15–17] that requires further detailed studies.

Recently a novel function of CoA in the antioxidant defense has been revealed. Using the previously generated highly specific anti-CoA mAbs (1F10) and mass spectrometry we demonstrated modification of cellular proteins by covalent attachment of CoA to cysteine thiols [18–19] induced by oxidizing agents. This novel type of post-translational modifica-

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tion was termed as protein CoAlation. Our study discovered that protein CoAlation is a widespread post-translational modification and over 2000 CoAlated proteins have been already identified in the prokaryotic and eukaryotic cells exposed to oxidative or metabolic stress. Protein CoAlation has been shown to protect the oxidized cysteine residues from irreversible overoxidation and to regulate the activity and subcellular localization of modified proteins by induction of conformational changes [18–19]. Immunohistochemistry using anti-CoA antibody revealed protein CoAlation in post-mortem brains from patients with Alzheimer's disease (AD) and Parkinson's disease (PD) [20], raising the fundamental question of the molecular mechanisms and functional consequences of covalent protein modification by CoA in pathologies associated with oxidative stress that remains to be investigated.

All these findings were possible first of all due to the unique anti-CoA mAbs (F10) that recognized CoA in various immunological assays including Western blot analysis, immunoprecipitation and immunohistochemistry. The antigenic epitope for 1F10 mAbs was mapped to the deoxyribose ring of CoA with the 3'-phosphate as a key part of it.

In spite of the existence of mAbs specific to CoA, the aim of the current work was to generate anti-CoA mAbs with the antigenic epitope specificity different to known one in order to obtain a new powerful tool for the protein CoAlation studies as well as for other CoA-associated investigations. Having such antibodies, for example, it would be possible to create an approach for the detection of free CoA level in different biological samples using immunological tests.

Materials and Methods

Generation of antigens. As the antigen for mice immunization we used CoA crosslinked to the Keyhole Limpet Hemocyanin (Sigma, USA) carrier protein according to the following protocol: 4.4 mg of KLH were dissolved in 0.6 ml of PBS, mixed with 0.2 mg of SPDP crosslinker (Pierce, USA) dissolved in 60 μl of DMSO and incubated at 37 °C for 1h. Then KLH was desalted on G25 column to remove unreacted SPDP and incubated with 5.6 mg of CoA in a total volume of 0.66 ml at 37 °C for 2 h followed by dialysis against 1000-fold volume PBS with two buffer changes. The prepared samples of KLH-S-S-CoA were aliquoted and stored at –20 °C.

As an antigen for the positive clones selection, we used CoA crosslinked to bovine serum albumin (Sigma, USA). Briefly, 3 mg of BSA were resolved in 100 μl of PBS, mixed with SPDP (0.36 mg) dissolved in 10 μl of DMSO and incubated at 37 °C for 1h. BSA was desalted on G25 column to remove unreacted SPDP and then incubated for 2h at 37 °C with 0.2 mg of CoA in a total volume of 110 μl. Unreacted CoA was removed by desalting on G25 column. Prepared samples of BSA-S-S-CoA were aliquoted and stored at –20 °C.

For generation of dephosphoCoA-S-S-BSA, dephosphoCoA was crosslinked to BSA using the protocol stated above.

Mice immunization. Female BALB/c mice (6–8 weeks old) were immunized with 20 μg of KLH-S-S-CoA in complete Freund's adjuvant (for the first injection) or in Freund's incomplete adjuvant (for booster injections) by intraperitoneal injections every two weeks. When the titer of anti-CoA antibody in the sera of immunized mice reached 10^{-4}, the production of hybridomas was performed.

Production of hybridomas. Generation of the hybridoma cells producing anti-CoA monoclonal antibodies has been performed using hybridoma technique according to a standard protocol [21] as described elsewhere [22].

ELISA screening of positive hybridoma clones. Ninety-six-well polystyrene plates were incubated with 0.3 μg/well of BSA-S-S-CoA in 100 μl of PBS (pH 7.4) for 2 h at 37 °C. The plates were then washed with PBS containing 0.1 % Tween-20 (PBS-T) (Sigma, USA) and incubated with 200 μl of 2 % bovine serum albumin in PBS (pH 7.4) for 1h at room temperature in order to block non-specific binding of antibodies. Subsequently, the plates were loaded with 100 μl aliquots of hybridoma media and were incubated for 1 h at 37 °C followed by three washes with PBS-T. The mouse polyclonal serum was used as positive control. After washing, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (1:8000 v/v, Jackson ImmunoResearch Laboratories, Pennsylvania, USA) were added to each well and incubated at 37 °C for 1 h. Plates were washed again three times, before 100 μl of substrate solution (0.02 % H_2O_2, 0.5 mg/ml 2.2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) sodium salt (ABTS, Sigma, USA), 0.1 M citrate-phosphate buffer (pH 5.8)) was added to each well. The absorbance at 450 nm was measured using a microplate reader after 15 min incubation at 37 °C.

Competitive ELISA. This analysis was performed according to the above protocol with minor modifications. The assay was carried out in 96 well plates, containing immobilized BSA-S-S-CoA (0.3 μg/well). The primary mAb A11 (10 μg in 100 ul PBS) was added together with CoA in different concentrations (5×10^{-1} mM, 5×10^{-2} mM, 5×10^{-3} mM, 5×10^{-4} mM, 5×10^{-5} mM, 5×10^{-6} mM) of CoA, dephospho-CoA, ATP or glutaryl-CoA. The mixture was incubated for 1 h at room temperature and the assay was developed as described above.

Western blot analysis. BSA-S-S-CoA (0.1 μg per well) was heat treated in the sample buffer with and without DTT, resolved by SDS/PAGE and electrotransferred to Immobilon-P membrane (Millipore, USA). The membrane was divided into strips and blocked with 0.5 % BSA in PBS for 1 h at room temperature followed by a single wash with PBS containing 0.1 % Tween 20. Strips were incubated with hybridoma media from selected clones for 2 h at room temperature. After three washes, a peroxidase-conjugated anti-mouse secondary antibody (1:8000 v/v, Jackson ImmunoResearch Laboratories, Pennsylvania, USA) was added to the strips and incubated for 1 h at room temperature. The strips were washed three times, and developed by ECL detection system (Amersham, Sweden).

Alternatively, the cell lysates were mixed with Laemmli Sample buffer with or without addition of dithiothreitol (DTT, 100 mM final) and heated at 99 °C for 5 min. Total proteins were separated by 12 % SDS-polyacrylamide gel electrophoresis (PAGE) and then wet-transferred to Immobilon-P membrane (Millipore, USA). Membrane was blocked with 5 % non-fat milk in PBS containing 0.1 % Tween 20. Protein bands were visualized using peroxidase-conjugated secondary antibodies (1:8000 v/v, Jackson ImmunoResearch Laboratories, Pennsylvania, USA) and developed by ECL detection system (Amersham, Sweden).

Generation and purification of MAbs from ascitic fluid. BALB/c mice were injected with 0.5 ml of Pristane and 7–10 days later inoculated with 2×10^6 of hybridoma cells (clone A11). The ascitic fluid was collected after 7–10 days. The fraction of immunoglobulins was precipitated from ascitic fluid with 50 % ammonium sulfate and used for affinity purification by Protein A Sepharose CL-4B (Amersham, Sweden) chromatography. The IgG fractions were pulled together and dialyzed in a PBS. The aliquots of purified antibodies were stored at –70 °C with 50 % glycerol.

Immunoprecipitation. Culture medium (1 ml) from A11 hybridoma clone was incubated with 25 µl of 50 % suspension of Protein A Sepharose CL-4B (Amersham, Sweden) at the rotation for 2 h at 4 °C. Then the beads were washed twice with PBS and incubated with 0.5 µg of BSA-S-S-CoA in PBS (500 µl) for 2 h at 4 °C. After incubation, the beads were washed three times with 1 ml of PBS containing 0.1 % Triton X-100. Immune complexes were removed from the beads by boiling in Laemmli sample buffer (with or without DTT), separated by SDS-PAGE and immunoblotted with culture medium from A11 hybridoma clone.

Mammalian cell culture. HEK293/Pank1b (Pank1b) cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza) supplemented with 10 % foetal bovine serum (FBS) (Hyclone), 50 U/ml penicillin and 0.25 µg/ml of streptomycin (Lonza). The cells were cultured in the atmosphere of 5 % carbon dioxide (CO_2) at 37 °C.

Treatment of cell lines with H_2O_2. Approximately 0.2 million of HEK293/Pank1b cells were seeded in 60 mm culture dishes and grown up for 24 h in complete DMEM with 10 % FBS. The medium was replaced with pyruvate-free DMEM supplemented with 5 mM glucose and 10 % FBS, and the cells were incubated for other 24 h with the following treatment by H_2O_2 (0.5mM) for 30 min at 37° C in the same media. After treatment, the cells were collected by pressure washing and centrifuged at 1,800 g for 5 min at RT. The medium was removed and cells were lysed in ice cold Lysis Buffer (PBS, pH 7.4, 5 mM EDTA, 1 % NP40, protein inhibitors cocktail (Thermo Scientific™), 25 mM N-Ethylmaleimide (Thermo Scientific™)). The lysates were centrifuged at 21,000 g for 5 min at 4° C and the supernatant was collected and analyzed by Western blotting.

Results and Discussion

Generation of monoclonal and polyclonal antibodies to hapten molecules is quite difficult because of their very low immunogenicity. To overcome this problem and generate specific anti-hapten antibodies it is a common practice to use for immunization the conjugates of haptents with carrier proteins that possess high immunogenicity. At the same time, it is believed that a linkage mode may affect antigenic properties of conjugated molecules. In our previous work, as the antigen for generation anti-CoA mAbs we used irreversible CoA conjugated via its thiol group to maleimide-activated KLH (KLH-S-CoA) [22]. The epitope of created anti-CoA mAbs (1F10) was mapped to deoxyribose ring of CoA with the 3'-phosphate as a key part of it. Such antibodies were very selective in the CoA-modified proteins recognition in numerous immunological assays.

In the presented work we aimed to generate anti-CoA mAbs specific to another CoA epitope and applied reversible CoA crosslinking to KLH by SPDP via CoA thiol group (Fig. 1 A) hoping that it will affect antigen presentation at immunization and as a consequence could be influenced on the specificity of anti-CoA antibodies pool. Consequently, KLH-S-S-CoA antigen was used for mice immunization and generation of hybridoma clones producing anti-CoA monoclonal antibodies using hybridoma techniques as described elsewhere [22].

To test the specificity of anti-CoA antibodies in ELISA, WB and immunoprecipitation assays, CoA was reversibly crosslinked to BSA with the use of SPDP as well (Fig. 1 B).

Following two rounds of ELISA screening and WB analysis only one positive hybridoma clone A11 was selected. According to the data presented in Fig. 2A, mAbs generated by A11 hybridoma clone showed strong recognition of BSA- conjugated CoA in WB that was dependent on the treatment with reducing agent DTT.

Next, we performed an immunoprecipitation assay of BSA-S-S-CoA with A11 mAbs. As a result, we show that A11 mAbs are capable to immunoprecipitate CoA-modified BSA and probably other CoA-modified proteins (Fig. 2 B).

To analyze the ability of A11 mAbs to detect endogenously CoAlated proteins we performed Western blot analysis of HEK293/

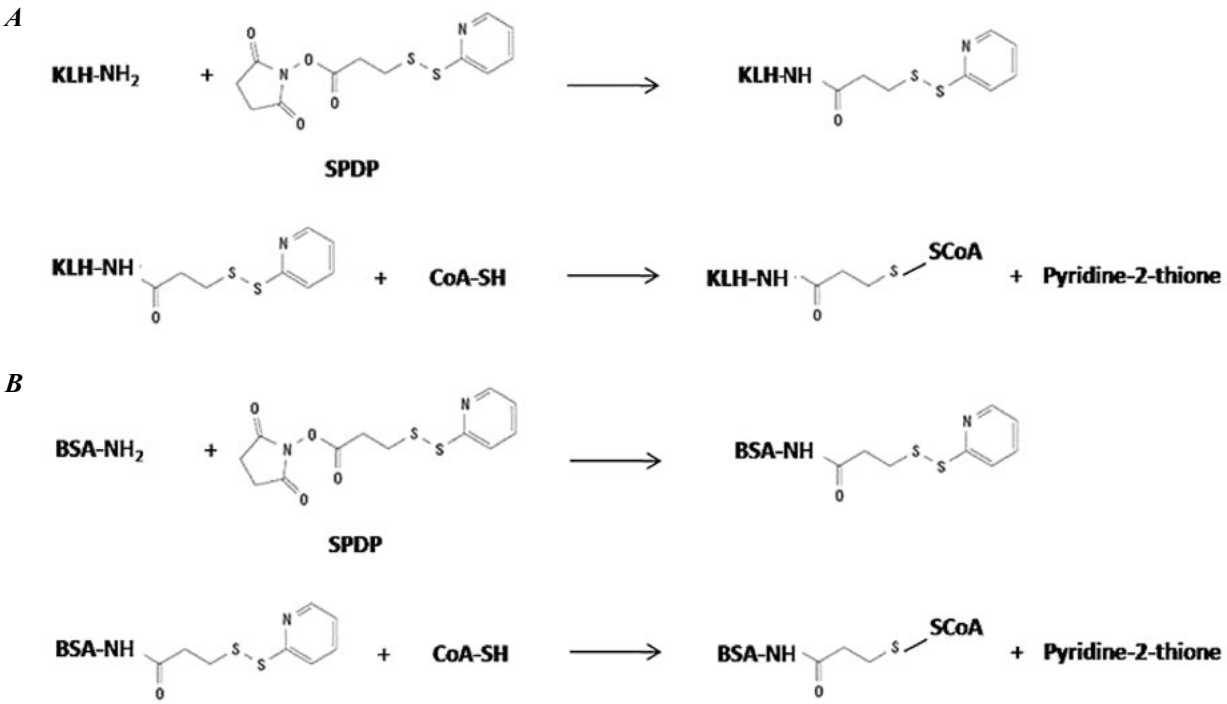

Fig. 1. Generation of antigens for mice immunization by crosslinking CoA with KLH (*A*) and for hybridoma screening by crosslinking CoA with BSA (*B*).

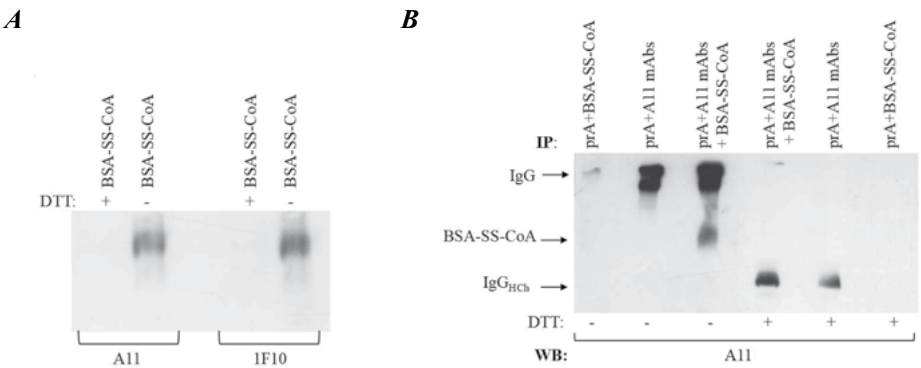

Fig. 2. Analysis of the specificity of anti-CoA mAb A11 towards BSA-S-S-CoA in WB analysis (*A*) or immunoprecipitation (*B*).

PANK1b cells under normal and oxidative stress conditions induced by H_2O_2 in the presence or absence of reducing agent DTT. It has been shown that both newly generated A11 and previously characterized 1F10 mAbs have very similar pattern of CoAlated proteins recognition in stressed HEK293/PANK1b cells lysates (Fig. 3).

In our previous studies we have demonstrated that antigenic epitope of 1F10 anti-CoA

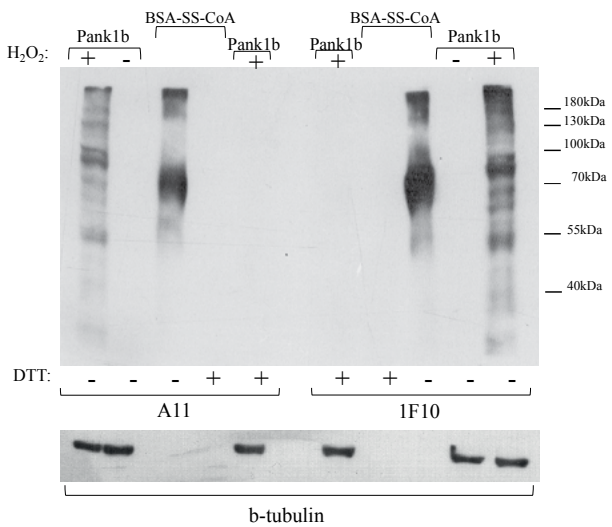

Fig. 3. Detection of endogenously CoAlated proteins using A11 and 1F10 anti-CoA mAbs. Western blot analysis of HEK293/PANK1b cell's lysates grown up at standard conditions or after the treatment with H_2O_2. Cell lysates were heat treated in sample buffer with or without addition of DTT. BSA-S-S-CoA was taken as a control.

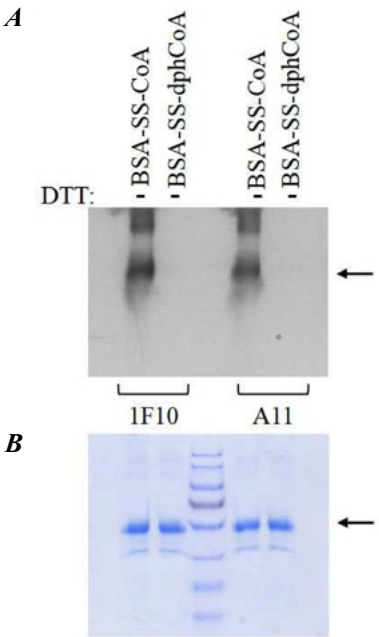

Fig. 4. WB analysis of the specificity of anti-CoA mAbs 1F10 and A11 towards BSA-S-S-CoA and BSA-S-S-dephosphoCoA (*A*). Coomassie stain of the gel (*B*). Both BSA-S-S-CoA and BSA-S-S-dephosphoCoA are indicated by arrows.

mAbs is located within CoA deoxyribose ring and is strongly dependent on its phosphorylation in 3' deoxyribose position. To verify the antigenic epitope of A11 mAbs we analysed their immunoreactivity toward dephosphorylated CoA. According to our data (Fig. 4) A11 mAbs similarly to 1F10 mAbs does not recognise dephosphoCoA conjugated to BSA suggesting that phosphate group in 3' position of CoA deoxyribose ring is crucial for antigenic epitope formation. In favour of this conclusion our data (Fig. 5) demonstrate that ATP, Cysteine and dephosphoCoA do not compete

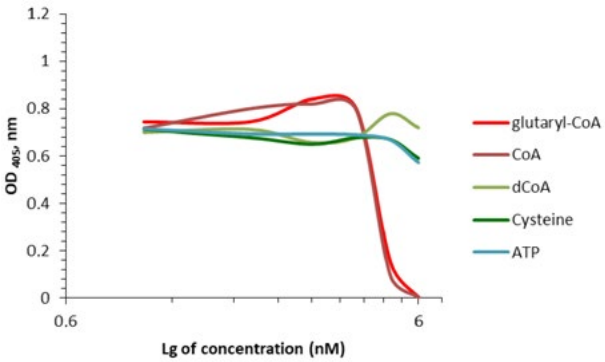

Fig. 5. Deprivation of anti-CoA mAbs A11 by ATP, cysteine, dephospho-CoA, CoA and CoA derivative glutaryl-CoA. Semi-logarithmic plot of competitive ELISA data

Table. **The comparative analysis of anti-CoA mAbs generated using different approaches for antigens preparation**

	1F10 mAbs	A11 mAbs
Type of carrier protein for immunization	KLH	KLH
Method of antigen preparation for immunization	CoA was conjugated to maleimide-activated KLH via thiol group (mKLH-S-CoA, irreversible conjugation)	CoA was crosslinked to the KLH via thiol group by SPDP crosslinker (KLH-S-S-CoA, reversible conjugation)
Type of carrier protein for hybridoma screening	BSA	BSA
Method of antigen preparation for hybridoma screening	CoA was crosslinked to the BSA by SPDP (BSA-S-S-CoA, reversible conjugation)	CoA was crosslinked to the BSA by SPDP (BSA-S-S-CoA, reversible conjugation)
Type of immunization	Intraperitoneal injections every two weeks	Intraperitoneal injections every two weeks
Immunogenicity	10^{-4}	10^{-4}
Hybridoma efficiency	High	medium
Antibody characteristics:		
ELISA	Specific recognition of BSA-S-S-CoA	Specific recognition of BSA-S-S-CoA
WB	Specific recognition of BSA-S-S-CoA and endogenously CoAlated proteins	Specific recognition of BSA-S-S-CoA and endogenously CoAlated proteins
IP	Immunoprecipitation of BSA-S-S-CoA	Immunoprecipitation of BSA-S-S-CoA
Epitope for mAbs	Antigenic epitope located within 3'-phosphate of CoA deoxyribose ring	Antigenic epitope located within 3'-phosphate of CoA deoxyribose ring

with BSA-S-S-CoA for the recognition by A11 mAbs in contrast to CoA and its derivative glutaryl-CoA. To sum up, in order to generate anti-CoA mAbs with different to earlier generated 1F10 mAbs epitope specificity we modified the protocol for antigen generation introducing reversible instead of previously irreversible crosslinking CoA with KLH using SPDP crosslinker that may provide different access to CoA moieties during immune response formation after immunization. We assumed that different antigen processing could open access to the additional antigenic epitopes of CoA if such exist. The efficiency of the optimized KLH-S-S-CoA antigen in eliciting CoA-specific antibodies was further investigated in various immunoassays: ELISA, WB, IP. According to the analysis of anti-CoA mAbs (1F10 and A11) generated using different approaches of antigen preparation we can conclude that CoA conjugation to maleimide-activated KLH as well as CoA crosslinking to the KLH by SPDP, can be used for high specific monoclonal antibodies generation, but with the similar specificity due to the same antigenic epitope recognition (Table). We assume that CoA most probably has only one antigenic epitope and the 3'-phosphate of the deoxyribose ring might be a key molecule in epitope formation for both types of mAbs. This knowledge would be very useful for further studies of protein CoAlation using both types of mAbs.

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Моноклональні антитіла до Коензиму А.

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Мета. Отримати моноклональні антитіла до специфічного антигенного епітопу Коензиму А (КоА), які б відрізнялися від попередньо розроблених анти-КоА мкАт (1F10), шляхом впровадження альтернативного підходу для створення антигену для імунізації тварин. **Методи.** Зоротнє крослінкування КоА з гемоціаніном молюска (Keyhole Limpet Hemocyanin, KLH) і з бичачим сироватковим альбуміном (Bovine serum albumin, BSA) за допомогою хімічного крослінкера SPDP (N-Succinimidyl 3-(2-pyridyldithio) propionate). Гібридомна технологія. Вестерн-блот аналіз. Імунопреципітація. ІФА. **Результати.** Було отримано моноклональні антитіла (А11), які специфічно розпізнають КоА в різних імунологічних аналізах і мають таку саму специфічність антигенного епітопу, як і у раніше отриманих анти-КоА мкАт (1F10). **Висновки.** Використання різних способів кон'югації КоА з білками-носіями для створення антигену для імунізації не впливає на специфічність антигенного епітопу отриманих анти-КоА антитіл, швидше за все тому, що молекула КоА має лише один можливий імуногенний епітоп.

Ключові слова: КоА, гібридомна технологія, моноклональні антитіла, крослінкування SPDP.

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