

DETECTION OF UNUSUAL HIGH MOLECULAR FORM OF ALBUMIN IN BLOOD SERUM OF COVID-19 PATIENTS

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Blood sera of 12 severe Covid-19 patients and 14 healthy human donors were subjected to original TCA-extraction/acetone-precipitation followed by SDS-PAAG electrophoresis and mass-spectrometry. 76 kDa protein was detected as one of the differentially expressed proteins in the samples of Covid-19 patients. This 76 kDa protein was identified with mass-spectrometry as human serum albumin. Such molecular form of albumin was absent in blood serum of healthy human donors. The potential ways of generation of the unusual form of human serum albumin and its probable diagnostic value were discussed.

Key words: Covid-19, biomarker proteins, unusual form of human serum albumin, TCA-extraction, acetone-precipitation, electrophoresis, mass-spectrometry.

Human serum albumin (HSA) belongs to the most abundant protein in human body. Liver is the main organ which produces HSA to the amount of approx. 35-50 g per liter of blood serum. HSA participates in transportation of hormones, fatty acids, as well as other biologically active compounds. It also allows buffering physiological pH and maintaining the oncotic pressure in blood serum. Besides, the detoxification function of HSA in the organism is considered, since albumin provides >50% of the antioxidant activity of normal plasma [1]. Due to this activity, albumin can scavenge various reactive oxygen species and nitrogen oxygen species. In addition, albumin can bind unconjugated bilirubin whose concentration is related to patients' mortality observed in many diseases accompanied by a decline in serum albumin [1].

Post-translational modifications (PTMs) of specific proteins are important indicators used to diagnose diseases of certain types, as well to assess a response to therapy, for example, the quantification of glycated hemoglobin and glycoalbumin for the diagnosis and efficiency of treatment of diabetes [2]. Other reported PTMs of HSA related to diseases

of certain types are truncation, dimerization, carbamylation and oxidation [2].

In our recent review, 123 O-phosphorylation, glycation, methylation, carbonylation, and acetylation PTMs of albumin were described.

Here, in a search for novel biomarkers in blood serum of Covid-19 patients, we unexpectedly detected earlier unknown high molecular (76 kDa) form of HSA in a part of those patients. For its isolation, an original TCA-extraction/acetone-precipitation technique which was earlier proposed by us for isolation of new biomarkers of autoimmune [3] was applied. The identification of this protein was carried out using SDS-PAAG electrophoresis and mass spectrometry analysis. Potential ways of generation of this new form of HSA are considered.

Materials and Methods

Patients diagnosed with Covid-19 were on stationary treatment at Regional Infection Clinical Hospital of Lviv. The BioEthics Committee of Danylo Halytsky Lviv National Medical University approved the procedures carried out at scientific investigations at the Hospital. For verification of Covid-19

diagnosis, samples of blood serum were tested in clinical diagnostic laboratory with Vitrotest® SARS-CoV-2 IgG and Vitrotest® SARS-CoV-2 IgM Kits (Vitrotest®, Ukraine). Other clinical indicators of patients under study were also taken into consideration.

Peripheral blood samples. Venous blood was obtained from 12 Covid-19 patients who were hospitalized because of this disease and 14 human individuals without clinical manifestations of Covid-19. Serum was obtained after blood coagulation for 30 min at 23°C, followed by centrifugation for 10 min at 5,000 g.

Isolation of proteins for electrophoretic study. In this study, an original TCA-extraction acetone-precipitation procedure that was proposed by us [3] for detection and isolation of new biomarkers of autoimmune diseases, was applied. Briefly, 200 µl of blood serum sample was diluted 2-fold with phosphate-buffered saline (PBS), then 100% 2,2,2-trichloroacetic acid (TCA) was added to 10% final concentration. After 30 min incubation on ice, samples were centrifuged for 15 min at 10,000 g. 200 µl of supernatant containing TCA-soluble compounds was transferred to a fresh Eppendorf tube (1.5 ml), and cold (-20°C) acetone was added to a final volume of 1.5 ml. The obtained mixture was kept for 18 h at -20°C and the precipitate was pelleted by centrifugation for 30 min at 10,000 g. Proteins of human peripheral blood were extracted with lysis buffer (20 mM Tris-HCl, pH 8.0, 1% Triton-X100, 150 mM NaCl, 50 mM NaF, 0.1% SDS) containing 1 mM phenylmethanesulfonyl fluoride (PMSF, Roche, Basel, Switzerland) and 10 µg/ml of protease inhibitors cocktail "Complete" (Roche, Basel, Switzerland), solution was boiled (100°C, 2 min), and samples were stored at -20°C until use but no longer than 2 weeks.

Electrophoresis. The obtained protein samples were subjected to SDS-polyacrylamide gel (SDS-PAAG) electrophoresis in 12% PAAG [4], followed by protein staining in gel with Coomassie brilliant blue G-250 (Sigma-Aldrich, USA). The individual electrophoretic protein bands were excised from the gel and subjected to in-gel digestion with trypsin (Promega, USA), followed the mass spectrometry.

Mass-spectrometry. Protein samples digested in gel with trypsin were subjected to matrix-assisted laser-desorption ionization (MALDI) mass spectrometry, as described earlier [5]. MALDI TOF mass spectrometry was performed on the Ultraflextreme

instrument (Bruker, Germany). Peptide mass fingerprinting with collected mass spectra was performed with use of Mascot tool. Significance of identification was set to $P < 0.05$, and corresponded to identifications with scores higher than 56. Database used in searches was SwissProt 2021_01, and taxonomy was set to "human".

Statistical analysis. The Analysis of Variance (ANOVA) was used as a statistical test for the comparison of the experimental groups. Two-way ANOVA with Bonferroni posttests was applied in order to compare replicated means by rows using GraphPad Prismv6.0 software.

BioEthics approval. Blood samples of Covid-19 patients were collected under the approval of the Bio-Ethics Review Board at the Danylo Halytsky Lviv National Medical University in accordance with the recommendations of the Ministry of Health of Ukraine and statements of National Bioethics Advisory Commission Research involving human biological materials: issues and policy guidance (1999), available at <www.bioethics.georgetown.edu/nbac>. Obtaining blood samples from healthy human volunteers was approved by the Bio-Ethics Commission at the Institute of Cell Biology, NAS of Ukraine, (Protocol No 4 dated by April 7, 2021).

Results and Discussion

We detected in blood serum of 5 of 12 Covid-19 patients under investigation a protein of 76 kDa molecular mass (Fig. 1, B), that was identified with mass-spectrometry as human albumin (Fig. 2, Table), while it is known that the processed HSA has a molecular mass of 66.5 kDa (Fig. 1, A) [6]. We suggest that individual peculiarities in the PTMs existed in Covid-16 patients, however, an approval of this suggestion requires conducting additional studies on a broader cohort of those patients. At the same time, none of 14 healthy human donors contained 76 kDa protein in blood serum (Fig. 1, B). That conclusion was confirmed by the results of quantitative analysis of the expression of the 76 kDa protein during densitometry of the electrophoregrams of Covid-19 patients and healthy donors (Fig. 1, C).

Two potent mechanisms of generation of this high molecular weight form of the HSA might be proposed: 1) PTMs of HSA molecule via its complexation with various compounds that appear in blood of Covid-19 patients; 2) abnormal synthesis and processing of the albumin molecule. We consider PTMs as most probable way of generating of

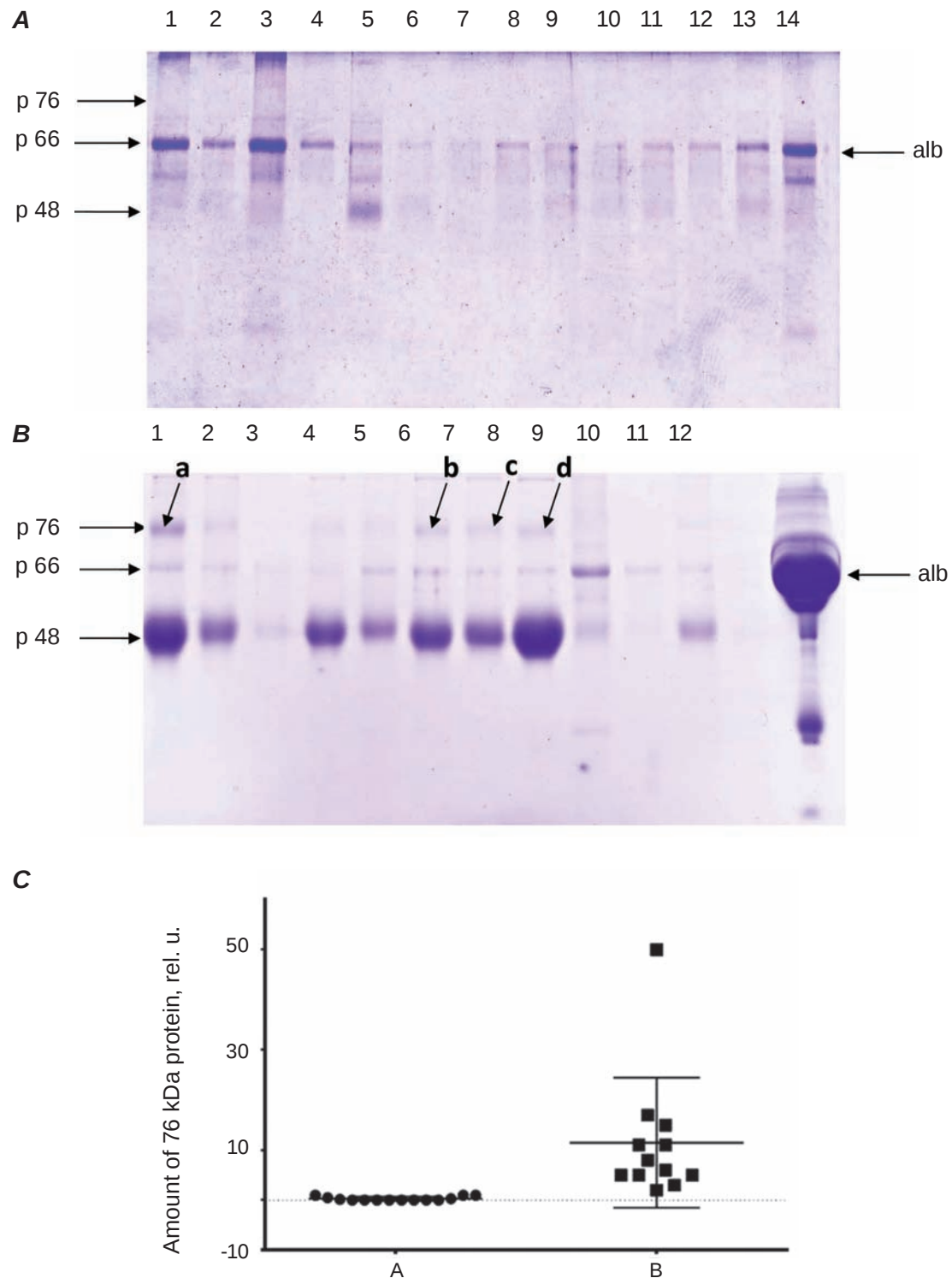


Fig. 1. Electrophoretic (SDS-electrophoresis in 12% PAAG) patterns (**A**, **B**) and results of quantitative densitometric evaluation (**C**) of proteins detected in TCA-extract of blood serum of Covid-19 patients (**A**) and healthy human donors (**B**). **A**: Lanes 1-14, blood serum samples of clinically healthy human donors. **B**: Lanes 1-12, blood serum samples of Covid-19 patients. **C**: Results of densitometric analysis of the amount of 76 kDa protein detected in blood serum of the Covid-19 patients compared with its amount in samples of clinically healthy human donors. **p48** – truncated form of the unconventional Myosin 1C (Kit et al., 2022, in press); **p66** – human serum albumin; **p76** – protein detected in blood serum of the Covid-19 patient

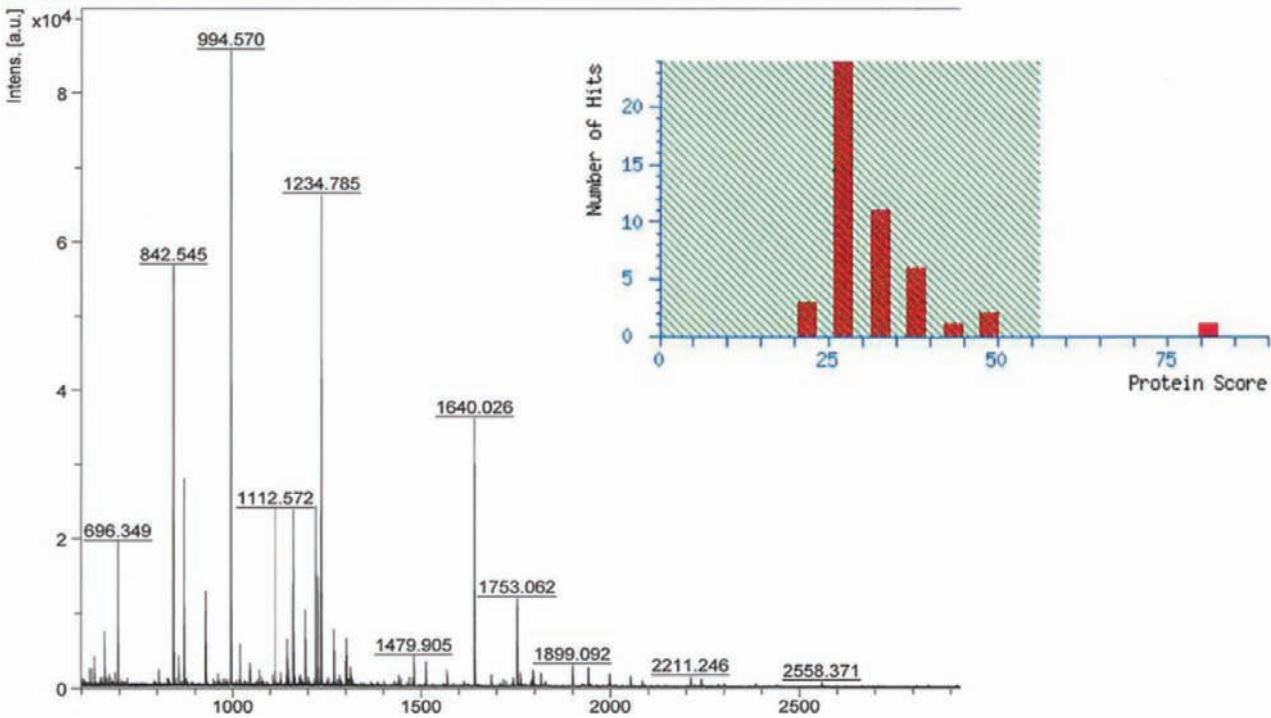


Fig. 2. Results of MALDI TOF MS of 76 kDa protein as human serum albumin

high molecular weight form of HSA. SUMOylation of albumin could add 10-12 kDa, and would increase the molecular mass of HSA to 76-78 kDa which corresponds to a size of HSA detected in the serum of Covid-19 patients. Tryptic digestion of SUMOylated albumin would generate branched peptides with 27 to 32 amino acids of SUMO attached to a lysine of albumin. The molecular mass of such a peptide would be higher than 3,000 Da, and would generate ions of masses beyond detection in the used MALDI TOF MS. Verification of PTMs and mutation of HSA as a reason for generation of the high molecular weight form of HSA requires conducting of additional experiments.

Values of score, expectation and number of matched peptides were indicated, as they were retrieved by searches with Mascot tool. Note that significance of identification is set at $P < 0.05$, that corresponds a score of 56. Identifications show scores above 76.

In a recent study, proteomes of Covid-19 patients and healthy donors were compared [7]. Global analysis of PTMs demonstrated a marked up- and down-regulation in such PTMs as phosphorylation, glycosylation, citrullination, and arginylation in Covid-19 patients compared to healthy donors. Thus,

Table. Identification of 76 kDa protein as human serum albumin

No	Protein code	Mass	Score	Expect	Matches
a	Albu Human	71317	81	0.00015	18
b	Albu Human	71317	76	0.00055	19
c	Albu Human	71317	89	2.5e-05	19
d	Albu Human	71317	80	0.00019	19

a question appears - why in that study no changes were detected in the PTMs of serum albumin? We consider that was because of the experimental protocol used by the investigators, who purposely depleted blood plasma samples of albumin and IgG using that special Albumin & IgG depletion Spin-Trap columns (GE healthcare). In such a way, they tried to avoid possible “contaminations” of these proteins that quantitatively dominate in blood plasma.

It was shown that the PTMs of HSA affected the biological activity of this protein, specifically

through oxidation of Cys34, glycation of Lys525, and C-terminal truncation of Leu585 that decreased interaction of HSA with the Fc receptor [8].

In a recent study, we identified with mass spectrometry in the HSA 61 new PTMs including phosphorylation, glycosylation, nitrosylation, deamidation, methylation, acetylation, palmitoylation, geranylation, and farnesylation. Three-dimensional modeling of albumin with selected PTMs was conducted. Specific PTMs were located in the regions involved in the interactions of the albumin with various medicines, metal cations, and fatty acids [9].

The SDS-PAAG electrophoresis demonstrated that the albumin produced by canavanine-treated Hep-G2 cells had a larger (by 4 kDa) molecular weight compared with serum albumin. These cells secreted 79% of proalbumin, 21% of processed albumin, and no preproalbumin, while the untreated Hep-G2 cells secreted 93% of fully processed serum albumin and only 7% of proalbumin [10].

Albumin Redhill was found in English family with a gene mutated at the N-terminus. Due to an aberrant amino-acid sequence, it cannot be processed into normal protein, thus, being 2.5 kDa bigger than native albumin [11].

Chicken preproalbumin identified taking into account the results of cloning of a double-stranded cDNA and immunoprecipitation of synthesized protein had a molecular mass of 72 kDa [12].

Thus, the presented data allow suggesting that the 76 kDa polypeptide revealed by us in blood serum of Covid-10 patients can be neither the preproalbumin nor proalbumin, since both of them are smaller in their size.

The oligomerization of HSA was described [13], however, the minimum molecular mass of the albumin dimer should be 133 kDa.

Since most of the described PTMs add to the main polypeptide approx. 200–300 Da per a single active group, it is possible to add 10,000 Da only with the help of bigger adduct, such as another protein or peptide (e.g., SUMOylation), or polymeric PTMs, such as poly-ADP/PARYlation or N-glycosylation.

Conclusion. Blood sera of 12 patients with severe form of Covid-19 were subjected to unique TCA-extraction/acetone-precipitation treatment combined with electrophoresis in SDS-PAAG electrophoresis. Further application of mass-spectrometry permitted us to identify in 5 of 12 patients the

unusual high molecular weight (76 kDa) form of HSA which could be a novel protein biomarker in Covid-19 patients. This protein was not detected in blood serum of any of 14 healthy human donors. The potent ways of generation and potential role of the unusual high molecular weight HSA in Covid-19 patients are discussed.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

ВИЯВЛЕННЯ НЕТИПОВОЇ ВИСОКОМОЛЕКУЛЯРНОЇ ФОРМИ АЛЬБУМІНУ У СІРОВАТЦІ КРОВІ ХВОРИХ НА COVID-19 ПАЦІЄНТІВ

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Зразки сироватки крові 12 важких пацієнтів з Covid-19 та 14 здорових донорів піддавали ТХО-екстракції/ацетон-преципітації з наступним SDS-PAAG-електрофорезом та мас-спектрометрією. У зразках крові пацієнтів із Covid-19 було виявлено один із диференціально експресованих протеїнів з молекулярною масою 76 кДа. Цей протеїн було ідентифіковано за допомогою мас-спектрометрії як сироватковий альбумін людини. Така молекулярна форма альбуміну була відсутня у сироватці крові здорових донорів. Обговорено можливі шляхи утворення нетипової форми сироваткового альбуміну людини та його ймовірне діагностичне значення.

Ключові слова: Covid-19, протеїни-біомаркери, нетипова форма сироваткового альбуміну людини, ТХО-екстракція, преципітація ацетоном, електрофорез, мас-спектрометрія.

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