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ANTICOAGULANT PROPERTIES OF RECOMBINANT ANNEXIN A5

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Animal genes, with fewer occurrences in plants and fungi [1], primarily encode Annexins, soluble proteins found widely across eukaryotic organisms. Among these, annexin A5 is noteworthy, as it belongs to the family of Ca^{2+}-dependent proteins that bind phosphatidylserine (PS), playing vital roles in trophoblast and vascular endothelium [2, 3].

Due to its involvement in membrane characteristics, annexin A5 significantly impacts membrane-dependent processes within hemostasis, including the initiation of the clotting cascade, activation of factor X and prothrombin, and protein C function on membrane surfaces. By binding to phosphatidylserine, annexin A5 serves as a protective shield, concealing exposed phospholipid surfaces and exerting a general anticoagulant effect [4]. Disruptions in these interactions, particularly during autoimmune processes leading to the formation of autoantibodies against annexin, can predispose individuals to thrombosis [5–7]. Furthermore, annexin A5 serves as a valuable marker for apoptosis and platelet activation [8].

Aim. Considering the significant influence of annexin A5 on the activation of hemostatic compounds on cell surfaces, it becomes crucial to delve deeper into its mechanisms. Therefore, the objective of our study was twofold: first, to produce recombinant annexin A5, and second, to explore its impact on platelet aggregation and blood plasma clotting. Through this investigation, we aim to shed light on the intricate interplay between annexin A5 and the hemostatic system, potentially uncovering novel insights into thrombosis and hemostasis regulation.

Materials and Methods. *Accumulation of cell biomass for the production of recombinant annexin A5. E. coli* strain Rosetta *(DE3)* p*ET*28a-EGFP-Annexin A5 was poured into 50 mL tube with 20 ml of lysogeny broth (LB) medium and the kanamycin were added to a concentration of 50 μg/ml of medium. The culture was allowed to grow overnight under active aeration and at 37 °C. Then 5 ml of the overnight culture was taken and 200 mL fresh LB medium was mixed and kanamycin to a final concentration of 50 μg/ml of medium was added. It was cultured at 37 °C under active aeration to an optical density (OD) of 0.3–0.5 at 600 nm. After reaching this optical density, temperature was lowered to 30 °C, an inducer of recombinant protein expression IPTG (Isopropyl β- d-1-thiogalactopyranoside) with a final concentration of 1 mM was added and culture flasks were rotated for 3 hours with active aeration. After that, the medium was centrifuged for 20 minutes at 3600 rpm. The supernatant was decanted, and the cells' precipitate was stored at –80 °C. A green precipitate was obtained, which at first glance indicates a successful protein expression.

Purification of polyHis-Tag proteins by immobilized metal affinity chromatography. The column with nickel-agarose was deconditioned by successive washing with 5-10 column volumes of ethanol, distilled water, 0.5 NaOH, and distilled water. Then it was equilibrated with 15–20 volumes of wash buffer (50 mM Na_2HPO_4, 0.3 M NaCl, 10 mM imidazole, pH 8.0) with 6 M urea. The precipitate of producer bacteria from 50 ml of bacterial suspension was dissolved in 1 ml of wash buffer. The mixture was sonicated 3 times for 20 seconds each using an ultrasonic disintegrator, and the microtubes were kept on ice. The mixture was centrifuged for 20 min at 13400 rpm. The supernatant was applied to a nickel-agarose column. It was washed with 5–10 volumes of wash buffer (50 mM Na_2HPO_4, 0.3 M NaCl, 10 mM imidazole, 6 M urea), after that concentration of urea was gradually

reduced with 1 M difference among solutions). Protein was eluted with wash buffer (50 mM Na_2HPO_4, 0.3 M NaCl, 250 mM imidazole).

SDS-PAGE of recombinant annexin A5. To samples were added sample buffer (4% SDS, 6 M urea, 20% glycerol, 5% 2-mercapto-ethanol and Bromphenol blue) with further running in 10% SDS-PAGE. Bands were visualized by Coomasie Brilliant Blue G-250. Concentration was measured by densitometry with help program software TotalLab Quant.

Platelet rich blood plasma (PRP) was prepared from human citrated blood by centrifugation at 1000 rpm during 30 min. Platelet poor blood plasma as obtained by spinning-down PRP at 1500 rpm during 30 min. Blood plasma was prepared from human citrated blood by centrifugation at 1200 rpm during 30 min.

Activated partial thromboplastin time (APTT) was performed according to the following procedure. 0.1 ml of blood plasma was mixed with equal volume of APTT-reagent (Siemens, Germany) and annexin A5 in final concentrations 10 and 40 ug/ml and incubated during 3 minutes at 37 °C. Then the coagulation was initiated by adding of 0.1 ml of 0.025 M solution of $CaCl_2$ (Siemens, Germany). Clotting time was monitored by the Coagulometer Solar CGL-2410 (Solar Technical Service, Kharkiv, Ukraine).

Prothrombin time (PT) was performed by adding of 0.1 ml of thromboplastin to the mixture of blood plasma (0.1 ml) with equal volume of 0.025 M solution $CaCl_2$ and annexin A5 in final concentrations 10 and 40 ug/ml. Results were presented as the ratio of blood plasma clotting time of experimental animal to the control clotting time. Clotting time was monitored by the Coagulometer Solar CGL-2410 (Solar Technical Service, Kharkiv, Ukraine)).

Platelet aggregation measurements were based on changes in the turbidity of platelet-rich plasma. Aggregation was registered for 10 min using Aggregometer Solar AP2110 (Solar Technical Service, Kharkiv, Ukraine)). We estimated the initial rate and final level of aggregation at 37 °C. In typical experiment 250 µl of PRP was activated by ristocetin with final concentration 1.2 mg/ml and added annexin A5 in final concentration 40 µg/ml.

Statistical analysis. Statistical analysis of the data was performed using Microsoft Excel. All analyzes were performed in a series of three replicates. Statistical significance at $P < 0.05$ was tested by paired t-test.

Results and Discussion. *Analysis of recombinant annexin A5 production and purification.* To analyze the purity of annexin A5 at different stages of purification, we tested lysate, eluted samples, and dialysis samples. In Fig. 1 we can observe presence of annexin A5 in the *E. coli* lysate. Affinity chromatography significantly purified the protein from impurities, but not completely.

The effect of annexin on blood clotting time. As shown in Fig. 2, recombinant annexin A5 prolonged time of blood clotting induced by APTT-reagent.

Annexin A5 also slightly prolonged blood clotting time induced be thromboplastin in PT test but not significantly.

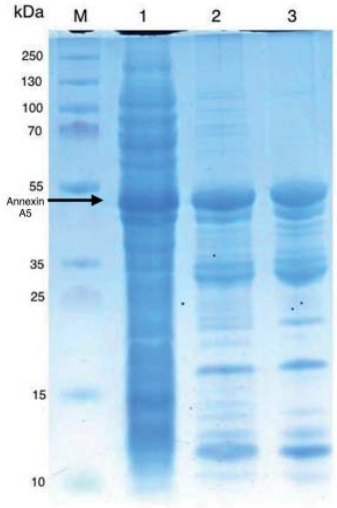

Fig. 1. **SDS–PAGE of samples taken at various stages of the process of bacterial expression and purification of recombinant annexin A5**

M — markers of molecular weight; 1 — indissoluble fraction of lysate before application to the column; 2 — eluted fraction; 3 — eluted fraction after dialysis

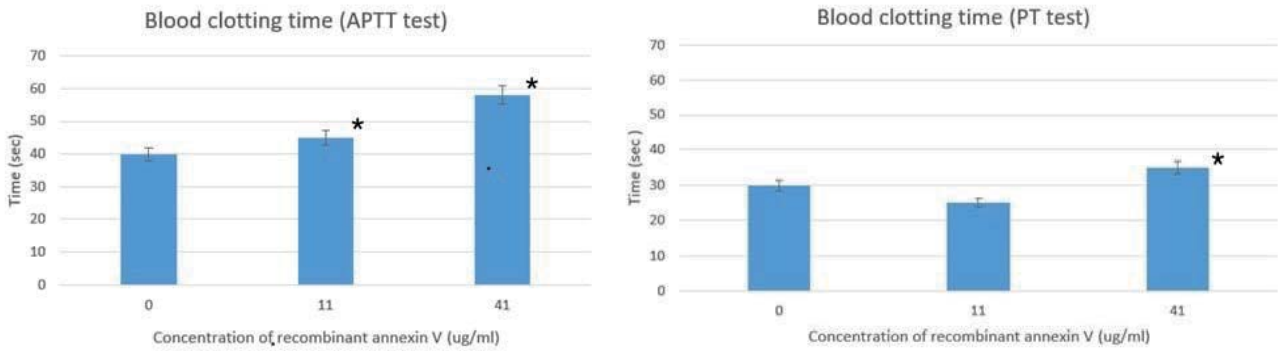

Fig. 2. **Effect of recombinant annexin A5 on the time of clotting of blood plasma activated by APTT-reagent (left). Effect of recombinant annexin A5 on the time of clotting of blood plasma activated by thromboplastin in the PT test (right)**
* — difference statistically significant

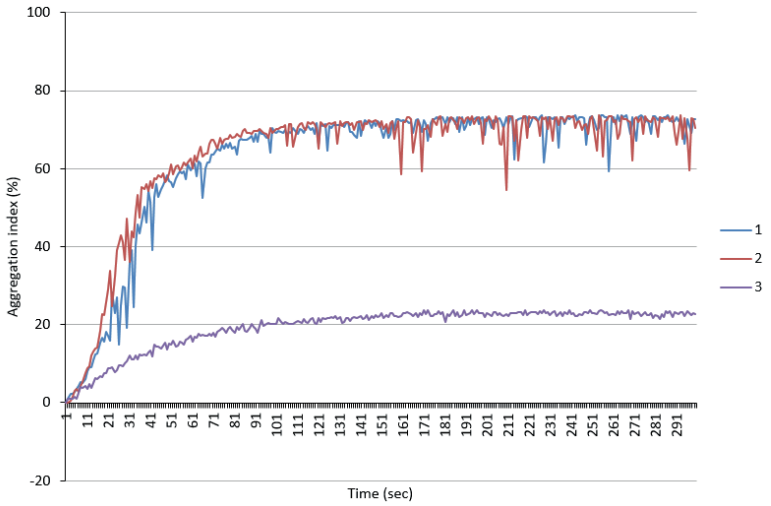

Fig. 3. **Effect of recombinant annexin A5 on the platelets aggregation:**
1 — control platelet aggregation induced by ristocetin; *2* — platelet aggregation induced by ristocetin and annexin A5 in final concentration 40 ug/ml; *3* — disintegrin from the venom of Echis multisquamatus

The effect of annexin A5 on platelet aggregation. Speed or rate of platelet aggregation with annexin A5 didn`t differ from control assays (Fig. 3).

Conclusions. Our EGFP-conjugated annexin A5 prolonged blood clotting time in the APTT test, so it had the biological effect of natural annexin, but did not inhibit platelet activation and aggregation. It can be assumed that its anticoagulant effect is directed to the enzyme complexes of the hemostasis system and does not extend to platelet receptors. The obtained protein can be used to study the activation of hemostatic components on cell surfaces.

Key words: annexin A5, blood coagulation, platelet aggregation, phosphatidylserine, platelets.

Authors' contribution. DS performed cell cultivation, protein purification, SDS-PAGE, blood clotting time by APPT test and blood clotting by PT test; AO performed platelet aggregation; AA maintained the study and orchestrated the work on it.

Conflict of interest. Authors declare no competing interests.

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