Bulatovski A.B.
Master of biology, junior researcher,
Institute of Microbiology, National Academy of Sciences of Belarus
Zinchenko A.I.
Dr. Sci, Professor, head of laboratory,
Institute of Microbiology, National Academy of Sciences of Belarus

PRODUCTION OF HUMAN ANNEXIN A5 FUSED WITH BACTERIAL ADENOSINE CATABOLIC ENZYMES

Булатовский А.Б.,
магистр биологических наук, младший научны сотрудник,
Институт микробиологии Национальной академии наук Беларуси
Зинченко А.И.,
доктор биологических наук, профессор, заведующий лабораторией,
Институт микробиологии Национальной академии наук Беларуси

ПОЛУЧЕНИЕ ЧЕЛОВЕЧЕСКОГО АННЕКСИНА А5, СЛИТОГО С ФЕРМЕНТАМИ КАТАБОЛИЗМА АДЕНОЗИНА

Summary. Submerged culture of recombinant *Escherichia coli* strains produced chimeric proteins «Annexin-ADase» and «Annexin-PNPase» composed of human annexin A5, fused with homologous adenosine deaminase and purine nucleoside phosphorylase of *E. coli*, respectively. Affinity chromatographic isolation of these proteins from ultrasonic cell lysates on Ni^{2+}-NTA-agarose yielded preparations of 95% purity grade (according to the results of polyacrylamide gel electrophoresis). The chimeric proteins «Annexin-ADase» and «Annexin-PNPase» have been shown to transform adenosine into adenine and inosine, respectively. Such properties of the target proteins, judging by literature data, allow to consider them as promising agents for use in cancer biotherapy.

Keywords: fusion protein, human annexin A5, adenosine deaminase, purine nucleoside phosphorylase, Escherichia coli.

Introduction. Cancer is one of the major lethal pathologies around the world. It accounts for heavier human mortality rate than overall death toll of malaria, TB and AIDS [1–3]. Solid tumors affecting lungs, mammary gland, rectum, prostate are referred to four most frequently diagnosed varieties of malignant diseases. Lung cancer takes the irrefutable lead both in incidence and fatality in the morbid tetrad. Global neoplastic advance is rapidly progressing. By 2030 new cancer cases are expected to exceed 21.7 million figure and 13 million deaths will be provoked by ageing and demographic boom [4, 5].

In Belarus, similar to other European countries, malignant tumors are the second worst plague of local population, leaving the notorious championship only to cardiovascular diseases [6]. Unfortunately, conventional oncotheraphy methods, like surgery, irradiation, chemical treatment have failed so far to cope with cancer challenge [7, 8]. At the same time rare events of spontaneous tumor regression and remarkable achievements of medical enthusiasts (dated back to the past century) who succeeded to cure some types of cancer with bacterial lysates [9–11], provide evidence that human immune system is capable to eliminate both the recurrent singular aberrant cells and massive tumors at the late stages of oncogenesis. As a rule, it does not happen in everyday practice due to the fact that each solid tumor is surrounded by special microenvironment inactivating anticancer immune cells [12, 13].

Ribonucleoside adenosine distinguished by 1000-fold elevated extracellular concentration in tumor microenvironment is known to act as one of key inhibitors of cancerostatic immune activity [14].

In the previous study [15] we proposed the idea to eliminate inhibitory effect of adenosine on antitumor immune response of oncopatients using adenosine-degrading enzymes, like adenosine deaminase (ADase) or purine nucleoside phosphorylase (PNPase). It was suggested to deliver the above-mentioned enzymes precisely into the tumor with the aid of proteins recognizing the target cancer cells. The role of such protein, for instance, may be played by annexin A5 expressed in human placenta. It is selectively linked with phosphatidyl serine – lipid compound arising in huge amounts on the surface of most cells subjected to neoplastic degeneration [16, 17]. Noteworthy, that the latest reports have indicated the inherent antitumor potential of annexin A5 [18].

Getting down to the experimental testing of the afore-postulated assumption, we were guided by the approach described by J. Krais et al. [19] who engineered the recombinant strain of E. coli capable to produce annexin A5 fused with homologous PNPase for further use of this chimeric protein as the element of the so-called suicidal gene therapy of cancer envisaging specific binding with tumor cells.

Our previous research efforts resulted in *E. coli* strains producing annexin A5 fused with homologous ADase [20] and PNPase [21], designated as «Annexin-ADase» and «Annexin-PNPase», respectively.

The present investigation is aimed at synthesis and characterization of recombinant chimeric proteins «Annexin-ADase» and «Annexin-PNPase».

Materials and methods. Strain *E. coli* BIM B-1223G – a source of protein «Annexin-ADase» and strain *E. coli* BIM B-1411D – a source of protein «Annexin-PNPase» deposited at Belarussian collection of non-pathogenic microorganisms served as objects of study.

Bacterial strains were cultured in Luria-Bertany liquid nutrient medium [22] at 37°C to reach OD value 0.6 (λ=600 nm), then enzyme synthesis was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) (0.2 mM) into the medium, and the cultivation continued for 4 h. Upon sedimentation of bacterial cells by centrifugation the pellet was treated to recover chimeric proteins. Cell biomass was resuspended in buffer containing 300 mM NaCl, 10 mM imidazole, 50 mM NaH_2PO_4 (pH 8.0). Ultrasonic cell disintegration was conducted at Sonifier-450 unit (Branson, USA). Cell lysate was clarified by centrifuging and supernatant was applied on chromatographic column with Ni^{2+}-NTA-agarose (Qiagen, USA).

Chimeric proteins were eluted with the buffer comprising 300 mM NaCl, 50 mM NaH_2PO_4 and 500 mM imidazole (pH 8.0). Fractions containing the target chimeric proteins were pooled, dialyzed against 400-fold volume of 50 mM Tris-HCl-buffer (pH 8.0), and their enzymatic activities were assayed.

ADase and PNPase activities of chimeric proteins were determined via the rate of adenosine transformation into inosine and adenine, respectively. Reaction mixture containing 40 mM adenosine, 50 mM Tris-HCl-buffer (pH 8.0) and 0.005% enzyme was incubated at 37 °C. The process was controlled using thin-layer chromatography (TLC) on Silica gel 60 F_{254} plates (Merck, Germany) in the solvent system: isopropanol-chloroform-25 % aqueous ammonia (10:10:1 volume ratio). The substances were detected on the plates in UV-light. They were eluted from the plates with 5 ml aliquots of 10 mM potassium phosphate buffer (pH 7.0). Concentrations of eluted substances were measured spectrophotometrically employing the known coefficient of molar extinction. The absorbance spectra of eluates were recorded at spectrophotometer PB2201A (Solare, Belarus). The amount of enzyme sufficient to convert adenosine into inosine or adenine at the rate 1 μmol/min was assumed as one unit of ADase and PNPase activity.

Electrophoretic analysis of proteins was performed by SDS-polyacrylamide gel electrophoresis [23].

Results and discussion. The applied flow sheet of producing chimeric proteins (fig. 1) embraced such principal stages as engineering of strains-producers of target proteins, microbial culture and accumulation of biomass, ultrasonic cell disintegration and finally recovery of end products from cell lysate and subsequent purification.

Since the engaged *E. coli* strains produce chimeric proteins carrying at C-terminus the additional Ni-affine octahistidine oligopeptide, such proteins may be recovered from the solution using metal affinity chromatography. That is why the clarified cell lysates were applied on the column with Ni^{2+}-NTA resin. Highly purified proteins were derived by multiple washing of the column with lysis buffer and the buffer fortified with Triton X-100 to promote removal of non-specifically bound by-proteins.

The presence of octahistidine oligopeptide at C-end of chimeric protein molecule coupled to repeated column treatment with buffer solutions enabled to yield biopreparations «Annexin-ADase» and «Annexin-PNPase» with purity grade over 95% (fig. 2).

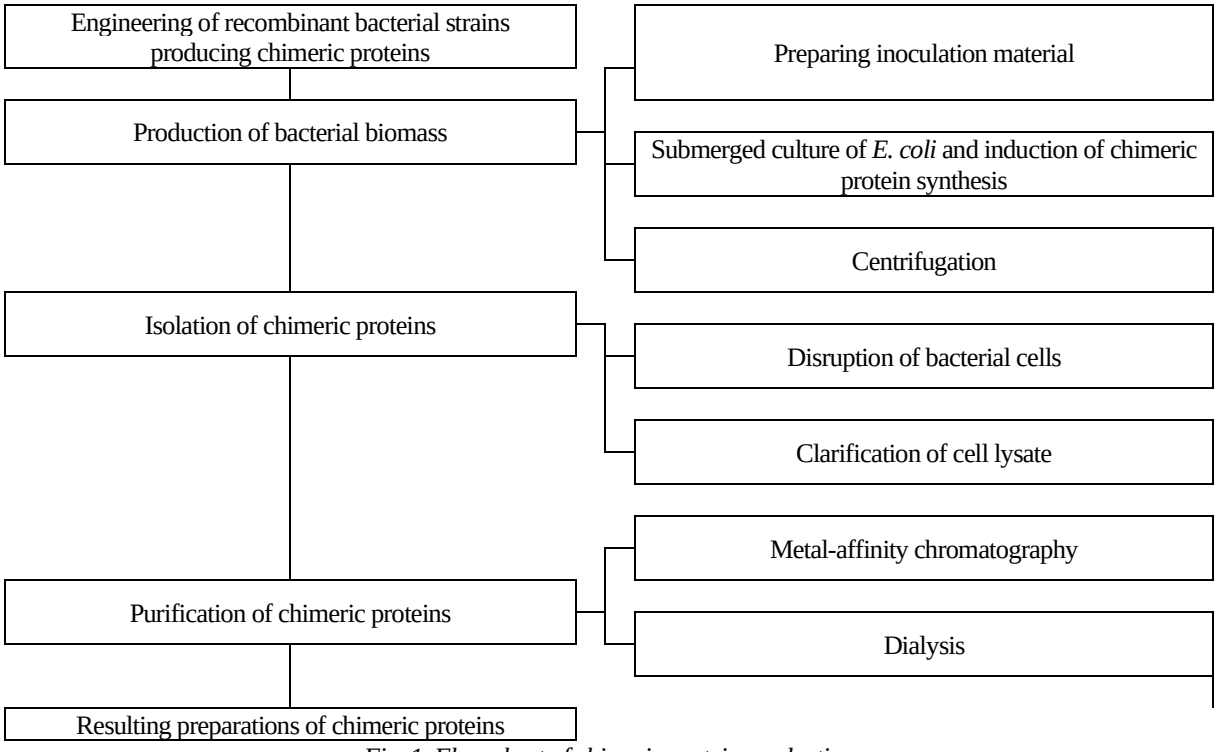

Fig. 1. Flow chart of chimeric protein production

It can be seen from the electrophoregram that isolated and purified chimeric proteins possess molecular weights about 64 kDa («Annexin-PNPase») and 74 kDa («Annexin-ADase»), corresponding to the theoretically calculated values.

In accordance with the end product synthesis and purification procedure, 54 mg and 36 mg of chromatographically pure chimeric proteins «Annexin-ADase» and «Annexin-PNPase», respectively, were recovered from 1 liter of cultural liquid.

At the final stage of research we tested the ability of chimeric proteins to express the required enzymatic activity, i.e. to carry out processes illustrated by fig. 3.

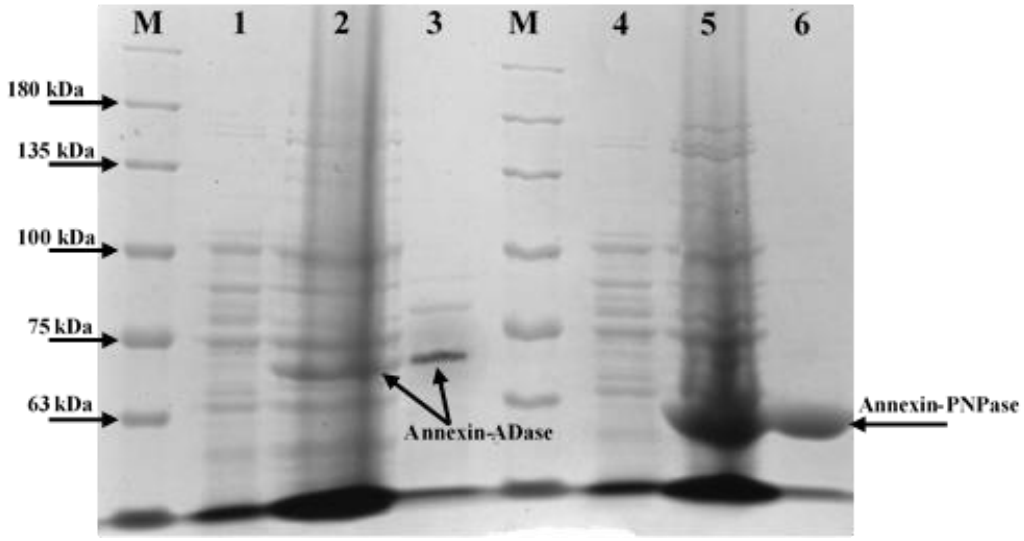

Fig. 2. SDS-PAGE image of chimeric proteins «Annexin-PNPase» and «Annexin-ADase»; M – protein molecular weight markers; 1 and 4 – cell lysates before protein synthesis induction; 2 and 5 – cell lysates after protein synthesis induction; 3 and 6 – purified chimeric proteins

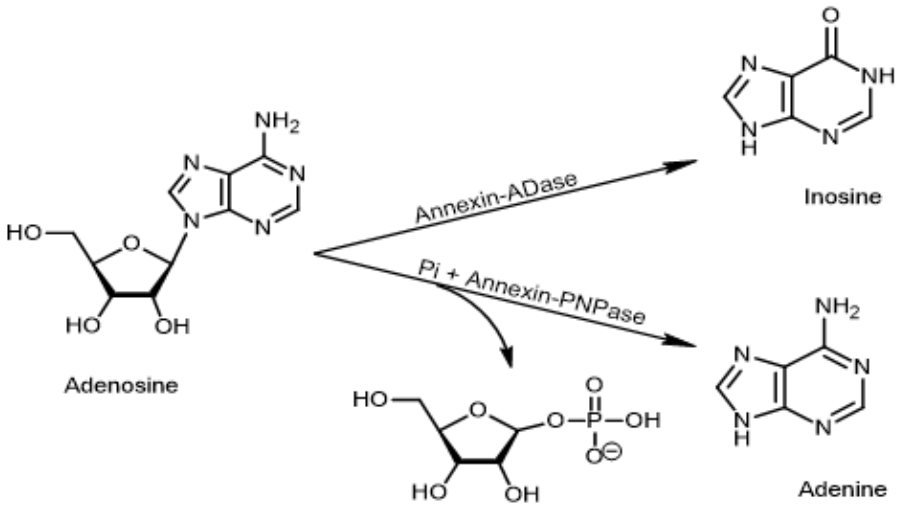

Fig. 3. Reactions catalyzed by chimeric proteins Annexin-ADase (upper) and Annexin-PNPase (lower)

Enzymatic transformation of adenosine by the chimeric proteins was controlled by thin-layer chromatography. It is evident from fig. 4 data that both reactions are accompanied by generation of adenosine dissimilation products – adenine and inosine.

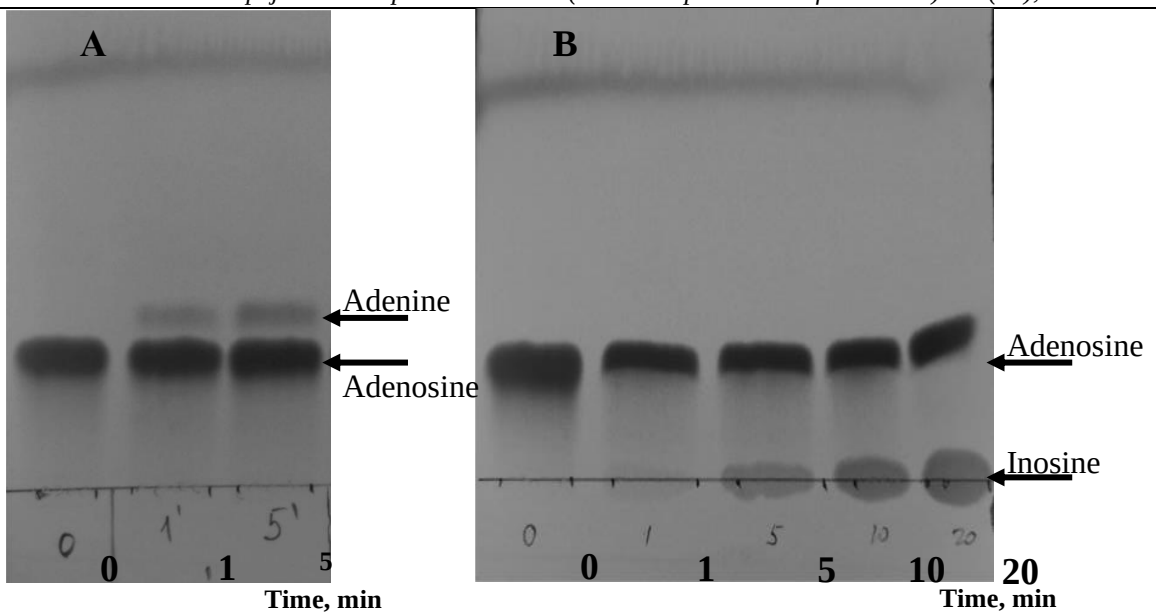

Fig. 4. Chromatogram of adenosine decomposition products released in the course of the reactions catalyzed by chimeric proteins

Taking into account literature reports reviewed in the section Introduction and our research data, it may be deduced that such chimeric proteins are attractive agents for further use in immunotherapy of cancer.

Conclusion. Summing up, submerged fermentation of recombinant *E. coli* strains resulted in 2 chimeric proteins composed of phosphatidylserine-binding human annexin A5 conjugated with homologous ADase or PNPase. The ability of these proteins to convert adenosine into inosine or adenine, respectively, was demonstrated. Sufficient grounds are available to regard such proteins as potential anticancer agents.

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[1]Podhaietskyi Anatolii Adamovich.
Doctor of Agricultural Science
[2]Matskevych Vyacheslav Viktorovich,
Candidate of Agricultural Science
[2]Filipova Larisa Mykolayivna,
Candidate of Agricultural Science
[3]Skripchenko Nadiya Vasilivna
Candidate of Biological Science
[1]Kravchenko Nataliya Volodimirivna.
Candidata of Agricultural Science

TROPHIC AND HORMONAL DETERMINANTS OF ONTOGENESIS ACTINIDIACHINENSIS VAR. DELICIOSA (A.CHEV.) IN VITRO AT THE CULTIVATION STAGE

[1]Подгаєцький Анатолій Адамович
д. с.-г. н., професор
[2]Мацкевич В'ячеслав Вікторович
к. с.-г. н., доцент
[2]Філіпова Лариса Миколаївна
к. с.-г. н., доцент
Скрипченко Надія Василівна
к. б. н., с. н. с.
[1]Кравченко Наталія Володимирівна
к. с.-г. н., доцент
[1]Сумський національний аграрний університет,
м. Суми, вул. Г. Кондратьєва 160, Україна, 40021
[2]Білоцерківський національний аграрний університет,
м. Біла Церква, Соборна площа, 8/1, Україна
[3]Національний ботанічний сад ім. М. М. Гришка НАН України,
м. Київ, вул. Тімірязєвська, 1, Україна

ТРОФІЧНІ ТА ГОРМОНАЛЬНІ ДЕТЕРМІНАНТИ ОНТОГЕНЕЗУ *ACTINIDIACHINENSIS* VAR. *DELICIOSA* (A.CHEV.) *IN VITRO* НА ЕТАПІ МУЛЬТИПЛІКАЦІЇ

Abstract. The results of research to determine the composition of the nutrient medium for the multiplication of Actinidiachinensis var. deliciosa (A.Chev.) in vitro to study trophic and hormonal determination of ontogenesis. The specific reaction of four varieties to different environments by plant height, reproduction rate was revealed. The use of the developed nutrient medium allowed to increase the value of the indicators during six subcultures,