PROTEOMICS AND PROTEIN FUNCTIONS

FEATURES OF DIFFERENTIATED GLIOMA C6 CELL CULTURE

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The management of neoplastic cell cultures of neuroectodermal origin, as a rule, is carried out in accordance with the clear protocols attached by the cell culture banks when acquiring cell lines from them. These requirements allow accurate reproduction of the results of studies on these cell lines, rapid production of cellular responses to the agent under study, while maintaining the standard culture conditions. However, in some cases, the introduction of changes in cultivation conditions makes it possible to achieve great success in *in vitro* studies in view of a more vivid extrapolation of phenomena to the living organism of mammals.

So the cell line of glioma C6 of neuroectodermal origin is widely used as a model of cellular responses of fibroblast and astrocyte-like cells. The general culture conditions for conducting the confluent culture of this line are described as the DMEM or RPMI medium containing 10% fetal bovine serum (FBS). However, given that this cell line consists of four subtypes of cells (astrocyte-, fibroblast-, oligodendrocyte-like, and epithelioid cells) that can be differentiated with specific antibodies to the glial acid fibrillar protein (GFAP) and galactocerebroside.

Then in some cases it is advisable to apply the conditions of the C6 line in which the majority of cells will differentiate according to the type of astrocyte-like cells.

ORTHOPHOSPHATE EFFECT ON PROTEOLYTIC ACTIVITY OF SUPERNATANTS OF *CHLORELLA VULGARIS* CELL HOMOGENATES

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It was earlier shown that the brain and liver mitochondrial fraction of mice did not cleave fibrin. The fibrinolysis was shown in the presence of inorganic orthophosphate – Pi. The increase of proteolytic activity in the presence of Pi was also demonstrated on some lymphoblast cell lines. And, judging by data of the inhibitory analysis, this effect was not bound to a resynthesis of the ATP. It allowed us to put forward the idea about existence of the ATP-independent pathway of proteolysis stimulation by Pi – "phosphatic effect". Further it was shown that inorganic orthophosphate (0.001-0.06 M) increased the activating function of streptokinase, urokinase, or tissue activator of a plasminogen by 50-250% and, in general, – 1.2-12.0 times lysis of a number of proteins by trypsin (T), α-chymotrypsin (CT), subtilisin (S), papain (Pap), metalloproteinase of bacilli (MP), and at ≤0.004 M pepsin (Pep) as well. In higher concentration phosphate activity of Pep was sharply decreased. It suppressed lysis of Pap gelatin, gelatin and casein of MP by 40-50%. It turned out that fibrinogenolitic activity of a number of opportunistic microorganisms strains was shown only in the presence of inorganic orthophosphate.

The aim of the present work – manifestation of the feature of Pi effect on proteolytic activity of cell water-soluble fraction of a photosynthesizing alga *Chlorella vulgaris*.

Researches are executed on *Ch. vulgaris* cells, the strain of IBCE C-19 (algas' collection of Institute of Biophysics and Cell Engineering of NAS of Belarus). *Ch. vulgaris* grew up in the conditions of periodic culture on the Tamiyya medium at the continuous bubbling of suspension of cells air – 25 l/h; t = 25–26 °C; illuminating intensities on a vessel surface – 32 W/sq.m; to a photoperiod (light/darkness) – 12 h/12 h. After the 7th day of culture growth, we measured the cells concentration, selected their aliquotas, washed with distilled water three times. Cells were homogenized with bidistilled water on ice, homogenates were centrifuged within 10 min, at 8000 rpm, at 4 °C. Proteolytic activity was determined by lysis of a fibrinogen or casein in a thin agar layer as it was earlier described. Concentration of proteins was 10 g/l, and agar – 10 g/l. As solvent for preparation of protein-agar plates we used deionized water to which Na_2HPO_4+KH_2PO_4 aliquots were added. All experiments were made no less than fivefold. Results are processed statistically with calculation of a *t*-student criterion (Statistica-6).

Supernatants of homogenates of *Ch. vulgaris* cells were capable of cleavage of both proteins and in the absence of inorganic orthophosphate though the casein was hydrolyzed less intensively, than fibrinogen: 20.3 ± 0.9 and 24.5 ± 0.9 mm^2, respectively. However, in the presence of inorganic orthophosphate the proteolysis significantly differed. At 0.001-0.009 M Pi concentration the fibrinogenolytic activity of supernatant was reduced by 12-37% whereas at effector concentration of 0.15 and 0.45 M it increased by 21 and 27%, respectively. Changes of caseinolytic activity had a three-phase character. At concentration of an effector of 0.001 and 0.003 M this activity increased by 68 and 84%, respectively, at concentration of Pi of 0.009 M it decreased by 37%. It was notet that at Pi concentration between 0.03-0.06 M the second phase of activity increase by 51–63% .

Therefore, as well as it is shown earlier, the effect of Pi depends on substrate protein. In this case, the complex concentration dependence on the effector action and the zone of proteolytic activity inhibition was observed. The causes of such picture need further researches.

ROLE OF THE proHB-EGF HEPARIN-BINDING DOMAIN IN DIPHTHERIA TOXIN BINDING

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Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family of growth factors. This protein binds heparin and heparan sulfate proteoglycans (HSPGs) with high affinity. Interaction with HSPGs provides HB-EGF recruitment to cell surface and stabilizes the complex with epidermal growth factor receptor (EGFR). Transmembrane precursor of HB-EGF (proHB-EGF) is the only known diphtheria toxin (DT) receptor in eukaryotic cells. It is known that the site for DT binding is located in the EGF-like domain of proHB-EGF. However, participation of the other structural parts of proHB-EGF in interaction with toxin is still unclear.

The aim of this work was to investigate the influence of heparin-binding site of HB-EGF on interaction with DT.

To reduce ability of proHB-EGF to interact with heparan sulfate, positively charged amino acids in heparin-binding domain were substituted with non-polar alanine using PCR-driven overlap extension technique. Mutant proHB-EGF sequence was cloned to plasmid vector pEGFP-N1 that allows eukaryotic cell expression of fluorescently-labeled proteins. The resulting (pEGFP-N1-proHB-EGF-mut) and reference (pEGFP-N1-proHB-EGF) constructions were used to transfect Vero cells to obtain stable expressing population of cells. Transfected cells were treated by recombinant *Eschrichia coli*-produced B-subunit of DT fused with red fluorescent protein mCherry.

It was determined that expression of heparin-binding deficient form of proHB-EGF was 15% lower compared to full-size form of proHB-EGF. The intensity of B-subunit binding to mutant form of proHB-EGF was 30% lower compared to non-mutated proHB-EGF.

Obtained results may suggest that the loss of heparin-binding ability of HB-EGF reduces the intensity of ligand-receptor complexes formation. We suppose that proHB-EGF interaction with HSPGs is responsible for efficient toxin internalization by mammalian cells.

PREPARATION AND CHARACTERIZATION OF HEMOGLOBIN ALGINATE MICROSPHERES

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Hemoglobin has been previously used as a model protein in numerous studies concerning calcium alginate microspheres preparation, their properties as well as effectiveness of their application as a delivery system were estimated. One of the restrictions in protein loaded microsphere usage is protein oxidation during hypothermic storage. Earlier it has been established that heme, which is known to be the main hemoglobin functional cite, undergoes oxidation under certain storage conditions. Hydrogen peroxide (H_2O_2) is known to be one of the reactive oxygen species acting as a key intermediate for oxidative stress development. Thus the reaction between hemoglobin and hydrogen peroxide results in degradation of the protein heme. Herewith the addition of antioxidant enzyme, catalase, to the hemoglobin solution has been shown to limit the extent of this reaction. Catalase was also reported to be successfully encapsulated in alginate microspheres without losing its functional activity. The constant ratio between hemoglobin concentration and catalase activity was demonstrated in erythrocytes. The isoelectric points of these proteins lay in the same pH area (6.8 for human hemoglobin, 7.0 for human blood catalase). Therefore, our assumption was that the effective encapsulation of hemoglobin and catalase into alginate microspheres will occur under the same conditions.

Hemoglobin and catalase loading in alginate microsphere were carried out by ionotropic gelation. Hemoglobin concentration in hemolysate was assessed spectrophotometrically at 540 nm. Hemoglobin degradation in microspheres in the presence of H_2O_2 (6 mM) was evaluated recording absorbance at 413 nm. H_2O_2 concentration was assessed using ammonium molybdate.

It has been established that the amount of encapsulated hemoglobin correlates with the hemolysate concentration. In these conditions the catalase activity, determined by the level of peroxide in the solution and by ability to prevent hemoglobin degradation in microspheres, strongly depends on hemoglobin concentration.

Experimental data have revealed the possibility of obtaining microspheres containing both, hemoglobin and catalase. In so doing catalase was shown to protect hemoglobin against peroxide deleterious action. Herewith, the usage of high hemoglobin concentration decreases the level of catalase incorporation into alginate microspheres.

MUTAGENESIS PROBE INTO EDITING MECHANISM OF D-AMINOACYL-tRNA DEACYLASE FROM *THERMUS THERMOPHILUS*

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D-aminoacyl-tRNA-deacylase (DTD) exists as an additional checkpoint in the machinery of protein biosynthesis control. It hydrolyzes the ester bond between D-amino acids and tRNA, having mistakenly bound during the activation step. DTD is specific toward different D-aminoacyl-tRNA substrates (D-Tyr, D-Phe, D-Trp, D-Asp-tRNA) (Calendar and Berg 1967; Soutourina, Plateau et al. 1999; Zheng 2009), strictly discriminating the chirality of amino acid (Ahmad, Routh et al. 2013). Interestingly it was recently found that this enzyme could also cut achiral Gly from its cognate tRNA and misacylated tRNAAla (Routh, Pawar et al. 2016; Pawar, Suma et al. 2017). This fact suggests DTD's role in glycine deacylation, preventing from its cellular toxicity. Despite the interest to this enzyme, its editing mechanism still requires analysis and investigation.

We performed molecular modelling by AUTODOCK and Modeller and molecular dynamics (MD) simulations by Gromacs and VMD based on reported crystal from *Plasmodium falciparum* bound to substrate analogue D-Tyr-3AA (Ahmad, Routh et al., 2013). The results after 5ns were analysed for site-directed mutagenesis studies. Mutagenesis was done by QuickChange Stratagene Kit. All substitution mutants were tested in deacylation assay with α-[32P]-radiolabelled-tRNATyr.

The comprehensive analysis of wild-type enzyme and its mutants' activity divided them into 3 groups: 1) with significantly decreasing editing activity (Q78A, F79A, Y125A, G137A, P138A, P138H); 2) with significantly increasing activity (Y125F, A127M); 3) with the same activity comparing to WT (S77A, V124A, A127V, V139A). Those mutants belong to several conservative elements from prokaryotes to eukaryotes: -SFQL- and -Gly-cis-Pro- (enantioselective) motif, and some separate amino acids from proposed catalytic site (Y125, A127). Steady-state kinetic parameters of the deacylation reaction were determined for WT, Y125A, Y125F and S77A, showing 10-fold less (Y125A) and 100-fold higher (Y125F) catalytic velocity than WT DTD.

Based on the results of mutagenesis experiments and MD data, a preliminary idea for deacylation mechanism catalysed by DTD was suggested.

FIBRINOGENOLYTIC ACTIVITY OF *AGKISTRODON HALYS HALYS* VENOM PROTEASE

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Snake venoms are the natural source of proteases targeted to fibrinogen and fibrin (fibrinogenases) that can be used for obtaining functionally active fragments of fibrinogen for further study *in vitro* as well as for the direct defibrination *in vivo*. The aim of the present study was to determine the target of proteolytic action of fibrinogenase from the venom of *Agkistrodon halys halys* (Halygenase) and study the effect of proteolysis on fibrinogen-dependent aggregation of platelets.

Halygenase was purified from the crude venom of *Agkistrodon halys halys* using two-step chromatography on Blue-Sepharose followed by Q-sepharose substrate specificity of Halygenase was determined by chromogenic substrate assay using S2251 (D-Val-Leu-Lys-pNA), S2238 (H-D-Phe-Pip-Arg-pNA), S2765 (Z-D-Arg-Gly-Arg-pNA). Products of hydrolysis were characterized by SDS-PAGE under reducing conditions with following Western-Blot using of monoclonal antibodies II-5C and 1-5A with epitopes in Aα20-78 and in Aα504-610, respectively. Fibrinogen fragments cleaved by Halygenase were detected by MALDI-TOF analysis using a Voyager-DE and identified using software "Peptide Mass Calculator". Aggregation of washed platelets was studied using aggregometer SOLAR-2110.

Amidase activity assay showed that the halygenase was mostly specific towards peptide bonds formed by C-group of lysine. It was shown that it preferentially cleaved the Aα-chain of fibrinogen splitting off the peptide with apparent molecular weight of 20 kDa. Western-Blot analysis using monoclonal antibody recognized the cleaved fragment as the C-terminal part of Aα-chain of fibrinogen. MALDI-TOF followed by in silico analysis with "Peptide Mass Calculator" identified this peptide as fragment Aα414-610 of fibrinogen molecule. It was shown that in the presence of fibrinogen desAα414-610 the rate and speed of platelet aggregation were decreased by 63 ± 11% and by 40 ± 7%, respectively in comparison to aggregation in the presence of native molecule.

It was demonstrated that protease from the venom of *Agkistrodon halys halys* preferentially cleaves the peptide bond AαThr-Glu-Lys413-Leu414 of fibrinogen Aα-chain thus cleaving-off the C-terminal half of αC-domain. Its proteolytic action dramatically impaired fibrinogen-dependent aggregation of washed human platelets. Thus we can consider protease from the venom of *Agkistrodon halys halys* as a prospective agent for study of interaction between fibrinogen and platelets and possibly as the useful tool for anti-aggregatory action *in vivo*.

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RED-EDGE EXCITATION FLUORESCENCE STUDIES OF THE FAST INTRAMOLECULAR DYNAMICS OF AIMP1/P43 PROTEIN

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Aminoacyl-tRNA synthetase complex-interacting multifunctional protein 1 (AIMP1/p43) is an auxiliary component of aminoacyl-tRNA synthetase complex of higher eukaryotes. Outside of the complex AIMP1/p43 shows pleiotropic cytokine activity, modulates the proliferation of different types of cells, suppresses angiogenesis and stimulates apoptosis and inflammation. Since the spatial structure of full-length protein has not been established yet, the specific physical nature of protein conformational changes and their contribution to the functional activity of the AIMP1/p43 protein remain largely unknown.

The purpose of this work is the AIMP1/p43 protein nanosecond dynamics studies at the temperature interval from 25 to 50 °C by steady state fluorescence spectroscopy technique. AIMP1/p43 was expressed in the bacterial system using pET28b vector and *Escherichia coli* BL21(DE3) cells. The protein has been purified to homogeneity by metal-chelating chromatography. In this work we used the intrinsic fluorescence of the single tryptophan residue Trp271 in the AIMP1/p43 structure as a probe to monitor the protein conformational change during thermal denaturation. The dynamics of the microenvironment of Trp271 was investigated by the red-edge excitation shift effects.

The fluorescence emission spectrum of AIMP1/p43 on excitation at 295 nm reveals a maximum position at 331 ± 1 nm that indicates the mainly buried state of fluorophore in the protein globule. A significant gradual red-edge excitation shift effect which is about 9 ± 1 has been detected in the range of the excitation wavelengths from 290 till 304 nm at 25 °C. The magnitude of the red-edge excitation shift begins decreasing at 37 °C and practically disappears at 48 °C. The disappearing of magnitude indicates the disruption of the Trp271 specific microenvironment in native protein globule in this temperature range.

Based on the obtained results it is proposed that such conformational rearrangement of the Trp271 microenvironment which began at physiological temperature may be essential for the following binding with the partner molecules during recognition process.

FUNCTIONING AND PHYSIOLOGICAL ROLE OF NICOTINIC ACETYLCHOLINE RECEPTORS IN MITOCHONDRIA

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Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels, which regulate synaptic transmission in muscles and neurons, as well as cell viability and proliferation in many non-excitable cells. Previously we reported the presence of α3β2, α4β2 and α7(β2) nAChRs in mitochondria, where they control the early stages of mitochondria-driven apoptosis like cytochrome *c* release under the effect of Ca^{2+} or H_2O_2. Mitochondrial nAChRs function in ion-independent manner by activating intramitochondrial kinases through conformational changes caused by the binding of either agonist or competitive antagonist. The aim of the present study was to delineate structural requirements for activation of different signaling pathways in mitochondria and to evaluate physiological role of mitochondrial nAChRs.

Experiments have been performed in mitochondria isolated from rodent liver, lung or carcinoma and from human thymus by differential centrifugation according to standard procedures. The level of nAChR subunits in mitochondria detergent lysates was studied by Sandwich-ELISA using subtype-specific antibodies and the apoptogenic effect of Ca^{2+}, H_2O_2 or wortmannin was evaluated based on the level of cytochrome c released from liver mitochondria.

It was found that the binding of nAChR-specific type 2 positive allosteric modulators (PAMs) was sufficient to attenuate cytochrome c release from mouse liver mitochondria in the absence of orthosteric agonist. Moreover, binding of a7 nAChR-specific PAMs PNU120596, PAM-2 or 4BP-TQS prevented the apoptogenic effect of Ca^{2+}, while b2-specific desformylflustrabromine was effective against wortmannin-induced cytochrome c release. We suggest that α7 nAChR subunits regulate CaK-MII-dependent, while β2 subunits control PI3K-dependent signaling pathways in mitochondria.

We have found increased levels of a7 nAChRs in mitochondria of human thymoma compared to normal thymus and in Lewis carcinoma compared to normal mouse lung. Experiments with mitochondria of regenerating liver in rats demonstrated the increase of not only a7-, but a3-, α4- and, especially, a9-containing nAChRs 3-6 h after partial hepatectomy resulting in increased mitochondria resistance to 0.1-0.9 μM Ca^{2+} and 0.1-0.5 mM H_2O_2. These data demonstrate a physiological significance of mitochondrial nAChRs to support the proliferating cell viability in the course of liver regeneration or tumor growth.

OXIDIZED LIPIDS SUPPRESS AMYLOID FIBRIL FORMATION: SEARCHING FOR A MECHANISM

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Preventing of amyloid fibril formation *in vivo* has long been a focus of extensive studies, since this process plays a key role in a molecular etiology of Alzheimer's disease, type II diabetes, systemic amyloidosis, etc. However, effective strategy of treating these diseases is still not developed.

This study was aimed at assessing the inhibiting effects of oxidatively modified phospholipids, viz. 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PazePC), and 1-palmitoyl-2-(9′-oxononanoyl)-sn-glycero-3-phosphocholine (PoxnoPC) on the protein fibrillization.

Lipid dispersions of PazePC and PoxnoPC in sodium phosphate buffer were prepared using a bath sonicator. Alternatively, the extrusion technique was employed to obtain liposomes composed of 80 mol% phosphatidylcholine (PC) and its mixtures with PazePC (20 mol%) or PoxnoPC (20 mol%), referred to here as PazePC20 and PoxnoPC20, respectively. The lysozyme (insulin) fibrillization was initiated at pH 2 (7.4), 60 °C, at lipid concentration 16 μM. The kinetic parameters for amyloid fibril formation, viz. lag time, apparent rate constant for the fibril growth (k) and maximal fluorescence of the dye (Fmax), were obtained by approximation of the time dependence of the Thioflavin T (ThT) fluorescence intensity at 480 nm with the sigmoidal curve.

It appeared that Fmax values of lysozyme- (insulin-) bound ThT were about 20% (86%) lower in the presence of PazePC20/PoxnoPC20, as compared to those in control samples. The revealed effects were attributed to the decrease in the extent of amyloid fibril formation. The inhibition of lysozyme aggregation was accompanied by the reduction of the lag time and increase of the k values, while the opposite effect was observed for insulin. Furthermore, lipid vesicles, containing PazePC and PoxnoPC, inhibited protein aggregation into mature fibrils, unlike lipid dispersions, highlighting the important role of the polar surfaces of the lipids in the reduction of the protein fibrillization extent. The obtained results point to significant impact of PazePC-lysozyme hydrophobic interactions on the inhibition of the protein fibrillogenesis. In turn, Shiff bases could be formed between insulin monomers and PoxnoPC domains of the lipid vesicle, thereby stabilizing an aggregation-resistant protein conformation.

In conclusion, our findings provide a basis for comprehensive testing of oxidized lipids as potential anti-amyloid agents.