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Structure and Functions of Biopolymers

Obtaining recombinant chaperon GroEL and its immunologic cross-reactivity with Hsp60

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The method for obtaining and purification of recombinant chaperon GroEL (prokaryotic homolog of eukaryotic chaperon Hsp60), including the protein expression in E.coli cells, precipitation with saturated ammonium sulphate from a producent lysate, gel-filtration on Sephacryl-300 column and ion-exchange chromatography on MonoQ HR 5/5 column, is described. The method allows effective obtaining a preparative amount of the GroEL protein of 95% purity. The recombinant protein was used for the anti-GroEL antibodies production and synthesis of an affine column.The immunologic cross-reactivity of polyclonal affine-purified anti-GroEL antibodies and members of Hsp60 family from different mammalian organs and cells – from mouse to human, has been revealed. This allows using these antibodies for research of the Hsp60 expression alterations and cell localization at human autoimmune and cancer pathologies.

***Key words**: chaperon, Hsp60, GroEL, chromatographic purification, bacteriological producer.*

Introduction. Different types of physiological stress (heat shock, virus infection, radiation, hemodynamic disorders, ischemia, oxidative stress, etc) are able to cause multiple changes within the cells, including those affecting the structure and functions of proteins. A number of data obtained in the recent years suggests that incorrect protein folding (packing of new synthesized polypeptide chains) is a molecular basis of many diseases [1-3]. One of the factors influencing correct folding is functioning of molecular chaperons – heat shock proteins (HSPs). HSPs are responsible for the fidelity of protein folding and the import of precursors of organnella proteins into the corresponding cell compartments. Chaperons participate in reparation of ionic channels, apoptosis, steroid receptors activation, regulation of signaling cascades, in the process of collagen synthesis at reparative fibrosis, presentation of intracellular antigenes [4-6].

HSPs are divided into several families depending on their molecular weight and specialized functions [7, 8]. The Hsp60 family are proteins of mitochondrial matrix that participate in assembling, transport of mitochondrial proteins

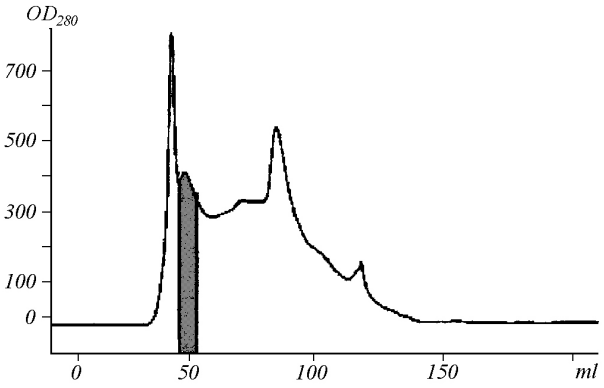

Fig.1. The profile of gel chromatographic purification of GroEl on the Sephacryl-S300 column (fractions containing GroEl, are marked dark).

and prevention of their aggregation. Both the level of expression and cell localization of Hsp60 change at different pathological states [9-12

Molecular chaperons Hsp60 of different taxonomic groups belong to the highly conservative protein family with homology level up to 50% [13]. In particular, *E.coli* GroEL is a prokaryotic homologue of a mammalian Hsp60. The aim of our work was the development of fast and effective method of purification of the bacterial protein GroEL, obtaining anti-GroEL antibodies, and the study on their possible application to research the eukaryotic Hsp60 expression and cellular localization at different human pathologies.

Materials and Methods. A recombinant plasmid pT-GroEL, constructed on the basis of pACYC vector and containing coding sequence of the GroEL gene under T7 promoter, p15a replicon and marker of chloramphenicol resistance; cell strain *E.coli* BL21(DE3), control protein GroEL (Sigma), and monoclonal antibodies to GroEL (Sigma) were used in the work. The transformation was performed by a standard method [14]. The purity of proteins was analyzed by electrophoresis in the presence of sodium dodecyl sulfate (DS-Na) in 12% polyacrylamide gel (PAAG) according to Lammly [15]. The concentration of protein was determined according to Bredford [16].The immunization of rabbits was performed by the method earlier developed by us [17]. The affine column was prepared as described in the work [17]. Anti-GroEL affinity was tested by immunoenzyme assay (ELISA) [18], the specificity was determined by the method of immunoblotting (Western-blot) [19]. Lysates of mammalian organs were obtained according to [20].

Results and discussion. Several clones of E.coli cells BL21(DE3), containing plasmid pT-GroEL, were obtained by the transformation method. The induction of protein expression in cells being in the logarithmic growth phase was performed by addition of 1mM IPTG (isopropyl-1-thio-в-D-galactosidase) with subsequent incubation for a night at 37EC. The cells were precipitated, resuspended in a lysis buffer A, containing 10mM Tris/HCl, pH=8.0, 50mM NaCl, and proteases inhibitors (1mM PMSF, 0.2% Aprotinin, 5mM Benzamidine), and destroyed by ultrasound in a disintegrator. The supernatant, obtained after centrifugation, was used to purify GroEL by several methods according to the following scheme: precipitation with ammonium sulphate, gel filtration, HPLC ion-exchange chromatography. The largest protein precipitate was obtained at saturation by ammonium sulfate in the range of 43-55%. Gel-filtration was conducted on the column of Sephacryl-S300, equilibrated with the buffer A. At gel-filtration the chaperons (GroEL) were eluted in front of the buffer (Fig.1).

The analysis of purity and specificity of fractions, containing GroEL, was performed by electrophoresis of proteins according to Lemmly (Fig.3a) and by Western-blot analysis with commercial anti-GroEL antibodies (Fig.3b). The fractions containing GroEL were collected and put on the column with MonoQ HR 5/5 for ion-exchange chromatography. The column was equilibrated with the buffer A and elution was performed by a linear gradient of NaCl [50-1000mM]. GroEL was eluted at NaCl concentration of 500-600mM (Fig.2). The fractions, containing the protein (not less than 95% of purity) were selected according to the results of the electrophoresis, and dialyzed against the buffer A. The specificity of obtained protein was determined by immunoblotting method, using commercial monoclonal anti-GroEL antibodies (Sigma) (Fig.3b).

Rabbits were immunized with GroEL obtained to get antibodies. After 7 days of the immunization of rabbits their blood was taken to obtain antiserum. Immunoglobulin precipitation was performed at 50% saturation of serum with ammonium sulfate. The antibodies purification was performed on DEAE cellulose. The column was equilibrated with a PBS-buffer (0.75 M NaCl, 0.025 NaH_2PO_4 H_2O, pH=7.4). The elution of antibodies was performed by PBS-buffer, fractions volume of 0.5ml. The fractions in peak area were collected and put on an affine column with conjugated GroEL. The column was equilibrated with PBS buffer. After the incubation with antibodies for 2 hours at the room temperature the column was washed with the PBS-buffer, antibodies were eluted by 0.2M glycine buffer, pH 2.5 with subsequent neutralization by 1M Tris/HCl, pH 11. The fractions containing antibodies were dialyzed against the PBS-buffer for a night at 4°C. The affinity of obtained anti-GroEL antibodies was tested by ELISA, the specificity was determined by Western-blot.

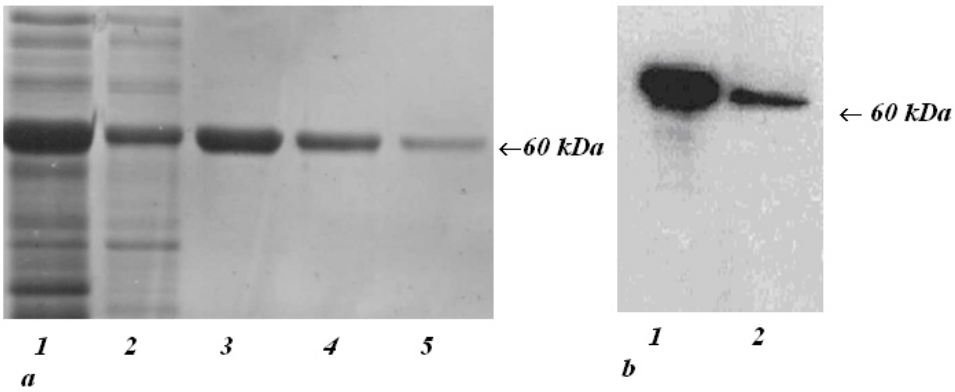

Fig.2. The analysis of purity and specificity of the protein GroEl: a– electrophoregramme of fractions, containing GroEL, at different stages of purification: 1-after precipitation; 2-after gel-filtration (peak fractions) on the Sephacryl-S300 column; 3,4 – after ion-exchange chromatography on the MonoQ column; 5-control GroEL (Sigma). b – Western-blot analysis of specificity of the obtained recombinant GroEL:1-purified GroEL (after ion-exchange chromatography on the Mono Qcolumn; 2-control GroEL (Sigma).

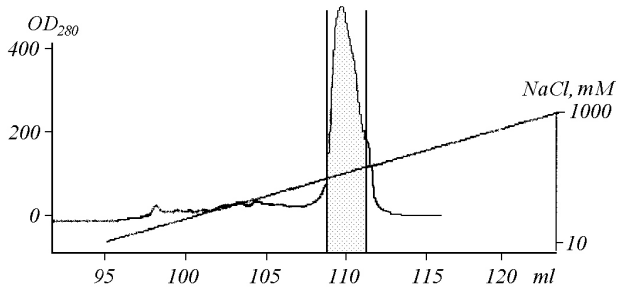

Fig.3. The profile of protein elution from the MonoQ column with linear gradient of NaCl (50-1000mM). GroEL was eluated in the range of NaCl concentrations of 500-600mM.

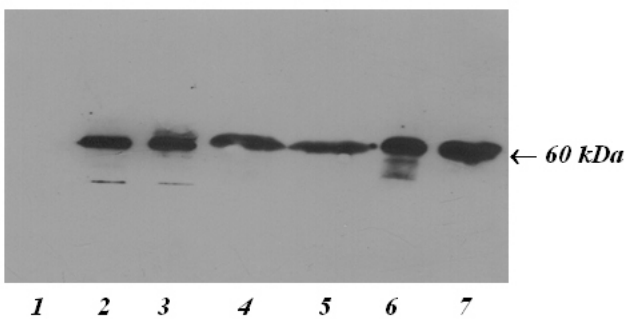

Fig.4.Immunologic cross-reactivity of the Hsp60 chaperons, determined by Western-blot analysis using affine purified polyclonal anti-GroEL antibodies in heart lysates of different species of mammals: 1-BSA, 2-mouse Balb\c, 3-rabbit, 4-bull, 5-human, 6-*E.coli*, 7-control GroEL (Sigma).

As we used the recombinant protein, obtained by means of the GroEL gene expression (bacterial chaperon homologue of mammalian Hsp60), it was necessary to show the immunologic cross-reactivity of the obtained antibodies with chaperons of this family from different organisms – from prokaryotes to various species of mammals, including human cells. The results of study on immunoreactivity of polyclonal affine-purified anti-GroEL antibodies are shown in Figure 4. It is evident that anti-GroEL antibodies recognize polypeptides with the molecular mass of about 60kDa in heart lysates of all investigated species (from mice to humans) and their immune reactivity is comparable with the immune reactivity of commercial anti-GroEL antibodies against a reference antigen (Fig.3b). These data and the literature data, proving high homology of the Hsp60 family chaperons and GroEL protein, allow using the recombinant prokaryotic protein GroEL and the antibodies obtained against it in the researches on eukaryotic material.

The analysis of time stability showed that the GroEL protein expression remained relatively stable for 4-6 months while storing the cells suspension in 50% glycerol and at -70°C.

The method of GroEL purification developed by us allows fast and effective obtaining preparative quantity of the high purity protein.

The recombinant prokaryotic protein GroEL is supposed to be used for investigating a role of its eukaryotic homologue Hsp60 at carcinogenesis and autoimmune human pathologies.

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ПОЛУЧЕНИЕ РЕКОМБИНАНТНОГО ШАПЕРОНА GROEL И ЕГО ИММУНОЛОГИЧЕСКАЯ КРОСС-РЕАКТИВНОСТЬ С HSP60

Резюме

Описан метод получения рекомбинантного шаперона GroEL (прокариотного гомолога эукариотного шаперона Hsp60), включающий экспрессию белка в клетках Escherichia coli с последующей очисткой белка градиентным высаливанием из лизата клеток-продуцентов, гель-фильтрацией на сефакриле-S300 и ионообменной хроматографи-

ей на MonoQ HR 5/5. Данный метод позволяет эффективно наработать белок GroEL 95 %-й степени очистки в препаративных количествах. Рекомбинантный белок затем использовали для получения поликлональных анти-GroEL антител и синтеза аффинной колонки. Показан иммунологический перекрест аффинно очищенных поликлональных анти-GroEL антител с представителями семейства Hsp60 в лизатах органов и клеток различных видов млекопитающих – от мыши до человека, что позволяет использовать данные антитела при изучении изменения уровней экспрессии и клеточной локализации Hsp60 в случае аутоиммунных и раковых патологий человека.

Ключевые слова: GroEL, Hsp60, ионообменная хроматография, гель-фильтрация, поликлональные антитела, аутоиммунные патологии человека.

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