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V.A. KHOMENKO, E.V. SIDORIN, S.I. BAKHOLDINA,
N.U. CHERNYSHEVA, N.U. KIM, M.P. ISAEVA, T.F. SOLOV'EVA

The culture temperature affects the properties of the *Yersinia pseudotuberculosis* porin inclusion bodies and the structure of the recombinant porin

The structure of inclusion bodies (IBs), in addition to the nature of the protein that forms them, depends on a number of parameters of the expression process, including the growth temperature of the cells of the producer strain. In this work we studied the effect of cell cultivation temperature on the properties and structure of Yersinia pseudotuberculosis porin IBs. The inclusion bodies synthesized in E. coli at different temperatures (37, 30 and 18 ° C) were isolated, purified and characterized. Using dynamic light scattering and optical spectroscopy, the solubility of IBs in SDS and urea and the state of the recombinant porin in the denaturants solutions (particle size, degree of polydispersity, secondary structure) were investigated. It was found that lowering the cell growth temperature resulted in a decrease in the stability of IBs in aqueous solutions of urea and SDS and an increase in the proportion of native-like conformations in IBs but also led to an increase in sensitivity of the recombinant porin to chemical denaturation. Mild methods of solubilization of IBs formed at reduced temperature are necessary for the preservation of the native-like porin structure.

Key words: inclusion bodies, recombinant porin, Yersinia pseudotuberculosis, porin folding intermediates, circular dichroism (CD) spectroscopy, dynamic light scattering

The recombinant proteins are widely used in various fields of industry (pharmaceutical, food, cosmetic), in scientific research and diagnostics, so interest in their production is constantly increasing. One of the most widely used bacterial systems expressing heterologous proteins remains *E. coli*. However, the production of recombinant proteins in *E. coli* is often accompanied by the formation of so-called inclusion bodies (IBs), which are aggregates of an almost pure and potentially ready-to-use target protein. In this connection, the task is to obtain IBs containing as much as possible correctly folded functionally and biologically active protein. The solution of this problem requires, among other things, a deep knowledge of the structure and mechanisms of the formation of IBs. The proportion of the functionally active protein in IBs is characteristic of the protein sequence, but also depends on many other factors such as the parameters of the expression process, including the cell growth temperature. A number of studies have shown that a decrease in the culture temperature effectively improved the quality of the protein in IBs [1, 3]. The purpose of this study is to determine the effect of cell cultivation temperature on the properties and structure of *Yersinia pseudotuberculosis* porin IBs.

* KHOMENKO Valentina Alexandrovana – PhD, Senior Researcher, SIDORIN Evgeny Viktorovich – PhD, Researcher, BAKHOLDINA Svetlana Ivanovna – PhD, Senior Researcher, CHERNYSHEVA Nadezhda Uryevna – Graduate student, KIM Natalya Yurievna - Researcher, ISAEVA Marina Petrovna – PhD, The Head of The Laboratory, *SOLOV'EVA Tamara Fedorovna – DSc, Principal Researcher (G.B. Elyakov Pacific Institute of Bioorganic Chemistry, FEB RAS, Vladivostok, Russia). *E-mail: soltaf@mail.ru

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The OmpF porin *Yersinia pseudotuberculosis* was expressed in *E. coli* at 37 °C (IB-37), 30 °C (IB-30) and 18 °C (IB-18) in the form of IBs, which were isolated, purified and characterized. The stability of inclusion bodies in aqueous solutions of urea (1-8 M) and SDS (0.02-0.1%) and the state of the solubilized recombinant protein in these solutions (particle size, degree of polydispersity, secondary structure) were investigated. Solubilization of IBs in urea or SDS was monitored by measuring the turbidity at 350 nm, the size and size distribution of the particles of the recombinant porin in the solutions were measured by dynamic light scattering (DLS).

All studied IBs were progressively more soluble with increasing urea concentration from 1 to 8 M. The turbidity of the IB-37 suspensions decreased sharply with an increase in the urea concentration from 3 to 5 M and remained almost unchanged in 7-8 M urea. The time of incubation of these IBs in urea in the interval 1 - 24 hours did not visibly affect their solubilization. Dissolution of TB-30 and TB-18 mainly occurred at lower urea concentrations: from 2 to 4 M and from 2 to 3 M when incubated in solution for 1 and 24 hours, respectively. At the same time, IB-18 in comparison with IB-30, are more soluble in urea, and the incubation time is a more significant factor for affecting them solubilization. Thus, IBs produced at lower temperature were less stable in urea. This suggests a higher content of the porin folding intermediates with native-like structure in these IBs [6].

According to the DLS data, in the IBs solutions in urea there were two populations of particles. One of them was represented by large particles (200-500 nm), which, apparently, were intact IBs and the particles close to them in size, the other - consisted of relatively small (<100 nm) particles that appeared as a result of solubilization of IBs and were very polydisperse. In solutions with a lower concentration of urea (1-4 M), the large particles were mostly present, while a population of the small particles with R_H 40-80 nm and 40-60 nm predominated (up to 70% of the total number of the particles) in more concentrated solutions of 5-8 M and 4-6 M for IB-37 and IB-30, IB-18, respectively. It should be noted that the large protein particles (secondary aggregates) accumulated in the concentrated urea solution, and their number increased with increasing incubation time. As is known, the fully unfolded OmpF porin has a hydrodynamic radius $R_H = 8.2 \pm 0.3$ nm measured by DLS [4], hence in urea solutions the recombinant porin was present in the form of oligomers (associates) and aggregates, but not monomers.

IBs dissolved well in dilute solutions of SDS: the turbidity of IBs suspensions dropped sharply with an increase in detergent concentration from 0.02 to 0.06 (0.08) %. An increase in IBs exposure in the detergent led to an increase in the amount of dissolved recombinant protein, while the concentration range of the detergent in which IBs solubilization occurs remained the same. The most effective IBs dissolved in the first hours of contact with SDS. Analysis of the IBs solutions in the detergent by DLS showed that the particle size decreased with increasing detergent concentration from 0.02 to 0.1% and the incubation time from 2 to 24 hours. The particles obtained by solubilizing IB-37 in 0.06-0.1% SDS for 24 hours had an average R_H of about 40 nm. The smallest particles, R_H 11-20 nm, were found in 0.08% SDS solutions. At the same time, the solution of IB-18 in 0.06% SDS incubated for 24 hours contained particles with R_H of 5.4-7.1 nm, the proportion of which was 86.9% and 81% of the total number and volume of particles, respectively. In the IBs solutions with a detergent concentration of 0.08-0.2%, smaller particles (R_H 3.7-4.6 nm) were present, whose content was about 99% both in number and volume, and they had mainly monomodal size distribution. Thus, in a detergent solution, the recombinant porin from IB-18 is in monomeric form, unlike that of IB-37, which under these conditions exists as an oligomer.

The structure of rOmpF porin solubilized from IBs and the effect of urea and detergent on it had been studied using CD-spectroscopy in the far UV region. Analysis of CD spectra allowed us to conclude that recombinant porin in IBs, expressed at 37, 30, and 18 °C, had a pronounced secondary structure with predominance of β-structure. However, differences in the type of β-structure between IBs synthesized at 37 °C and at lower temperatures are likely to occur. This assumption was supported by the CD spectra of recombinant porin in urea, which had negative maxima at 230 and 210-220 nm and positive bands at 202 and 198-199 nm for IB-37

and IB-30, IB-18, respectively. The denaturants that were used to solubilize IBs caused changes in the conformation of the porin. The resistance of the secondary structure of recombinant porin to SDS and urea decreases with a decrease in the temperature of its expression. The porins from IB-37 and IB-18 had the greatest and the least structural stability, respectively. The process of porin denaturation occurred in two stages: the accumulation of the α-helix by decreasing the β-structure (the ratio of ellipticities at 216-217 and 207-209 nm in the porin CD spectra decreased with increasing concentration of SDS) and the subsequent increase in the content of the disordered structure (the short-wavelength minimum in the CD spectrum shifted from 207-209 to 204-203 nm and the band intensity increased). In the case of rOmpF from IB-37, an increase in the concentration of denaturants and incubation time in solution did not accompanied by a marked increase in the content of the disordered structure.

Thus, a decrease in the expression temperature is accompanied by an increase in the solubility of IBs in SDS and urea and in the content of the recombinant porin with native-like β-sheet structure in IBs but leads to a decrease in the resistance of the secondary structure of the porin folding intermediates to the denaturants. The use of mild solubilizing agents and additives that stabilize the conformation of proteins could preserve of the existing native-like protein structure during solubilization of IBs formed at low temperatures andensure a high recovery of the properly folded porin [2, 5]. The novel information obtained in the study contributes to the understanding of the structural organization and mechanisms of formation of IBs and other protein aggregates and to the development of approaches to produce biologically active IBs.

REFERENCES:

1. de Groot N.S., Ventura S. Effect of temperature on protein quality in bacterial inclusion bodies // FEBS Lett. 2006. Vol. 580.P. 6471–6476.

2. Leibly L.J., Nguyen T.N., Kao L.T., Hewitt S.N., Barrett L.K., Van Voorhis W.C. Stabilizing additives added during cell lysis aid in the solubilization of recombinant proteins // PLoS ONE. 2012. Vol. 7. e52482.

3. Peternel S., Grdadolnik J., Gaberc-Porekar V., Komel R. Engineering inclusion bodies for non-denaturing extraction of functional proteins // Microb. Cell Fact. 2008. 7:34. Vol. 7. P. 34.

4. Sidorin E. V., Khomenko V. A., Kim N. Yu., Dmitrenok P. S., Stenkova A. M., Novikova O. D., and Solov'eva T. F. Self-Organization of Recombinant Membrane Porin OmpF from Yersinia pseudotuberculosis in Aqueous Environments // Biochemistry (Moscow). 2017. Vol. 82. P. 1304-1313.

5. Singh A., Upadhyay V., Upadhyay A.K., Singh S.M., Panda A.K. Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process // Microb. Cell Fact. 2015. 14:41.

6. Upadhyay A. K., Murmu A., Singh A., Panda A. K. Kinetics of Inclusion Body Formation and Its Correlation with the Characteristics of Protein Aggregates in *Escherichia coli* // PLoS ONE. 2012 Vol. 7. e33951