Scientific paper

Inclusion Bodies Contraction with Implications in Biotechnology

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Abstract

Irreversible contraction of inclusion bodies was found to have a huge impact on sedimentation velocity, solubility and extractability of target protein from non-classical inclusion bodies. The downstream process during protein isolation from non-classical IBs is also affected. Based on extractability studies accompanied by electron microscopy, a hypothetical model of IBs formation mechanism is presented, which is supported by usually observed single IB per *E. coli* cell.

Keywords: Non-classical inclusion bodies; *E. coli*; low pH contraction; solubility; extractability; inclusion bodies formation

1. Introduction

Formation of inclusion bodies (IBs) is a common event during over-expression of proteins in bacterial cells.[1,2] Some physiological factors that influence the formation of IBs are already known, but a great deal still remains unclear.[3,4] Aggregation occurs due to interactions among the *de novo* formed folding intermediates, which expose hydrophobic residues at their surface.[5]

IBs are now recognized as being more plastic structures than thought in the past.[6] For a long time it was supposed that IBs are formed as insoluble particles in the cell and remain there as an unreactive deposit.[2] However, there are recently documented cases when IBs are partially or completely degraded due to proteolysis.[7,8]

In some past years, several authors' reported about the presence of properly folded protein precursors inside IBs.[9–12] Independent reports on enzymatic activity of recombinant enzymes forming IBs have also been noted, although the biotechnological relevance remained unexplored.[10,13–15]

Understanding the mechanisms of IB formation is important not only in biotechnology, but also in medicine. As the aggregation of proteins is involved in the so-called conformational diseases (prionic and amyloid), understanding the basic mechanisms, even in *E. coli*, could be helpful for designing new drugs and developing new ideas for therapy.[16]

Jevsevar et al.[11] previously described a new subtype of IBs, that contain a high proportion of correctly folded protein precursor which can be extracted under non-denaturing conditions. As they expressed several significantly different properties from IBs described previously[17] we designated them "non-classical inclusion bodies" (ncIBs).

Transferring ncIBs from pH 7 to pH 4 resulted in a significant reduction of IBs size.[17] The size reduction was seen to be irreversible, so that when the IBs were returned to pH 7, their volume remained the same as it used to be at pH 4. In the present work, the consequences of the IBs contraction are described and in addition, the impact of this phenomenon on biotechnology is discussed.

2. Experimental

2. 1. Strain, Plasmid and Bacterial Culture Conditions

Escherichia coli BL21(DE3) production strain with plasmid pET3a (Novagen) carrying a codon optimized gene for high expression of human G-CSF was used for the study. Details about the production strain development have been described previously by Jevsevar et al.[11] All the production processes were preformed with shaked flask cultures growing at 25 °C in GYSP medium.[11,17] The culture was induced with IPTG at OD_{600} 2, and incubated at 25 °C and 160 rpm (Kühner linear shaker) until the culture reaching stationary phase.

2. 2. Isolation of Inclusion Bodies

Bacterial cells were disrupted with a high-pressure homogeniser Emulsiflex® – C5; Avestin. IBs were isolated by centrifugation (pellet), washed twice with pure water and used for further analysis. The supernatant containing the soluble protein fraction as well as the pellet (IBs) were analyzed by SDS-PAGE.

2. 3. Contraction of IBs at Low pH

Isolated IBs were divided into two aliquots and resuspended in two different buffers (0.1 M phosphate buffer pH 7.0 and 0.1 M acetate buffer pH 4.0). They were incubated for 15 minutes at room temperature and then thoroughly washed with pure water. Supernatants corresponding to buffers 0.1 M phosphate pH 7.0 and 0.1 M acetate pH 4.0, respectively, were analyzed by SDS-PAGE.

2. 4. Scanning Electron Microscopy

Isolated IBs were prepared in a corresponding buffer (see contraction of IBs at low pH) and then washed in pure water. They were prepared on a gold-coated polycarbonate Isopore™ membrane filter (filter pore size 0.22 μm) (Millipore). Samples were observed under a Zeiss SUPRA 35 VP electron microscope.

2. 5. Sedimentation of IBs

IBs previously treated with two different buffers (see contraction of IBs at low pH) were washed in pure water and resuspended in 10 mM Tris/HCl, pH 7. Suspension was put into a cuvette and its optical density was recorded on Agilent 8453 Spectrophotometer on every 20 minutes for 8 hours. The last spectrum was recorded after 24 hours.

2. 6. Solubility of Inclusion Bodies

IBs previously treated with neutral and low pH buffers (see contraction of IBs at low pH) were washed with deoxicholate, the detergent generally used for washing the inclusion bodies. Two detergent concentrations were prepared respectively (0.1% and 1% deoxicholate in pure water). IBs were incubated in deoxicholate for 5 minutes and than centrifuged at 4400 g for 10 minutes. The supernatant (solubilized proteins from IBs) and the pellet (IBs) were analyzed by SDS-PAGE.

2. 7. Extractability of Inclusion Bodies

The wet pellet mass of IBs previously treated with two different buffers (pH 4, pH 7) was determined and resuspended in a ratio of 1:40 with solubilizing buffer (40 mM Tris/HCl with 0.2% N-lauroyl sarcosine, pH 8.0). The suspension was shaken for 24 hours at 20 °C and centrifuged at 4400g for 15 minutes. The supernatant (solubilized target protein from IBs) and the pellet (insoluble fraction of IBs) were analyzed by SDS-PAGE. Target protein was determined densitometrically as a fraction of the total proteins by profile analysis using a BIO-RAD imaging densitometer – model GS-670.

3. Results and Discussion

3. 1. Contraction of IBs at Low pH and Sedimentation Velocity

Transferring IBs from neutral (pH 7) to acidic pH (pH 4) buffer resulted in a significant decrease in IBs volume (by at least one third) as was first observed at macro level after centrifugation and later confirmed under electron microscope. The contraction was found to be irreversible.[17] As a consequence of contraction, the density of

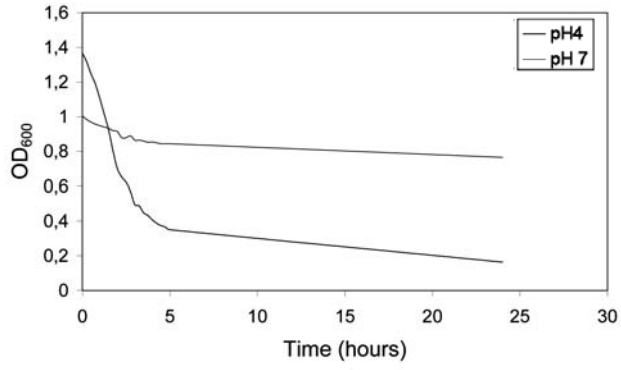

Figure 1: Sedimentation of IBs measured as a change in optical density at 600 nm (OD_{600}) of IBs suspension. Contraction of IBs treated with low pH buffer results in higher density of IBs, therefore they sediment faster.

IBs increased. This resulted in the sedimentation velocity changes during centrifugation. After centrifugation under the same conditions the pelet become more compact when IBs were previously treated with low pH buffer. Sedimentation of IBs due to gravitation in cuvette was also observed. Results presented at Figure 1 shows that contracted IBs sedimented faster.

3. 2. Solubility of ncIBs

The contraction of IBs at low pH affected not only IBs density but also other IBs properties. Results show, that non-classical IBs were very soluble in common washing solutions containing detergents such as 1% deoxycholate.[11] Results show, that up to 20% of target protein can be lost from IBs in a single washing step. We found that even lower detergent concentrations, e.g., 0.1% deoxycholate, resulted in significant protein loss. However, ncIBs previously treated with low pH buffer were less soluble in detergents, which resulted in reduced amount of protein extracted from IBs during washing (Figure 2).

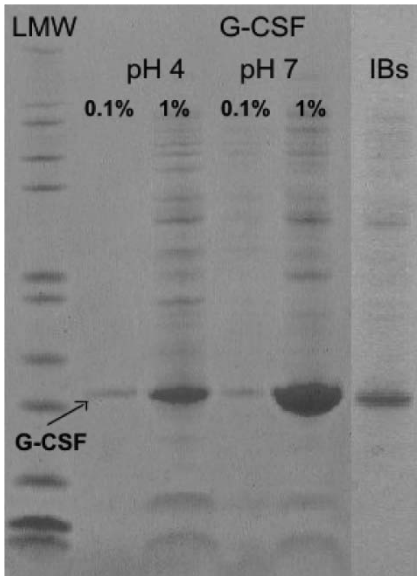

Figure 2: At neutral pH the IBs containing G-CSF are very soluble in common washing solutions containing detergents, such as 1% deoxycholate and even in lower detergent concentrations, e.g., 0.1% deoxycholate, which result in significant loss of the protein. When IBs are previously treated with low pH buffer, less protein is lost during washing.

Densitometry analysis of SDS-PAGE gel showed, that even washing of contracted IBs with 1% deoxycholate, could wash out up to 5% of target proteins in a single washing step. Classical washing, using relatively high concentrations of detergents, should therefore be carefully applied, otherwise in some cases complete loss of IBs can occur during washing.

3. 3. Extractability of Target Protein from ncIBs

The solubility of ncIBs into mild detergents could however also be used as an advantage in isolation of target proteins from ncIBs. G-CSF IBs produced at 25 °C were easily soluble in 0.2% N-lauroyl sarcosine. More than 95% of G-CSF could be dissolved from IBs. Using no renaturation procedure, up to 50% of the extracted target protein molecules were present in the biologically active form.[17] When IBs were treated in low pH buffer before protein extraction, only up to 70% of G-CSF could be dissolved from IBs.

3. 4. Porosity of IBs and Suggested Models

Electron microscopy figures show, that IBs are highly porous structures (Figure 3) as previously described by several authors.[3,17,18] During rapid washing of IBs with 0.2% N-lauroyl sarcosine, internal structure of IBs, composed of smaller granules (proto-aggregates) imbedded in a cotton-like matrix became clearly visible under electron microscope.[17]

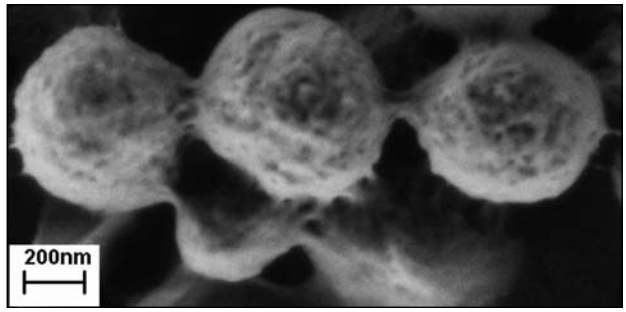

Figure 3: IBs from *E. coli* cultivated at 25 °C, prepared by cell disruption in a homogenizer, and then thoroughly washed in pure water.

Following the results presented above, a model was proposed to explain IBs formation and internal structure. An Attractor Model is presented in Figure 4. A similar model has previously been proposed by Kopito.[16] for IBs formation in animal cells, describing inclusion bodies as aggregates of aggregates. In Kopito's model aggregates are actively transported on microtubules to join together, whereas in our case, in bacterial cells, aggregation occurred passively (IBs were one by one attracted together). We believe that after fusion of proto-aggregates the IBs continued to grow spontaneously to form the final IBs aggregates.

It appears that granules (proto-aggregates) are imbedded into a cotton-like amorphous matrix, which probably belongs to a mass of misfolded proteins. Reports in recent articles suggest that these aggregates share impor-

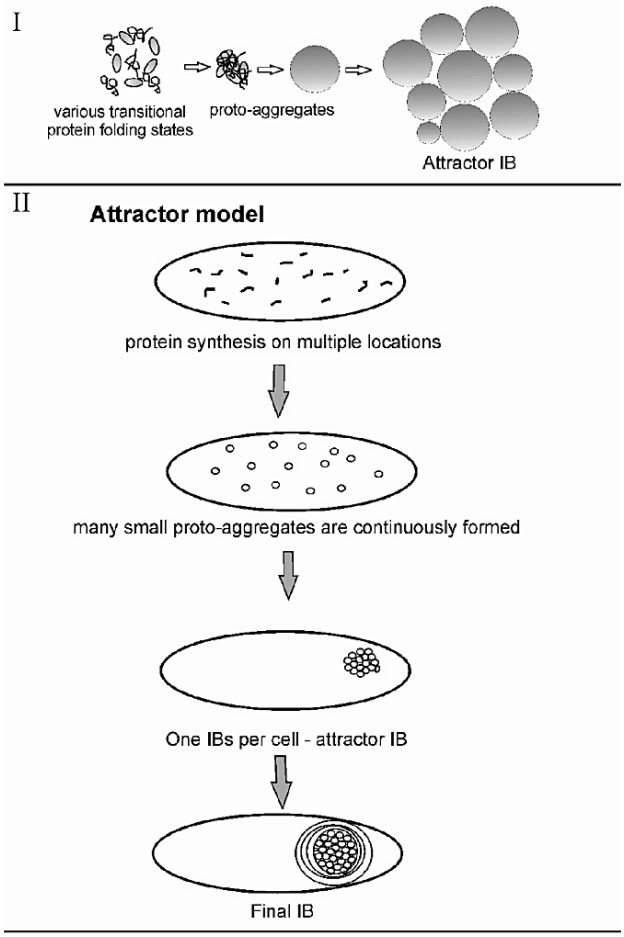

Figure 4: Suggested models for IBs formation. During over-expression of homologous as well as heterologous genes, protein misfolding commonly occurs. Therefore various transitional folding states of the target protein are simultaneously present in the cell. Due to the hydrophobicity and rapidly exceeded solubility of these transitional forms, many small proto-aggregates are continuously formed (Fig. 4 I). In a proposed Attractor Model these proto-aggregates are glued together in an »attractor IB« which will then grow further to form the final IB as depicted in Fig. 4 II.

tant structural and biological features with amyloids.[19] However, Congo red dye, which is usually used for amyloid detection, did not bind to our IBs, which denied the amyloid-like structure of our IBs.[17] It may be the case that the spaces both among and inside the granules are filled with a similar amorphous matrix but composed mostly of unfolded proteins associated by hydrophobic interactions. Inside the network, there may thus be amount of spaces large enough to allow properly folded protein precursors to be trapped (Figure 5). At pH values around 7 the whole network is loosely bound, allowing easy extraction of native-like precursor molecules as well as some still soluble proteins having biologically inactive conformations.[11] In contrast, by transferring IBs into pH 4 buffer, the high proton concentration could induce a change in unfolded proteins, leading to strong contraction of the network and formation of more compact IBs where properly folded precursors are trapped and their extraction prevented. This hypothesis was supported by faster sedimentation rates of IBs observed at low pH, which indicated a higher density of IBs. Finally, we excluded the possibility that properly folded G-CSF molecules made a significant contribution to contraction, as it is well known that conformation of native G-CSF is very stable at low pH,[20] as seen by the two-year shelf life of the protein drug formulated at pH 4 and stored at temperatures around 5 °C.

4. Conclusions

Important implications of new properties of ncIB in biotechnology

Preliminary experiments with some other proteins, such as N-terminally truncated form of tumor necrosis factor alpha, N-terminally truncated form of tumor necrosis factor beta and green fluorescent protein show that the new properties described here were also found in other structurally unrelated proteins.

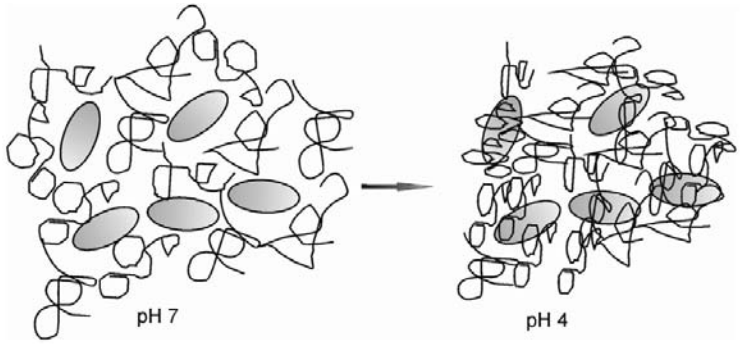

Figure 5: A putative model for the internal structure of IBs. At pH around 7, the whole network is loosely bound. By transferring IBs into pH 4 buffer, strong contraction of the network results in more compact IBs from which properly folded protein can no longer be efficiently extracted.

 – Unfolded proteins that putatively form a network and thus compose the cotton-like material of the IBs.

 – Properly folded protein precursor – the protein molecule in its native conformation, with S–S bonds not yet formed. After extraction from the IBs, disulfide bonds are formed spontaneously in contact with oxygen.

In biotechnology, downstream processes, such as centrifugation, can be affected by density of IBs. For instance sedimentation velocity of compact IBs (prepared at pH 4.0) in comparison to less dense IBs (prepared at pH 7.0) is different and can be modulated by pH of the buffer. Even more importantly, the solubility/extractability of IBs depends very much on the buffer pH. As demonstrated with G-CSF, extraction of proteins from ncIBs is much less efficient in the pH region 3–5 than extraction undertaken in conditions of pH 7–8. On the basis of this knowledge, an economically advantageous biotechnological process for G-CSF production and purification was designed.[21,22]

To summarize, the newly described properties of ncIBs could have a profound effect on the efficiency of technological operations in biotechnology.

5. Acknowledgements

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Povzetek

Ireverzibilno krčenje inkluzijskih teles vpliva na njihovo posedanje, raztapljanje v blagih detergentih in ekstrakcijo tarčnih proteinov iz neklasičnih inkluzijskih teles. Krčenje tako vpliva tudi na potek izolacije proteinov iz inkluzijskih teles. Na podlagi rezultatov elektronske mikroskopije ter ekstrakcije proteinov iz inkluzijskih teles smo predlagali model tvorbe inkluzijskih teles.