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REVIEW PAPER

Friendly production of bacterial inclusion bodies

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Abstract−Protein aggregation is commonly observed in genetically engineered bacteria over-expressing foreign genes, in the context of protein production processes. Very often, recombinant polypeptides deposit as insoluble protein clusters named inclusion bodies, whose formation is driven by stereo-specific cross-molecular interactions between partially folded polypeptide chains. The formation of inclusion bodies has been historically considered as the main bottleneck in industrial production processes of proteins, since a wide diversity of protein species tend to aggregate in bacteria. As the formation of inclusion bodies can be eventually minimized but rarely prevented, aggregated polypeptides of industrial interest need to be refolded in vitro before use. However, the progressive understanding of the molecular and physiological mechanisms regulating aggregation has revealed that inclusion bodies contain significant amounts of biologically active protein species making them suitable for the straightforward use in different in vitro processes, as functional, particulate entities. Therefore, when formed by enzymes, inclusion bodies are catalytic particles ready for industrial use. As discussed here, the genetic background of the host bacteria and the protein production conditions can be adjusted to tune the biological and biophysical properties of bacterial inclusion bodies, to gain manipulability and to make them more biotechnologically friendly.

Key words: Inclusion Bodies, Biosynthesis, Proteases, Chaperones, *E. coli*

INTRODUCTION

Bacterial protein aggregation in the form of electro-dense protein clusters was primarily described when growing bacteria in presence of non natural amino acids [1]. Since the late seventies, when recombinant DNA technologies were implemented and *Escherichia coli* was selected as universal cell factory to produce heterologous polypeptides for biotechnological purposes, the deposition of recombinant proteins as large clusters of insoluble polypeptides has been observed as a common event [2], associated with the conformational stress suffered by cells overproducing foreign proteins [3]. The so-called inclusion bodies (IBs) occurred when producing a wide diversity of recombinant protein species, and their formation represents the main obstacle for the production of soluble protein species in protein production processes ruled by bacteria [4]. Usually, IBs are formed in the bacterial cytoplasm as a single or few protein clusters with diameters usually limited by the bacterial cell size (usually up to around 500 nm). Cytoplasmic IBs are refractile, roundish particles when observed under optical microscopy [5,6], electro-dense when determined by transmission electron microscopy (TEM) [7-9] and spherical or rod-shaped under scanning electron microscopy (SEM) [10-12] or atomic force microscopy (AFM) [13]. In all these cases, IBs are devoid of detectable inner structure, although a sub-particulate architecture has been envisaged during controlled proteolytic digestion of isolated IBs [12]. IBs can also emerge in the periplasm when the recombinant, aggregation-prone protein is secreted through the fusion of a leader peptide [11,14-16].

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COMPOSITION AND MOLECULAR STRUCTURE OF BACTERIAL IBs

IBs are insoluble protein aggregates, essentially constituted by the overexpressed recombinant protein that might represent up to 95% of the total protein content [17]. Very often, chaperones [18] and other cellular proteins [7,8,19] have been observed during proteomic studies in minor amounts. Nowadays, it is widely accepted that, although they are seen as refractile [6] and electrodense particles [11,20] when observed by optical and transmission electronic microscopy respectively, IBs are highly hydrated [21] and porous [12] aggregates.

Being the formation of bacterial IBs associated with protein misfolding events [4], the conception of IB architecture, structure and biology has dramatically evolved. It had been largely assumed that IBs were non-ordered protein structures devoid of any internal organization. However, several evidences suggest that, despite IB macroscopic amorphous appearance, these protein aggregates have, not only a high degree of intrinsic organization, but also many similarities with the highly structured amyloid fibrils [22-25]. In this context, nowadays, the amyloid-like nature of inclusion bodies is widely accepted [22,26]. First, IBs analyzed by Fourier transform infrared spectrometry (FTIR) show a peculiar secondary structure, characterized by a signal around 1,620 cm^{-1} in the amide I region [27-29]. In fact, FTIR spectroscopy of IBs reveals the existence of both an important fraction of highly ordered, amyloid-like, β-sheet enriched structure and also a significant fraction of both native-like secondary structure and disordered conformations [24,30], which interestingly coexist. In accordance with this data, the circular dycroism (CD) spectrum of IBs in the far UV-region also points out the domi-

nance of a β-sheet secondary structure [23]. Regarding secondary structure, nuclear magnetic resonance (NMR) and X-ray diffraction data strongly supports the formation of amyloid-like structures inside IBs as a general phenomenon [23]. Additionally, Wasmer and collaborators, using solid-state NMR and H/D exchange, have shown indistinguishable spectra for both IBs and amyloid fibrils [31], proving a matching organization of IBs and fibrils on a molecular level. In support of all these observations, both Thioflavin T (Th-T) and Congo Red (CR), which are amyloid specific dyes, clearly bind IBs [22,23,25,32], this fact being a further evidence of the highly ordered, amyloid-like structure supporting the IB organization [25]. Moreover, it has also been reported that 2-(p-toluidinylnaphthalene)-6-sulfonate (TNS), which interacts with exposed hydrophobic surfaces, also binds IBs [22]. Thus, such a set of recent results strongly prompts one to conclude that conformational versions of the same polypeptide, with different degrees of native-like conformation and amyloid structure, nicely coexist in IBs.

Regardless of the target recombinant protein, IBs are formed through self-association protein processes governed by the seeding capacity of growing aggregate cores [22,26], thus commonly resulting in only one IB per cell [6]. Interestingly, when co-producing different aggregation-prone proteins in single cells co-aggregation is not observed [33], showing that IB formation, far from being a diffusion-limited process, is a selective process driven by stereospecific interactions, as suggested by King and co-workers [34] and discussed below. It must be noted, however, that the volumetric growth of IBs in producing cells is a highly dynamic event, as protein deposition is accompanied by a steady protein removal [5], mediated by the combined activity of cell chaperones and proteases [18,35-38].

Finally, it has also been recently reported that IBs can be even more stable and resistant to adverse chemical environments than their soluble counterparts [23], although the stability against chemical denaturation or proteinase K attack might be different depending on the specific protein species forming the IBs [39]. Furthermore, IBs are mechanically stable and resistant to ultrasonic treatment, lyophilization and long-time storage at different temperatures [13]. All these features, make these protein clusters suitable for use as fully biocompatible, particulate materials for different applications, including the engineering of nanoscale topography for the control of mammalian cell proliferation in regenerative medicine [13].

IB PURIFICATION AND PROTEIN REFOLDING OR EXTRACTION

Upon cell disruption, usually mediated by mechanical methods, IBs can be separated from soluble proteins and other cell components by differential sedimentation upon high speed centrifugation [40]. Pellets resulting from this process are usually contaminated with cell membranes that can be further removed by repeated washing with detergent-containing buffers [12]. Although gradient ultracentrifugation would provide highly pure IBs [40], detergent washing seems to be sufficient for most of the further uses of IBs. Since in contact with mammalian cells, such purified IBs do not shown any sign of cytotoxicity [13], this simple procedure is probably removing most of the significant cell derived, IB surface contaminants. From such isolated IBs, the forming polypeptide can be rescued in vitro by chemical denaturation, usually by adding chaotropic agents such as urea at high concentrations, followed by a progressive recovery of the conditions allowing protein folding, usually by several step dialysis [17]. Refolding protocols have been refined and adapted to specific products [41], and are often used by pharmaceutical industries to obtain protein drugs from bacterial cell factories [42] in a form suitable for use.

Lately, it has been shown that IBs produced under specific conditions (usually when producing bacteria are cultured at sub-optimal temperatures) contain functional polypeptides that can be released as active products by mild denaturing treatments [10,43,44]. This is probably due to the enhanced abundance of loosely embedded protein species showing native-like secondary structure and high biological activity, when IBs are formed at low temperatures, being the general quality of the cellular proteins (both soluble and insoluble) then enhanced [45]. It might be also possible that the in situ protein refolding processes identified within IBs [29] could be favored during IB formation at low temperatures.

PROTEIN PURITY AND BIOLOGICAL ACTIVITY IN IBs

The occurrence of one or very few IBs in the cytoplasm of a single bacterial cell, in which the protein synthesis is expected to take place rather homogeneously, indicates a seeding-driven aggregation process as discussed below. According to the high purity of IBs regarding protein composition, aggregation could be, in addition, sequence-specific. This was firstly strongly supported by elegant studies carried out in vitro by King and co-workers [34] and confirmed later in vivo [22,33]. In both kinds of setting ups, only homologous polypeptides co-aggregated in the same clusters while heterologous species were excluded and deposited separately. Since there is no molecular basis supporting association of homologous amino-acid regions, that could account for homogeneous composition of aggregates, protein deposition during IBs formation could be stereospecific. Such a possibility would be clearly opposite to the model of IBs as being formed by completely unfolded polypeptides. In fact, native-like secondary structure was firstly determined in interleukin IBs by FTIR [46]. The presence of folded or partially folded polypeptides in IBs was later confirmed for different unrelated proteins using FTIR [22,27-30,35,47-49] and other techniques [50]. Interestingly, the native-like secondary structure of IB proteins is linked to biological activity exhibited by these particles, as shown in IBs formed by both enzymes and fluorescent proteins [51]. The fluorescence emitted by GFP-forming IBs is not restricted to the IB surface [51, 52], indicating that it is not related to contamination of IBs by soluble species from the soluble cell fraction. In fact, the IB external layer is less fluorescent than the core [52] indicating that properly folded species are truly structural components of IB particles. In this context and from a practical point of view, the biological activity of enzyme-forming IBs has recently pushed to explore with success IBs as carriers of immobilized enzymes suitable to be used in catalysis, without any refolding process on purified IBs [51-58] (Table 1).

Interestingly, the genetic background in which IBs are formed can dramatically influence the size and number of IBs per cell [38] and, in addition, the folding state and biological activity of the embedded proteins [13,29,35,49]. In particular, the absence of the main chaperone DnaK or the Lon or ClpP proteases result in larger IB

Table 1. Representative examples of biologically active recombinant enzymes produced as inclusion bodies and straightforward used in bioprocesses

Enzyme	Process	Reference
β-galactosidase (β-gal)	ONPG and CPRG hydrolysis	[51,52]
Human Dihidrofolate Reductase (hDHFR)	Conversion of NADPH to $NADP^+$	[51]
Polyphosphate Kinase (PPK)	ATP/NTP synthesis	[54]
D-Amino acid Oxidase (DAO)	Catalysis of the O_2-dependent transformation of an α-amino acid substrate into α-keto acid, H_2O_2, NH_3	[55]
Maltodextrin Phosphorylase (MP)	Degradation of soluble starch	[53]
Sialic Acid Aldolase (SAA)	Production of neuraminic acid (sialic acid, Neu5ac)	[57]
Polyphosphate Kinase (PPK3)	Synthesis of cytidine monophosphate N-acetylneuraminic	[56]
Cytidilate Kinase (CMK)	acid(CMP- NeuAc) and 3'-sialyllactose	

particles in which the biological activity of the forming protein is enhanced [13,49]. This indicates that DnaK, apart from its disaggregation activities, promotes the proteolysis of aggregated but functional protein species (mainly mediated by the proteases Lon and ClpP), probably acting on the IB surfaces during protein removal [20]. In fact, deficiencies in several key proteins of the cytoplasmic heat shock system, including GroES, GroEL, ClpA and ClpB, result in an increase of the half life of the aggregation-prone recombinant protein, protein yield and often, also, in IB size [20]. This indicates that the bacterial quality control tends to drive aggregated or aggregation-prone proteins into proteolytic pathways. Interestingly, in absence of the small heat shock proteins IbpA and IbpB, usually found associated to IBs [59], recombinant proteins are not stabilized but, on the contrary, their yield is slightly reduced when comparing with the wild type strain [20]. This suggests that, contrarily to other heat shock proteins, IbpA and IbpB inhibit proteolysis as previously suggested [60] and their role in protein removal on the IB surface [61], could be keeping protein integrity as antagonists of DnaK.

The appropriate genetic control of IB activity and the determination of IB size (genetically or by selecting and appropriate harvesting time), should permit a fine tuning of particulate (IB) clusters of enzymes to be adapted to specific technological setting-ups of industrial interest.

PROTEIN SOLUBILITY AND QUALITY: ROLE OF THE QUALITY CONTROL IN IB FORMATION

In recombinant protein production, solubility of recombinant proteins has been paradigmatically pursued, under the assumption that occurrence of the recombinant protein in the soluble cell fraction would be indicative of a good conformational quality [62]. In this regard, the virtual soluble and insoluble cell fractions would mechanistically separate active and inactive polypeptides in a production process and, therefore, different approaches have been explored to minimize IB formation and increase the yield of soluble proteins. Chaperone co-production along the target protein is probably the most employed genetic strategy to increase solubility [63]. However, two separate sets of observations have severely challenged this model. First, as discussed in previous sections, IBs contain significant amounts of functional protein species [36,43,51]. Second,

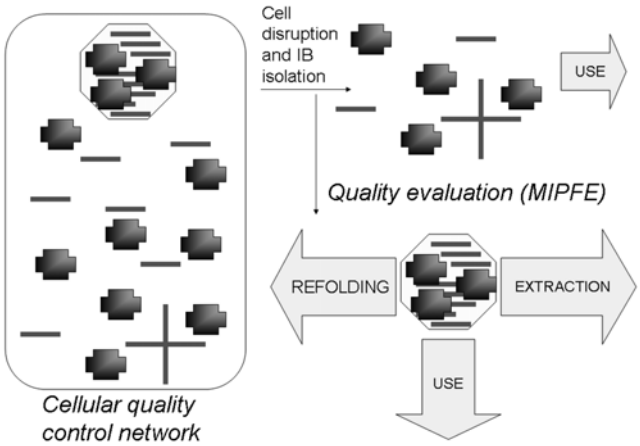

Fig. 1. In the cytoplasm of bacterial cells (left), a recombinant protein can be produced in a diversity of forms, summarized here as properly folded functional species (green squares), or misfolded, less active or inactive protein versions (red lines) that can oligomerize (red squares). Any of these forms can be found as soluble species or embedded as IBs (indicated as octagonal forms). The quality control cell machinery monitors both cell fractions and also the interface between IBs and the cytosol, in which relevant processes such as protein deposition but also disaggregation and removal and in situ proteolysis seem to occur [20,49,61,74-77]. Upon cell disruption and fractioning, both soluble protein and IBs can be isolated (right). As protein solubility does not guarantee functional quality [62], the minimal information for protein functional evaluation (MIPFE) should be obtained before use [73], specially for fine analytical purposes. On the other hand, IB proteins can be recovered by denaturing-refolding protocols [41], non denaturing protocols [43], or directly used in catalytic processes [51-58].

the recombinant proteins present in the soluble cell fraction can form a spectrum of soluble aggregates (adopting globular or fibril-like morphologies), that might be poorly functional [64,65]. Therefore, the folding machinery of the hosting cell must be observed as equally monitoring soluble and insoluble cell fractions [52,66] (Fig. 1), whose quality, in terms of the recombinant protein, seems to be gained or lost in parallel by conditions affecting protein folding, such as temperature or chaperone availability [45,67]. In addition, when exploring the range of different conditions affecting protein quality, namely

the bacterial genetic background or the external supply of chaperones, it has been recently observed that the higher the yield of soluble protein species poorer is the biological quality of the obtained protein [49,68]. This fact, that has been also recently reported in eukaryotic cell factories (namely insect cells and insect larvae) [69,70], prompts one to carefully explore the specific requirements requested for a given protein and to evaluate in advance the form in which it must be most conveniently obtained for its further use. In this context, IBs could turn from undesired by-products to industrially appealing clusters of functional proteins, from which functional proteins can be refolded by conventional procedures [41], released by non denaturing conditions [43] of straightforward used as immobilized enzymes (within the same IBs) in catalytic processes [53,57, 58,71] (Fig. 1).

Equally important, the identification of the soluble population of recombinant proteins as formed by an heterogeneous spectrum of conformational variants (including soluble aggregates) [64,65], strongly pushes one to deeply evaluate the functional quality of soluble preparations, specially if to be used for interactomic of other fine proteomic analysis. This need has been recently discussed in international forums [72] and revised in the context of recombinant protein production [73]. In this regard, a set of minimal information that should be provided when producing a soluble recombinant proteins has been recently defined [73], to evaluate its functional performance and to better interpret any functional data obtained upon its use in analytical in vivo experiments (Fig. 1).

The continuum of conformational and functional forms (soluble and insoluble) that a recombinant protein can adopt, upon its controlled in vivo production, pictures the cell factory as an extremely complex environment, in which intricate machineries coordinately act to minimize the negative impact of the conformational stress on the cell. These activities, among which protein degradation and aggregation are main players, can be controlled, at some extent, to drive the cell factory towards the production of a narrowed set of forms of interest for defined purposes. For diverse applications, and specially if involving enzymatic catalysis, such more convenient forms might unexpectedly be IBs, and bacterial cells and target proteins are already being engineered by biotechnologists in a way that controlled aggregation and the formation of highly active IBs are favored.

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