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Quality comparison of recombinant soluble proteins and proteins solubilized from bacterial inclusion bodies

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ABSTRACT

Recombinant protein production in bacteria is often accompanied by the formation of aggregates, known as inclusion bodies (IBs). Although several strategies have been developed to minimize protein aggregation, many heterologous proteins are produced in aggregated form. For these proteins, purification necessarily requires processes of solubilization and refolding, often involving denaturing agents. However, the presence of biologically active recombinant proteins forming IBs has driven a redefinition of the protocols used to obtain soluble protein avoiding the protein denaturation step. Among the different strategies described, the detergent n-lauroylsarcosine (NLS) has proved to be effective. However, the impact of the NLS on final protein quality has not been evaluated so far. Here, the activity of three antimicrobial proteins (all as GFP fusions) obtained from the soluble fraction was compared with those solubilized from IBs. Results showed that NLS solubilized proteins from IBs efficiently, but that protein activity was impaired. Thus, a solubilization protocol without detergents was evaluated, demonstrating that this strategy efficiently solubilized proteins embedded in IBs while retaining their biological activity. These results showed that the protocol used for IB solubilization has an impact on final protein quality and that IBs can be solubilized through a very simple step, obtaining fully active proteins.

Introduction

Since the advent of recombinant DNA technologies, the recombinant protein production field has experienced significant progress [1]. In this regard, microorganisms are still one of the most widely used expression systems, with *Escherichia coli* being by far the preferred choice [2]. Although some heterologous proteins of pharmaceutical or biomedical interest are mainly produced in a desirable soluble form, many others are produced as cytoplasmic aggregates, also known as inclusion bodies (IBs) [3–6]. To avoid, or at least minimize, IB formation and increase the amount of the soluble protein fraction, different approaches have been proposed [7]. The strategies include the optimization of expression conditions (e.g. temperature, inducer concentration, or media composition), the use of solubility enhancing tags (e.g. maltose-binding protein (MBP), thioredoxin A (TrxA) or glutathione S-transferase (GST)), the secretion of the heterologous protein to the culture medium or *E. coli* periplasm, the co-expression of chaperones during the production process and the use of mutant strains [8]. However, in many cases, this is not sufficient to reach the desired soluble protein production yields. For such cases, different protocols have been developed for the extraction of soluble proteins from IBs.

Traditionally, IBs have been solubilized by application of harsh denaturing and high concentrations (6–8 M) of chaotropic agents, such as urea or guanidine hydrochloride (GdnHCl), along with reducing agents such as β-mercaptoethanol and dithiothreitol [9]. Consequently, the protein released from the aggregates undergoes complete denaturation, requiring a refolding step to recover the bioactive native conformation of the protein of interest [10]. However, the progress made over the last decade concerning the nature of IBs has evidenced that these protein aggregates are structured amyloid-like nanoparticles containing biologically active and properly folded recombinant protein [11–14]. This has driven different groups to redefine the methodologies used to obtain soluble protein using IBs as a protein source. The use of a high concentration of chaotropic agents has been substituted by non-denaturing protocols that avoid complete denaturation and usually refolding steps [15–17]. The use of mild detergents such as

Abbreviations: DMSO, dimethyl sulfoxide; GdnHCl, guanidine hydrochloride; GFP, Green Fluorescent Protein; GST, glutathione S-transferase; HD5, human α-defensin 5; IB, inclusion body; IPTG, isopropyl β-D-1-thiogalactopyranoside; LAP, lingual antimicrobial peptide; LB, Luria-Bertani; MBP, maltose-binding protein; NLS, n-lauroylsarcosine; TrxA, thioredoxin A.

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n-lauroylsarcosine (NLS) or lauroyl-L-glutamate takes advantage of the nature of IBs, enabling release of correctly folded protein without the need for expensive and time-consuming refolding procedures [18,19]. In addition, low concentrations of organic solvents, such as n-propanol, trifluoroethanol and isopropanol [20–23], as well as dimethyl sulfoxide (DMSO), have also been demonstrated to be suitable as IB solubilizing agents, without affecting the native structure of the released protein [24]. Alcohols are well described for not only protecting but also promoting the secondary structure of the protein [25,26]. Other approaches combine low amounts of denaturing reagents with the adjustment of either physical parameters, including heat [27], high hydrostatic pressure [28], and freeze-thaw cycles [29] or chemical factors, such as pH oscillations [30], to accomplish solubilization of IBs in a non-denaturing manner.

Remarkably, despite the extensive description of novel solubilization methods and their inherent benefits, where NLS is one of the detergents most widely used, the comparison of the solubilized protein quality with its soluble counterpart remain unexplored, although being crucial for the evaluation and validation of the whole IB solubilization process. In this study, three short antimicrobial peptides, namely lingual antimicrobial peptide (LAP), human α-defensin 5 (HD5), and human cathelicidin LL-37, fused to Green Fluorescent Protein (GFP) to minimize proteolysis, have been produced in *E. coli* and either purified directly from the soluble fraction or using IBs as a source of soluble protein through a mild solubilization protocol, seeking to determine any impact of the protocol used on the final protein quality.

Materials and methods

Bacterial strains and growth media

Escherichia coli BL21 (DE3) strain was used for recombinant protein production. The strain selected for antimicrobial activity evaluation was *E. coli* DH5α. Both strains were grown in Luria-Bertani (LB) medium (10 g/L NaCl (Merck, Darmstadt, Germany), 5 g/L yeast extract ((ThermoFisher, Kandel, Germany), 10 g/L tryptone (VWR International, Leuven, Belgium)).

C. onstruction of expression plasmids

The active forms of bovine lingual antimicrobial peptide (LAP; Uniprot Q28880, V25-K64), human α-defensin 5 (HD5, Uniprot Q01523, A63-R94), and cathelicidin LL-37 (Uniprot P49913, L134-S170) were fused to GFP using the linker sequence GGSSRSS. Each protein sequence was C-terminally fused to a hexahistidine (H6) tag for purification purposes. The resultant DNA sequences (LAP-GFP-H6 (32.53 kDa), HD5-GFP-H6 (31.79 kDa), and LL-37-GFP-H6 (32.66 kDa)) were chemically synthesized while optimizing codon usage for *E. coli* expression platform (GeneArt®, Life technologies, Regensburg, Germany). Each construct was cloned into a pET22b (Amp^R) vector and transformed by heat shock in competent *E. coli* BL21 (DE3) cells.

Protein production kinetics

E. coli BL21 (DE3)/pET22b-LAP-GFP-H6, *E. coli* BL21 (DE3)/pET22b-HD5-GFP-H6, and *E. coli* BL21 (DE3)/pET22b-LL-37-GFP-H6 were grown overnight (O/N) in LB broth supplemented with 100 μg/mL ampicillin (VWR International LLC, Sanborn, NY, USA), for plasmid conservation, at 37 °C and 250 rpm. O/N cultures were inoculated in 200 mL of LB media with 100 μg/mL ampicillin in 1 L shake flasks (at an initial OD of 0.05) and grown at 37 °C and 250 rpm until reaching an OD_{600} of 0.4–0.6. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, PanReac AppliChem, Barcelona, Spain). Thereafter, cultures were grown at 37 °C and 250 rpm, and 25 mL samples were withdrawn at 0, 1, 3, and 5 h post-induction. Cells were then harvested and recovered by centrifugation at 6000 × *g* for 15 min at 4 °C. These cultures were performed in triplicate.

To determine protein fractionation, pellets from 500 mL culture were resuspended in 30 mL phosphate-buffered saline (PBS) with an EDTA-free protease inhibitor cocktail (cOmplete EDTA-free, Roche Diagnostics GmbH, Manheim, Germany). Ice-jacketed samples were disrupted by sonication (2 cycles of 3 min at 10 % amplitude under 0,5 s cycles) (Branson SFX550 Sonifier, Branson Ultrasonics Corporation, Danbury, CT, USA). The soluble and insoluble fractions were separated by centrifugation (15,000 × *g*, 15 min, 4 °C), and both fractions were stored at − 80 °C until quantification by Western blot and Coomassie blue staining (BioRad Laboratories, Hercules, CA, USA) (Supplementary Fig. 2).

Protein production and purification

For production purposes, two shake flasks of 2.5 L with 500 mL of LB media supplemented with 100 μg/mL of ampicillin were inoculated with O/N cultures at initial OD_{600} of 0.05, and each culture was incubated at 37 °C and 250 rpm until reaching an OD_{600} of 0.4–0.6 when protein expression was induced with 1 mM of IPTG (IPTG, PanReac AppliChem, Barcelona, Spain). After 3 h of induction, the whole culture was harvested by centrifugation at 6000 × *g* for 15 min at 4 °C, the supernatant was discarded, and the pellet was stored at − 80 °C.

Pellets were resuspended in binding buffer (500 mM NaCl, 20 mM Tris, 20 mM Imidazole) with EDTA-free protease inhibitor cocktail (cOmplete EDTA-free, Roche Diagnostics GmbH, Manheim, Germany) and disrupted by sonication (4 cycles, 5 min, at 10 % amplitude under 0,5 s cycles) (Branson SFX550 Sonifier, Branson Ultrasonics Corporation, Danbury, CT, USA). After cell disruption, samples were centrifuged at 15,000 × *g*, 45 min, 4 °C, and soluble and insoluble (pellet with inclusion bodies (IBs)) fractions were separated.

To solubilize protein from the insoluble fraction (IBs), the pellet obtained after centrifugation was washed with dH_2O, centrifuged (15,000 × *g*, 45 min, 4 °C) and the supernatant was discarded. The pellet was weighed and 40 mL of solubilization buffer (0.2 % n-lauroylsarcosine (NLS, Sigma-Aldrich, St.Louis, MO, USA), 40 mM Tris (VRW chemicals, Solon, OH, USA) pH = 8.2) were added per gm of pellet. The mixture was solubilized for 40 h at room temperature (RT) under gentle stirring. The samples were equilibrated prior to the purification step by addition of NaCl (500 mM final concentration, Merck, Darmstadt, Germany) and imidazole (20 mM final concentration, Merck KGaA, Darmstadt, Germany). Finally, samples were centrifuged at 15,000 × *g*, 45 min, 4 °C, recovering the supernatant with the solubilized protein. Both soluble and solubilized proteins were purified using the protocol described below.

Samples were filtered (Ø 0.2 μm filters (VWR north America, USA) and purified by Immobilized Metal Affinity Chromatography (IMAC) in an ÄKTA Start (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using 1 mL HisTrap chelating HP columns (GE Healthcare AB, Uppsala, Sweden). Protein was loaded into the column in binding buffer and eluted with an increasing gradient of imidazole, mixing both binding and elution buffer (500 mM NaCl, 20 mM Tris, pH 7.4, 500 mM Imidazole). For the solubilized proteins, 0.2 % NLS was also added to the binding and elution buffers. Finally, protein buffer exchange was performed with 5 mL HiTrap Desalting columns (GE Healthcare AB, Uppsala, Sweden), using phosphate buffer (10 mM KPi (3:1 K_2HPO_4: KH_2PO_4) pH 7.4, 12.5 mM NaCl). The amount of purified protein was determined by NanoDrop™ (ThermoScientific, Wilmington, DE, USA) and the integrity and purity by SDS-PAGE (Supplementary Fig. 2).

Antimicrobial activity assay

The effect of the different antimicrobial candidates was evaluated with the Bactiter-Glo™ Microbial Cell Viability kit (Promega, Madison, WI, USA). Briefly, the selected strain to assess the bactericidal activity (*E. coli* DH5α) was grown O/N at 250 rpm and 37 °C and then diluted

1:100 in KPi buffer (10 mM KPi buffer pH 7.4). 150 µL of the bacterial dilution were aliquoted and centrifuged at 6200 × *g*, 15 min at 4 °C. The supernatant was removed, and the bacterial pellet was resuspended in 150 µL of either antimicrobial treatment (soluble or solubilized LAP-GFP, HD5-GFP, or LL-37-GFP) or KPi buffer as negative control. Samples were incubated in a sterile polypropylene 96-well (Costar, Kennebunk, ME, USA) microtiter plate for 5 h at 37 °C without agitation. 100 µL of each well were transferred on a sterile 96-well opaque microtiter plate (ThermoFisher, Roskilde, Denmark) and mixed with 100 µL of the BacTiter-Glo™ reagent. The plate was incubated for 5 min, and subsequently, luminescence was measured using a microplate luminometer (LumiStar, Omega Ortenberg, Germany). The registered arbitrary luminescence was normalized against the control (KPi treatment).

Evaluation of N-lauroylsarcosine effect in soluble and solubilized protein

To evaluate the effects of NLS on the performance of the soluble and solubilized protein preparations, different conditions were assessed. All the tested combinations are summarized in Table 1. With the soluble protein, two binding buffers were examined for pellet resuspension before sonication, namely the standard buffer (500 mM NaCl, 20 mM Tris, pH7.4, 20 mM imidazole) described in protocol 1 (S) and another with the same composition plus 0.2 % NLS, protocol 2 (S-NLS). For the solubilized proteins, three different protocols were evaluated, two with NLS during solubilization, but differing in the purification buffer composition (protocol 3 (ST-NLS) and protocol 4 (ST-pNLS)). And the last protocol without using detergent (ST) with solely 40 mM Tris buffer was also used to solubilize proteins during 40 h at RT under gentle agitation.

Statistical analysis

For all assays, each condition was performed in triplicate and the results are expressed as the means of non-transformed data ± standard error of the mean (SEM). Data were checked for normality (JMP software, SAS Institute Inc., Cary, NC, USA) and transformed when required (SQRT transformation of data from Fig. 3A and LN transformation of data from Fig. 4). The p-values and letters correspond to the ANOVA and Tukey test analyses, respectively.

Table 1
Experimental conditions used to purify LAP-GFP-H6, HD5-GFP-H6, and LL-37-GFP-H6 from both soluble and insoluble fractions. S: soluble; ST: solubilized; NLS: n-lauroylsarcosine.

Protocol no.	Sonication buffer	Purified fraction	Solubilization buffer	Purification buffers	
				Binding buffer	Elution Buffer
1- S	500 mM NaCl 20 mM Tris 20 mM Imidazole	Soluble	–	500 mM NaCl 20 mM Tris 20 mM Imidazole	500 mM NaCl 20 mM Tris 500 mM Imidazole
2- S-NLS	500 mM NaCl 20 mM Tris 20 mM Imidazole 0.2 % NLS				
3- ST-NLS	PBS	Insoluble	40 mM Tris, 0.2 % NLS	500 mM NaCl 20 mM Tris 20 mM Imidazole 0.2 % NLS	500 mM NaCl 20 mM Tris 500 mM Imidazole 0.2 % NLS
4- ST-pNLS				500 mM NaCl	500 mM NaCl
5- ST			40 mM Tris	20 mM Tris 20 mM Imidazole	20 mM Tris 500 mM Imidazole

Results

Protein production

The distribution of the three proteins used in this study (LAP-GFP-H6, HD5-GFP-H6, and LL-37-GFP-H6) in the soluble and insoluble fractions of recombinant *E. coli* cultures was determined (Fig. 1A). LAP-GFP-H6 and LL-37-GFP-H6 were equally distributed between both fractions, especially at longer production times (Fig. 1A,C). In contrast, HD5-GFP-H6 produced was mainly insoluble, reaching aggregation values of 75–85 % (Fig. 1B).

Despite these fractionation differences, all the proteins were produced in sufficient quantity in both soluble and insoluble form, such that it was possible to purify them from both fractions (cytoplasmic and solubilized from IBs). The soluble form was purified using protocol 1 (Table 1), and the solubilized forms were purified after incubation of IBs with NLS to solubilize the protein aggregates (Table 1, protocol 3). In the purification process, LAP-GFP-H6 and HD5-GFP-H6 elution profiles were distributed among three peaks, and LL-37-GFP-H6 in two peaks, independent of whether the protein was obtained from the soluble (S) or insoluble fraction (ST-NLS) (Table 2, Supplementary Fig. 1).

Antimicrobial activity

Analyzing the antimicrobial activity of the protein eluted in each peak, in general the soluble protein was significantly more active than the protein purified from the solubilized IBs (Fig. 2, A ($p = 0.05$); B ($p = 0.0079$); C ($p < 0.0001$)). This was particularly clear at 5 µM, where the highest activity was reached. Different elution peaks of the soluble version did not show differences in antimicrobial activity, except for LL-37-GFP-H6, for which peak 1 was considerably more active at 5 µM (Fig. 2). Although no relevant differences were observed for the activities of LAP-GFP-H6 and HD5-GFP-H6 elution peaks, protein yield revealed differences, the protein amount of peak 2 being the highest one for both soluble proteins (Table 2).

To determine if the mild detergent (NLS) used to solubilize the protein had a negative impact on the antimicrobial activity, IBs of two proteins (LAP-GFP-H6, as an example of protein with low aggregation levels, and HD5-GFP-H6, as an example of prone-to aggregate protein (Fig. 1)) were solubilized in Tris buffer without NLS (Table 1, protocol 5) and the solubilized (ST) protein activity was compared with that obtained from the soluble fraction (S) and the protein solubilized using detergent (ST-NLS) (Fig. 3, A ($p = 0.0001$); B ($p = 0.002$)). Interestingly, the protein solubilized without any detergent (ST) had an activity comparable to that observed for the soluble version (S) for LAP-GFP-H6 (Fig. 3A) or even greater for HD5-GFP-H6 (Fig. 3B).

To validate these results, different combinations were compared. The activities of the soluble protein (S) purified using protocol 1 or with buffers containing NLS (S-NLS) (protocol 2), solubilized protein using NLS in the entire process (ST-NLS) (protocol 3), or with NLS but purified with buffers free of detergent (ST-pNLS) (protocol 4), and solubilized and purified protein without NLS (ST) (protocol 5) were compared (Fig. 4, ($p < 0.0001$)). This experiment showed that only the soluble protein (S) and the protein solubilized without using detergent in the whole process (ST) showed good levels of activity (Fig. 4). In contrast, when NLS was used in the solubilization and/or purification process the antimicrobial activity significantly decreased (Fig. 4, ($p < 0.0001$)).

Discussion

Since many proteins of interest produced recombinantly are prone to aggregate as IBs, their production in bacterial expression systems as soluble and native forms is often challenging. Different strategies are

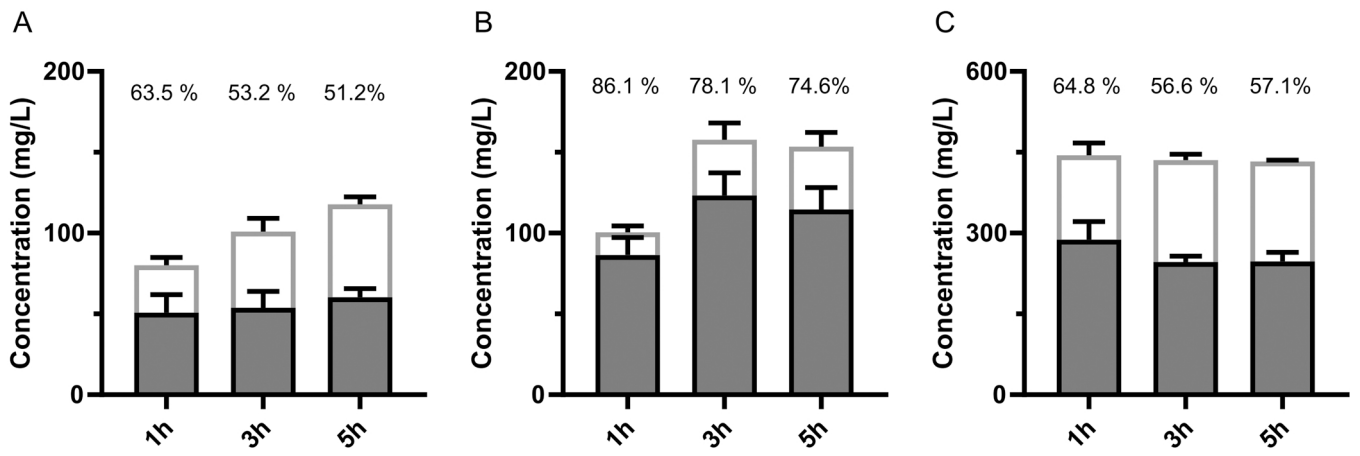

Fig. 1. Production kinetics and soluble/insoluble protein distribution of LAP-GFP-H6 **(A)**, HD5-GFP-H6 **(B)**, LL-37-GFP-H6 **(C)** at 1, 3 and 5 h post-induction. The stacked bars indicate the total amount of protein produced at each time distributed between aggregated fraction (grey) and soluble (white). Values of % aggregation are represented on the top of each condition. Error bars indicate SEM.

Table 2
Peak distribution and yield of the protein obtained from the soluble fraction (soluble (S) and solubilized from IBs with n-lauroylsarcosine (ST-NLS). The % of elution buffer is indicated for each peak.

Protein	Format	Fraction	Yield (mg/L)	% B elution
LAP-GFP-H6	S	peak 1	0.62	15
		peak 2	7.02	27
		peak 3	1.86	49
	ST-NLS	peak 1	0.18	14
		peak 2	2.07	18
		peak 3	5.58	32
	ST	peak 1	0.72	33
HD5-GFP-H6	S	peak 1	1.40	14
		peak 2	4.26	30
		peak 3	1.18	100
	ST-NLS	peak 1	1.93	14
		peak 2	4.20	24
		peak 3	1.89	40
	ST	peak 1	1.08	30
		peak 2	0.83	47
LL-37-GFP-H6	S	peak 1	0.96	13
		peak 2	0.87	26
	ST-NLS	peak 1	5.57	10
		peak 2	1.37	25

used to favor the production of these proteins in soluble form [7,8] but there are still many proteins only produced as IBs, making it necessary to use various protocols to extract soluble protein. Although denaturing and refolding procedures have been used for several years, the presence of active protein forms in the IBs has evidenced the need to develop mild strategies for their recovery. Among the different strategies, NLS has been employed for this purpose, using both *E. coli* [19,29,31,32] and *Lactococcus lactis* [16,33] IBs, and has been shown to be a good strategy to obtain soluble and active protein from bacterial aggregates through a simple solubilization process. However, so far, no detailed comparison of the same protein obtained from the soluble and insoluble fraction has been reported.

Here, three different proteins that are produced in both soluble and aggregated forms (Fig. 1) were used to compare the activity of each protein obtained from the soluble fraction or solubilized from IBs with NLS. The proteins are host defense peptides (HDPs) fused to GFP, for which it has already been shown that when produced recombinantly in *E. coli* form active (fluorescent) IBs [34]. In all three cases (LAP-GFP-H6, HD5-GFP-H6, and LL-37-GFP-H6), the purification profile of the protein obtained from the soluble fraction or IBs, solubilized with NLS, was the same (Table 2, Supplementary Fig. 1). However, the antimicrobial activity revealed significant differences (Fig. 2). The soluble form (S) was highly active, especially at 5 μM, whereas proteins purified from the solubilized fraction with NLS (ST-NLS) showed low levels of bactericidal activity (Fig. 2). These results agree with those reported elsewhere, where it was found that in two of the proteins used (GFP and His7ΔN6 TNF-α), a lower percentage of proteins extracted from IBs using NLS was active compared with that purified from the soluble cell fraction [31]. Others have described a similar effect for GST with 0,3 % NLS [35]. This indicated that NLS may interfere with the activity of the purified protein, which was shown by the negative impact of NLS traces on the protein activity (Figs. 3 and 4). After solubilization, proteins are usually purified and dialyzed using standard procedures, but it is known that, after dialysis, traces of detergent can be still present in the solution, which could have an impact on the activity of the purified protein. To minimize the detergent effect, others have previously reported that it is possible to reduce NLS concentration reaching good levels of solubilization at 0.05 % NLS [29]. However, the complete removal of the detergent during the solubilization process and its impact on protein quality were not previously tested. Thus, IB solubilization was evaluated using Tris buffer, pH 7.4, without detergent (protocol 5), demonstrating not only that the solubilized proteins (ST) showed an activity comparable (or higher) to that isolated from the soluble fraction (S), but also that the detergent was not required for the solubilization process (Fig. 3). Although some proteins solubilized with NLS from IBs such as G-CSF have been shown to retain their biological activity [19,31], others, such as those described in this study, and those previously reported [19,31], are affected by the use of detergents. These findings suggest that when non-denaturing protocols are applied for IB solubilization, it is necessary to validate that the solubilization agent does not interfere with the protein's mode of action; for that, both protein yield and activity need to be monitored. Alternatively, for those proteins with an impaired activity when solubilized, a solubilization process could be applied without detergent (Fig. 4) [36]. In this study, it was found that, through this simple process, it is possible to obtain properly folded and active proteins from bacterial aggregates. The purification yields are lower than that obtained from the soluble fraction (Table 2), indicating that this process is less effective than the same process using NLS. However, for those proteins that are only produced as IBs and negatively affected by the presence of detergents, this protocol could be an optimal strategy. Thus, this work is a further step in the exploitation of IBs as a source of soluble proteins, indicating that the organization of IBs, in which active proteins are embedded in an amyloid-like scaffold [37], is dynamic enough to allow the recovery of soluble protein without use of solubilizing agent. Moreover, in this study, we also demonstrated that the solubilization protocols without detergents are effective in IBs produced at 37 °C and not only for those formed at lower temperatures [38]. In

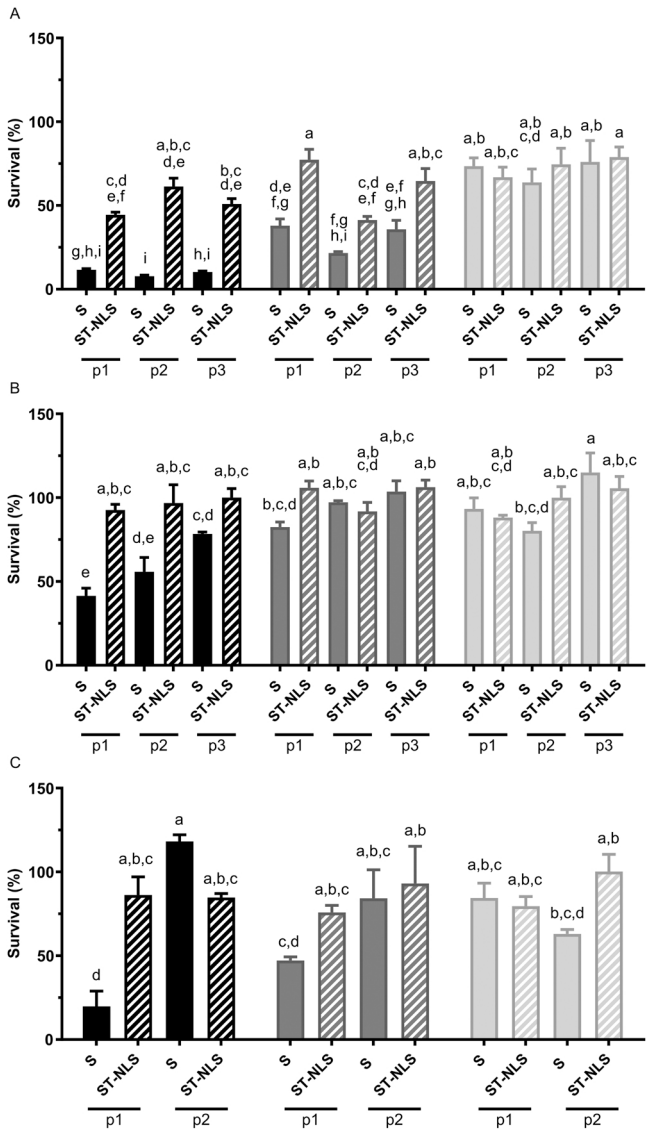

Fig. 2. Antimicrobial activity of the different peaks (p1–p3) of LAP-GFP-H6 **(A)**, HD5-GFP-H6 **(B),** and LL-37-GFP-H6 **(C)** against *E. coli* DH5α at 5 μM (black), 1 μM (grey) and 0.1 μM (light grey). The bars indicate the protein origin of soluble (S), represented with solid bars, or solubilized with n-lauroylsarcosine (ST-NLS), with striped bars. Error bars indicate SEM. Different letters depict significant differences between format (S or ST-NLS), peak, and concentration A ($P = 0.05$); B ($P = 0.0079$); C ($p < 0.0001$).

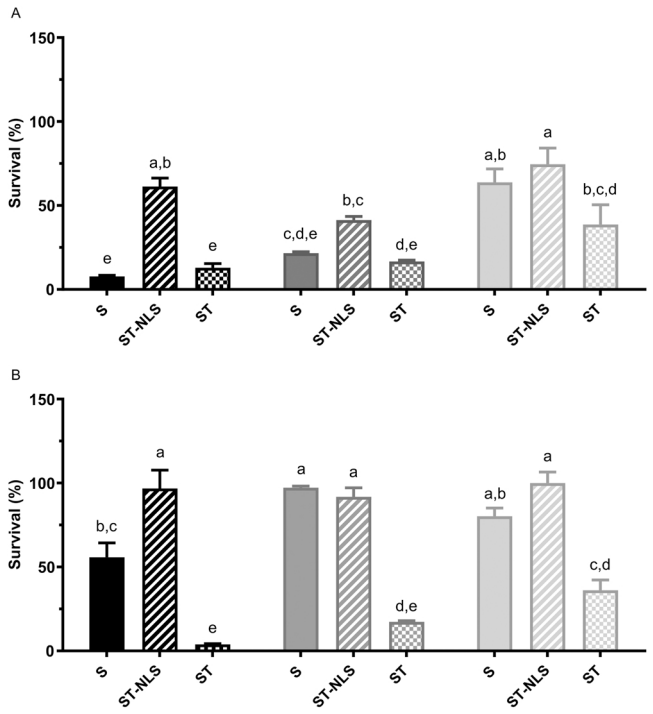

Fig. 3. Bacterial survival (%) of *E. coli* DH5α in presence of LAP-GFP-H6 **(A)** or HD5-GFP-H6 **(B)** peak 2 at 5 μM (black), 1 μM (grey) and 0.1 μM (light grey). The bars indicate the protein origin as either soluble (S), represented as solid bars, or solubilized with (ST-NLS) or without (ST) n-lauroylsarcosine (NLS) represented as stripped or mosaic bars, respectively. Error bars indicate SEM. Different letters depict significant differences between format (S, ST-NLS, or ST) and concentration. A ($p = 0.0001$); B ($p = 0.002$).

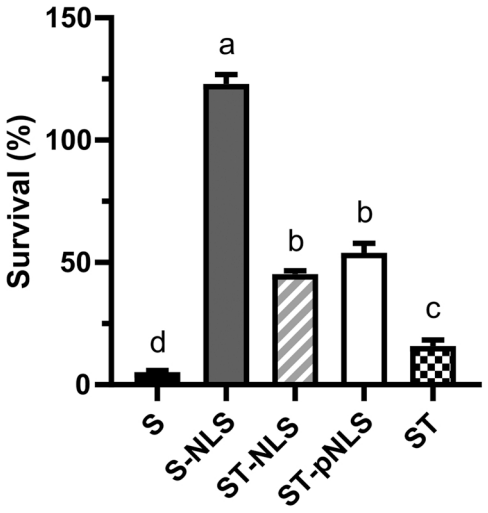

Fig. 4. Bacterial survival (%) of *E. coli* DH5α in presence of LAP-GFP-H6 at 5 μM in different formats: soluble (S); soluble protein purified with NLS buffers (S-NLS); solubilized protein either with NLS in the entire process (ST-NLS) or solely during solubilization (ST-pNLS); and solubilized and purified free of NLS (ST). Error bars indicate SEM. Different letters depict significant differences between treatments ($P < 0.0001$).

agreement with this, it was previously reported that the activity of epimerase, recovered by mild solubilization from IB, is identical when IBs are produced at 37 °C and 25 °C [36].

Conclusion

The comparison of the activity of three different antimicrobial peptides-GFP-fusion proteins, either directly purified from the soluble fraction or solubilized from IBs with a non-denaturing protocol shows that the use of NLS as solubilization agent can have a negative impact on protein activity. Thus, monitoring not only the purified protein yield but also protein activity is necessary to determine the optimal protocol for IB solubilization.

CRediT authorship contribution statement

Adrià López Cano: Investigation, Methodology, Formal analysis, Writing – original draft. **Paula Sicilia:** Investigation. **Clara Gaja:** Investigation. **Anna Arís:** Conceptualization, Supervision, Writing – review & editing. **Elena Garcia-Fruitós:** Conceptualization, Supervision, Writing – review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.09.003.

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