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Time to consider ruling out inclusion bodies denaturing protocols for spontaneous solubilization of biologically active proteins

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The formation of inclusion bodies (IBs) in microbial cell factories is a very common process occurring during recombinant protein production. Different protocols have been developed for the extraction of soluble proteins from IBs using several strategies, ranging from the use of harsh denaturing and high concentrations of chaotropic agents and reducing agents to the use of mild protocols based on the use of non-denaturing detergents. However, in recent years, the biological vision of IBs has changed and research studies have demonstrated that these protein aggregates contain biologically active and properly folded recombinant proteins. This drives us to redefine the methodologies currently used to obtain soluble protein using IB as a protein source. Hence, we propose the extraction of IB protein via the simple spontaneous solubilization of IB as a strategy broadly applicable to all kinds of recombinant proteins without the negative effects of detergents and chaotropic agents on final biological activity. We prove the wide applicability of spontaneous solubilization processes to different types of IBs and that protocols can be easily customized for each protein in terms of timing and incubation temperature by monitoring the protein activity of the solubilized fraction.

Keywords Inclusion bodies, Solubilization, Activity assay, Recombinant production

Recombinant proteins (e.g., enzymes, antibodies, antimicrobials, growth factors, hormones, and vaccines) are becoming very important products with industrial, biomedical and agricultural applications. Microorganisms are the most frequently used hosts for recombinant protein production due to the low cost associated with their fast growth and easily scalable procedures[1]. At present, it is broadly accepted that during recombinant protein processes the formation of inclusion bodies (IBs) in microbial cell factories is very common, not only in the widely used *Escherichia coli* but also in lipopolysaccharide (LPS)-free bacterial recombinant systems, such as *Lactococcus lactis*[2] and *Lactiplantibacillus plantarum*[3] or even in yeast such as *Pichia pastoris*[4]. IBs are protein-based aggregates naturally formed due to cell stress associated with the overproduction of heterologous proteins. Although IBs have long been considered residual by-products of such processes, over the last two decades, it has been extensively demonstrated that they are protein-based nanoparticles formed by biologically active recombinant proteins which have huge potential in biotechnological and biomedical context[5,6]. In fact, they can be directly used as anti-tumorals[7], biocatalysts for enzymatic reactions[8,9], immunostimulants[10] or antimicrobials[11]. However, it is important to consider that IBs can also contain impurities from producer cells such as nucleic acids, lipids, carbohydrates, and other proteins.

For instances in which aggregation occurs and a soluble format is needed, different approaches have been proposed to avoid, or at least minimize, IB formation and increase the soluble protein fraction. These strategies include the optimization of expression conditions, the use of solubility-enhancing tags, the secretion of a heterologous protein in the culture medium or *E. coli* periplasm, the co-expression of chaperones during the production process and the use of mutant strains[12]. However, in some cases, these strategies are not enough to reach the desired soluble protein production yield, and IB solubilization procedures have been developed to extract soluble protein using IB as a protein source. It has also been proven that proteins with difficult purification patterns can be obtained in a purer form when extracted from IBs because these aggregates suppose a pre-

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concentrated sample of recombinant protein[13]. In this context, different protocols have been developed for the extraction of soluble proteins from IBs. Traditionally, IBs have been solubilized applying harsh denaturing and high concentrations (6–8 M) of chaotropic agents, such as urea or guanidine hydrochloride (GdnHCl), along with reducing agents like β-mercaptoethanol and dithiothreitol[14]. After these treatments, the protein released from these aggregates undergoes a complete denaturation and requires a refolding protocol to recover its bioactive native conformation, which is usually highly inefficient. However, in recent years, the biological vision of IBs has changed and research studies have demonstrated that these protein aggregates contain biologically active and properly folded recombinant protein[15]. This droves different groups to redefine the methodologies used to obtain soluble protein using IBs as a protein source. In this context, the use of a high concentration of chaotropic agents has been substituted with non-denaturing protocols that avoid complete denaturation and the need to apply refolding protocols. This has been achieved using mild detergents like n-lauroylsarcosine (NLS) or lauroyl-L-glutamate, which enables the release of correct folded proteins from IBs[13,16–18]. As an alternative, low concentrations of organic solvents have also been demonstrated to be suitable as IB solubilizing agents[19]. Finally, combinations of low amounts of denaturing reagents with the adjustment of either physical parameters, like heat[20], high hydrostatic pressure[21], and freeze–thaw cycles[22] or chemical factors[23] have been used to accomplish the solubilization of IBs in a non-denaturing manner.

Despite the more recent milder solubilization methods described above, some studies demonstrate that non-denaturing detergents can impact the final quality and biological activity[24]. Although some proteins solubilized with NLS from IBs have been shown to retain their biological activity, others are affected using detergents[17]. The main reason for this seems to be those detergent traces, which are difficult to remove, that could impair the final biological activity of proteins recovered from IBs. Recombinant antimicrobial proteins whose activity is significantly reduced by residual traces of NLS are an example[17].

The present study takes a step forward in finding universal strategies for recovering functional recombinant proteins from IBs and ruling out detergents from solubilization protocols. In summary, we describe a screening method to select the optimal conditions for the spontaneous solubilization of different types of recombinant proteins from IBs by testing a broad range of detergent-free solubilizing conditions and correlating them with the final specific activity.

Results

To evaluate the optimal solubilization conditions for IBs formed during the recombinant production of different proteins, a solubilization screening assay was performed using JAMF2, BMAP27GFP and M-SAA3 as model proteins. The proteins M-SAA3 and BMAP27GFP were produced using *L. lactis* as recombinant host whereas for JAMF2 production *E. coli* was used. Both BMAP27GFP and M-SAA3 showed a moderate aggregation rate of 36% and JAMF2 was produced completely as IBs (Table 1). An initial step of IB purification using standard protocols was applied, and the IBs were then resuspended in the optimal buffer for each protein without adding any kind of denaturing agent or detergent (Fig. 1). Herein, we used phosphate buffers (10 mM KPi buffer for JAMF-2 and PBS for M-SAA3) and acetic acid 0.01% (v/v) for BMAP27GFP because they were the most appropriate for performing activity assays for the three model proteins. We first evaluated the solubilization of the BMAP27GFP and JAMF2 IBs by monitoring the antimicrobial activity of the protein present in the recovered supernatant under different conditions (Figs. 2 and 4). Also, another type of assay was performed for each protein, using the enzymatic activity of JAMF2 (Fig. 5) and the fluorescent activity of BMAP27GFP (Fig. 3).

In the case of BMAP27GFP, the two activity assays conducted, the antimicrobial assay against MRSA (Fig. 2) and the assay of the fluorescence emission of the GFP carrier protein (Fig. 3), evidenced that maximum IB solubilization was achieved at 37 °C for 48 h. The assay based on BMAP27GFP's fluorescence emission (Fig. 3) was more sensitive and also easier to carry out than the antimicrobial assay (Fig. 2), showing much greater differences between the activity of the protein solubilized after 48 h, 24 h or 16 h (Fig. 3), differences not observed for the bactericidal activity (Fig. 2). In the case of JAMF2, the solubilization efficiency was assessed by measuring the antimicrobial activity against the target bacteria carbapenem-resistant *Klebsiella pneumoniae* (Fig. 4) and the enzymatic activity of the $sPLA_2$ domain (Fig. 5) included within the protein. A comparison of both assays showed that $sPLA_2$ activity was not sensitive enough (Fig. 5), but bacterial survival indicated the release of active JAMF2 protein from IBs very well (Fig. 4). In this case, the selected conditions were also 37 °C but an incubation time of 16 h was enough to obtain the maximum amount of solubilized protein (Fig. 4).

Finally, the IBs of the third model protein, M-SAA3, were solubilized but using the temperature that performed best for the other two proteins. Several activities stimulating the release of IL-8, IL-6, IFN-γ and IL-10 (Fig. 6) and inhibiting *S. aureus* invasion (Fig. 7) in mammary cells were evaluated. Although the cytokine profiles were similar in all cases, the most sensitive assay was IL-8 because it showed a progressive increase in activity that could be correlated to protein solubilization at an increasing incubation time at 37 °C (Fig. 6).

	Expression system	Soluble protein	Insoluble protein
M-SAA3	*L. lactis*	65 ± 4%	36 ± 4%
JAMF2	*E. coli*	-	> 99%
BMAP27GFP	*L. lactis*	65 ± 1%	34 ± 1%

Table 1. Description of the proteins produced, expression system used, solubility and aggregation yield and protein yield reached using solubilization protocols using three independent samples of IBs as starting material. In the case no standard error is presented because no soluble protein was detected.

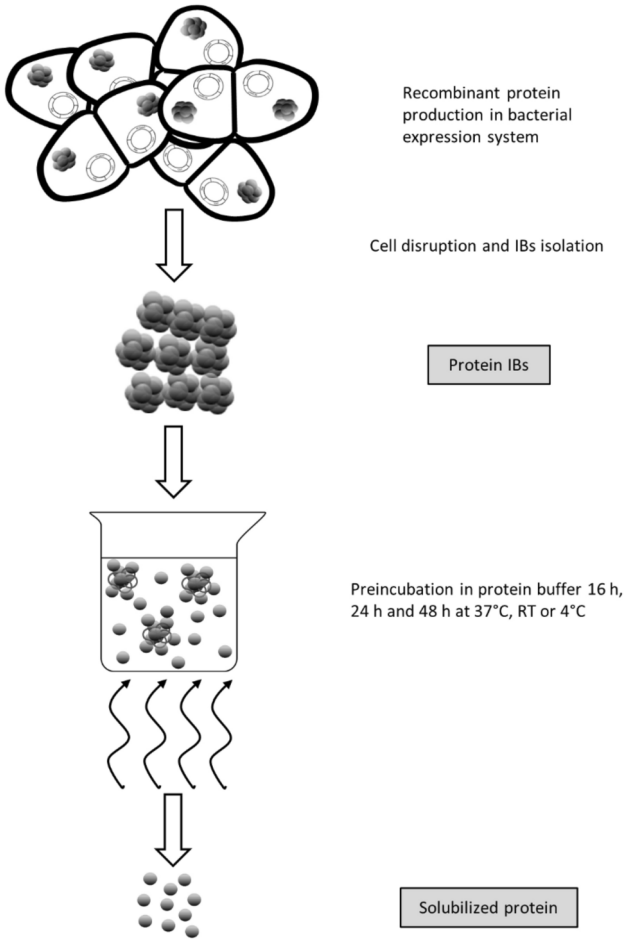

Fig. 1. Schematic representation of the protein solubilization protocol.

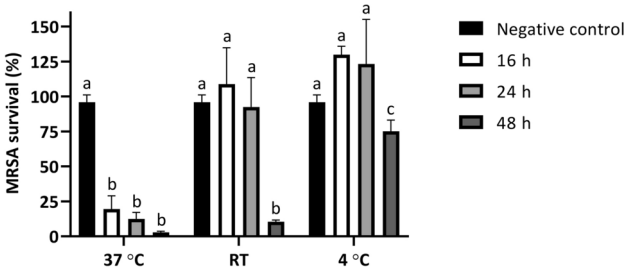

Fig. 2. Antibacterial activity of BMAP27GFP solubilized from IBs against methicillin resistant *Staphylococcus aureus* (MRSA). Bars show MRSA's survival percentage in the presence of different treatments. The results represented are significantly different from the control bacteria treated with solubilizing buffer ($p < 0.001$) by ANOVA analyses. Different letters depict significant differences among treatments ($p < 0.001$). Error bars depict the SEM of three different experiments.

Discussion

The objective of this study was to prove that a spontaneous solubilization protocol carried out in a standard buffer without the presence of any detergent could be used as a protocol to easily recover active soluble protein from IBs. To achieve this, we screened different solubilization conditions considering temperature and timing as the main variables to be evaluated for each recombinant protein with relevant biological functions. JAMF2 is an antimicrobial multidomain protein constructed via the combination of a human α-defensin-5 (HD5), a bacterial binding domain (gelsolin), and an enzymatic antimicrobial peptide ($sPLA_2$), flanked by two aggregation-seeding domains (a fragment of c-Jun and c-Fos leucine zippers at the N- and C-terminals, respectively) in a single polypeptide[25]. BMAP27GFP is also an antimicrobial protein, and it is based on the fusion of the bovine cathelicidin BMAP-27 and a carrier fluorescent protein (GFP). Finally, M-SAA3 is an acute phase protein from

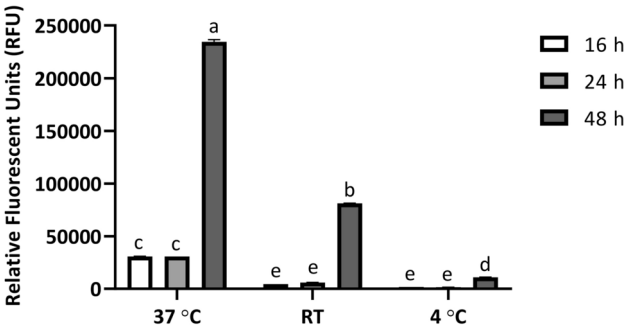

Fig.3. Fluorescence of BMAP27GFP solubilized from IBs. Bars show GFP fluorescence of the protein solubilized under different conditions. Error bars depict the SEM of three different experiments. Different letters depict significant differences among samples ($p < 0.05$). The results represented are significantly different from the negative control ($p < 0.001$) by ANOVA analyses.

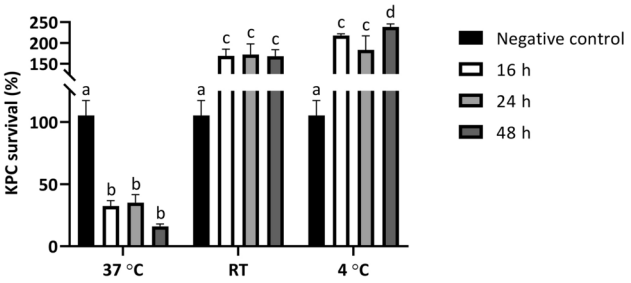

Fig.4. Antibacterial activity of JAMF2 solubilized from IBs against *Klebsiella pneumoniae* carbapenem-resistant (KPC). Bars show KPC's survival percentage in the presence of the different treatments. The results represented are significantly different from the control KPC treated with solubilizing buffer ($p < 0.001$) by ANOVA analyses. Different letters depict significant differences among treatments ($p < 0.001$). Error bars depict the SEM of three different experiments.

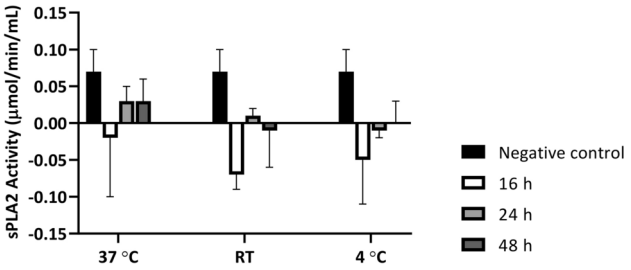

Fig. 5. Enzymatic activity of JAMF2 solubilized from IBs. Bars show the enzymatic activity of the secretory phospholipase A2 domain contained in JAMF2 for 20 min. The results represented are not significantly different from the control (solubilizing buffer) by ANOVA analyses. Error bars depict the SEM of three different experiments.

a caprine mammary gland with the ability to modulate the innate immune response and prevent mammary infections[26].

In agreement with what was previously described, we proved that the aggregation rate is a protein-dependent parameter (Table 1). Sometimes, as in the case of JAMF2, the solubilization of IBs is the only viable approach to isolate the soluble protein of interest. However, in other situations in which aggregation rates are lower, and consequently, the amount of soluble protein in the bacterial cytoplasm is higher, the soluble fraction can be used to purify the recombinant protein. However, high solubility rates do not guarantee the obtention of the soluble form of a protein of interest. M-SAA3 is an example of a protein with a high solubility rate (Table 1) but with low production yields and which is copurified with other host proteins, yielding a low-quality final product[13]. In this case, the use of IBs as a source of recombinant protein allows one to obtain a highly pure, soluble form of M-SAA3 through IB solubilization using a non-denaturing detergent[13]. For three different model proteins, we have demonstrated that it is possible to solubilize IBs spontaneously without the use of chaotropic agents or

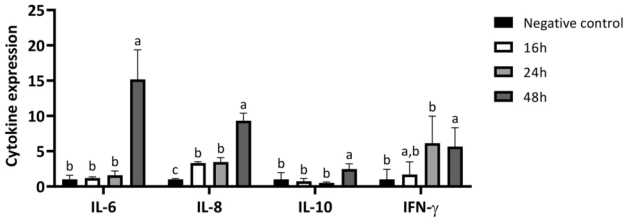

Fig. 6. Activity determination of M-SAA3 solubilized from IBs. RT-PCR quantification of IL-6, IL-8, IL-10, and IFN-γ expression in mammary epithelial cell culture. The results represented are significantly different ($p < 0.01$) from the control (cells treated with solubilizing buffer) by ANOVA analyses. Different letters (a-c) denote differences ($p < 0.05$) among treatments. Error bars depict the SEM of the different treatments.

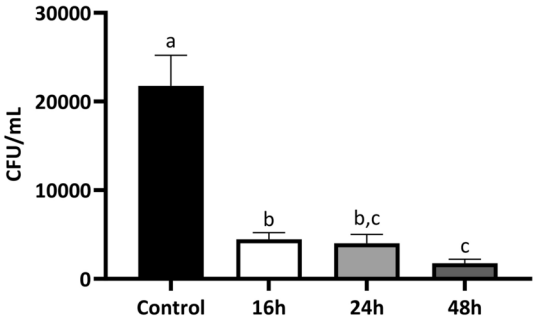

Fig. 7. Antibacterial activity of M-SAA3 solubilized from IBs. Bacterial counts of infective bacteria (expressed as CFU/mL) associated with mammary gland cell cultures after infection with 2×10^7 CFU/mL of *S. aureus* and treated with solubilized M-SAA3 at 150 µg/mL. The results represented are significantly different ($p < 0.001$) from the control (*S.aureus* with solubilizing buffer) by ANOVA analyses. Different letters (a–c) denote differences ($p < 0.05$) among treatments. Error bars depict the SEM of the different treatments.

detergents and obtain soluble and active proteins. It is likely that 37 °C is the most appropriate temperature for all cases but it is also possible to achieve solubilization at a lower temperature, as demonstrated for BMAP27GFP (Figs. 2 and 3). Herein, neither RT nor 4 °C was enough to solubilize JAMF2 IBs (Fig. 4), probably because the presence of jun and fos seeding domains producing compact aggregates[25]. We did not test incubation times longer than 48 h to avoid the risk of protein degradation and because the main goal of this study was to demonstrate that spontaneous solubilization is possible at least for three different model proteins forming IBs and that the protocol can be customized by monitoring protein activity.

We demonstrated that it is worth conducting several activity assays, if possible, to establish the solubilization conditions of each IB, although after this study we could recommend that it is possible to use a general condition based on 37 °C for 48 h. This is because an activity assay can have limitations regarding sensitivity or concerning a maximum threshold, not one allowing to finely detect differences in the activity of solubilized protein. Sometimes, such as in the case of BMAP27GFP, several assays work with similar efficiencies such as antimicrobial (Fig. 2) and fluorescence (Fig. 3) assays that allowed us to detect that 48 h of 37 °C solubilization was the best condition for BMAP27GFP. However, the detection of GFP carrier protein fluorescence is the easiest and can be extrapolated to other fusion proteins carrying GFP. In the case of JAMF2 the antimicrobial assays (Fig. 4) offered higher sensitivity and allowed us to detect solubilized active protein at 37 °C, whereas the enzymatic activity assay (Fig. 5) did not. The increase in KPC survival observed with the solubilization obtained at 4 °C and RT is in accordance with the bacterial growth observed in other studies using antimicrobial peptides that do not work correctly[25,27]. Probably the selected conditions are not appropriate to allow the solubilization of this protein, which showed high aggregation rates (Table 1), being probably more difficult to solubilize than proteins with lower aggregation values. Alternatively, we could not discard that in these supernatants, there is unfolded and inactive protein. Again this corroborates that the best temperature is 37 °C for the solubilization of this protein achieving an active folding. Also, JAMF2 results suggested that some complex proteins containing disulfide bonds and high-prone aggregation rations could also be recovered in soluble form using this approach while keeping their biological activity. HD5 domain contained in JAMF2 have 3 disulfide bonds and the protein is active once solubilized (Fig. 4). Finally, in the case of M-SAA3, the antibacterial assay (Fig. 7) did not detect solubilized protein, whereas IL-8 stimulation clearly showed different solubilization rates along the incubation time at 37 °C (Fig. 6).

We also performed Western blot analyses (data not shown) for the three model proteins, but they were not sensitive enough to detect protein in the solubilized supernatants of the screening experiments. In a further step, selected solubilization conditions can be applied to a greater volume of IBs, followed by carrying out IMAC or

appropriate purification and concentration protocols in a way that Western blot or other qualitative analyses can be performed to characterize the final biologically active protein.

In conclusion, we clearly showed that IBs from three model proteins with different characteristics can be solubilized without the use of denaturing agents or non-denaturing detergents. Whereas the experiments do show that active protein can be solubilized from IBs using the investigated solubilization protocols, a comparison to the state-of-the-art protocols mentioned within the introduction was beyond the scope of this study.

Monitoring protein activity and screening different solubilization conditions allowed us to establish the optimum timing and temperature with greater sensitivity than a Western Blot or standard protein detection protocols and to ensure the customization of the protocol to achieve maximum protein activity.

Methods

Bacterial cultures and media

Lactococcus lactis sub. cremoris NZ9000[28] was used for the expression of M-SAA3 and BMAP27. *E. coli* BL21 (DE3) was used for the expression of JAMF2. *L. lactis* strains were grown at 30 °C in M17 media (Merck) supplemented with 0.5% glucose and with 5 µg/ml Cm for plasmid maintenance. The *E. coli* strain was grown in Luria-Bertrani (LB) medium with 100 µg/ml of ampicillin for plasmid maintenance.

The strain used for eukaryotic cell culture infection was *Staphylococcus aureus*. The strains used for antimicrobial activity assays were carbapenem-resistant *Klebsiella pneumoniae* (KPC) and methicillin-resistant *Staphylococcus aureus* (MRSA). The three pathogenic strains were grown in brain–heart infusion (BHI) broth (Scharlau).

Genetic construct design

The sequence of goat mammary serum amyloid A3 (M-SAA3) was previously designed[29], cloned in a pNZ8148 plasmid (MoBiTech, Cm^R) and transformed in *L. lactis sub. cremoris* NZ9000 (NZ9000/pNZ8148:M-SAA3)[13]. The sequence of JAMF2 was previously designed, cloned in a pET22b (Amp^R) plasmid and transformed in *E. coli* BL21 (DE3) (*E. coli* BL21 (DE3)/pET22b:JAMF2)[25]. The sequence of bovine myeloid antibacterial peptide 27 (BMAP27) was based on the sequence UniProtKB—P54228 (CTHL6_BOVIN) fused to the Green Fluorescent Protein (GFP) gene through the linker sequence GGSSRSS. The BMAP27GFP sequence was cloned in a pNZ8148 plasmid (MoBiTech,Cm^R) and transformed in *L. lactis sub. cremoris* NZ9000 (NZ9000/pNZ8148:BMAP27GFP) as previously described in[2]. All proteins had a 6 histidine (H6)-tag at C-terminal for protein purification purposes.

Aggregation yield determination

Cultures of 500 mL volume were produced for aggregation yield determination. To produce both M-SAA3 and BMAP27GFP, cultures of *L. lactis* NZ9000/pNZ8148:M-SAA3 or *L. lactis* NZ9000/pNZ8148:BMAP27GFP in 0,5 L bottles were grown at 30 °C without agitation and induced with 12.5 ng/mL of nisin at an $OD_{600nm} = 0.4–0.6$. For the production of JAMF2, cultures of 500 mL of *E. coli* BL21 (DE3)/pET22b:JAMF2 in 2 L Erlenmeyer flasks were grown at 37 °C and 250 rpm and induced with 1 mM of isopropyl-β-d-thiogalactoside (IPTG) at $OD_{600nm} = 0.4–0.6$. The whole volume of the three cultures was recovered after 3 h of production and centrifuged at 6000×*g* for 15 min at 4 °C. Pellets of 500 mL of culture were suspended in 300 mL of PBS with protease inhibitors (cOmplete™ EDTA-free, Roche). Bacteria were mechanically disrupted using a cellular disruptor (CF1 Model, Constant Systems Ltd) with 2 cycles at 40 kpsi for *L. lactis* and one cycle at 27 kpsi for *E. coli*. Soluble and insoluble fractions were split by centrifugation (15,000×*g*, 15 min, 4 °C). The soluble and insoluble protein fractions were analyzed by Western Blot. A 15% denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was prepared. All samples were resuspended in PBS with Laemmli loading buffer (100 mM Tris base, 8% glycerol, 55 mM SDS, 4% β-mercaptoethanol, 1.6 M urea). Soluble and insoluble (inclusion bodies) fractions were boiled for 10 and 40 min before electrophoresis, respectively. Protein bands were electroblotted into polyvinylidene difluoride (PVDF) membranes at 2.5 A and 25 V for 10 min, followed by a blocking step with BSA O/N at 4 °C (5% BSA in TBST buffer: 10 mM Tris, 150 mM NaCl, 0.05% Tween 20). Anti poly-histidine (Santa Cruz Biotechnology; mouse) was used as the primary antibody at a 1/1000 dilution in BSA-TBST buffer, in which membranes were incubated for 2 h at RT, followed by 3 washes in TBST buffer. Then, membranes were incubated in a 1/20,000 dilution in TBST of an anti-mouse IgG-alkaline phosphatase (Sigma), used as secondary antibody, along 1 h at RT followed by 3 washes in TBST buffer. Protein bands were developed after adding the alkaline phosphatase substrate solution NBT/BCIP (Thermo Scientific). A protein marker, PageRuler™ Prestained Protein Ladder, has been loaded in all the gels (ref. 26616, ThermoFisher Scientific). Bands were quantified with a standard curve of purified soluble GFP. Densitometry analyses were performed with ImageJ software.

M-SAA3, BMAP27GFP and JAMF2 IBs production and purification

To produce both M-SAA3 and BMAP27GFP IBs, cultures of 1 L of *L. lactis* NZ9000/pNZ8148:M-SAA3 or *L. lactis* NZ9000/pNZ8148:BMAP27GFP in 1 L bottles were grown at 30 °C without agitation and induced with 12.5 ng/mL of nisin at an $OD_{600nm} = 0.4–0.6$. For the production of JAMF2 IBs, cultures of 500 mL of *E. coli* BL21 (DE3)/pET22b:JAMF2 in 2 L Erlenmeyer flasks were grown at 37 °C and 250 rpm and induced with 1 mM of isopropyl-β-d-thiogalactoside (IPTG) at $OD_{600nm} = 0.4–0.6$. The whole volume of the three cultures was recovered after 3 h of production and centrifuged at 6000×*g* for 15 min at 4 °C. Pellets of 500 mL of culture were suspended in 300 mL of PBS with protease inhibitors (cOmplete™ EDTA-free, Roche). Bacteria were mechanically disrupted using a cellular disruptor (CF1 Model, Constant Systems Ltd) with 2 cycles at 40 kpsi for *L. lactis* and one cycle at 27 kpsi for *E. coli*. Then, a freeze/thaw cycle was performed O/N at − 80 °C to disrupt the partially digested bacteria and release the IBs protein contents. After bacteria disruption, an extensive sample

washing was performed in the presence of mild detergents. Firstly, lysozyme was added at 0.01 mg/mL and the mixture was incubated for 2 h at 37 °C and 250 rpm. Then, the samples were frozen at − 80 °C O/N and thawed the next day. IBs were then washed in 4 µL/mL of sample Triton X-100 for 1 h at RT. After that, 100 µL portions of the sample were plated on M17 agar plates supplemented with 0.5% glucose and incubated O/N at 30 °C for *L. lactis* or platen on LB plates and incubated O/N at 37 °C for *E. coli,* to evaluate the presence of viable cells. If bacterial colonies were observed, extra freeze/thaw cycles were performed to eliminate those bacteria. Once no colonies were observed, the IBs were washed in 0.25 µL/mL Nonidet P40 for 1 h at 4 °C. The sample was then treated with $MgSO_4$ at 6 µL/mL from a stock of 0.1 M and with DNAse at 0.6 µL/mL from a stock of 1 mg/mL at 37 °C and 250 rpm for 1 h. IBs were centrifuged at 6000×*g* for 30 min. Each pellet of 50 mL of culture was suspended in 5 mL of lysis buffer (Tris 50 mM, NaCl 100 mM, 1 mM EDTA) + 0.05% Triton X-100. Once suspended, samples were frozen O/N at − 80 °C and thawed the next day. After thawing, the samples were centrifuged at 6000×*g* for 30 min and suspended in 5 mL of PBS. Finally, pure IBs were centrifuged at 15,000×*g* for 15 min discarding the supernatant. The integrity and the amount of protein in each IB were determined via SDS-PAGE, with a posterior densitometry analysis conducted using ImageJ software.

Protein solubilization from IBs

JAMF2 and BMAP27GFP solubilization from IBs

Purified JAMF2 and BMAP27GFP IBs were suspended in 10 mM of KPi buffer (K/PO_4 buffer pH 7.4) or acetic acid 0.01% (v/v) respectively at a final concentration of 1 mg/mL. Then, different samples were incubated at 37 °C, RT or 4 °C for 16 h, 24 h or 48 h. After the incubation, samples were centrifuged at 15,000×*g* for 15 min and 4 °C. The supernatants were recovered and frozen at − 80 °C until later use.

M-SAA3 solubilization from IBs

Purified M-SAA3 IBs were suspended in PBS at a final concentration of 150 µg/mL. Then, different samples of the suspension were incubated at 37 °C for O/N, 24 h or 48 h. At the end of the incubation period, they were centrifuged at 15,000×*g* for 15 min at 4 °C. The supernatants were recovered and frozen at − 80 °C until later use.

Eukaryotic cells cultures and media

The cell cultures used in this study were primary bovine mammary gland cultures and the human mammary gland cell-line MCF-12A (ATCC® CRL-10782™). All cultures were grown in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) enriched with insulin (8 µg/mL), hydrocortisone (50 µg/mL), penicillin–streptomycin (100 U/mL), Fungizone™ (2.5 µg/mL), gentamycin (50 µg/mL) and 10% fetal bovine serum (FBS). In the case of MCF-12A cell growth, epidermal growth factor (EGF) was added at 20 ng/mL. All cultures grew at 37 °C in 5% CO_2.

Induction of cytokines expression in mammary gland primary cultures

Bovine mammary gland primary cultures were obtained as previously described[30] and expanded before the experiment. Once the culture reached 80% of confluence, 44,000 cells/well were cultured in 24 well plates with 500 µL of complete DMEM/F-12. The cells grew O/N at 37 °C in 5% CO_2. The day after, solubilized M-SAA3 was added to mammary gland primary cultures in a final volume of 1 mL (500 µL M-SAA3 + 500 µL medium), for an O/N incubation. Negative controls were treated with solubilizing buffer + 500 µL medium. At the end of the experiment, cells were washed twice with 500 µL of PBS and 500 µL of Trizol reagent (Thermo Fisher Scientific) was added to each well to collect and lysate the cells. The extraction of RNA was performed using the Trizol reagent (Thermo Fisher Scientific) and the RNA was processed for qPCR analyses of IL-6, IL-8, IL-10 and IFN-γ expression. Relative gene expression was calculated using the $2^{\Delta Ct}$ method using RpS9 as a reference gene.

Staphylococcus aureus infection inhibition assay in mammary gland cultures

The MCF-12A mammary cells were grown at 80% confluence, and 44,000 cells/well were cultured in 24-wells plates with 500 µL of complete DMEM/F-12. The cells grew O/N at 37 °C and 5% CO_2. The day after, solubilized M-SAA3 was added to the mammary gland cultures in a final volume of 1 mL (500 µL M-SAA3 + 500 µL medium) for an O/N incubation. Negative control were treated with solubilizing buffer + 500 µL medium. After the incubation, the medium was discarded. The cells were washed twice with 500 µL of PBS. Then, the cells were infected with 500 µL of infection medium (*S. aureus* suspended in DMEM/F12 without additives) at 2×10^7 CFU/mL for 2 h at 37 °C and in 5% CO_2. The cells were washed twice with 500 µL of PBS, and incubated for 2.5 h at 37 °C and in 5% CO_2 with 500 µL of gentamycin at 100 µg/mL. The cells were washed twice with 500 µL of PBS 1X, trypsinized with 150 µL of Trypsin–EDTA for 10 min, neutralized with 300 µL of DMEM/F-12 containing 10% FBS and recovered. The samples were then centrifuged for 6.5 min at 200 × *g* and suspended in 500 µL of Triton 0.1% for 10 min at 37 °C. Finally, each sample was diluted 1/100 in NaCl 0.9%, plated on a BHI-agar plates and incubated O/N at 37 °C for viable cell counting.

Bactericidal assay against pathogenic bacterial strains

A bactericidal assay based on the BacTiter-Glo™ Microbial Cell Viability assay (Promega) was performed. Firstly, an O/N culture of the selected strain, KPC for JAMF2 or MRSA for BMAP27GFP, was diluted (10^6 CFU/ml) in 10 mM KPi buffer. Then, 150 µL from the diluted bacterial stock was centrifuged at 6,200 × *g* for 15 min and 4 °C. The supernatant (SN) was removed and the bacterial pellet was suspended with 150 µL of either 10 mM KPi buffer (negative control) or the SN resulting from IBs solubilization for JAMF2, or either acetic acid 0.01% (v/v) (negative control) or the SN resulting from IBs solubilization for BMAP27GFP. All samples were transferred to a sterile polypropylene 96-well plate (Costar) and incubated for 5 h at 37 °C. After, 100 µL was withdrawn and tested with 100 µL of the BacTiter-Glo™ reagent following the manufacturer's instructions.

Secretory phospholipase A2 ($sPLA_2$) enzymatic activity assay

An enzymatic activity assay was performed based on an $sPLA_2$ Assay Kit (Cayman) to quantify the enzymatic activity of the $sPLA_2$ domain of the solubilized JAMF2. Solubilizing buffer was used in the negative control. The enzymatic activity of the SN obtained after the solubilization of JAMF2 IBs was analyzed for 20 min following the manufacturer's instructions.

Fluorescence determination

The fluorescence of the GFP of the BMAP27GFP solubilized from the IBs was determined as follows: 100 µL of each SN obtained after IB solubilization was transferred to a black and opaque 96-well plate (Thermo Scientific). Solubilizing buffer was used in the negative control. The fluorescence was measured using a LUMIstar Omega plate reader (BMG-LabTech). Samples were excited at 480 nm and the emission was recorded at 510 nm.

Statistical analysis

All data were analyzed using an ANOVA that accounted for the effect of solubilization time (16 h, 24 h and 48 h) in comparison with the controls. Differences between the solubilization temperatures (37 °C, RT and 4 °C) were established using Tukey's multiple mean separation test. Results are expressed as the means of non-transformed data ± the standard error of the mean (SEM).

Data availability

Sequence data of proteins used in this study have been deposited in GenBank database under accession numbers GenBank PP693209 for M-SAA3 protein, GenBank PP583564 for BMAP27GFP and GenBank PP575758 for JAMF2 protein.

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Author contributions

EGF and AA conceived the original idea and supervised the project. RBF carried out the experiments. RBD, EGF and AA wrote the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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