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ORIGINAL PAPER

The Optimisation of the Expression of Recombinant Surface Immunogenic Protein of *Group B Streptococcus* in *Escherichia coli* by Response Surface Methodology Improves Humoral Immunity

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Abstract

Group B Streptococcus (GBS) is the leading cause of neonatal meningitis and a common pathogen in livestock and aquaculture industries around the world. Conjugate polysaccharide and protein-based vaccines are under development. The surface immunogenic protein (SIP) is a conserved protein in all GBS serotypes and has been shown to be a good target for vaccine development. The expression of recombinant proteins in *Escherichia coli* cells has been shown to be useful in the development of vaccines, and the protein purification is a factor affecting their immunogenicity. The response surface methodology (RSM) and Box–Behnken design can optimise the performance in the expression of recombinant proteins. However, the biological effect in mice immunised with an immunogenic protein that is optimised by RSM and purified by low-affinity chromatography is unknown. In this study, we used RSM for the optimisation of the expression of the rSIP, and we evaluated the SIP-specific humoral response and the property to decrease the GBS colonisation in the vaginal tract in female mice. It was observed by NI–NTA chromatography that the RSM increases the yield in the expression of rSIP, generating a better purification process. This improvement in rSIP purification suggests a better induction of IgG anti-SIP immune response and a positive effect in the decreased GBS intravaginal colonisation. The RSM applied to optimise the expression of recombinant proteins with immunogenic capacity is an interesting alternative in the evaluation of vaccines in preclinical phase, which could improve their immune response.

Keywords Group B streptococcus · Response surface methodology · Protein expression

Introduction

Streptococcus agalactiae or Group B Streptococcus (GBS) can cause severe diseases in both animals and humans. In animals, GBS mainly affects the livestock and aquaculture industry sectors. In the livestock sector, it causes bovine mastitis, an infectious disease that reduces milk production due to inflammation of the mammary gland of the animal [1–3]. In the aquaculture sector, it is characterised by generating sepsis and mortality in some varieties of fish such as Gulf killifish, grouper and tilapia [4]. In humans, GBS presents in 20–40 per cent of women and is the main causative agent of neonatal sepsis [5] and meningitis in neonates, the immunocompromised and the elderly [6, 7]. Prophylactic antibiotics are used when GBS is detected in the vaginal tract between 35 and 37 weeks of pregnancy. However, this procedure is not an ideal approach to treating GBS infection, as antibiotic resistance has been reported [8, 9].

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Vaccines against GBS are under development and in clinical trials. A vaccine based on three capsular polysaccharides (CP) conjugated to a protein carrier is undergoing Phase II testing, and there is a vaccine in Phase I based on a protein of GBS [10]. The protein-based vaccine has the advantage of inducing immune protection against all GBS serotypes, not only the serotype included in the formulation of the vaccine. Moreover, the GBS protein is being used in the development of clinical testing and may be useful as a molecular target based on a biological interaction between GBS and human epithelial cells. Among the main proteins used for these studies, the C5a peptidase, a serine protease [7, 11] and the surface immunogenic protein (SIP) have been characterised as highly immunogenic [7, 12–16].

Recombinant vaccines rely on the capacity of one or multiple defined antigens to induce immunity against the pathogen. The use of biotechnological tools has led to a number of new approaches for the development of vaccines [17]. The classic way for the optimisation of a biotechnological process, defined as the transformation of nutrients and components of the medium culture into biomass and molecular products of interest [18], may vary one factor at a time and analyse its effect on the protein expression until an optimum is reached. This form of analysis is laborious and time-consuming; moreover, it is not possible to analyse the interactions between factors, and therefore, several researchers have chosen to use experimental approximations or statistics in experimental design techniques such as response surface methodology (RSM), which consists of a number of mathematical and statistical analysis techniques [19]. RSM has been widely used for studying the interactions of several parameters during bioprocess optimisation in different biotechnological processes [18]. However, the biological effect of immunisation with protein vaccines optimised by RSM and purified by low-pressure chromatography has not been studied. The SIP from GBS is a conserved protein in all GBS serotypes [15] and has been shown to be a good target for vaccine development. The recombinant SIP (rSIP) has been expressed by different authors; however, there is no consensus protocol on the expression of this recombinant protein. In this study, we applied a Box–Behnken design (BBD) with three central points to optimise the expression of rSIP in *E. coli* cells. Three factors were studied: temperature ($T°$), optical density (OD_{600}) and isopropyl β-D-1-thiogalactopyranoside (IPTG). The optimisation of rSIP expression was evaluated by measuring the amount of rSIP with respect to total bacterial proteins. Also, we evaluated whether the increment over protein expression influences the purity and the induction of immune response in mice, measuring the induction of SIP-specific IgG and the effect on GBS vaginal colonisation.

Materials and Methods

Production of the Recombinant SIP

The SIP gene was cloned from a Chilean GBS strain serotype III available in our laboratory (KU736792.1). The sip gene was amplified by PCR and cloned into *pET21*a plasmid DNA to obtain the *pET21a-sip* plasmid DNA. The cloned sip gene was analysed by DNA sequencing and submitted to GenBank (KX363665.1).

The *pET21a-sip* plasmid DNA was introduced into the *Escherichia coli* strain BL21-CodonPlus (DE3) by electrotransformation using a MicroPulser™ (Bio-Rad). The transformed bacterial cells were spread on LB agar plates containing ampicillin (100 µg/ml), streptomycin (75 µg/ml) and chloramphenicol (34 µg/ml). The bacterial plates were incubated at 37 °C overnight (O/N).

The rSIP expression was induced as described by Xu et al. [13] with slight modifications in the time of induction. A single bacterial colony was used for rSIP expression. The bacterial colony was inoculated in 5 ml LB medium containing glucose (0.4%), ampicillin (100 µg/ml), chloramphenicol (34 µg/ml) and streptomycin (75 µg/ml), which was then incubated O/N at 37 °C and 220 rpm. The saturated bacterial culture was used at dilution factor 1:100, and 100 ml of fresh medium was incubated at 37 °C and 220 rpm. When the bacterial culture reached OD_{600} 0.5, the bacterial culture was induced with 0.8 mM of IPTG for 2 h. The bacterial pellets were recovered by centrifugation at 2500*xg* for 20 min at 4 °C and stored at − 80 °C until use.

To purify rSIP under native conditions, we used Pro-Bond™ Nickel-Chelating Resin (ThermoFisher Scientific, catalogue no. R80115), which is designed for the purification of 6xHis-tagged recombinant proteins expressed in bacteria. Briefly, we used 1.5 g of bacterial pellets and resuspended in binding buffer (20 mM NaH_2PO_4—pH 8.0; 500 mM NaCl, 10 mM imidazole), then added lysozyme and RNase (each 10 mg/ml) and incubated on ice for 30 min. Then, we centrifuged the mixture at 2500×*g* for 20 min at 4 °C and recovered the supernatant. The soluble protein extraction was purified and loaded into the column packed with NI–NTA resin (Pro-Bond). The column was washed with 50 ml washing solution (20 mM NaH_2PO_4—pH 8.0; 500 mM NaCl; 20 mM imidazole) and eluted with elution buffer (20 mM NaH_2PO_4—pH 8.0, 500 mM NaCl, 250 mM imidazole). Finally, the purified protein samples were dialysed with 1× buffer (50 mM NaH_2PO_4 —pH 8.0; 0.5 MNaCl) for 24 h under constant agitation at 4 °C. The purified rSIP was analysed by SDS-PAGE and quantified by Bio-Rad Protein Assay (BIO-RAD).

Analyses of rSIP by MALDI-TOF Mass Spectrometry

The identity of the rSIP was analysed by MALDI-TOF MS. Briefly, 40 µg of purified rSIP was concentrated on a 12.5% SDS-PAGE. The rSIP was extracted and exposed to a proteolysis solution [50 mM ammonium bicarbonate/10% (v/v) acetonitrile and 0.042 µg/µl trypsin (Promega Corp., WI, USA)], and the resulting solution was concentrated in a SpeedVac (Savant Instruments Inc., NY, USA) at room temperature (RT). The dried samples were reconstituted in 10 µl of 0.1% (v/v) formic acid and 3% (v/v) methanol. Then, 1 µl aliquot of the analyte was mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution 1 µl on a MALDI target plate.

The acquisition of mass spectra was performed in MALDI-TOF Microflex (Bruker Daltonics Inc., MA, USA) in positive ion mode and calibrated with calibrant II of the InvitroMass LMW calibrant Kit (Invitrogen). For the control of the spectrometer the flexControl 3.0 program (Bruker Daltonik GmbH, Germany) was used. Individual spectra were produced from 30 laser shots, and 15 spectra were obtained for each sample. The analysis of the spectra was generated by the program mMass, version 5.5.0 [20]. The data were compared with the NCBI database. The search was limited to tryptic peptides with a maximum of one cleavage of loss. Carbamidomethylation of cysteine was considered a fixed modification, whereas oxidation of methionine residues was considered as a variable modification. The mass values were restricted to monoisotopic signals, and the tolerance of the peptides was restricted between 0.1 and 0.3 Da. The search for the peptide mass fingerprinting was performed using a restriction search by taxonomy (all entries) and restricted to Firmicutes. The mass spectra for rSIP were performed in triplicate.

Detection of rSIP by Immunoblotting

The identity of the purified rSIP was analysed by western blot using a specific antibody against SIP produced in rabbit in our laboratory (data not shown). Briefly, purified rSIP was run on SDS-PAGE and transferred to a nitrocellulose membrane for 1 h at 200 mA. The membrane was blocked using PBS-5% BSA O/N and incubated for 1 h at RT with rabbit anti-SIP antibody (1:1000). The membrane was washed with PBS-0.05% Tween (PBST) and incubated for 1 h at RT with anti-rabbit IgG antibody (1:1000) conjugated to alkaline phosphatase (Sigma). The membrane was washed with PBST, and the detection was performed using BCIP/NBT (Calbiochem) as the substrate for alkaline phosphatase. The western blot image was recorded using a Bio-Imaging System (miniBis Pro).

As control for polyclonal antibody, the efficacy of the generated antibody from the rabbit serum was tested against the SIP from *S. agalactiae* lysate. Briefly, to detect SIP from *S. agalactiae* lysate, gram-positive was pelleted from a Todd-Hewitt broth (Difco Laboratories). The Y-PER reagent (Cat# 78990, Thermo Scientific) was employed for protein extraction. Lysate was then centrifuged at 100,000*xg* for 20 min at 4 °C. The supernatant was resolved by SDS-PAGE and western blot as described above.

Evaluation of Protein Concentration and HPLC Analysis

Total protein quantification was conducted using the Bradford methodology, following the manufacturer's instructions. Briefly, a protein standard curve was generated using a linear range of BSA (0.2–0.9 mg/ml). Protein samples were mixed Bradford reagent and incubated at RT for 5 min. The absorbance was measured at 595 nm using an Evolution 60 spectrophotometer (Thermo Scientific).

A sandwich enzyme-linked immunosorbent assay (ELISA) was performed to quantify specific rSIP in complex mixtures of *E. coli* total protein. ELISA plate was coated with anti-histidine monoclonal antibody (Millipore, dilution 1:8000) and incubated O/N at 4 °C. Plates were then washed with PBST and blocking solution (3% BSA in PBS) added to each well for 1 h. Samples were added to each well for 1 h at RT, followed by addition of the rabbit polyclonal anti-SIP (1:1000). The plates were washed with PBST and alkaline phosphatase-conjugated anti-rabbit antibody (Sigma, dilution 1:1000) was added and incubated for 1 h at RT. The wells were washed with PBST and *p*-nitrophenylphosphate substrate (pNPP) was added and incubated for 30 min. The enzymatic reaction was stopped by adding 3 M NaOH and plate reads in an ELISA reader SPECTROstar Nano ELISA plate reader (BMG Labtech) at a wavelength of 405 nm. The assays were performed in triplicate, and we used samples of purified rSIP with known concentration to construct a calibration curve with a plot of absorbance versus concentration.

The purified protein from NI–NTA resin (20 µg) was analysed on a HPLC, Smarline UV detector 2520 (Knauer, Wissenschaftliche Geräte GmbH, Germany). The system consisted of GPC/SEC column (5 µm, 300 mm × 8 mm, 100 Å), a mobile phase composed of phosphate buffer (34 mmol/l) and sodium chloride (0.5 M) at pH 6.6. The flow rate was 0.8 ml/min, and detection was performed at 210 nm using a UV detector at ambient conditions.

Table 1 Values of variables and the levels used in Box–Behnken design

Condition	Variable	Levels			Unit
		− 1	0	1	
A	Inducer concentration	0.1	0.8	1.5	mM
B	Cellular concentration	0.3	0.5	0.7	Abs_{600nm}
C	Temperature	25	31	37	°C

Optimisation of the Expression of rSIP by RSM

To optimise rSIP expression, we used RSM and BBD with three centre points. As described by De Leon et al. [21], we evaluated the effect of temperature, cell concentrations and inductor concentration to establish the best culture conditions for expression of recombinant protein. A total of 15 experimental runs with different combinations of the three factors and three replicates of the centre point were performed (Table 1). Experiment number 16 had the experimental basal conditions as described above, but it was not considered in the statistical analysis. The statistical program Statgraphics Centurion XVI [22] was used for the development of the model. As shown in Table 1, the independent variables were IPTG concentration in the range 0.1–1.5 mM (factor *A*), OD_{600} in the range 0.3–0.7 (factor *B*) and temperature in the range 25–37 °C (factor *C*).

We evaluated the optimisation procedure based on the experimental conditions that induced the greatest amount of expressed rSIP with respect to the total bacterial protein. We evaluated the rSIP expression by sandwich ELISA and the total bacterial protein by Bradford methodology. To analyse all the experimental values of IPTG, OD and *T*° and the resulting behaviour, we constructed an RSM graphic using the program R [23].

Evaluation of Immune Response Induced by rSIP in Mouse Model

In order to evaluate the immune responses induced by purified rSIP, we immunised female mice and challenged them with GBS. C57BL/6 female mice originally obtained from Jackson laboratories (Bar Harbor, ME) and maintained at the pathogen-free facilities of the Instituto de Salud Pública de Chile (Institute of Public Health in Santiago, Chile) were utilised. All animals were handled in accordance with protocols approved by the Instituto de Salud Pública de Chile. For immunisations, mice from 6 to 8 weeks in age (five mice per group) received a subcutaneous injection for each treatment. The vaccinated group received 20 µg of rSIP, and the control groups received PBS-1X (PBS group).

The female mice were immunised 3 times, on days 0, 14, 28, and 4 days after the last immunisation, the animals were challenged by GBS intravaginal infection. The protocol of vaginal infection was adapted from Randis et al. [24]. The animals were challenged with 1×10^6 UFC/ml of GBS strain serotype III (Genbank: KU736792.1). We evaluated GBS vaginal colonisation and circulating IgG 96 h post-infection. Also, the experimental animals were killed 4 days post-challenge with lethal dose of ip anaesthetic (120 mg/kg ketamine; 10 mg/kg xylazine).

Anti-SIP IgG plasma samples were analysed by ELISA. Plates were coated with rSIP (1 µg) O/N at 4 °C. ELISA plates were blocked with blocking solution (3% BSA in PBS) for 1 h at RT. Serum samples were added to each well at sixfold dilutions for 1 h at RT. Plates were washed with PBST, followed by the addition of alkaline phosphatase-conjugated rat anti-mouse IgG (1:3000) incubated for 1 h. pNPP substrate was used to reveal ELISA as described above. The assays were performed in triplicate.

Vaginal rinsings were collected using a 40-µl of PBS-1X and agitated resuspended in 300 µl of Todd Hewitt broth supplemented with nalidixic acid, with serial dilutions plated to quantify organism recovery. The samples were patched onto 5% sheep blood for quantification of β-hemolytic colonies.

Results

Recombinant SIP Expression

The aim of this research was to find the best experimental conditions for rSIP expression in *E. coli* cells in batch mode using RSM and BBD, and the improvement in the immune response against GBS. We began our study with rSIP expression in accordance with the experimental conditions described in "Materials and Methods" section. The rSIP was expressed in *E. coli* cells and analysed by SDS-PAGE 12.5% (Fig. 1), using the same amount of bacterial total protein in each gel lane. We observed an overexpression of rSIP in samples corresponding to 1 and 2 h after IPTG induction.

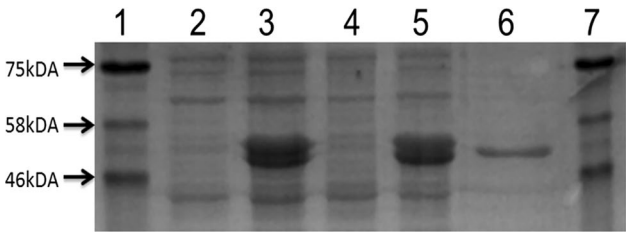

Fig. 1 Analysis of rSIP expression in *E. coli* cells using SDS-PAGE 12.5%. Lanes 1 and 7: MW P77SO4 (New England). Lanes 2 and 4: bacterial protein before induction with IPTG. Lanes 3 and 5: bacterial protein after 1 and 2 h of IPTG induction. Lane 6: purified rSIP

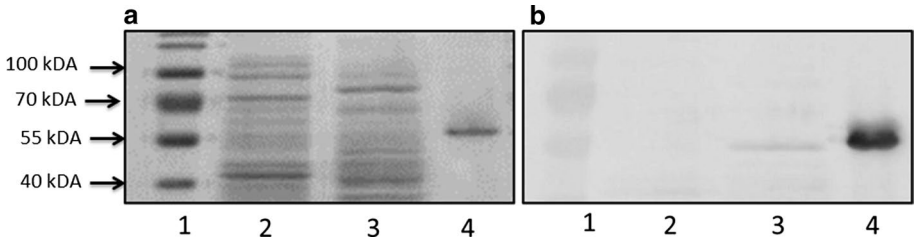

Fig. 2 Analysis of rSIP expression by SDS-PAGE 12.5% (**a**) and western blot (**b**). Lane 1 PageRuler prestained protein ladder (Thermo Scientific). Lane 2: *E. coli* protein without expression of rSIP. Lane 3: GBS protein. Lane 4: purified rSIP

The rSIP was purified using Pro-Bond Ni–NTA purification system following the protocol described in "Materials and Methods" section and analysed by SDS-PAGE 12.5% gel (Fig. 2a). A single protein band was observed in the samples eluted from the column. A pool of samples with a single rSIP was made and used in the subsequent experimental procedures. Western blot analysis was performed using an anti-SIP antibody made in rabbit, polyclonal antibodies made therefrom were as described [15, 25, 26] (Fig. 2b). The results showed rSIP expression throughout the period of protein induction, and no protein degradation was observed. A protein with a molecular weight of approximately of 53 kDa [15], consistent with rSIP, was observed. Having established the basal conditions for rSIP expression and purification, we confirm that the band of 53 kDA of purified protein in SDS-PAGE analysis belongs to rSIP. Its identity was confirmed by peptide mass fingerprinting (Fig. 3). The purified rSIP was quantified and used as a standard for determination of rSIP concentration expressed under different conditions (Table 1).

Response Surface Methodology and Box–Behnken Design

Ensuring that the rSIP expressed in *E. coli* cells under basal conditions was characterised, we next optimised the rSIP expression using RSM and BBD. For this purpose, we evaluated the yield of the rSIP expression against the total native bacterial protein. The yield was calculated using Eq. 1.

$$\text{Yield } (\%) = \frac{\text{rSIP Concentration } \left(\frac{\mu g}{ml}\right)}{\text{Total protein Concentration } \left(\frac{\mu g}{ml}\right)} \times 100\%. \quad (1)$$

The results of the experiments described in Fig. 4a, under No. 13–15, correspond to the midpoints of the experimental variables (Table 1), where we observed great variability between the values of total protein and specific rSIP, despite being treated under the same conditions. These results may

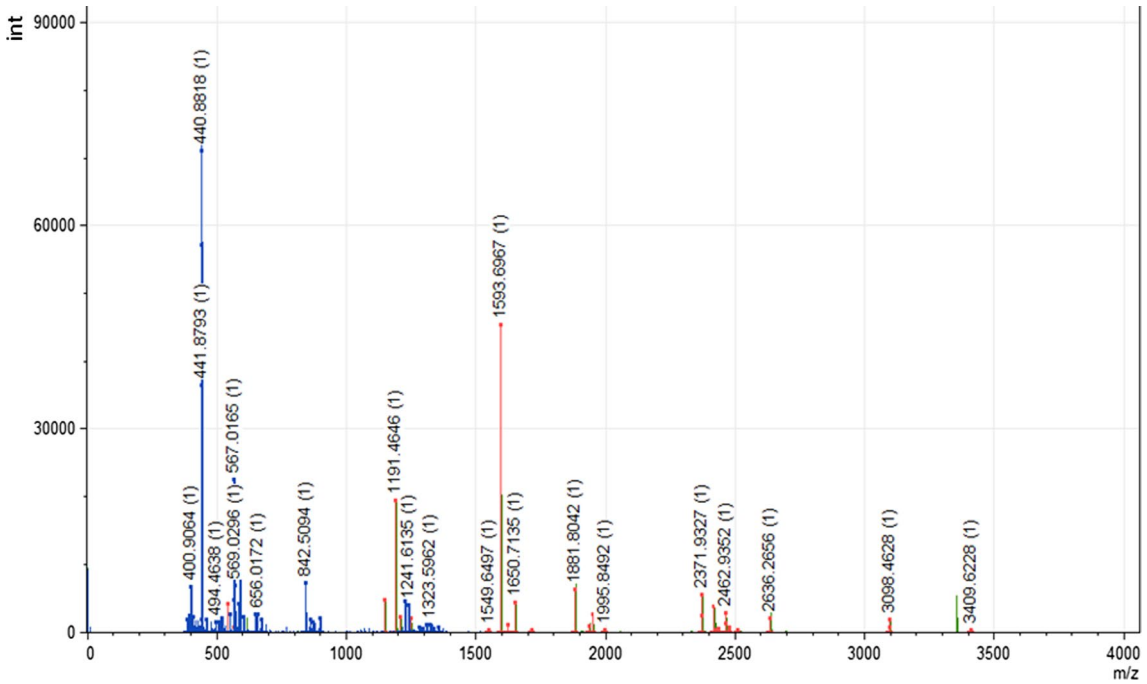

Fig. 3 Mass spectrum analysis of purified rSIP on a MALDI-TOF mass spectrometer. The rSIP was characterised by the LysM domain, which was compared to the NCBI database, and restricted to Firmicutes

a

Experiment (Nº)	Factor A (IPTG[mM])	Factor B (OD_{600})	Factor C (°C)	Total Protein [ug/ml]	rSIP protein [ug/ml]	Yield % (Y)
1	0.1	0.3	31	1751±19	81±5	4.60
2	1.5	0.3	31	1648±50	749±6	45.5
3	0.1	0.7	31	2059±45	896±3	43.5
4	1.5	0.7	31	1182±21	354±8	29.9
5	0.1	0.5	25	2028±13	15±2	0.80
6	1.5	0.5	25	1165±25	749±3	64.3
7	0.1	0.5	37	2932±11	1254±3	42.8
8#	1.5	0.5	37	2052±48	1585±2	77.2
9	0.8	0.3	25	3571±13	652±4	18.3
10	0.8	0.7	25	3674±46	415±3	11.3
11	0.8	0.3	37	1506±46	516±3	34.3
12	0.8	0.7	37	2612±19	1515±4	58.0
13	0.8	0.5	31	3196±25	1751±5	54.8
14	0.8	0.5	31	1693±47	753±4	44.5
15	0.8	0.5	31	2168±50	1095±5	50.5
16¤	0.8	0.5	37	1462±28	731±3	50.0

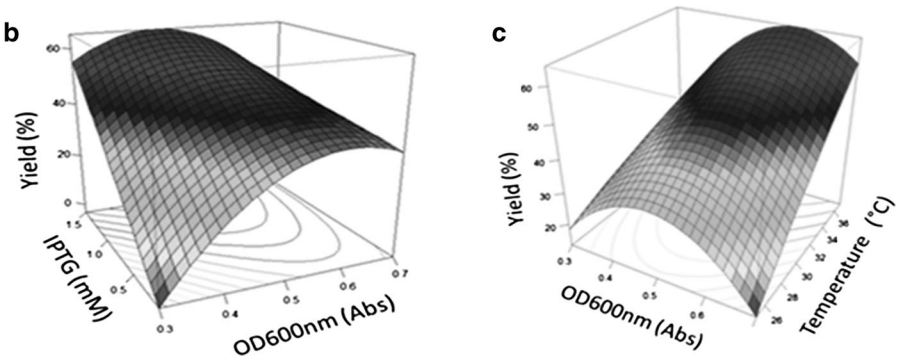

Fig. 4 Response surface methodology and Box–Behnken design in the optimisation of the expression of the rSIP. **a** Box–Behnken design matrix and experimental results for the different factors and their effect on the responses. Experiment 16¤ express rSIP under basal conditions and experiment 8# are the best conditions determined by RSM. **b** The response surface graph represents the influence of the concentration of IPTG and the optical density on the rSIP yield with constant temperature (31 °C). **c** The response surface graph represents the influence of the optical density and temperature on rSIP yield with a constant concentration of IPTG (0.8 mM). The R program was used to create the graphics. The ANOVAs determined a standard error in our model of 5.17333 and an R^2 of 89.99%

be attributed to the techniques used for protein quantification; however, despite the varying experimental values of the protein, the yields are similar, indicating that the relationship between the specific and the total protein expression remains within a close range, despite the differences in the amounts of protein obtained. Experiment No. 16 (Fig. 4a) corresponds to the initial experimental conditions, with 50% yield (basal condition). Among the experimental conditions defined in Fig. 4a, in No. 8, we observed an increase in the specific rSIP expression to 77%, the highest value observed in our experimental model. To determine the significance of the effect on rSIP expression, we performed an analysis of variance (ANOVA) [(Supplemental File, Table S1)]. Our mathematical analysis showed that the lack of fit value ($p = 0.1141$) was not significant, indicating that the mathematical model is applicable to our approach and validates our result. Also, we observed a standard error in our model of 5.17333 and an R^2 of 89.99%, which is less than 90% (the ideal result) and may explain the experimental variability in the yield.

To analyse the effect of the experimental factors on the rSIP expression, we used the regression model polynomial indicated by Statgraphics Centurion XVI (Eq. 2) and the equation with significant values (Eq. 3), where Yi (yield) is the predicted response, and the independent factors correspond to A = IPTG concentration; $B = OD_{600}$; C = temperature. The values of the different factors analysed using Eq. 3 were plotted using the R program, where we may observe the factors that increased and decreased rSIP expression (Table S1).

$$\begin{aligned} \mathrm{Yi} = {} & 49{,}933 + 15{,}65A + 5{,}0B + 14{,}7C - 1{,}62917A^2 \\ & - 13{,}625AB - 7{,}275AC - 17{,}4292B^2 \\ & + 7{,}675BC - 2{,}02917C^2 \end{aligned} \tag{2}$$

$$\mathrm{Yi} = 49{,}933 + 15{,}65A + 5{,}0B + 14{,}7C - 13{,}625AB - 17{,}4292B^2. \tag{3}$$

Using the Statgraphics Centurion XVI program, we obtained the best experimental condition for rSIP expression (Fig. 4a), defined as 1.5 mM IPTG, $OD_{600} = 0.5$ and $T = 37$ °C, with a theoretical yield of 69% (Table 2). Following the theoretically defined experimental conditions, we observed a 77% yield. The difference observed between the theoretical and practical performance may be described

Table 2 Validation of the experimental model

Experiment (no)	Factor *A* (IPTG [mM])	Factor *B* (OD_{600})	Factor *C* (°C)	Yield (%)
Basal condition	0.8	0.5	37	50
Predicted by RSM	1.5	0.5	37	69
Optimised condition	1.5	0.5	37	77

by $R^2 = 89.9\%$, and this variability in performance can be observed in the analysis of variance (ANOVA; Table S1).

To analyse the effect of IPTG and OD_{600} on the expression of rSIP, we used the graphic RSM (Fig. 4c), where we observed that the highest yield was achieved at the peak of curvature corresponding to 1.5 mM IPTG OD_{600} 0.5. In the same way, we analysed the relationship between OD_{600} and *T* with regard to rSIP expression. We observed the peak of curvature corresponding to OD_{600}0.5 and 37 °C (Fig. 4d). The relationship between IPTG and *T* belongs to a plane curve (data not shown). The graphic shows the relationship with $p = 0.1066$ (Table S1).

HPLC Analysis of rSIP Purified by Ni–NTA

In order to evaluate the impurities associated with the purification of the recombinant protein by NI–NTA column and the improvement in the yield by the RSM, we performed an HPLC analysis of the protein expressed in basal and optimised condition. The rSIP expressed in basal (rSIP-basal) and optimised conditions (rSIP-optimised) was purified in accordance with "Materials and Methods" section. The purified rSIP contains varying amounts of an unidentified impurity migrating with a minor mobilised (Fig. 5; peak 2–4 from the chromatographic run). The purified rSIP-optimised (Fig. 5a) generates smaller amounts of impurity in comparison with the rSIP-basal (Fig. 5b). These results demonstrate that the optimisation in the expression by the RSM generates an increase in the amount of specific rSIP by Ni–NTA due to the decrease in impurities.

Assessment of Immunogenicity of rSIP

The rSIP under optimised conditions was used to evaluate the immune response against vaginal GBS colonisation. An important aspect in the expression of recombinant proteins

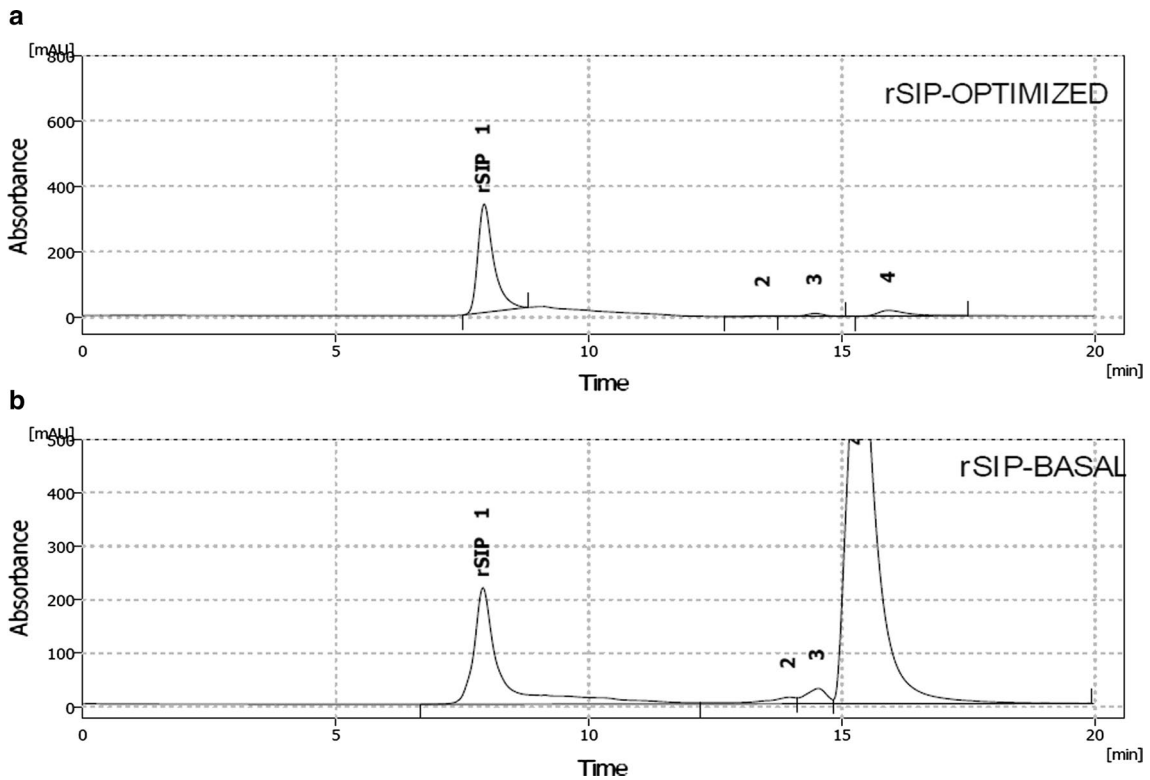

Fig. 5 HPLC analysis of purified rSIP under basal and optimised conditions. Size exclusion chromatography was performed on rSIP purified by Ni–NTA in 34 mM sodium phosphate and 0.5 M NaCl at pH = 6.6. The rSIP-optimised proteins (**a**) and the protein (**b**) rSIP-basal were analysed by HPLC. The peak 1 corresponds to rSIP, and peak 2–4 correspond to impurities of the chromatographic run

is the correct folding to preserve their immunogenic capacity [27]. In addition, conformational epitopes could generate antibodies less responsive to the pathogen and decrease immune response [28]. Otherwise, expression of recombinant proteins could co-purify with *E. coli native* proteins that bind to metal-chelating sorbents [29]. Therefore, the optimisation of rSIP expression and purification using RSM, and its immunogenic capacity against GBS is an important aspect of this work.

In order to evaluate the immunological function of our rSIP, we immunised female mice with rSIP-basal and rSIP-optimised and measured the specific IgG-SIP antibodies (Fig. 6). The rSIP-optimised allows an increased circulating IgG over the immunisation with rSIP-basal (Fig. 7a). The female mice were then vaginally inoculated with GBS, and 4 days after the second booster immunisation. The group immunised with rSIP-basal and rSIP-optimised had a decreased GBS burden after bacterial colonisation (Fig. 7b). Otherwise, the rSIP-optimised has a fold reduction over the colonisation that is non-significant compared with the rSIP-basal. However, the rSIP-optimised has a positive effect ($p = 0.0571$) to decrease intravaginal colonisation by GBS (Fig. 7c).

Discussion and Conclusion

The SIP from GBS has been shown to be an important target for the development of vaccines. Several studies have expressed the protein rSIP in *E. coli* cells; however, there is no consensus on its experimental expression conditions (Table 3). We investigated the contribution in the optimisation of rSIP expression using RSM on the immune response against GBS colonisation. The RSM and the BBD were used to improve the expression performance of the rSIP. The optimisation of the expression of recombinant proteins has been

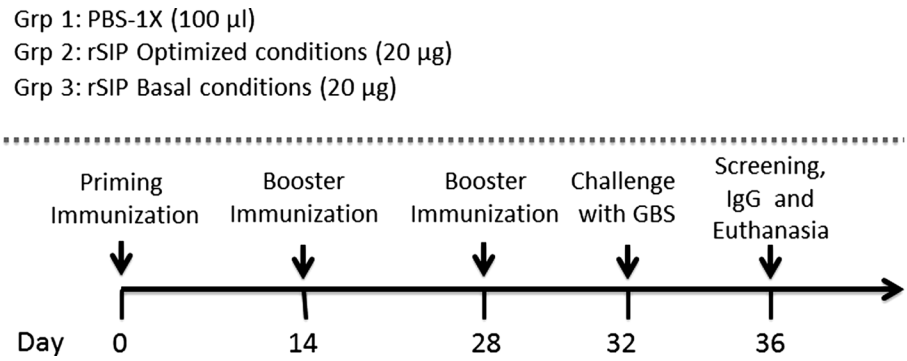

Fig. 6 Animal immunisation schedule. We applied three subcutaneous immunisations before the challenge with GBS. Four days post-infection, we performed the screening and IgG analysis

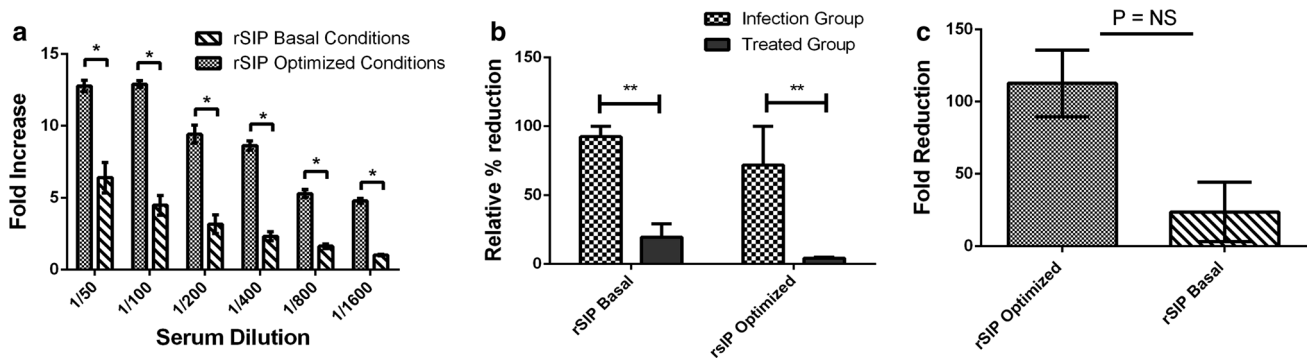

Fig. 7 Subcutaneous rSIP immunisation induces an IgG immune response and percentage reduction in GBS colonisation from the vaginal tract of C57 BL/6 mice. **a** Serum anti-SIP IgG antibody absorbance levels of groups immunised with rSIP in basal and optimised conditions. Serum dilutions were significantly elevated in mice immunised with rSIP in optimised conditions compared with rSIP expressed in basal conditions after GBS colonisation. Fold increase was calculated over serum of control group (PBS-1×). The results are expressed as the mean (± SD) of the fold increases in five mice from each group (*$p < 0.05$; unpaired Student's t test). **b** Relative percentage reduction in the vaginal CFU GBS at 4 days post-colonisation of immunised mice versus control mice. The bacterial colonisation was calculated over a range of dilutions and averaged. Each dilution was plated in triplicate. Data are representative of two independent experiments (**$p < 0.01$, Mann–Whitney U analysis). **c** Vaginal CFU GBS at 4 days post-colonisation of mice immunised with rSIP under optimised conditions versus rSIP-basal conditions. Fold reduction was calculated over control group (PBS-1X). The results are expressed as the mean (± SD) of the fold increases in five mice from each group ($p = 0.0571$; *NS* non-significant; unpaired Student's t test)

Table 3 Experimental conditions in induction of recombinant SIP

Author	*E. coli* strain	Vector	Expression conditions			
			IPTG (mM)	*T* (°C)	OD_{600}	Time (h)
Zhang et al. [16]	BL21 (DE3)	pET-32a	–	–	–	–
He et al. [12]	Rossetta	pET32a	0.2	37	–	4
Xu et al. [13]	BL21 (DE3)	pET30a	0.8	37	–	6
Xue et al. [14]	BL21 (DE3)	pET28a	–	–	–	–
Brodeur et al. [15]	BLR	pURV32	–	39	–	4–5
The present work	BL21 (DE3)	pET21a	1.5	37	0.5	2

studied through various experimental approaches. If we do not know the experimental factors involved in the fermentation and expression of recombinant proteins, we must use a screening design by Plackett–Burman [30], and Taguchi [19], and then applying a BBD. If we know the experimental factors, we may omit this step, saving time and resources [31]. Similar approaches have been considered by other authors for the expression of recombinant proteins [32–35]. In our study, a screening was not performed because we used the protocol described by Xu et al. [13]; this protocol was defined herein as the basal experimental condition. To the best of our knowledge, the RSM for the optimisation in the expression of recombinant proteins that could improve the immune response has not been studied until now.

The expression of the rSIP was characterised based on the presence of "Peptigoglycan-binding protein LysM" motif [36]. The MALDI-TOF MS analysis determined that the peptide imprint of the rSIP contains a LySM motif, which correlates with in silico studies [37]. The analysis was performed by Xue et al. [14] to characterise the identity of the rSIP expression and used in intranasal immunisation in BALB/c mice. In the present study, we performed the rSIP characterisation by SDS-PAGE, western blot, and MALDI-TOF MS, indicating that the protein is fully expressed in *E. coli* BL21 (DE3). CodonPlus studies have reported that SIP is exposed on the surface of intact cells of every GBS serotype [15, 25], and immunisation with recombinant SIP elicited specific antibodies that confer protection against GBS strains of different serotypes [14, 26, 38]. Otherwise, immunisation with inactivated bacteria, has been shown to generate an increase in antibody titres and decreased vaginal colonisation by GBS [39]. In the present study, the protein immunisation develops a SIP-specific humoral response and also generates a decreased intravaginal GBS colonisation in female mice. Moreover, the rSIP expression using RSM generates an increase on specific SIP-IgG antibodies compared with the expression of rSIP expressed under basal conditions; this increase may have generated a positive effect to decrease vaginal colonisation in mice. Further investigations are required to clarify the mechanism(s) by which rSIP decreases the intravaginal colonisation by GBS colonisation for the development of a vaccine against GBS infection.

The subunit vaccines that include purified proteins as antigens, which are either purified from the pathogen of interest, produced the majority using heterologous expression systems [40]. *Escherichia coli* cells have long been used for the expression of foreign protein and the His-tag technique is unarguably the most commonly used, and prepacked nickel columns for capturing His-tagged target proteins are widely available [41]. Otherwise, the presence of process-related impurities can modify and augment the immunogenic potential of proteins [42].

We demonstrated that the optimisation of the recombinant protein by RSM generates an increase in the amount of specific rSIP against the amount of impurities of the purification by NI–NTA. In addition, immunisation with purified rSIP optimised by RSM generates an increase in the immune response against GBS. The implication is that this is the first report in which the RSM is used in order to improve the protein purification and an immune response, which could be useful for the evaluation of vaccines based on recombinant proteins. Further, if the recombinant protein is used as pharmaceutical product, the purity can influence both patient safety and product efficacy. Because most of the adverse effects resulting from the elicitation of an excessive immune response to a therapeutic protein product appear to be mediated by effector mechanisms based on humoral immunity [43].

The RSM proved to be an effective tool for identifying significant variables and experimental conditions to optimise expression of rSIP in *E. coli* cells. Our study allowed us to increase the yield of rSIP expression from 50% (basal condition) to 77% (optimised condition). Furthermore, RSM optimisation leads to a decrease in the amount of impurities associated with the NI–NTA purification process. Besides, the rSIP-optimised generates an increase humoral response, displayed by high levels of SIP-specific IgG. Indeed, there was a tendency to decrease GBS vaginal colonisation by the immunisation with rSIP-optimised in comparison with rSIP-basal. These observations suggest an important consideration for the purity of protein vaccines used in immunisation, and also for the immunological evaluation by ELISA. Finally, the RSM applied to optimise the expression of recombinant proteins with immunogenic capacity on the

purification process influences experimental observation on the immune response.

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Authors' Contributions JJ, JS and DD-D performed all experimental procedures, data collection, analysis, interpretation and the writing of this paper. JS, DD-D and DS developed the experimental rSIP purification procedures and the ELISA procedure. RV performed the statistical analysis and interpretation. AMK and AEV analysed and interpreted the results. AEV designed and directed the experiments and wrote and edited this paper. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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