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ORIGINAL ARTICLE

Expression and refolding of the protective antigen of *Bacillus anthracis*: A model for high-throughput screening of antigenic recombinant protein refolding

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Abstract *Bacillus anthracis* protective antigen (PA) is a well known and relevant immunogenic protein that is the basis for both anthrax vaccines and diagnostic methods. Properly folded antigenic PA is necessary for these applications. In this study a high level of PA was obtained in recombinant *Escherichia coli*. The protein was initially accumulated in inclusion bodies, which facilitated its efficient purification by simple washing steps; however, it could not be recognized by specific antibodies. Refolding conditions were subsequently analyzed in a high-throughput manner that enabled nearly a hundred different conditions to be tested simultaneously. The recovery of the ability of PA to be recognized by antibodies was screened by dot blot using a coefficient that provided a measure of properly refolded protein levels with a high degree of discrimination. The best refolding conditions resulted in a tenfold increase in the intensity of the dot blot compared to the control. The only refolding additive that consistently yielded good results was L-arginine. The statistical analysis identified both cooperative and negative interactions between the different refolding additives. The high-throughput approach described in this study that enabled overproduction, purification and refolding of PA in a simple and straightforward manner, can be potentially useful for the rapid screening of adequate refolding conditions for other overexpressed antigenic proteins.

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PALABRAS CLAVE
Bacillus anthracis;
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Evaluación de alto rendimiento del replegado

Expresión y renaturalización del antígeno protector de *Bacillus anthracis*: un modelo para evaluar el replegado de proteínas antigénicas recombinantes a gran escala

Resumen El antígeno protector de *Bacillus anthracis* (protective antigen, PA) es una importante proteína inmunogénica, en la que se basan tanto las vacunas contra el ántrax/carbunclo como varios métodos diagnósticos. Para estas aplicaciones es esencial que el PA mantenga sus propiedades antigénicas, para lo cual debe estar correctamente plegado. En este estudio se obtuvieron altos niveles del PA en *Escherichia coli* recombinante. Inicialmente, la proteína se acumuló desnaturalizada en cuerpos de inclusión, lo que facilitó su eficiente purificación en simples pasos de lavado, pero no fue reconocida por anticuerpos específicos. Se analizaron las condiciones de replegado con un sistema de alto rendimiento, evaluando simultáneamente casi un centenar de condiciones diferentes. La recuperación de la capacidad del PA de ser reconocido por los anticuerpos se evaluó por *dot blot* utilizando un coeficiente que proporcionó una medida de los niveles de proteína correctamente plegada, con un alto grado de discriminación. Las mejores condiciones de renaturalización permitieron un aumento de diez veces en la intensidad de los *dot blots* con respecto del control. El único aditivo que produjo buenos resultados de forma constante fue la L-arginina. El análisis estadístico de las interacciones entre los diferentes aditivos de replegado permitió identificar tanto interacciones cooperativas como negativas. El enfoque de alto rendimiento descripto en este trabajo, que permitió la sobreproducción, purificación y plegado del PA de una manera sencilla y directa, puede ser potencialmente útil para el rápido *screening* de las condiciones adecuadas de replegado cuando se sobreexpresan otras proteínas antigénicas.

Introduction

From ancient times (fifth Biblical plague) to the present day (bioterrorism), *Bacillus anthracis* continues to be a specially relevant human and veterinary pathogen[27,30]. Its protective antigen (PA) is the basis for different anthrax vaccines, including second generation recombinant PA and third generation modified rPA vaccines[3,7,10,23,31]. This antigen is also essential for the evaluation of the serological status of vaccinated humans and animals[6,17,21,25]. Examples of these applications are diverse, and can range from field serodiagnosis of human cutaneous anthrax[14] to serological evaluation of wild animals[16,35].

PA is a protein of 83 kDa organized in four functional domains[27], for a long time was purified from *B. anthracis* culture supernatants[15,28]. In an effort to increase its yield, while avoiding the inconvenience of working with this pathogen, PA was produced in *Escherichia coli* expressing *pagA*, the gene responsible for PA synthesis in *B. anthracis*. The first studies that attempted to obtain this protein from recombinant *E. coli* reported that it suffered extensive degradation during the purification process, and also that the presence of large amounts of contaminant proteins made time-consuming procedures necessary[32]. Later, full-length PA was expressed in *E. coli* as a polyhistidine-tagged fusion protein, yielding insoluble protein aggregates[18]. More recently, untagged PA obtained as inclusion bodies (insoluble aggregates of misfolded proteins) was purified by hydrophobic-interaction chromatography yielding active PA[22]. Biologically active PA was also produced in the periplasm of recombinant *E. coli*[1,19,20].

Nowadays, the expression of recombinant proteins is essential for many biotechnological applications that generally require that these proteins conserve their native folding characteristics to remain functionally active. When high expression vectors are used for the overexpression of intracellular proteins, these are usually accumulated as inclusion bodies that lack biological activity. Expression as inclusion bodies facilitates protein purification, as they can be easily separated from cell debris[13]. However, hurdles arise when renaturation is attempted in order to obtain a properly refolded active protein[34]. Multiple refolding conditions have to be assayed because there is not a universally applicable protocol: specific conditions must be met for each protein and these cannot be determined *a priori*. As a result, finding the conditions for the efficient refolding of recombinant proteins can be a laborious task.

On the other hand, when expressed as inclusion bodies, some antigenic proteins can still be recognized by specific antibodies in the denatured state. Others need to be refolded, and in these cases antibody recognition can be useful to monitor the efficacy of the refolding protocol used.

In this work, a high throughput approach was used to efficiently monitor the correct refolding of recombinant PA, initially obtained as inclusion bodies. The recovery of the capability of PA to be recognized by antibodies was quantified allowing the assessment and detailed analysis of multiple different refolding conditions.

Materials and methods

Cloning of PA

B. anthracis reference strain Sterne $34F_2$ used in this study carries the virulence plasmid pXO1 and lacks pXO2. It had

been previously typified using the *vrrA* locus, as belonging to the $VNTR_4$ variant[26]. Boiled crude lysates were used as templates for PCR (polymerase chain reaction): several colonies grown overnight at 37 °C in Tryptic Soy Agar (Difco) were resuspended in Tris-EDTA buffer (10 mM Tris–HCl, 1 mM EDTA) and boiled at 98 °C during 10 min, centrifuged at 14,000 × *g* for 30 s and the supernatant directly used as DNA template for *pagA* amplification. DNA purification steps were avoided to reduce microorganism manipulation to a minimum. It was important to use a fresh non-sporulated culture as a DNA source for PCR. The PCR mixture contained 1× AccuPrime *Pfx* Reaction Mix, 1 mM $MgCl_2$, 1.5 U of AccuPrime *Pfx* DNA polimerase (Invitrogen), 1 μM each primer (pagA-forward and pagA-reverse) and 5 μl of DNA template in a total volume of 50 μl. The oligonucleotides pagA-forward (5′-CACCTTATTAAATGAATCWGAATCAAGTTCC-3′) and pagA-reverse (5′-TTATCCTATCTCATAGCCTTTTTTAGA-3′) were designed using the Oligo program[29], with manual optimization. The template was replaced by nuclease-free water for the negative PCR control. PCR assay was carried out as follows: 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s and extension at 68 °C for 3 min, with a final cycle at 68 °C during 20 min. Following the PCR, 10 μl of amplified product were purified using the Wizard SV gel and PCR clean-up System kit (Promega). Gateway pENTR/D-TOPO (Invitrogen) was used as an entry vector for cloning *pagA* and transformed into competent *E. coli* One Shot TOP10 (Invitrogen), according to the manufacturer's instructions. Expression plasmids, pEXP17-pagA, were generated by site-specific recombination, using LR clonase II, between the pENTR/D-TOPO-pagA plasmid previously obtained and the destination vector pDEST17, using the *E. coli* Expression System kit from the Gateway Technology (Invitrogen), with subsequent transformation of competent *E. coli* One Shot TOP10 (Invitrogen). The expression plasmids were used to transform competent *E. coli* BL21 Star (DE3) pLysS One Shot (Invitrogen) to obtain expression clones. Plasmids from two different expression clones were verified by sequencing.

Theoretical molecular weight and protein isoelectric point (pI) of the fusion recombinant protein PA were computed using ProtParam on the ExPASy Server[12].

Expression, purification and refolding of PA

PA was expressed by culturing expression clones in 200 ml MagicMedia *E. coli* Expression Medium (Invitrogen), in the presence of ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml), in shake flasks (250 r.p.m.) at 37 °C during 24 h (OD_{595} = 2.1). To analyze inclusion bodies *versus* soluble fraction proteins, bacteria were centrifuged during 2 min at 16,000 × *g* resuspended in BugBuster Protein Extraction Reagent (Novagen) and Benzonase Nuclease (Novagen), and incubated at room temperature for an hour with gentle agitation, according to the manufacturer's instructions. Inclusion bodies were separated from soluble proteins by centrifugation at 5200 × *g* for 5 min. Analysis of total, soluble and insoluble proteins was performed in 15% SDS-PAGE, followed by Coomassie Brilliant G-250 staining.

To purify inclusion bodies, cells were collected and frozen at −20 °C until processed. One gram of bacteria was suspended in 5 ml of BugBuster Protein Extraction Reagent and 125 U of Benzonase Nuclease. Previous to refolding, inclusion bodies were solubilized overnight at room temperature in 50 mM Tris–HCl, 200 mM NaCl, 2 mM EDTA, 10 mM tris (2-carboxyethyl) phosphine (TCEP), 7 M guanidine pH 8.0, with shaking at 250 r.p.m. Insoluble debris was removed by centrifugation at 16 000 × *g* for 15 min at 4 °C, and PA purity was checked by SDS-PAGE.

Protein concentration was measured by the Bradford method[4] in a microplate format. Samples (25 μl) were diluted in 155 μl water, and 45 μl reagent concentrate of the Bio-Rad Protein Assay (Bio-Rad) were added.

The iFOLD Protein Refolding System 2 (Novagen) was used to simultaneously assess multiple refolding conditions in a 96-well plate: 10 μl of denatured protein were added to each well containing 0.5 ml of refolding buffer, obtaining a final concentration of 100 ng/μl of PA. A multichannel pipettor was used to rapidly mix each well by pipetting up and down ten times. The refolding buffers were: MOPS pH 7.0, HEPES pH 7.5, EPPS pH 8.0, TAPS pH 8.5 or CHES pH 9.0, 50 mM each, with diverse combinations of salts, redox agents or refolding additives: NaCl:KCl (24:1 mM or 240:1 mM); TCEP 1 mM; reduced: oxidized glutathione (9:1 mM or 6:4 mM); EDTA 1 mM; L-arginine 0.5 M; PEG3350 0.06% w/v; non-detergent sulfobetaines NDSB-201 or NDSB-256 0.5 M or 1 M each; trehalose 0.58 M; sorbitol 1.5 M; methyl-β-D-cyclodextrin 10 mM; $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$: 0.25 mM each one. Incubation was carried out at room temperature for 16 h, with gentle agitation. After that, it was left to stand for 1 h and the soluble protein refolding level was monitored by dot blotting. Soluble protein was quantified by Bradford.

Assessment of PA refolding and antibody recognition

Antibody recognition of PA was assayed by Western or dot blots using nitrocellulose membranes (Pall Biodyne). For dot blots, 2 μl of the protein solutions were directly spotted onto the membranes. Membranes were blocked with 5% nonfat milk in TS buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl) and incubated for 45 min with 1:200 dilution of a mix of goat anti-C-terminal and anti-internal PA region polyclonal antibodies, PA (bc-19) and PA (bE-16) respectively (Santa Cruz Biotechnology, Inc.). Afterwards, membranes were incubated with a 1:2000 dilution of donkey anti-goat IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology, Inc.).

Quantification and computational analysis of dot blots

Dot blots were used to assess multiple refolding conditions at the same time. To avoid uncertainties due to the irregular dispersion of each droplet onto the nitrocellulose membrane, a method to quantify dot blot results was used. This method was based on an image processing technique that extracted, from an image of the multiwell plate, a matrix of numerical coefficients that was used to sort reagent refolding capabilities. An index standing for the image intensity level of each dot and its respective area was defined. This index, henceforth called coefficient *D*, was automatically

computed using the tools available in Matlab (Mathworks, Natick, USA). First, a grayscale, high resolution digital image of the whole plate layout was recorded. Then, a contour detection algorithm automatically recognized the edge of each individual dot, distinguishing it from the plate background, and computed the dot area (A) defined by the pixels inside the previously found edge. For each separate well, a relative density value (Δ density) was computed by subtracting the mean density value of the dot from the mean density of the image background. Finally, D was computed for each dot by multiplying its relative density by the respective area ($D = \Delta$ density $\times A$). Coefficient D was used as an index of antibody recognition, reflecting proper refolding of PA.

In order to evaluate the refolding capability of the different buffers and additives studied, statistical methods were used to evaluate results variability. An exploratory data Analysis of Variance (Kruskal–Wallis non-parametric one-way ANOVA, Matlab Statistical Toolbox) was first performed to identify those factors leading to higher coefficient D values. As in the multiwell plate assay, different factors were simultaneously considered, two-way ANOVA was used to search for possible interactions between them. In both analyses, results were considered statistically significant if the significance level (p) was less than 0.05.

Results and discussion

Expression and purification of recombinant PA

The N-terminal His-tagged fusion PA (84.98 kDa) constructed in this work has 756 aminoacids, no cysteines and a theoretical pI of 5.97. Expression of high levels of this recombinant protein (200 μg per ml of bacterial culture) was achieved in an expression medium that does not require induction. When the crude extract was analyzed by SDS-PAGE, a dominant protein band of the expected size was observed (Fig. 1a, lanes 1 and 2). Analysis of the soluble and insoluble fractions demonstrated that PA appeared exclusively in the insoluble fraction (Fig. 1a, lanes 3 and 4), and that most of the contaminant proteins remained in the supernatant as soluble proteins during cellular lysis (Fig. 1a, lanes 5 and 6). The T7 lysozyme produced by the host strain, combined with a special reagent that causes the gentle disruption of the cell wall, facilitated bacterial lysis permitting a rapid and easy recovery of inclusion bodies, which after several washes formed a distinct white pellet. Analysis of the guanidine solubilized pellet by SDS-PAGE (Fig. 1b) revealed a remarkable high purity, considering that no purification steps other than simple washes were performed. Anti-C-terminus or anti-internal PA region polyclonal antibodies did not recognize the recombinant protein in Western blots (data not shown), indicating that PA did not retain the capability to be recognized by specific antibodies in this stage.

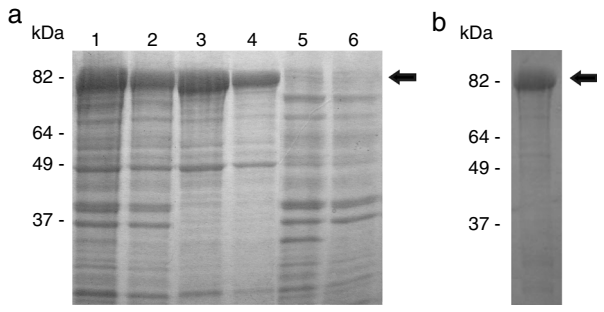

Figure 1 (a) SDS-PAGE of recombinant PA (indicated by the arrow) expressed in *E. coli* BL21 Star (DE3) pLysS carrying plasmid pEXP17-pagA. Lanes 1 and 2: total protein, lanes 3 and 4: insoluble fraction, lanes 5 and 6: soluble fraction. Results are shown for two different clones, corresponding to odd and even lanes respectively. (b) SDS-PAGE of washed inclusion bodies solubilized in 6 M guanidine.

Previous studies reporting the production of recombinant PA in *E. coli* involved multiple purification steps. In some studies in which PA was obtained as inclusion bodies, it was subjected to several purification steps including metal chelate affinity chromatography[18], or ammonium sulfate precipitations followed by hydrophobic-interaction chromatography[22]. Other researchers obtained PA in the periplasm, and used several different chromatographic steps to partially purify PA, such as ion exchange and hydrophobic interaction chromatography[1], or ion exchange and hydroxyapatite chromatography[20]. The high expression of PA obtained in the present work, combined with the low background and the enrichment of PA in the lysis step, facilitated PA purification using only washes, without the need for chromatographic purification steps.

Assessment of PA renaturing conditions

As PA was obtained in inclusion bodies, we assumed that its lack of antibody recognition was due to improper folding. Multiple renaturing conditions were subsequently tested for guanidine solubilized inclusion bodies using a refolding system that provides nearly a hundred unique buffers, allowing a high-throughput refolding screening. This system consists of a 96-well plate, each containing a different refolding solution. The renaturing conditions are generated through different buffers with the addition of diverse concentrations and combinations of the following components: NaCl and KCl, to supply different ionic forces; TCEP and/or reduced-oxidized glutathione as redox agents; L-arginine, PEG, non-detergent sulfobetaines NDSB-201 and NDSB-256, EDTA, trehalose, sorbitol, β-cyclodextrin and several cations as renaturing additives. We expected that dilution of PA in some of the multiple refolding solutions would lead to the correct refolding, restoring its ability to be recognized by antibodies.

As the properly refolded PA should remain in the soluble state, renaturing was monitored both by assaying the concentration (Bradford) and antibody binding (dot blots) of the protein that stayed soluble in each well (Table 1 and Fig. 2). The data presented in this work result from a single replicate testing 96 different buffers and additives conveniently contained in a single refolding plate. Protein quantification was useful to estimate soluble protein in most conditions assayed, however, some buffer components interfered with the Bradford method. For

Table 1 Assessment of all refolding conditions

No.[a]	Well	Coefficient *D*	Bradford[b]	Buffer	pH	Redox agents[c]	Additives[d]	NDSB[e]	Salts[f]	Metals/ EDTA[g]
1	C4	1178	0.69	HEPES	7.5	GSH + GSSG(1)	–	NDSB-256(2)	+	EDTA
2	F12	1172	0.63	CHES	9	TCEP	–	NDSB-256(2)	+	–
3	C10	1143	0.71	TAPS	8.5	GSH + GSSG(2)	–	NDSB-256(2)	+	EDTA
4	F6	1139	0.72	EPPS	8	–	–	NDSB-256(2)	–	–
5	G9	1128	0.80	TAPS	8.5	–	–	NDSB-256(1)	+	–
6	B7	1102	0.68	EPPS	8	GSH + GSSG(2)	–	NDSB-256(2)	+	–
7	D6	1087	0.72	EPPS	8	GSH + GSSG(1)	–	NDSB-256(1)	–	EDTA
8	D10	1085	0.75	TAPS	8.5	GSH + GSSG(2)	–	NDSB-256(1)	+	–
9	G6	1081	0.67	EPPS	8	–	PEG3350	–	+	–
10	H8	1047	0.71	TAPS	8.5	–	–	NDSB-201(1)	+	–
11	D9	1044	0.82	TAPS	8.5	GSH + GSSG(1)	L-arginine	–	+	EDTA
12	D4	995	0.72	HEPES	7.5	GSH + GSSG(1)	–	NDSB-256(2)	–	–
13	F4	991	0.74	HEPES	7.5	–	–	NDSB-201(2)	+	–
14	C9	987	0.80	TAPS	8.5	GSH + GSSG(1)	–	NDSB-201(2)	+	–
15	A7	980	0.70	EPPS	8	GSH + GSSG(2)	–	NDSB-201(1)	–	EDTA
16	B9	969	0.75	TAPS	8.5	GSH + GSSG(1)	–	NDSB-201(2)	–	EDTA
17	A2	961	0.75	MOPS	7	GSH + GSSG(2)	L-arginine	–	+	EDTA
18	G11	951	0.75	CHES	9	TCEP	–	NDSB-201(2)	+	–
19	C5	944	0.70	HEPES	7.5	GSH + GSSG(2)	–	NDSB-256(1)	–	EDTA
20	E11	941	0.78	CHES	9	–	–	NDSB-256(1)	–	–
21	E5	934	0.85	HEPES	7.5	–	L-arginine	–	–	–
22	B4	933	0.66	HEPES	7.5	GSH + GSSG(1)	–	NDSB-201(1)	+	–
23	C3	887	0.69	MOPS	7	TCEP	–	NDSB-256(2)	+	Metals
24	E8	879	0.79	TAPS	8.5	TCEP	Sorbitol	–	+	–
25	B10	877	1.00	TAPS	8.5	GSH + GSSG(2)	PEG3350	–	+	EDTA
26	G8	877	0.66	TAPS	8.5	TCEP	PEG3350	–	–	–
27	G10	875	0.61	CHES	9	–	Trehalose	–	+	Metals
28	E4	874	0.86	HEPES	7.5	GSH + GSSG(1)	L-arginine	–	+	–
29	D7	872	0.65	EPPS	8	GSH + GSSG(2)	–	NDSB-201(2)	+	EDTA
30	A10	866	0.75	TAPS	8.5	GSH + GSSG(2)	sorbitol	–	+	–
31	A6	850	0.71	EPPS	8	GSH + GSSG(1)	–	NDSB-256(1)	–	–
32	G7	849	0.78	EPPS	8	TCEP	L-arginine	–	+	–
33	E7	836	0.60	EPPS	8	GSH + GSSG(2)	Trehalose	–	+	–
34	F11	835	0.62	CHES	9	TCEP	–	NDSB-201(1)	+	Metals
35	D5	833	0.58	HEPES	7.5	GSH + GSSG(2)	PEG3350	–	–	–
36	C8	819	0.85	TAPS	8.5	TCEP	L-arginine	–	+	Metals
37	H9	803	0.69	TAPS	8.5	–	–	–	–	–
38	B1	798	0.79	MOPS	7	GSH + GSSG(1)	L-arginine	–	+	–
39	F10	777	0.59	CHES	9	–	Sorbitol	–	+	–
40	D8	768	0.62	TAPS	8.5	TCEP	Trehalose	–	–	–
41	H4	765	1.32	HEPES	7.5	–	β-Cyclodextrin	–	–	–
42	B11	761	0.60	CHES	9	GSH + GSSG(1)	–	NDSB-256(2)	+	EDTA
43	E10	760	0.76	TAPS	8.5	GSH + GSSG(2)	–	NDSB-201(1)	–	–
44	B12	759	0.60	CHES	9	GSH + GSSG(2)	L-arginine	–	+	EDTA
45	G2	759	0.66	MOPS	7	–	–	–	+	–
46	E6	753	0.66	EPPS	8	GSH + GSSG(1)	PEG3350	–	+	–
47	A8	751	1.26	EPPS	8	TCEP	β-Cyclodextrin	–	+	–
48	C1	744	0.52	MOPS	7	GSH + GSSG(1)	PEG3350	–	–	EDTA
49	G12	732	0.79	CHES	9	TCEP	L-arginine	–	–	Metals
50	D3	732	0.95	HEPES	7.5	TCEP	Sorbitol	–	+	–
51	A4	721	0.61	HEPES	7.5	GSH + GSSG(1)	Trehalose	–	+	EDTA
52	F3	705	1.35	HEPES	7.5	TCEP	β-Cyclodextrin	–	–	–
53	H5	705	0.60	EPPS	8	–	–	–	–	–
54	F5	703	0.55	HEPES	7.5	–	–	–	–	–
55	B2	702	0.56	MOPS	7	GSH + GSSG(2)	–	NDSB-256(1)	+	EDTA
56	G5	692	0.51	EPPS	8	–	–	NDSB-201(2)	+	Metals
57	A9	674	0.67	TAPS	8.5	GSH + GSSG(1)	–	–	–	–

Table 1 (*Continued*)

No.[a]	Well	Coefficient *D*	Bradford[b]	Buffer	pH	Redox agents[c]	Additives[d]	NDSB[e]	Salts[f]	Metals/ EDTA[g]
58	B5	670	0.60	HEPES	7.5	GSH + GSSG(2)	–	–	—	EDTA
59	C6	663	1.30	EPPS	8	GSH + GSSG(1)	β-Cyclodextrin	–	+	–
60	C7	646	0.59	EPPS	8	GSH + GSSG(2)	Trehalose	–	—	EDTA
61	A5	607	0.62	HEPES	7.5	GSH + GSSG(2)	–	–	+	–
62	H12	599	0.65	CHES	9	TCEP	PEG3350	–	—	Metals
63	H1	581	0.54	MOPS	7	–	–	–	—	–
64	F7	577	0.61	EPPS	8	TCEP	Sorbitol	–	+	Metals
65	G1	568	0.59	MOPS	7	–	Trehalose	–	+	–
66	A1	544	1.26	MOPS	7	GSH + GSSG(1)	β-Cyclodextrin	–	+	EDTA
67	H11	535	0.59	CHES	9	TCEP	–	–	+	–
68	E1	503	0.55	MOPS	7	GSH + GSSG(1)	Trehalose	–	—	–
69	H10	489	0.59	CHES	9	–	–	–	—	–
70	D1	485	0.51	MOPS	7	GSH + GSSG(1)	–	–	+	EDTA
71	B6	481	0.51	EPPS	8	GSH + GSSG(1)	Sorbitol	–	—	EDTA
72	B3	469	0.44	MOPS	7	TCEP	–	NDSB-201(2)	—	Metals
73	F9	467	0.46	TAPS	8.5	–	Trehalose	–	+	Metals
74	A3	465	0.46	MOPS	7	TCEP	–	NDSB-256(1)	+	Metals
75	F2	463	0.70	MOPS	7	–	–	NDSB-256(2)	+	Metals
76	E2	453	0.56	MOPS	7	GSH + GSSG(2)	–	NDSB-201(2)	—	–
77	E3	449	0.48	HEPES	7.5	TCEP	–	NDSB-201(1)	+	Metals
78	E9	423	1.31	TAPS	8.5	–	β-Cyclodextrin	–	+	Metals
79	H2	410	0.42	MOPS	7	–	–	NDSB-201(1)	+	Metals
80	D2	328	0.48	MOPS	7	GSH + GSSG(2)	–	NDSB-201(1)	—	–
81	A12	308	0.41	CHES	9	GSH + GSSG(2)	Sorbitol	–	+	EDTA
82	G3	303	0.42	HEPES	7.5	TCEP	Trehalose	–	+	Metals
83	G4	293	0.45	HEPES	7.5	–	–	NDSB-201(2)	+	Metals
84	F1	252	0.43	MOPS	7	–	Sorbitol	–	—	Metals
85	H3	226	0.38	HEPES	7.5	–	PEG3350	–	+	Metals
86	C12	212	0.41	CHES	9	GSH + GSSG(2)	β-Cyclodextrin	–	+	–
87	E12	208	0.79	CHES	9	GSH + GSSG(2)	PEG3350	–	+	–
88	D11	195	0.44	CHES	9	GSH + GSSG(1)	–	NDSB-256(1)	+	–
89	C2	189	1.08	MOPS	7	GSH + GSSG(2)	β-Cyclodextrin	–	+	EDTA
90	H6	184	0.39	EPPS	8	–	–	–	+	Metals
91	F8	160	1.31	TAPS	8.5	TCEP	β-Cyclodextrin	–	–	Metals
92	D12	136	0.46	CHES	9	GSH + GSSG(2)	β-Cyclodextrin	–	+	–
93	B8	123	0.40	EPPS	8	TCEP	–	–	+	Metals
94	H7	119	0.40	H_2O	7	–	–	–	–	–
95	A11	32	0.47	CHES	9	GSH + GSSG(1)	Sorbitol	–	+	–
96	C11	13	0.41	CHES	9	GSH + GSSG(1)	–	NDSB-201(1)	+	EDTA

[a] Order by decreasing coefficient *D* values.
[b] Absorbance values at 595 nm.
[c] Redox agents: reduced and oxidized glutathione (GSH + GSSG) 9:1 mM(1) or 6:4 mM(2); TCEP 1 mM.
[d] Additives: L-arginine 0.5 M; PEG3350 0.06% w/v; trehalose 0.58 M; sorbitol 1.5 M; methyl-β-D-cyclodextrin 10 mM.
[e] NDSB: NDSB-201(1) 0.5 M or NDSB-201(2) 1 M/NDSB-256(1) 0.5 M or NDSB-256(2) 1 M.
[f] Salts: + indicates wells containing NaCl and KCl (24:1 mM or 240:1 mM).
[g] EDTA 1 mM; metals: $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$: 0.25 mM each one.

example, nearly all of the wells containing β-cyclodextrin gave an intense light-blue color, greatly increasing absorbance.

The antibody recognition levels of soluble protein were analyzed with a high-throughput dot blot screening (Fig. 2b). A simple visual analysis revealed dots with different intensities; however, in order to achieve an accurate quantification, a computer program was used to analyze the density of the dots using a digitized image of the membrane containing the 96 spots. In addition, this program allowed us to perform a detailed analysis of the effect of the different renaturing conditions on PA antibody binding.

Table 1 and Fig. 3 show coefficient *D* values for each dot in decreasing order. Coefficient *D* values for the best refolding conditions were ten times higher than the value corresponding to the control (water, $D = 119$), allowing good discrimination between the different conditions analyzed. When coefficient *D* values were compared with soluble

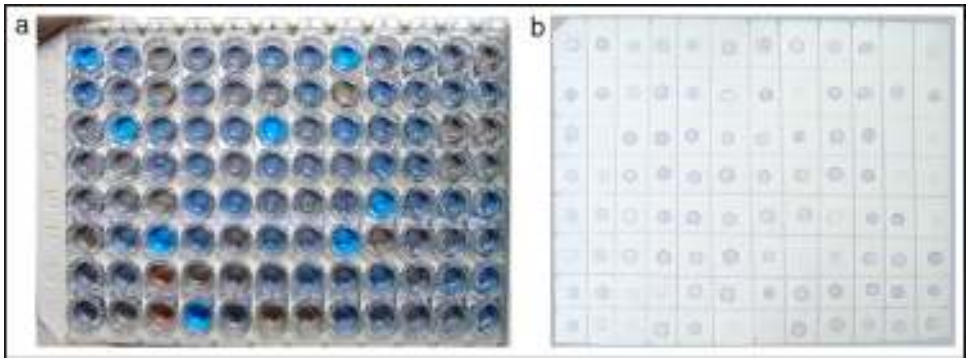

Figure 2 High-throughput screening for PA refolding conditions. (a) Soluble protein concentration Bradford assay. (b) Soluble PA dot blot assay. The buffer and additives corresponding to each well in the 96-well plate are shown in Table 1.

protein concentrations determined by Bradford, a good correlation was observed (Table 1 and Fig. 4), except for nearly all (8/10) the wells containing β-cyclodextrin due to the intense blue color developed that gave high absorbance readings, as previously mentioned. When these eight wells were excluded, the highest coefficient *D* corresponded to samples with high soluble protein concentrations. The two exceptions to beta-cyclodextrin interference were C12 and D12, in which the refolding buffer was CHES pH 9.0.

Statistical analysis of antibody recognition of PA

Considering a coefficient *D* cut-off of 900 to define the strongest signals, no obvious combination of buffers and refolding additives stands out as the best (Table 1). The two highest *D* values were obtained in wells containing HEPES pH 7.5 and CHES pH 9.0 buffers; however, the remaining conditions in the wells above the 900 cut-off value included either TAPS pH 8.5 or EPPS pH 8.0, and only one contained MOPS pH 7.0. When the coefficient *D* of all conditions was plotted against the different buffers, a great dispersion was observed for all of them (Fig. 5), although buffer TAPS pH 8.5 had the best overall performance, with 80 % of the wells that contained it (15/19) showing high antibody recognition (coefficient $D > 760$).

When the coefficient *D* corresponding to the wells containing each of the different additives were compared, the only one that appeared to yield consistently good results was L-arginine, as all (9/9) wells containing this additive had values above 700, three of which were higher than 900 (Fig. 5). Moreover, in one of the wells with the highest coefficient *D*, L-arginine was the only component present apart

	1	2	3	4	5	6	7	8	9	10	11	12
A	D=544 66	D=961 17	D=465 74	D=721 51	D=607 61	D=850 31	D=980 15	D=751 47	D=674 57	D=866 30	D=32 95	D=308 81
B	D=798 38	D=702 55	D=469 72	D=933 22	D=670 58	D=481 71	D=1102 6	D=123 93	D=969 16	D=877 25	D=761 42	D=759 44
C	D=744 48	D=189 89	D=887 23	D=1178 1	D=944 19	D=663 59	D=646 60	D=819 36	D=987 14	D=1143 3	D=13 96	D=212 86
D	D=485 70	D=328 80	D=732 50	D=995 12	D=833 35	D=1087 7	D=872 29	D=768 40	D=1044 11	D=1085 8	D=195 88	D=136 92
E	D=503 68	D=453 76	D=449 77	D=874 28	D=934 21	D=753 46	D=836 33	D=879 24	D=423 78	D=760 43	D=941 20	D=208 87
F	D=252 84	D=463 75	D=705 52	D=991 13	D=703 54	D=1139 4	D=577 64	D=160 91	D=467 73	D=777 39	D=835 34	D=1172 2
G	D=568 65	D=759 45	D=303 82	D=293 83	D=692 56	D=1081 9	D=849 32	D=877 26	D=1128 5	D=875 27	D=951 18	D=732 49
H	D=581 63	D=410 79	D=226 85	D=765 41	D=705 53	D=184 90	D=119 94	D=1047 10	D=803 37	D=489 69	D=535 67	D=599 62

Figure 3 Composite image of the dots, showing Coefficient *D* values above each dot while the number at the bottom indicates the order by decreasing coefficient *D* values. Letters and numbers on the top and left margins indicate original dot location, corresponding to the multiwell plate and the dot blot assay shown in Fig. 2.

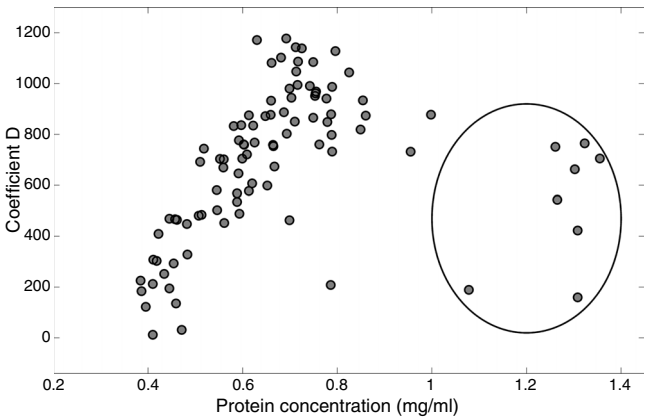

Figure 4 Correlation between antibody recognition (represented by coefficient *D*) and soluble protein concentration (determined by Bradford), for all conditions tested. Values lacking correlation, all of them corresponding to wells containing β-cyclodextrin (8/10), are circled.

from the buffer. Arginine is known to improve protein solubility and inhibit aggregation (it is a moderate chaotrope), although the molecular mechanisms behind this effect are still unclear[39]. The high signals obtained with L-arginine agree with previously reported results[5,39,40] indicating that it regularly allows efficient renaturation, making L-arginine an additive of choice for routine antigenic recombinant protein refolding protocols.

All wells (10/10) containing β-cyclodextrin had low coefficient *D* values, with no wells above 765 (Fig. 5). This result indicated that β-cyclodextrin was not efficient to increase PA antibody recognition, although it has been reported to be effective in the refolding of some proteins under other conditions[2,24,41]. Cyclodextrins are known to inhibit protein aggregation during the refolding process, as they can bind to hydrophobic protein surfaces increasing folding yields[39]. As our refolding efficiency evaluation was based on the capability of PA to be recognized by antibodies, we cannot rule out that β-cyclodextrin affects antibody binding in a way not related to protein folding, such as hindering the accessibility of the antibodies to PA epitopes. Although a great dispersion was observed for the rest of the additives, almost all wells (82 %) with coefficient *D* above 900 contained non-detergent sulfobetaines (Table 1). Two different variants, NDSB-256 and NDSB-201, were analyzed at two different concentrations. One-way analysis of variance indicated that NDSB-256 has a statistically significant ($p = 0.0016$) beneficial effect on PA refolding only when used at 1 M. No significant correlations were observed for NDSB-201, although previous reports have indicated that 1 M NDSB-201 reduced the aggregation of unfolded or partially unfolded proteins[8,11].

Wells containing sorbitol, trehalose or metals also showed great dispersion, but none had coefficient *D* values over 900. A similar trend was observed for PEG3350, with only one well above the cut-off value. Polyethylene glycol (PEG), sorbitol and trehalose have been proposed to act as stabilizers of protein structures[39].

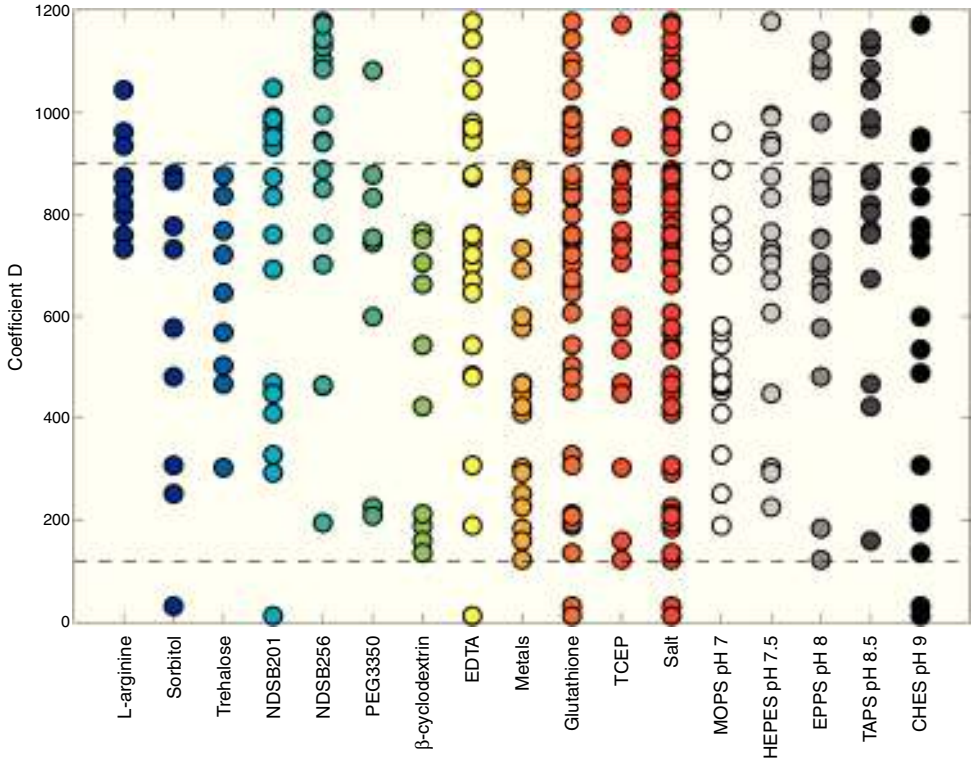

Figure 5 Coefficient *D* values corresponding to each refolding additive and buffer system. The cut-off value of 900 is shown with a dashed line, as well as the value for the control well (containing water), nearly 120. Different concentrations of the following additives were plotted together: NDSB-256, NDSB-201, salts, and glutathione (reduced and oxidized).

No statistically significant effects were observed for the addition of the commonly used reduced/oxidized glutathione redox agent[13,33] and wells containing this additive could be observed along the whole range, including the highest and lowest coefficient *D* values (Fig. 5). This is probably due to the lack of cysteines in PA, which could be involved in the formation of disulfide bridges in the protein. Wells containing TCEP or EDTA did not reveal an effect of these compounds on PA refolding.

Possible interactions between the different buffers and components were assessed using a two-way analysis of variance. Glutathione was observed to interact with CHES pH 9.0 buffer ($p = 0.001$), reducing its effect. On the other hand, although in the presence of glutathione the wells containing EPPS, HEPES and TAPS buffers had slightly higher *D* values, these differences were not statistically significant. When the interaction between the buffers and metals was analyzed, metals were observed to reduce the effects of EPPS and HEPES. No significant interactions were observed between buffers and L-arginine, NDSB or other components such as TCEP, sorbitol, trehalose, PEG3350, EDTA, salts and β-cyclodextrin.

Previous studies that focused on the high-throughput identification of optimal conditions for protein renaturation have identified different sets of conditions for different proteins, improving yields of biologically active proteins[9,36–38]. The determination of correct folding in these studies is generally based on *in vitro* assays of biological activity. A very important property for many biotechnologically relevant proteins used in a variety of applications, including healthcare and diagnostics, is their ability to be recognized by specific antibodies. This property is essential for bacterial antigens used in vaccines and/or pathogen detection, such as PA. The results obtained in the present work with this protein demonstrated that the different combinations of buffers and additives used for refolding can significantly affect its ability to interact with antibodies, and identified the conditions that resulted in a tenfold increase of its antibody binding properties with a high degree of discrimination. The statistical analysis of the interactions between different components allowed to determine the contribution of each of them on PA refolding and the identification of both cooperative and negative interactions. The high-throughput approach described in this study can be potentially useful for the rapid screening of adequate refolding conditions for other overexpressed antigens.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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