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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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*Bacillus anthracis*;
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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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**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