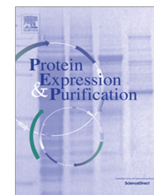
Protein Expression and Purification 108 (2015) 30–40

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep



An efficient method for the purification of proteins from four distinct toxin–antitoxin modules

Yann G.-J. Sterckx [a,b], Steven De Gieter [a,b], Valentina Zorzini [a,b], San Hadži [a,b,c], Sarah Haesaerts [a,b], Remy Loris [a,b,*,1], Abel Garcia-Pino [a,b,*,1]

[a] Structural Biology Brussels, Department of Biotechnology, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium
[b] Structural Biology Research Center, VIB, Pleinlaan 2, B-1050 Brussels, Belgium
[c] Department of Physical Chemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, 1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 12 November 2014
and in revised form 27 December 2014
Available online 9 January 2015

Keywords:
Toxin–antitoxin
Persisters
Protein purification
Refolding protocol

ABSTRACT

Toxin–antitoxin (TA) modules are stress response elements that are ubiquitous in the genomes of bacteria and archaea. Production and subsequent purification of individual TA proteins is anything but straightforward as over-expression of the toxin gene is lethal to bacterial and eukaryotic cells and over-production of the antitoxin leads to its proteolytic degradation because of its inherently unstructured nature. Here we describe an effective production and purification strategy centered on an on-column denaturant-induced dissociation of the toxin–antitoxin complex. The success of the method is demonstrated by its application on four different TA families, encoding proteins with distinct activities and folds. A series of biophysical and *in vitro* activity tests show that the purified proteins are of high quality and suitable for structural studies.

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Introduction

TA modules are ubiquitous in the genomes of prokaryotes in which they play an important role in a myriad of biological functions [1–4]. These include stress response, multi-drug tolerance, biofilm formation and the generation of persister cells [5–12]. Of all known TA loci, members of type II systems have been the most extensively studied.

Type II TA modules are typically organized in small operons containing one antitoxin gene lying upstream of one toxin gene, although variations on this theme may occur [13–15]. The toxin may be a monomer or a dimer and derives its name from the observations that it inhibits essential cellular processes such as transcription or translation by corrupting the function of essential molecules such as DNA gyrase, tRNA or the ribosome [14,16–25]. This 'poisoning effect' of the toxin will lead to growth arrest and ultimately to cell death when unregulated [26]. The antitoxin typically consists of two functionally distinct domains. The N-terminal domain adopts a well-defined fold and is a DNA-binding and dimerization domain. The C-terminal part is usually intrinsically disordered and neutralizes its cognate toxin via the formation of a tight complex [14,27–31]. The intrinsic unstructured nature of this neutralization domain makes the antitoxin highly susceptible for proteolytic degradation, reducing its *in vivo* lifetime [32–34] and also allows for a tight link between regulation of transcription and protein activity [31,35,36]. TA modules have found widespread applications in biotechnology and show potential in medicine and drug design [37–40].

Despite these interests, obtaining large quantities of TA proteins remains challenging. Obtaining recombinant wild-type toxins is difficult due to their toxicity in bacterial and eukaryotic cells [41,42]. Often their genes cannot even be cloned without introducing unwanted mutations [43]. Several strategies have been successfully formulated to bypass toxicity issues during toxin production, although they each contain drawbacks. The first strategy to produce toxin is based on knowledge of bacterial strains resistant to the action of the toxin [44,45]. This approach has successfully been used to acquire large amounts of the CcdB and VapC toxins, but such resistant strains are not available for other toxin families [46]. A second tactic uses the neutralising activity of the antitoxin. Pure free toxin can be produced when the antitoxin is provided in *trans* as a protection against accidental expression of low amounts of toxin prior to induction [47]. However, the authors reported that subsequent purification of the toxin is thorny because over-expression of the toxin remains limited and a

* Corresponding authors at: Structural Biology Brussels, Department of Biotechnology, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. Tel.: +32 2 629 18 53; fax: +32 2 629 19 63.

E-mail addresses: remy.loris@vib-vub.be (R. Loris), agarciap@vub.ac.be (A. Garcia-Pino).

[1] Contributed equally to this work and should be considered joint senior authors.

http://dx.doi.org/10.1016/j.pep.2015.01.001
1046-5928/© 2015 Published by Elsevier Inc.

significant portion of the produced toxin co-elutes with its cognate antitoxin, which notably reduces the yield of free toxin. A third procedure consists in more stringently controlling the amount of produced protein *in vivo* through the use of specialized expression vectors (e.g. PBAD [48]). This approach eliminates the issue of the toxin being 'trapped' in the toxin–antitoxin complex after production, but toxin yields after purification remain very low [46]. In a number of cases, point mutations rendering the toxin incapable of killing the cell are required to produce and purify large amounts of non-toxic versions of the toxin suitable for structural and biophysical studies [29,49,50]. However, this strategy usually limits one to the study of toxin and antitoxin interactions since the mutant is no longer catalytically active or capable of binding its cellular target.

Similarly production of full length antitoxins is challenging due to unwanted proteolytic trimming of their unstructured domains, even when working with protease-deficient bacterial strains [51,52].

A way to circumvent toxicity issues on one hand and to protect the antitoxin from proteolytic degradation on the other is by producing the toxin–antitoxin complex and separating both proteins via a denaturant-induced dissociation of the toxin–antitoxin complex. Denaturants such as urea and guanidine HCl (GnHCl)[2] have been used in many cases as tools in the purification of biological macromolecules [53–55]. Thermodynamic studies on many different TA systems have indicated that the thermal and chemical denaturation of the antitoxins is usually reversible [52,56–60]. For the toxin, however, return to the native state upon denaturation is less trivial. Most toxins seem to unfold irreversibly [61–63] and renaturation often results in multimerization or heavy precipitation. Therefore, yields of active toxin have been low or the resulting protein preparation showed a very low solubility and poor NMR spectra [61], indicating improper folding. Nonetheless, purification strategies focusing on a denaturant-induced dissociation of the toxin–antitoxin complex have been devised with successful outcome. Most involve the production of the toxin–antitoxin complex containing full-length, wild-type proteins. Because of the tight interaction between a toxin and its cognate antitoxin, high concentrations of denaturant are often necessary [16,28,64–67]. By truncating the antitoxin, the interaction between toxin and antitoxin can be weakened such that the antitoxin can still bind and alleviate toxicity *in vivo*, but that less denaturing agent is required to dissociate the complex [68].

In this paper we describe a purification strategy that, given case-dependent modifications, can be applied to four distinct toxin–antitoxin modules and produce significant quantities of both intact and active wild-type toxin and antitoxin suitable for structural and biophysical studies. We apply the procedure to members of the *phd/doc*, *mazEF*, *parDE*, and *higBA* families.

Materials and methods

Cloning

The cloning procedures for bacteriophage P1 *phd/doc*, *Escherichia coli* O157 *paaA2–parE2*, *E. coli mazEF* and *Vibrio cholerae higBA2* have been described [69–72]. All expression clones used have the coding regions of toxin–antitoxin operon behind an IPTG-inducible promotor [73]: pET21b-*phd/doc* (Doc equipped with a C-terminal His-tag), pET21b-*paaA2-parE2* (PaaA2 equipped with an N-terminal FLAG-tag and ParE2 equipped with a C-terminal His-tag), pET28a-*mazEF* (MazF equipped with a C-terminal His-tag). The expression vectors were transformed in *E. coli* strain BL21(DE3) [74] using the $CaCl_2$ method [75]. Transformed cells were selected on LB-plates containing the appropriate antibiotic and 1–2% glucose and grown overnight at 37 °C.

Purification of bacteriophage P1 Phd (antitoxin) and Doc (toxin)

Individual colonies were picked to start an overnight pre-culture of 50 ml incubated in LB medium [76] containing 100 μg/ml ampicillin and 1% glucose at 37 °C with aeration. A main culture was then started by inoculating 12 bottles containing 1 L of LB medium supplemented with 100 μg/ml ampicillin and 0.5% glucose with a 100-fold dilution of the pre-culture. Induction of expression of the operon was obtained by addition of 1 mM IPTG after an $O.D._{600}$ = 0.8 had been reached. Cultures were grown for 4 h at 37 °C with aeration and harvested by centrifugation for 20 min at 4 °C (5000 rpm using a JLA-8.1000 rotor).

The bacterial pellets were re-suspended in lysis buffer (50 mM TRIS pH 8.0, 500 mM NaCl, 0.1 mg/ml AEBSF, 1 μg/ml leupeptin) and aliquoted in volumes of 50 ml. The aliquots were stored at −80 °C and two aliquots were taken at a time for purification of the protein. Cells were lysed using a cell cracker at 15 kPa and 4 °C and the cell lysate was centrifuged for 30 min (16,000 rpm using a JA-20 rotor; 4 °C). The soluble fraction was kept aside and filtered (0.45 μm) and the pH adjusted to 8.0 prior to purification.

Purification of Doc and Phd then occurs though four major steps. In the first step, the Phd–Doc complex is loaded on the NiNTA column and Phd is eluted. For this, a 5 ml HisTrap™HP nickel–Sepharose column (GE Healthcare, Washington, USA) was equilibrated with buffer A.1 (50 mM TRIS pH 8.0, 500 mM NaCl) for at least five column volumes. The cell lysate containing the Phd–Doc complex is then loaded on the column in the same buffer. After loading the NiNTA column is washed with buffer A.1 (2 column volumes) followed by a wash with buffer A.2 (50 mM TRIS pH 7.5, 1 M NaCl, 10% ethylene glycol) until the OD_{280nm} stabilises to remove weakly bound contaminants. Next, Phd is eluted by disrupting the Phd–Doc interaction with a step gradient of guanidine hydrochloride (GnHCl): first a 50–50 mixtures of buffers A.2 and A.3 followed by 100% buffer A.3 (50 mM TRIS pH 7.0, 500 mM NaCl, 5.0 M GnHCl). The Phd-containing fractions are pooled and dialysed (MWCO 3.5 kDa) overnight against buffer A.4 (25 mM TRIS pH 7.5, 150 mM NaCl, 5% glycerol).

The second step concerns on-column refolding of Doc and needs to happen immediately after the elution of Phd. The unfolded Doc monomer, still attached to the Ni–Sepharose matrix, is refolded on the column with 10 column volumes of buffer A.5 (25 mM TRIS pH 7.5, 25 mM NaCl, 5% glycerol) followed by 10 column volumes of buffer A.6 (25 mM TRIS pH 7.5, 25 mM NaCl, 1% glycerol). Subsequently, the column is washed with 10 column volumes of buffer A.7 (50 mM MES pH 6.5, 250 mM NaCl) and finally with 1 column volume of buffer A.1.

In the third step, refolded Doc is eluted from the column. Therefore a linear imidazole gradient (0–0.5 M in 50 mM TRIS pH 7.5, 500 mM NaCl; buffer A.8) is applied over five column volumes. Collected fractions are analyzed on an 15% SDS–PAGE and those containing Doc are concentrated by centrifugation (Amicon Ultra-15 concentrators with MWCO 3.5 kDa and spun at 3,800 rpm) to a total volume of 2 ml for further purification and stored overnight at 20 °C.

In a fourth and final polishing step, some remaining impurities as well as possible (misfolded) Doc multimers are removed via SEC. The concentrated Doc sample is loaded onto a Superdex 75 16/60 HR-gel filtration column (GE Healthcare, Washington, USA) pre-equilibrated with buffer A.9 (100 mM Na_2HPO_4 pH 6.5, 200 mM NaCl). Collected fractions were analyzed on 15% SDS–PAGE and those containing Doc were pooled together. Protein concentrations

[2] *Abbreviations used:* TA, toxin–antitoxin; GnHCl, guanidine HCl; SEC, size-exclusion chromatography; DLS, dynamic light-scattering; ORFs, open reading frames.

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