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Cloning and Expressing Recombinant Protective Antigen Domains of *B. anthracis*

by Deborah A. Sarkes, Joshua M. Kogot, Irene Val-Addo, Dimitra N. Stratis-Cullum, and Paul M. Pellegrino

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14. ABSTRACT
Protective antigen (PA) of *Bacillus anthracis* is a four-domain protein that is required for transport of edema factor (EF) and lethal factor (LF) into host cells during anthrax infection. Each of the four domains of PA plays a role in infection: host cell receptor binding, domain 4, PA heptamerization and host membrane insertion, domains 2 and 3, and EF and LF recognition and intracellular transport, domain 1. This report details our efforts to produce single- and multi-domain combinations of the PA protein. All multi-domains (1-2, 2-3, 3-4) and three of the four single domains (1, 2, 4) have been cloned, expressed, and purified and will be used for future efforts in domain mapping of PA-binding antibodies and peptide reagents.

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Foreword

The U.S. Army Research Laboratory (ARL) was awarded a 3-year 6.1 project from the Defense Threat Reduction Agency (DTRA) titled "Iterative Modeling of Peptide-Protein Interaction for 'Smart' Reagent Development," BRCALL-08-Per3-2-0028, and includes investigators from ARL-Weapons and Materials Research Directorate (WMRD) and Edgewood Chemical Biological Center (ECBC). The main objectives of the 6.1 project are: (1) to develop a comprehensive, multi-scale toolkit for modeling target-peptide interactions improving binding prediction; (2) to predict and measure smart peptide binders for enhanced affinity and specificity to protective antigen of *B. anthracis*; and (3) to extend modeling capabilities to enable predictions of other biochemical/ligand systems. The purpose of this report is to highlight the production of single- and multi-domain proteins from protective antigen (PA) of *B. anthracis*. The domains produced in this work will be used to precisely map peptide-protein interactions experimentally for development of future predictive modeling toolkits.

1. Introduction

The use of *Bacillus anthracis* as a bio-weapon in the United States in 2001 affirmed the need for improved sensing and detection of biological weapons of mass destruction (WMD). Protective Antigen (PA) protein of *Bacillus anthracis* is the common link in both anthrax toxins, and is responsible for shuttling both edema factor and lethal factor into the host cell. PA is an 83 kDa protein monomer and, once activated by furin proteases on the cell surface of the host, forms a 63 kDa truncated protein that is capable of forming a heptameric pore complex. PA protein is non-toxic and only acts as a transport for edema factor (EF) (forming edema toxin), and lethal factor (LF) (forming lethal toxin). LF and EF both interact with PA through domain 1, following furin cleavage and release of the 20 kDa N-terminal fragment of PA (PA_{20}) to form domain 1‘-4 (PA_{63}), which is necessary for PA heptamerization, LF/EF binding, and receptor-mediated-endocytosis for cytosolic entry. Protective antigen is so-labeled because this protein is often used for vaccine development for anthrax since it is required for any toxic response to the host (*1*).

The full-length PA_{83} has been crystallized to 2.10 Å (*2*) and is a four-domain protein. Domains are single-protein subunits that can exist separately from the full protein while retaining structural and functional integrity (*3*). The four PA domains have specific function in the full PA protein and can be structurally demarcated by the flexible loop regions connecting each domain (figure 1). The cleaving of domain 1 at residue 167 is required to form the heptameric complex with PA_{63} monomers. Domain 1 also contains the region of LF/EF interaction (*4–6*), as well as two Ca^{2+} binding sites (responsible for PA stability) (*2, 7*).

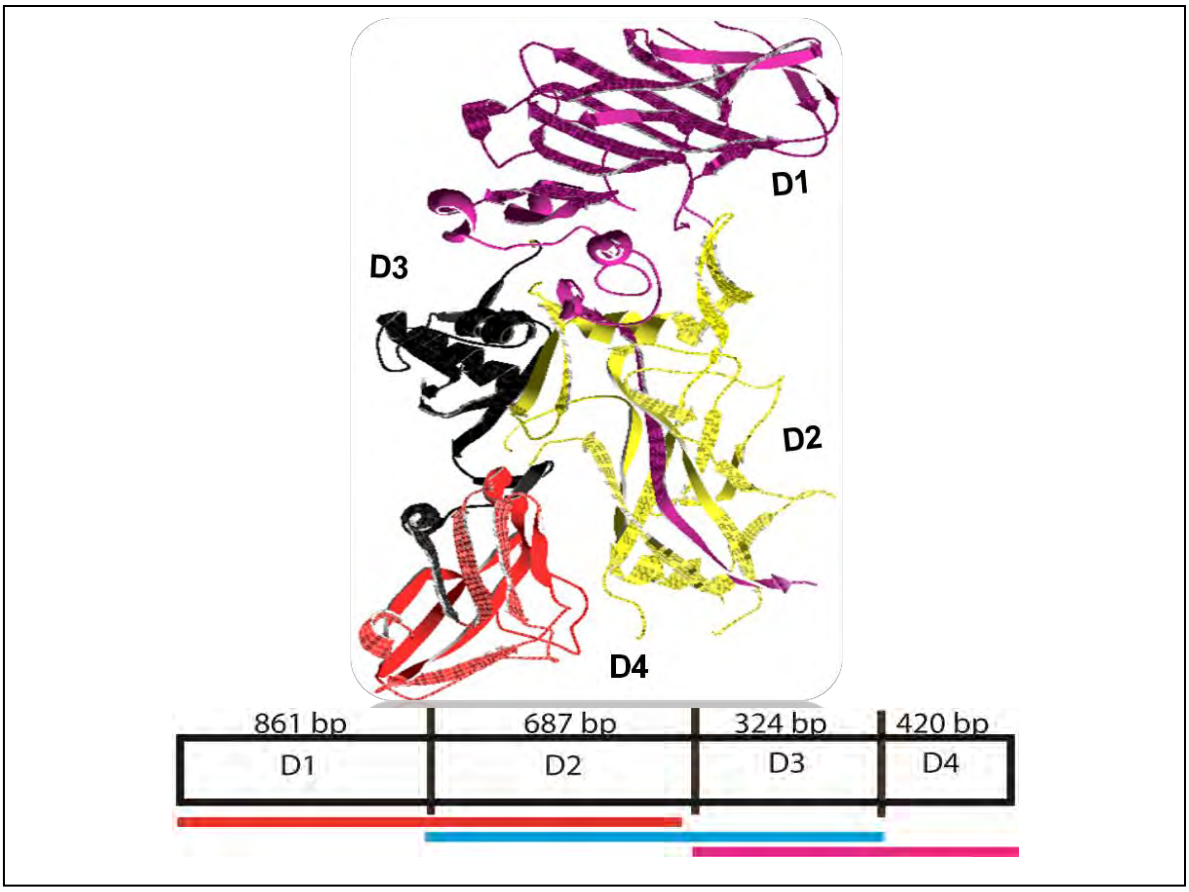

Figure 1. (top) Crystal structure of PA (PDB ID: 1ACC) outlining the location of each of the four domains of the protein. (bottom) A cartoon schematic showing the total length (in number of base pairs) of each domain to be produced. The strategy to produce multiple domains, including D1-2 (red), D2-3 (cyan), and D3-4 (magenta), to preempt any solubility or stability issues during single domain production.

Domain 2 is the membrane insertion and heptamerization domain, along with domain 3. During heptamerization, domain 2 becomes buried and inaccessible, along with domain 1, while domain 3 remains accessible with domain 4 (*2*). Domain 4 is not closely associated spatially with the other domains (*2*) and is responsible for host cell receptor binding in the heptameric form. Domain 4 was shown to be the most critical for protecting mice against anthrax infection, compared to other domains, due to the greatest overall exposure compared to other domains in the heptamer, and more specifically, the accessibility of an exposed loop region (703–722) (*8*).

Due to the greater accessibility and exposure of domain 4, most isolated and domain-mapped antibodies have been shown to bind this domain, with some instances of domain 2-selective antibodies. An affinity-matured scFV (M18) developed from the monoclonal antibody 14B7 (*9*), which has been humanized and is in advanced clinical development, was recently co-crystallized with PA_{83} and shows specific binding to domain 4 (*10*). Antibodies directed to domain 2 have been shown to block the formation of PA_{63}, whereas antibodies to domain 4 block the receptor binding; blocking either domain would neutralize the anthrax toxin (*1, 10, 11*).

Besides using single domains for vaccine development, producing, expressing, and purifying a full complement of single or multi-domain PA variants could be used for epitope mapping strategies, especially for more rapid domain mapping of antibodies or antibody alternatives (peptides, aptamers, etc.) using the standard immunoassay techniques enzyme-linked immunosorbent assay (ELISA) or surface plasmon resonance (SPR). Expressing individual domains for protein-mapping avoids complicated competition assays with sequential antibody addition (*12*), as these only compare results within a set of antibodies and require further biochemical analysis of activity to determine the outright binding location. An alternative method to producing single domain proteins is to use proteolysis strategies to selectively cleave PA, in domain 1, to form the PA_{20} and PA_{63} fragments (*13*), or using selective enzymatic digestion of PA_{83} beyond the furin protease site, which would disrupt an intact domain (*11*) and could expose residues typically buried in the protein core.

This report outlines the strategies used to design, clone, express, and purify domains 1, 2, 4, 1-2, 2-3, 3-4, and full-length PA that will be used for antibody epitope mapping, peptide-protein modeling studies, and peptide display library selections in our laboratory. The full-length recombinant protective antigen is most often expressed recombinantly using *E.coli*, with yields as high as 125mg per liter reported (*14*). Other expression systems, such as *Bacillus subtilis* (*15*) and Baculovirus and Vaccinia Virus (*16*), have been used to successfully produce recombinant PA. N-Glutathione S-transferase (GST) fusion proteins of the PA single and multi-domains have also been produced in *E.coli* (D1, D1‘-2, GST1-2, GST1‘-3, GST1-3, GST2-4, GST3-4, GST4, and GST1-4), but were expressed in inclusion bodies and required refolding with 8M urea and arginine buffer stabilizer (*8*). Instead of a GST fusion protein, we use a His_6-fusion to minimize unknown interactions between the small, single domains and the affinity purification. The GST-tag is approximately 26kDa, which is larger than both domain 3 and domain 4, so there is concern that the GST could interfere with the native activity of these domains. Maintaining near-native structure and function for these domains is critical to deriving an accurate empirical model for the exact domain binding site of our peptide reagents (*17*). The model will ultimately aid in designing smart reagents using computational docking and molecular dynamics modeling on the DoD high performance computing platform.

2. Experimental

2.1 PCR for Production of Single and Multiple Domain Clones

PCR primers (Integrated DNA Technologies) were designed for cloning PA domains into pET-22b(+) (Novagen), which adds a C-Terminal His_6-affinity tag to the product, via EcoRI and NotI restriction sites. PCR primers, annealing temperature, and extension time used for each PA domain combination are listed in table 1. Each 50 µl PCR reaction contained 0.4 µM final primer concentration of each of the forward and reverse primers listed, as well as 40 ng pUC57

PA_{83} template (GenScript, codon-optimized sequence) and 25 µl iQ SYBR Green Supermix (Bio-Rad). The PCR program was as follows: an initial, single 2-min step at 94 ºC (melt), then 40 cycles of 30 s at 94 ºC (pUC57 PA_{83} template melt), 15 s at 60 ºC (primer anneal), and polymerase extension time of 1–2 min at 72 ºC, depending on the length of the desired product (see table 1 for extension time), followed by a single step at 72 ºC for 10 min and a 4ºC holding step.

Table 1. Primers and temperatures for PCR amplification of PA domains.

PA Domain (Size)	Forward Primer Sequence	Reverse Primer Sequence	Annealing Temp	Extension Time
Domain 1 (861 bp)	5'-CGGATCCGAATTCGATGAAAA AACGCAAAGTCCTGATTCC-3'	5'-CTCGAGTGCGGCCGCTGCCGC AACCAGCGGATG-3'	60ºC	1 min
Domain 2 (687 bp)	5'-CGGATCCGAATTCGTATCCGA TCGTGCACGTTGATATGG-3'	5'-CTCGAGTGCGGCCGCGGTTTC TTGAATCTGCGGCAG AAC-3'		
Domain 3 (324 bp)	5'-CGGATCCGAATTCGACGGCAC GCATTATCTTTAATGGC-3'	5'-CTCGAGTGCGGCCGCGCGTTT GTCACGGATCAGAATGTT C-3'		
Domain 4 (420 bp)	5'-CGGATCCGAATTCGTTTCATT ACGATCGTAACAATATTGCTG-3'	5'-CTCGAGTGCGGCCGCACCGAT TTCATAGCCTTTTTTGG-3'		
Domain 1-2 (1548 bp)	5'-CGGATCCGAATTCGATGAAAA AACGCAAAGTCCTGATTC C-3'	5'-CTCGAGTGCGGCCGCGGTTTC TTGAATCTGCGGCAGAAC-3'		1.5 min
Domain 2-3 (1011 bp)	5'-CGGATCCGAATTCGTATCCGA TCGTGCACGTTGATATGG-3'	5'-CTCGAGTGCGGCCGCGCGTTT GTCACGGATCAGAATGTTC-3'		
Domain 3-4 (744 bp)	5'-CGGATCCGAATTCGACGGCAC GCATTATCTTTAATGGC-3'	5'-CTCGAGTGCGGCCGCACCGAT TTCATAGCCTTTTTTGG-3'		
Full Length (2292 bp)	5'-CGGATCCGAATTCGATGAAAA AACGCAAAGTCCTGATTCC-3'	5'-CTCGAGTGCGGCCGCACCGAT TTCATAGCCTTTTTTGG-3'		2 min

2.2 Vector Incorporation of PCR Products

The PCR products were run on gels containing 1% agarose (Bio-Rad) in 1x TAE buffer (Trizma base, acetic acid, ethylenediaminetetraacetic acid [EDTA]) with a 1 kb DNA Ladder (New England Biolabs) or 100 bp Molecular Ruler DNA Size Standard (Bio-Rad) for comparison. Appropriate bands were cut (figure 1), and the products were purified using a Gene Clean Spin Kit (Qbiogene, Inc) or Purelink PCR Purification Kit (Invitrogen) according to manufacturer‘s instructions. The purified products, as well as the pET-22b(+) vector (Novagen), were digested with the high fidelity restriction endonucleases EcoRI-HF™ and NotI-HF™ (New England Biolabs) with supplied Buffer 4 and bovine serum albumin (BSA) at 37 ºC for at least 2 h. The enzymes were heat-inactivated for 20 min at 65 ºC. The pET-22b(+) vector was dephosphorylated for 1 hr at 37 ºC by adding Antarctic Phosphatase and its supplied buffer,

containing zinc (New England Bio-labs), directly to the previous reaction. The phosphatase was heat-inactivated for 20 min at 65 ºC prior to ligation. For each ligation, T4 DNA ligase and its supplied buffer (New England Bio-labs) were combined with 50–100 ng (0.015-0.03 pmol) of digested, dephosphorylated pET-22b(+) vector and approximately 0.2 pmol of appropriate digested PA domain PCR product, as outlined in the pET System Manual, 11th ed. and incubated at room temperature for 1 h. This ligation reaction was transformed into BL-21 (DE3) bacteria (New England Biolabs) according to manufacturer's instructions and, after recovery, 100 µl of cells were streaked on LB agar plates (Luria Broth and agar, Fisher Scientific) containing 50 µg/ml ampicillin (Sigma), for selecting pET-22b(+)-transfected clones, and incubated overnight at 37 ºC. All positive clones confirmed by sequencing with T7 primers (Genewiz).

2.3 Expression of PA Proteins

Positive transformants were grown in 5 ml LB supplemented with 50 µg/ml ampicillin (LB-Amp^{50}) in a 37 ºC shaker to approximately OD_{600} = 0.5. Then PA Domain expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) for 3 h at 30 ºC, or 37 ºC and 250 RPM shaking. Expression at each temperature was confirmed by centrifuging 1 ml of cells at 16,000 x g; resuspending the cell pellet in 50 µl PBS (BupH Modified Dulbecco's Phosphate-Buffered Saline pH 7.4, Thermo Scientific), plus 50 µl 2x Laemmli Buffer (Bio-Rad) with 5% β-mercaptoethanol (Sigma); boiling for 5 min; and separating by denaturing SDS-PAGE using pre-packaged 8–16% polyacrylamide gels and Precision Plus Protein™ Dual Color Standards, SDS-PAGE Standards Low Range, or Polypeptide SDS-PAGE Standards (Bio-Rad) to determine molecular weight. Gels were stained using the eStain™ 2.0 Protein Staining device and R-250 eStain™ Protein Staining Pads (GenScript).

2.4 Determining Solubility of Expressed PA Proteins

Cultures with confirmed expression were scaled-up to 1 L of LB-Amp^{50} and were expressed and induced following the aforementioned protocol, then centrifuged at 5500 x g for 10 min to pellet cells. Each 1L cell pellet was lysed as follows: the pellet was resuspended in 24 ml of lysis buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1.3 mg/ml lysozyme, 1 mM dithiothreitol (DTT), and 1x Halt Protease Inhibitor Single-Use Cocktail, EDTA-free (Sigma)) and incubated on ice for 20 min to break the cell wall. To rupture the cell membrane, 200 µl of 160 mg/ml sodium deoxycholate—diluted in lysis buffer—was added to each reaction, and the solution was incubated at room temperature for about 1 h on a rotating platform until very viscous. To degrade the DNA, 1 mM $MgSO_4$ and 40 µl of 2500 units/µl DNAse I (Thermo Scientific) were added to each reaction and incubated at room temperature for about 90 min on a rotating platform until not viscous (adapted from Molecular Cloning: A Laboratory Manual) (*18*). Each solution was centrifuged at 45,000 x g for 30 min, and the supernatant (soluble lysate) and pellet (insoluble fraction) were analyzed by denaturing SDS-PAGE as previously mentioned. Osmotic Shock was also tried as an alternative to complete lysis, to isolate only those proteins expressed in the periplasm, since pET-22b(+) has an N-terminal pelB leader

sequence. For each protein that was completely insoluble or present primarily in inclusion bodies, the cell pellet was washed twice with 5 ml 50 mM Tris pH 7.5, 10 mM EDTA, 5 mM DTT, 2% Triton® X-100 (Fluka), and 500 mM NaCl, then once with 50 mM Tris pH 7.5, 10 mM EDTA, centrifuging at 15,000 x g for 15 min and removing the supernatant in between each wash, to further isolate inclusion bodies as described in the Thermo Scientific Pierce® Protein Refolding Kit Guide, Revision 2. The resulting pellet was resuspended in approximately 1 ml 6M guanidine hydrochloride (Gdn-HCl), 50 mM Tris pH 8.0 for each 20–40 mg of isolated inclusion bodies, vortexed to homogenize as completely as possible, and incubated at room temperature on a rotating platform for at least 2 h to completely solubilize inclusion bodies. Cell debris and non-solubilized material were removed by centrifugation at 45,000 x g for 20 min. Material from the inclusion body isolation and solubilization steps was analyzed by denaturing SDS-PAGE, as previously described, diluting samples containing 6M Gdn-HCl by 50% in PBS before adding 2x Laemmli Buffer with DTT, and *loading the gel as quickly as possible after boiling to avoid precipitation*.

2.5 Purification of PA Domains from Inclusion Bodies

Solubilized protein from inclusion bodies was purified using a HisTrap FF column and the ÄKTAprime Plus system (GE Healthcare), with 6M Gdn-HCl, 50 mM Tris pH 8.0, 20 mM imidazole as "Buffer A," and 6M Gdn-HCl, 50 mM Tris pH 8.0, and 500 mM imidazole as "Buffer B." The protein solution was brought to 20 mM imidazole and loaded or injected onto the column (pre-washed and equilibrated according to manufacturer's instructions) at 1 ml/min to 2.5 ml/min (depending on system pressure and sample volume). Following the A_{280} readout, the column was washed to baseline level and the protein was eluted with a linear gradient from 0% to 10% Buffer B over 10 ml, an isocratic gradient at 10% B until the entire peak eluted, a linear gradient from 10% B to 100% B over 20 ml, and another isocratic gradient at 100% B until baseline level was reached. Fractions with a 280 nm absorbance were analyzed by denaturing SDS-PAGE, as described, diluting the Gdn-HCl by 50% in PBS. The fractions containing purified PA domains were concentrated, if necessary, using Amicon Ultra Centrifugal Devices (Millipore) or a Stirred Ultrafiltration Cell (Millipore Model 8050) with Ultrafiltration Membranes (Millipore) at appropriate NMWLs (nominal molecular weight limits). Further purification was achieved using a HiLoad™ 16/60 Superdex™ 75 prep grade (pg) column (GE Healthcare) equilibrated according to manufacturer's instructions. Using the Gel Filtration method template and the settings suggested specifically for the HiLoad™ 16/60 Superdex™ 75 pg column (see ÄKTAprime Plus User Manual), the protein was injected onto the column and eluted with 180 ml of 6M Gdn-HCl, 50 mM Tris pH 8.0 at 0.5 ml/min. Fractions with a 280 nm absorbance were analyzed by denaturing SDS-PAGE, as described. Initially, this purification by gel filtration column was completed after refolding the proteins in either PBS or the refolding buffer used (see below), with no significant difference in the final purified product. Soluble protein (only achieved for PA domain 1 induced at 30 ºC) was purified by HisTrap FF and Superdex columns in a similar fashion, as well by loading the protein onto the HisTrap FF

column in lysis buffer, using PBS 20 mM imidazole as "Buffer A" and PBS 500 mM imidazole as "Buffer B," and eluting with steps at 30% B (rather than 10% B, due to tighter binding) and 100% B. The protein-containing fractions were concentrated, if necessary, and purified by Superdex column in PBS.

2.6 Refolding of Purified PA Domains

The purified PA proteins from inclusion bodies were first refolded in small volumes using the Pierce* Protein Refolding Kit, containing nine refolding buffers, with varying formulations as outlined in the Primary Screen (see table 3 of the Thermo Scientific Pierce® Protein Refolding Kit Guide, Revision 2) to determine the optimal refolding conditions. Visual inspection of the protein solution for lack of precipitates, along with results of the ELISA functional test (table 2), determined the appropriate refolding condition. The remaining purified protein was diluted 1:20 in ice-cold refolding buffer (no reducing agents added) containing 1 mM EDTA by adding approximately one-fifth of the sample at a time in a closed container, vortexing and placing on ice in between additions. The reaction was stored at 4 ºC overnight during refolding.

Table 2. Confirmation of proper protein refolding by ELISA.

PA Domain	Refolding Condition	ELISA Result	Comments
Domain 1	Buffer 1 + 1mM EDTA	Functional	Refolds in all 9 Buffers tested. Addition of Ca2+ recommended for stability[19].
Domain 2	Buffer 1 + 1mM EDTA	Functional	Refolds in all 9 Buffers tested.
Domain 4	Buffer 1 + 1mM EDTA	Functional	Refolds in all 9 Buffers tested.
Domain 1-2	Buffer 1 + 1mM EDTA	Functional	Refolds in all 9 Buffers tested. Addition of Ca2+ recommended for stability[19].
Domain 2-3	Buffer 6 + 1mM EDTA	Functional	Unstable in Buffer 1.
Domain 3-4	Buffer 1 + 1mM EDTA	Functional	Refolds in all 9 Buffers tested.
Full Length	Buffer 1 + 1mM EDTA	Functional	Refolds in all 9 Buffers tested. Addition of Ca2+ recommended for stability[19].

2.7 Functional Analysis by ELISA

ELISAs were set up by adding 100 µl of each refolding reaction (in Buffers 1–9 from above), undiluted and diluted serially by 50% in PBS, to the wells of a Maxisorp polystyrene 96-well, flat-bottom immunoplate (Nunc) and incubating at room temperature for 2 h on a shaking platform. A row of PBS alone was included as a negative control. Sample wells were blocked with 300 µl PBS 0.1% Tween-20 (Acros) containing 2% (w/v) non-fat milk for 1 hr, washed three times with this buffer, and then incubated with 100 µl of 2 µg/ml rabbit polyclonal anti-PA_{83} secondary antibody in blocking buffer for 1 h. Sample wells were washed again three

*Pierce is a registered trademark of Thermo Scientific.

times with blocking buffer, then incubated with 0.2 μg/ml donkey anti-rabbit IgG-HRP (Santa Cruz) in blocking buffer for 30 min at room temperature. The plates were washed three times with PBS before developing with 1-Step Ultra TMB ELISA (Thermo Scientific) for 15 min. The reactions were stopped with 2M sulfuric acid and measured using a Synergy HT plate reader (Bio-Tek).

3. Results and Discussion

3.1 Domain Amplification and Cloning into pET-22b(+)

Each of the eight single and multiple domain clones of PA_{83} were successfully amplified by PCR, digested with restriction endonucleases, ligated into pET-22b(+), and transformed into BL-21 (DE3) bacteria. An example agarose gel depicting the PCR products for PA domains 3, 4, and 3-4 at 324, 420, and 744 bp, respectively, can be seen in figure 2. Note that in each case, a single, prominent band is seen. This was the case for all other domains, as well (data not shown). The sizes of the other PA domains are shown in table 1. The composition of each domain was confirmed by colony sequencing (Genewiz) prior to expression tests.

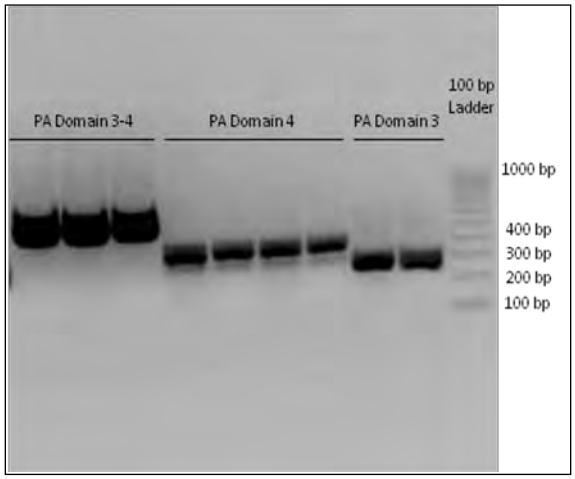

Figure 2. Agarose gel (1% w/v in 1x TAE) showing successful production of PCR products for PA domains 3 (324 bp), 4 (420 bp), and 3-4 (744 bp).

3.2 PA Single and Multiple Domain Clones Expressed Successfully, Except for Domain 3

PA domains 1, 2, 4, 1-2, 2-3, 3-4, and full length PA_{83} were successfully expressed at both 30 ºC and 37 ºC with 1 mM IPTG. In general, expression was better at 37 ºC. The expression of PA Domains 2, 1-2, and 2-3 at 37 ºC is shown in figure 3. Compared to the low molecular weight protein standard, each of these domains ran slightly higher than their expected molecular weights of 25.2, 57.6, and 37.5 kDa, respectively, in part due to the His_6-affinity tag at the C-terminus and any pelB leader sequence that remained uncleaved. This was also the case for domains 1, 4,

3-4, and full-length PA_{83}, with expected molecular weights of 32.4, 15.9, 28.2, and 85.8 kDa, respectively (not shown). All attempts to express PA domain 3 (12.3 kDa) have been unsuccessful, including transforming into alternate bacteria and varying concentrations of IPTG (data not shown). This is, however, consistent with the findings of Flick-Smith *et al.*, who isolate various PA domain combinations as GST-fusion proteins in a similar manner, but show no data for PA domains 2 or 3 alone (*8*). Methods to successfully express and purify PA domain 3 are ongoing.

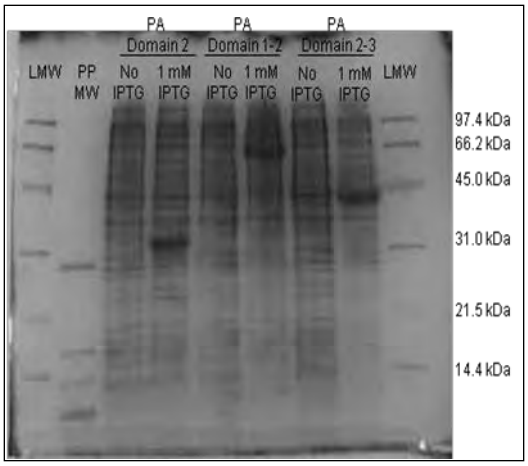

Figure 3. 8–16% polyacrylamide gel demonstrating successful expression of PA domains 2 (25.2 kDa), 1-2 (57.6 kDa), and 2-3 (37.5 kDa) with 1 mM IPTG at 37 ºC. LMW = Low-Range Molecular Weight Standard and PP MW = Polypeptide Molecular Weight Standard.

3.3 Determination of Domain Solubility

Bacteria from each successfully expressed PA domain were lysed completely to analyze protein solubility. All domains were predominantly expressed as inclusion bodies when induced at 37 ºC, with some solubility of domain 1. At 30 ºC, only PA domain 1 was predominantly soluble. Figure 4 shows a comparative analysis of PA domain 1 versus domain 3-4 solubility. Note that greater than 50% of over-expressed domain 1 is soluble when induced at 30 ºC (present in the lysate fraction), while little to no PA domain 3-4 is soluble when induced at 37 ºC (entirely in the pellet fraction). Similar results were seen for all other PA domains (data not shown), and this result is consistent with the findings of Flick-Smith *et al.*, except that their GST-domain 1 is also insoluble. However, induction temperature and method are not mentioned in this article, so it is possible Flick-Smith and colleagues did not vary IPTG concentrations or temperature. Expression of these domains in inclusion bodies was advantageous since the inclusion body-containing pellet fraction had fewer contaminating proteins. Although we were successful in expressing and purifying soluble PA domain 1, the final product had greater purity when PA domain 1 was expressed with 1 mM IPTG at 37 ºC and isolated from inclusion bodies. Osmotic

shock was also tried as an alternative method to obtain soluble PA domain 1 at a higher level of purity, since only periplasmic proteins are released from the cell with this method, but the level of purity achieved from refolding the protein was significantly higher (data not shown).

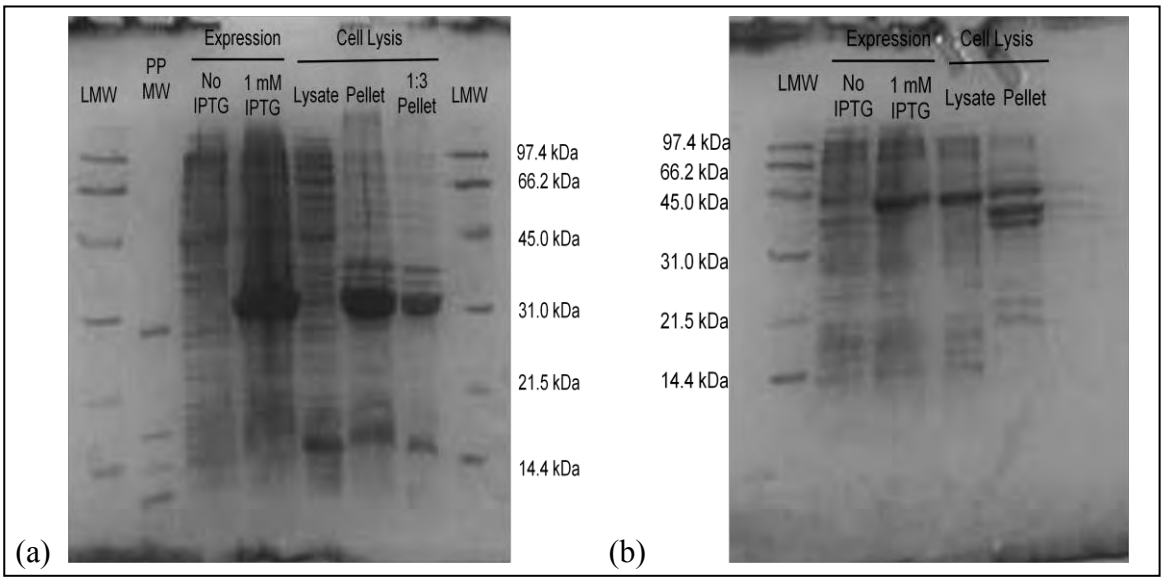

Figure 4. SDS-PAGE (8–16% gradient) gels comparing expression and solubility after induction with 1 mM IPTG. Compare PA domain 1, induced at 30 ºC (a) to PA domain 3-4, induced at 37 ºC (b). The results of the solubility test indicate that domain 1 is clarified intact primarily in the cell lysate, while domain 3-4 is clarified solely in the cell pellet after lysis. LMW = Low-Range Molecular Weight Standard and PP MW = Polypeptide Molecular Weight Standard.

3.4 Purification and Refolding of All Expressed Domains

For each PA domain, the lysed cell pellet was washed twice with a 2% Triton X-100 solution and once with 50 mM Tris pH 7.5, 10 mM EDTA to isolate inclusion bodies. The inclusion bodies were solubilized with Gdn-HCl and purified by HisTrap FF and Superdex™ columns as described in sections 2.4 and 2.5. The results of this purification process for full-length PA_{83} can be seen in figure 5, and similar levels of purification were seen for all isolated PA domains (not shown). In figure 5a, note that the inclusion body isolation washes remove a significant amount of non-specific proteins: membrane proteins, proteins from any unbroken cells or cellular debris that remain after lysis (Pierce Protein Refolding Kit Guide, Thermo Scientific), and proteins associated directly with the inclusion bodies. After solubilization with 6M Gdn-HCl, the insoluble pellet contains a very specific set of contaminating proteins, consistently seen between 31 and 45 kDa with each purification, that are not triton-soluble. The bulk of these contaminants are removed by centrifugation (figure 5A, Gdn Pellet). Greater than 90% of the remaining contaminating proteins are removed during the HisTrap FF purification. One example chromatogram outlining the HisTrap FF purification of PA_{83}, with fractions corresponding to those in figure 5a, is shown in figure 6. Although the flow-through fractions (figure5a, fraction 4) contain some of the His_6-tagged PA product, much of this material can be recovered by passing through the HisTrap FF column a second time (data not shown). This may be attributed

to the binding capacity of the column. The HisTrap FF-purified fractions were concentrated and loaded on a HiLoad™ 16/60 Superdex™ 75 pg column. The elution profile is shown in figure 7, and SDS-PAGE analysis of the corresponding fractions is shown in figure 5b. Note that although the chromatogram shows that PA_{83} elutes as an inseparable doublet, the purity achieved in this final purification step is greater than 90%; only minor contaminants are visible by SDS-PAGE (figue 5B). There are no apparent or obvious differences in the contaminants seen in the first half of the doublet (fractions 18 and 19), as compared to the second half (fractions 20 and 21). We speculate that these contaminants are proteins that are somehow associated with PA, even when denatured in 6M Gdn-HCl, since degradation products or non-associated contaminants should have been removed during the Superdex™ purification, which separates by size.

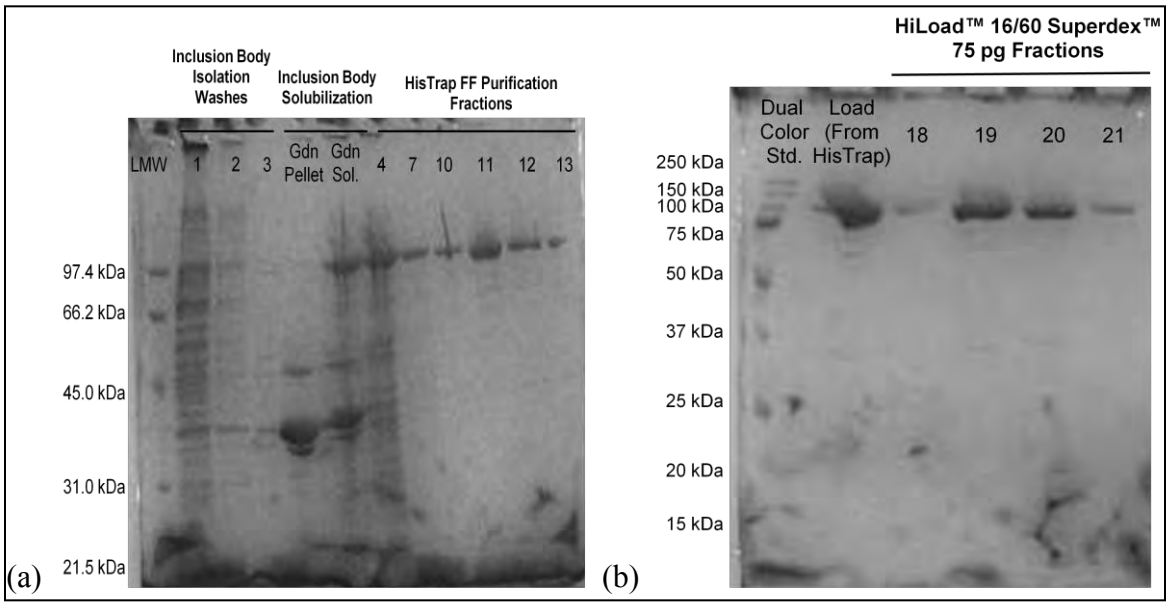

Figure 5. Polyacrylamide gels demonstrating the purification process used for each PA domain, with full-length PA_{83} presented as an example. (a) Inclusion body isolation, solubilization, and HisTrap FF purification. Fraction 4 represents loading the column while fractions 10-12 represent elution with 50 mM imidazole and fraction 13 represents elution with 500 mM imidazole (see figure 6 for chromatogram). (b) Purification by gel filtration column. Fractions 18-21 represent an inseparable, doublet peak (see figure 7 for chromatogram). Note the high level of purity achieved with this method.

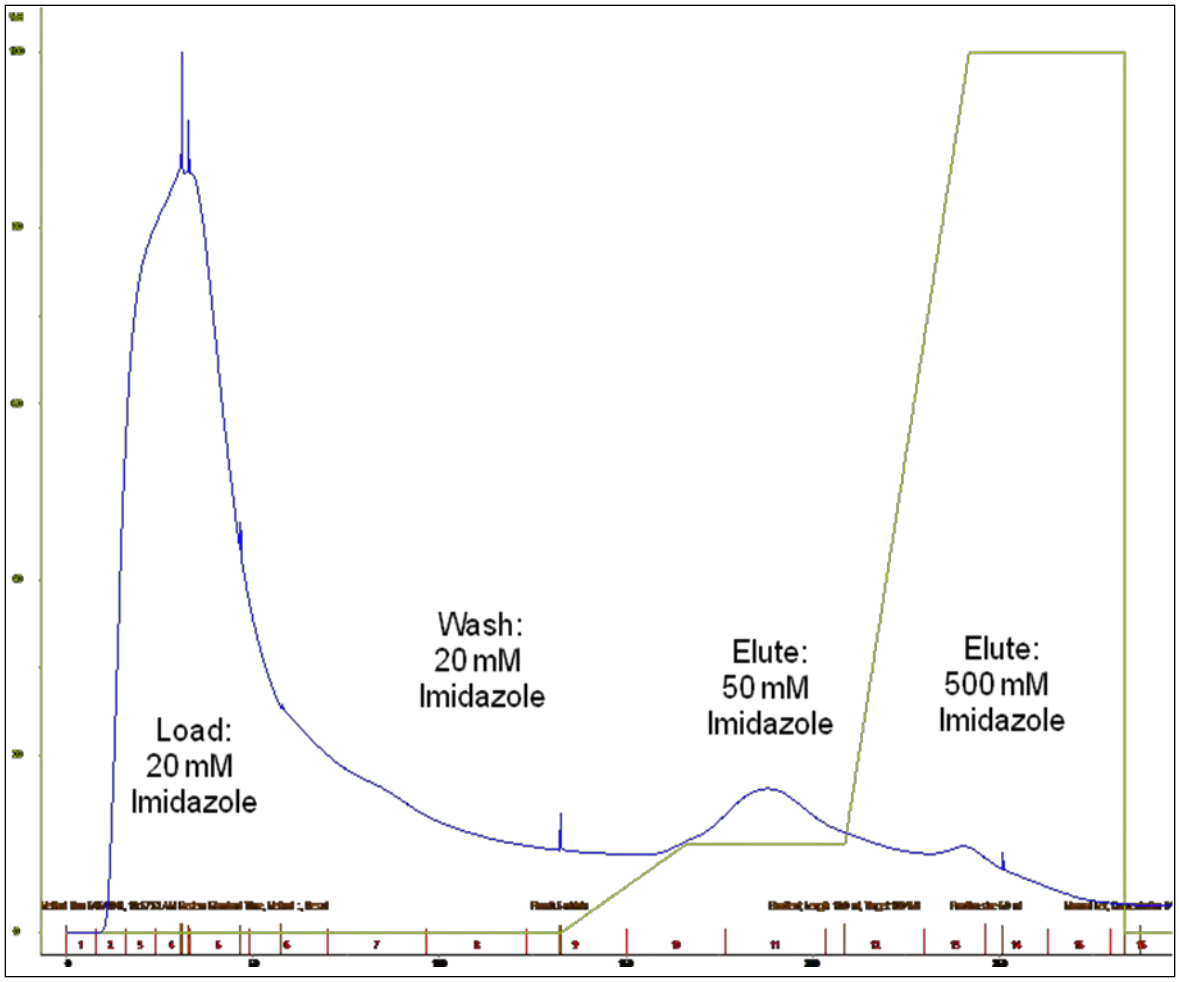

Figure 6. Chromatogram for HisTrap FF poly-histidine affinity column showing the typical purification results, with full-length PA_{83} presented as an example. Blue = A_{280}, Green = %B, and Red = fractions collected. See figure 5a for analysis of fractions by gradient SDS-PAGE. Note that the bulk of the purified PA_{83} elutes in fraction 11 at 50 mM imidazole.

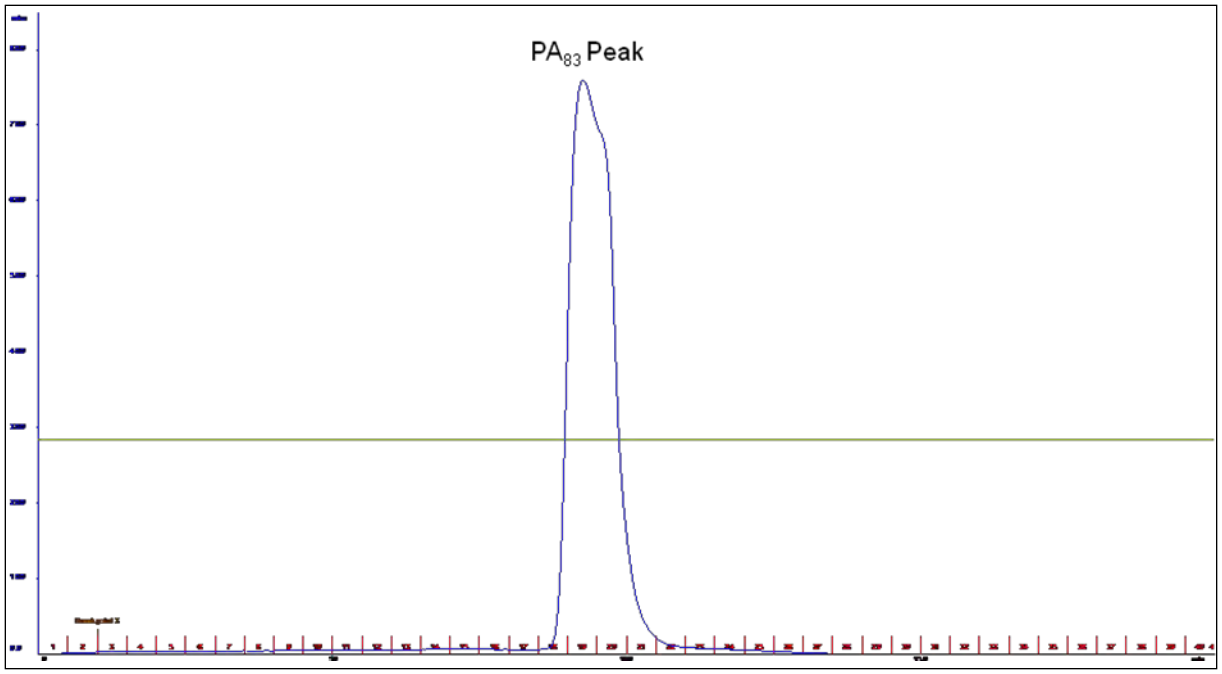

Figure 7. Chromatogram for HiLoad™ 16/60 Superdex™ 75 pg column showing the typical purification profile when run in 6M Gdn-HCl 50 mM Tris pH 8.0, with full-length PA_{83} presented as an example. Blue = A_{280}, Green = %B, and Red = fractions collected. See figure 5b for analysis of fractions by 8–16% gradient SDS-PAGE. Note that full-length PA_{83} elutes as a doublet, which we are unable to separate, but the gel shows a >90% pure end product.

Each highly pure PA domain was refolded in its optimal refolding buffer, based on an initial screen using small amounts of protein and the Thermo Scientific Pierce® Protein Refolding Kit, as outlined in section 2.6. Refolding was deemed successful if the PA domain did not precipitate in the buffer and if a functional ELISA screen showed that the protein binds to a PA polyclonal antibody. Refolding is currently being assessed by Circular Dichroism Spectroscopy, as well, to look at secondary structure. For most domains, all nine buffers were successful. With all else equal, the optimal refolding buffer was considered that with the simplest formulation. The results of these refolding tests and the functional ELISA assay are outlined in table 2. PA domain 2-3 was the only domain that was visibly precipitating in buffer 1 during refolding, and its optimal buffer is buffer 6 (550 mM guanidine, 880 mM L-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl; pH 8.2). The optimal buffer for all other domains is buffer 1 (55 mM Tris, 21 mM NaCl, 0.88 mM KCl; pH 8.2). The stability of PA domains 1, 1-2, and full-length PA_{83} in buffer 1 decreases over time when stored at 4 ºC, noted by an increase in the precipitation and aggregation upon visual inspection, and increased degradation upon analysis by SDS-PAGE (not shown). To overcome this, we limit storage at 4 ºC to short periods of time, which is preferred for proteins in general, and have also tried the addition of 100 μM $CaCl_2$ during refolding and storage, which has been shown to limit proteolysis of domain 1 (*2, 7, 19*). Flick-Smith et al. noted that degradation was apparent in all of the fusion proteins they investigated, which was

also notable for rPA expressed in *Bacillus subtilis*[1]; therefore, this is likely a general challenge for isolating stable, high purity PA protein and not an artifact of our methods.

4. Conclusion

Protective antigen protein is the critical component for anthrax infection since the formation of the heptameric PA_{63} through furin protease cleavage of domain 1 is necessary for shuttling edema and lethal factors into the host-cell. Each domain plays a specific role in the transfection event: domain 1 is proteolysed for hepatmer formation and EF and LF binding, domain 2 is necessary for membrane insertion and heptamerization, domain 3 is involved in heptamerization, and domain 4, the most accessible during heptamerization, binds to the host receptor. The recombinant His_6-tag full-length PA and domains 1, 2, 4, 1-2, 2-3, and 3-4 have been successfully produced in our lab using an *E.coli* expression host. Each of these proteins, except domain 1, was expressed solely in an inclusion body, which required unfolding, purification, and refolding in each case. Refolding of each insoluble protein was successful, as determined by the limited precipitation/aggregation noted after refolding and the ELISA function test.

Production of single and multi-domains as fusions has been shown to permit direct mapping of domain 4 as the critical domain recognized by a mouse host during *B. anthracis* vaccination (*8*). The production of His_6-tag PA domains rather than GST-PA fusions (Flick-Smith, et al.) was an attempt to recreate these domains in a near-native configuration since the GST protein itself is larger than either domain 3 or 4 of PA. The production of these domains in our lab will enable future work to accurately determine the domain binding region for antibodies and antibody alternatives (scFv‘s, peptides, aptamers) to develop a comprehensive modeling toolkit to predict smart binders in the future.

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List of Symbols, Abbreviations, and Acronyms

bp	base pair
BSA	albumin, bovine serum
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetrataacetic acid
EF	edema factor
ELISA	enzyme-linked immunosorbent assay
GdnHCl	guanidine-HCl
GST	N-Glutathione S-Transferase
IPTG	isopropyl β-D-1-thiogalactopyranoside
PA	protective antigen
LB	Luria Broth
LF	lethal factor
LMW	low molecular weight marker
NMWL	nominal molecular weight limit
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPMW	polypeptide molecular weight marker
scFv	single-chain variable fragment
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
TAE	tris acetate EDTA
TMB	3,3,5,5-tetramethylbenzidine
WMD	weapons of mass destruction

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