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The Development of Multimeric Bioscavenger Complexes

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14. ABSTRACT
To enhance the circulatory stability of candidate organophosphorus nerve agent bioscavenger enzymes, we aimed to develop an approach based on the assumption that increasing the molecular weight (MW) of smaller proteins could prolong in vivo retention. Our goal was to create a new type of complex with an avidin core with the ability to support up to four bioscavengers, increasing the overall size while maintaining access to enzyme sites. This approach is in contrast to multimeric bioscavenger-containing liposomes and nanoparticles, where the protein is not necessarily accessible until it is released. Our strategy requires biotinylation of the bioscavenger, followed by multimeric crosslinking mediated by a compound such as streptavidin, which can bind to multiple biotin molecules. In summary, we generated an enzymatically active PON1 variant that can be specifically biotinylated at a single site, completing the first steps to allow controlled assembly of multimeric bioscavenger complexes containing between one and four different bioscavenger proteins per complex.

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Abstract

To enhance the circulatory stability of candidate organophosphorus nerve agent bioscavenger enzymes, we aimed to develop an approach based on the assumption that increasing the molecular weight (MW) of smaller proteins could prolong *in vivo* retention. Our goal was to create a new type of complex with an avidin core with the ability to support up to four bioscavengers, increasing the overall size while maintaining access to enzyme sites. This approach is in contrast to multimeric bioscavenger-containing liposomes and nanoparticles, where the protein is not necessarily accessible until it is released. Our strategy requires biotinylation of the bioscavenger, followed by multimeric crosslinking mediated by a compound such as streptavidin, which can bind to multiple biotin molecules. To overcome the issue of non-specific biotinylation, we have engineered PON1-based bioscavengers with a biotin acceptor peptide (BAP) for site-directing peptide biotinylation by biotin protein ligase (BirA). His-tagged biotin-acceptor protein PON1 VII-D11 genes were produced for expression in *E. coli* and purified using Ni-NTA chromatography. In *E. coli*, His-BAP-PON1 (P4) was expressed and Michaelis-Menten parameters were determined for P4 with paraoxon: K_M = 2.704 mM, V_{max} = 6.82 x 10^{-5} M·min^{-1}, and k_{cat} = 2741 min^{-1}. The enzymatic efficiency (k_{cat}/K_M) was calculated to be 1.01 x 10^6 $M^{-1}min^{-1}$. This value is similar to previously reported values for *E.coli*-produced VII-D11 with paraoxon, demonstrating that the addition of BAP has no apparent effect on enzymatic activity. The *E. coli* BAP-containing protein appears to be efficiently biotinylated, which will allow for complex formation with streptavidin (SA). However, the BAP/PON1 variants may require solubility aids, as PON1 appears to aggregate, and the addition of carrier proteins may be beneficial. In summary, we generated an enzymatically active PON1 variant that can be specifically biotinylated at a single site, completing the first steps to allow controlled assembly of multimeric bioscavenger complexes containing between one and four different bioscavenger proteins per complex.

Introduction

Following a nerve agent (NA) incident with mass civilian or military casualties, initial treatments would include the current clinically approved therapeutics of an acetylcholine receptor blocker (atropine sulfate), acetylcholinesterase (AChE) reactivators (pralidoxime methyl-sulfate, 2-PAM), and symptom modulators (diazepam) [1-4]. These therapeutics can provide some protection, but they are less useful for persistent NAs such as the V agents and some pesticides (i.e., parathion and chlorpyrifos). Pesticide intoxication typically is treated by bolus chemical therapeutic, followed by continued infusion for up to 5-7 days [5]. The issue of how to treat more persistent NA and pesticide exposures with a minimum of physiological and cognitive consequences has led to the concept and development of organophosphorus compound bioscavengers [2, 6-11]. Bioscavengers are proteins that can bind to and hydrolyze NAs and are either stoichiometric (non-hydrolyzing) or catalytic (hydrolyzing) types. In either case, the elimination of free NAs spares AChE, leading to faster recoveries and beneficial outcomes.

Desirable characteristics for prophylactic bioscavengers include easy administration (i.e., intramuscular [IM] rather than intravenous injection), broad specificity, lack of physiological, cognitive, behavioral, or immunological effects, and sustained stability in the circulation [12]. The bioscavenger candidate furthest in development currently is the stoichiometric type, human butyrylcholinesterase (Hu BChE, BChE) [13, 14]. For the most part, Hu BChE has met these requirements; however, it has several limitations. Hu BChE is a large protein with a MW 340 kDa tetramer (monomer 85 kDa MW), and the amount of protein required to protect against two median lethal doses of NAs is estimated to be 200-300 mg [14, 15]. In addition, it does not meet the requirement for early onset of protection as it is slow to enter the blood stream from an IM administration and requires up to 24 hours to achieve t_{max} in rodents and 10 hours in nonhuman primates (NHPs) [14]. Once in the circulation, native BChE has a mean retention time of approximately 5 days in nonhuman primates, although it rapidly drops to one-third of the peak level within a few hours [16]. This small window of protection from a single dose of enzyme limits its utility. It is clear that the use of catalytic bioscavengers would be advantageous over the stoichiometric type of BChE. Catalytic bioscavengers would hold several advantages: 1) they would be smaller and would, therefore, release from muscle faster than BChE following IM administration, and 2) they would require less protein to neutralize an equivalent amount of NA or pesticide because of their catalytic nature. We therefore chose to build our multimeric complex using paraoxonase 1 (PON1), a catalytic bioscavenger with improved activity vs. G agents [17].

Retention of protein medical countermeasures is typically short-lived because they are cleared and catabolized by any number of mechanisms as a result of exposure to different cells, enzymes, and routes of extravasation [18, 19]. Therefore, technologies are needed to improve their *in vivo* stability and retention time. We propose to evaluate a strategy to improve catalytic bioscavenger stability by creating a new type of oligomeric bioscavenger with an avidin core that would bind up to four bioscavengers. This strategy will allow the flexibility to create designer bioscavengers using enzymes with different but complementary activities. It has already been shown that increasing the size of poly(ethylene glycol) (PEG) moieties on proteins lowers the renal clearance of the proteins [20, 21]. We theorize that increasing a bioscavenger's size through the use of multimeric binding may yield similar lower clearance rates. Additionally, the creation of a larger protein complex for the catalytic bioscavengers could in and of itself improve stability vs. glomerular filtration [22]. To maintain the stability of the structure of the multimeric bioscavenger, estimates of binding affinities on the order of 10^{-15} M are needed to achieve binding $t_{1/2}$ values of 7.4 days [23]. The use of biotin/avidin complexes may achieve that since the interaction between biotin and streptavidin is the one of the strongest non-covalent bindings known [24]. Typical chemical biotinylation reactions target primary amines (ε-amine of Lys), and the biotinylation is

non-directed, with any accessible amine potentially being modified. The result is that the biotinylation can occur anywhere on the enzyme surface (including in the active site), often leading to inactivation or reduction of enzyme activity. To overcome these issues, we have engineered PON1-based bioscavengers with a biotin acceptor peptide (BAP), also known as AviTag (GLNDIFEAQKIEWHE), for site-directing peptide biotinylation by biotin protein ligase (BirA) [25], which would decrease the likelihood of non-specific biotinylation.

Our goal was to create a new type of complex with an avidin core and up to four bioscavengers, increasing the overall size while maintaining access to enzyme sites. Our strategy requires biotinylation of the bioscavenger, followed by multimeric crosslinking mediated by the multiple biotin-binding compound streptavidin. To overcome the issues of non-specific biotinylation we engineered PON1-based bioscavengers with a biotin acceptor peptide (BAP) for site-directing peptide biotinylation by biotin protein ligase (BirA). A His-tag was incorporated for ease of purification, and the His-tagged biotin-acceptor protein PON1 VII-D11 genes were produced for expression in *E. coli*.

Methods

Enzyme Expression and Purification. Gene synthesis and plasmid production were performed by GenScript USA, Inc. (Piscataway, NJ) for expression in *E. coli*. The BAP (biotin acceptor peptide) [25] gene sequence was fused in line to the paraoxonase (PON) variant VII-D-11 separated by a GSGS linker. A His-tag (His) was part of the vector sequence to aid in purification of the expressed proteins. The One Shot BL21(DE3) Chemically Competent *E. coli* strain (ThermoFisher Scientific, Waltham, MA) was used for general protein expression, and strain CVB101 (Avidity, LLC., Aurora, CO) was used for Bir ligase co-expression studies. Cells were transformed with the indicated plasmids, and expression was induced with IPTG using standard molecular biology techniques and manufacturer's protocols. Co-expression cultures were supplemented with biotin (ThermoFisher) before induction. After harvest the cells were lysed with BugBuster™ Master Mix (Novagen, Inc., Madison, WI), and the His-tagged proteins were purified by Ni-NTA chromatography (Millipore, Inc., Burlington, MA). The following enzymes were expressed: His-PON (P1), PON-BAP-His (P2), His-BAP-PON (P4), and His-biotin-BAP-PON (bP4).

Paraoxon Activity Assay. The enzyme activity was assessed using a spectrometric paraoxonase activity assay [26]. The assay was run in a 96-well plate format with a final reaction volume of 200 µl per well. PON1 fusion proteins were added at a volume of 10 µl to the plates, along with 180 µl of assay buffer consisting of 50 mM HEPES pH 7.2 and 10 mM CaCl2 (Sigma-Aldrich, St. Louis, MO). Increasing concentrations of paraoxon (Chem Service Inc., West Chester, PA) were added at volumes of 10 µl to each well. Immediately following the addition of paraoxon the plate was read using a SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA) in kinetic mode at a wavelength of 412 nm. Following a 30 s plate shake, the plate was read in 20 s intervals for 10 min. The velocity of the reaction in milli-absorbance units per minute (mAU/min) was calculated by the instrument's software. Using the Beer-Lambert Law and the extinction coefficient of 1.8×10^4 M^{-1} cm^{-1}, the reaction rate was converted from mAU/min to M/min and plotted vs. paraoxon concentration. V_{max} and k_m were calculated using Prism v5.04 software (GraphPad Software, La Jolla, CA), and k_{cat} and the catalytic efficiency (k_{cat}/k_m) were calculated from those values.

Biotin Quantification Assay. The amount of biotinylation of proteins manufactured through *E. coli* expression was quantified using a Pierce Biotin Quantification kit (ThermoFisher). The amount of biotin in proteins bP4 and A743 was determined using biotinylated horseradish peroxidase (HRP) as a standard. The assay was run according to the manufacturer's protocol, and plate absorbance was read at 500 nm

using a SpectraMax Plus 384 microplate reader (Molecular Devices). The moles of biotin per mole of protein were calculated using the manufacturer's protocol.

SDS Page. 2x SDS sample buffer (Bio-Rad Laboratories, Hercules, CA) was added to 5 µg of protein at 1:1 and then heated at 95°C for 5 minutes. Samples were loaded onto a 4-15% precast mini-PROTEAN gel (Bio-Rad), and Bio-Rad running chambers were filled with a TRIS-buffered saline (137 mM NaCl [Sigma-Aldrich], 2.7 mM KCl [Sigma-Aldrich], 19 mM Tris base [Sigma-Aldrich]) with 0.1% Tween 20 (Sigma-Aldrich) (TBST) running buffer. The gel was run at 115 V until the markers ran to the end of the gel (approximately 1 hour). Gel was removed and washed 3x with ultrapure water. Gel was stained with SimplyBlue SafeStain (ThermoFisher) for 1 hour at room temperature with rocking. Gel was then imaged on a Bruker In-Vivo FX PRO Imager (Bruker, Billerica, MA) on bright field.

Western Blotting. 2x SDS sample buffer (Bio-Rad) was added to 5 µg of protein at 1:1 and then heated at 95°C for 5 minutes. Samples were loaded onto a 4-15% precast mini-PROTEAN gel (Bio-Rad), and Bio-Rad running chambers were filled with a TBST running buffer. The gel was run at 115 V until the markers ran to the end of the gel (approximately 1 hour). The gel was transferred to a membrane using the iBlot system (ThermoFisher) according to the manufacturer's protocol. The membrane was removed and blocked overnight in I-Block buffer (ThermoFisher) at 4°C with rocking. The membrane was removed and washed 3x with phosphate-buffered saline (PBS) before being incubated with IR-labeled streptavidin (Li-Cor Biosciences, Lincoln, NE) in TBST with 5% bovine serum albumin at 1:100 at 4°C with rocking. Membrane was then again washed 3x with PBS and imaged on an Odyssey CLx Imaging System (Li-Cor Biosciences) according to the manufacturer's protocol.

Size Exclusion Experiments. Expressed enzymes were placed into an Amicon 100K MW Centrifugal Filter Unit (Sigma-Aldrich). The samples were then spun at 14,000 × g for 30 minutes at 4°C. The retentate and filtrate fractions were then collected and analyzed by SDS PAGE and Western blot as stated above.

Aggregate Treatment Experiments. bP4 aggregates were treated with 0.5 M urea (Sigma-Aldrich), 0.2% Triton X100 (Sigma-Aldrich) and 0.5 M urea, Triton X100 and 1M urea, 0.2 M KCl, 10% glycerol (Sigma-Aldrich), 1 M guanidine HCl (Sigma-Aldrich), and 0.2% Triton X100 and 1 M guanidine and then processed by size exclusion as stated above. Samples were analyzed by SDS PAGE as stated above.

Results

Our strategy to increase the *in vivo* stability of NA bioscavengers (Figure 1) was to develop a multimeric bioscavenger using avidin/biotin that would have an extended bioavailability due to a decrease in first pass metabolism. To produce a biotinylated BAP-PON enzyme, we chose a strategy of biotinylation during protein expression. *E. coli* containing the vector for P1, P2, and P4 was transformed with pBirAcm, an engineered pACYC184 plasmid with an ITPG-inducible birA gene to overexpress biotin ligase. The transformed *E. coli* was grown to early log phase and then induced for protein expression with IPTG in the presence of 50 µM biotin. The cultures were grown until they reached an OD_{600} of ~1.1 and were then lysed by the detergent BugBuster™. The cell lysate was passed over a Ni-NTA column to isolate our His-tagged protein.

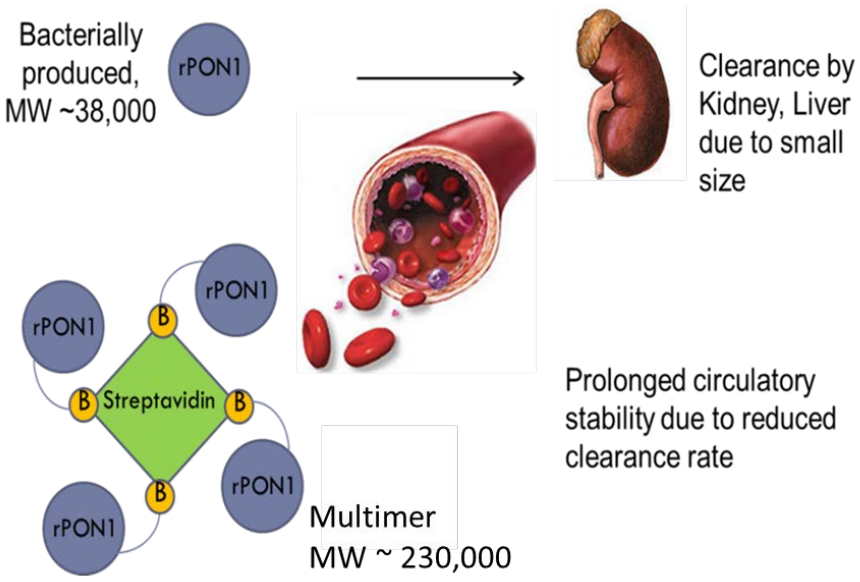

Figure 1. Multimeric Strategy

The protein yield for each was approximately 1-5 mg, with P4 having the highest yield. SDS-PAGE analysis of all enzymes revealed bands running at the equivalent position of approximately 38 kDa (not shown). The kinetic parameters of P1, native P4 and bP4 were determined for the pesticide paraoxon from the kinetic experiment shown in Table 1. Native bP4 had a catalytic efficiency (1.01×10^6 $M^{-1}min^{-1}$) slightly better than expressed PON1 (1.00×10^6 $M^{-1}min^{-1}$) with $K_M = 2.704$ mM, $V_{max} = 6.82 \times 10^{-5}$ $M \cdot min^{-1}$, and $k_{cat} = 2741$ min^{-1}. The catalytic efficiency of P2 was poor (4.80×10^4 $M^{-1}min^{-1}$) and was therefore abandoned as a platform for the multimeric bioscavenger. Biotinylation of P4 does reduce the catalytic efficiency of the enzyme (2.96×10^5 $M^{-1}min^{-1}$), but we feel that the efficiency of the enzyme is still sufficient to proceed with further development. To confirm that our biotinylation strategy was successful we analyzed biotin content of bP4 with a commercially available biotin assay kit (Table 2). The assay reported biotinylation of our enzyme, although at a level lower than the provided standard (0.62 mmol biotin/mmol protein vs. 1.37 mmol biotin/mmol protein) with His-PON returning a negative result.

	His-PON (P1)	His-PON-BAP (P2)		His-BAP-PON (P4)	
		native	biotinylated	native	biotinylated
K_{cat}/K_M ($M^{-1}min^{-1}$)	1.00×10^6	1.01×10^6	2.96×10^5	4.80×10^4	ND

Table 1. Summary of catalytic efficiencies of BAP-PON constructs produced by *E. coli*.

	mmol biotin/mmol protein	Standard Deviation
Biotin-HRP (Standard)	1.37	0.07
bP4	0.62	1.54
P1	-31.29	13.13

Table 2. Summary of biotinylation of expressed enzymes

The results of the assay were supported by probing a Western blot of the biotinylated-BAP-PON, PON, and bovine serum albumin (BSA) (Figure 2). The blot was probed with IR-labeled streptavidin, which only bound to our biotinylated BAP-PON enzyme, confirming the results of the biotin assay.

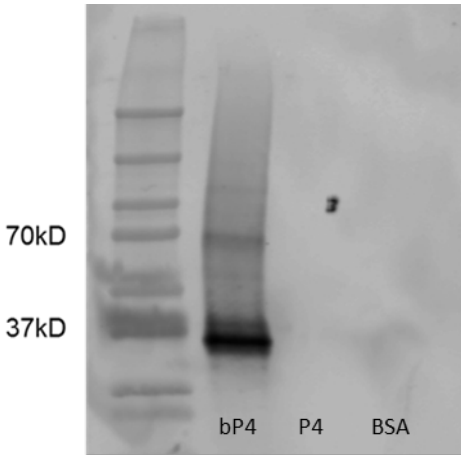

Figure 2. Western blot of E. coli-produced PON variants probed with IR-labeled streptavidin. A Western blot was run to confirm the success of our biotinylation strategy. Probing with IR-labeled streptavidin produced a band ~38 kD in the bP4 lane alone. This result suggests that our biotinylation strategy was able to produce biotinylated-BAP-PON.

In our previous attempts to develop a multimeric bioscavenger using PON1 produced in an expression system using *Trichoplusia ni* larval system, we observed that BAP-PON proteins had a tendency to form high MW aggregates post-biotinylation. To test whether our *E. coli* strategy produced similar enzyme aggregates, we performed a size exclusion study. Purified biotinylated-BAP-PON was passed through a centrifugal filtration device with a 100 kDa membrane, and both the filtrate and the retentate were collected. We then analyzed both the filtrate and retentate by SDS PAGE (Figure 3A) and Western blot with an IR-labeled streptavidin probe (Figure 3B). The results suggest that the *E. coli*-produced biotinylated enzyme form aggregates in the same fashion that the larval-produced enzymes did.

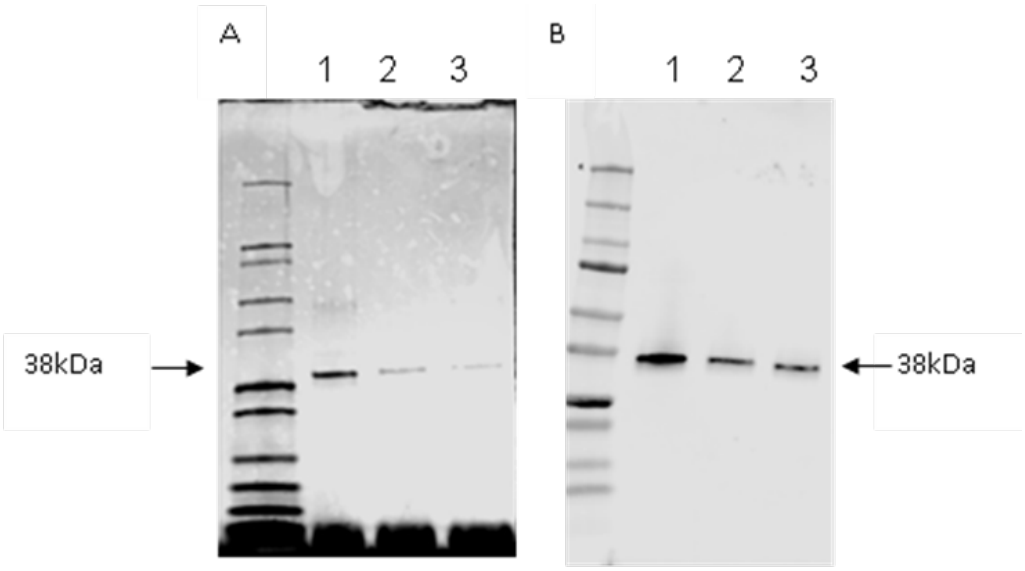

Figure 3. SDS-PAGE and WB analysis of bP4 protein. A) SDS-PAGE of bP4 ultrafiltration fractions; lanes: 1. unprocessed sample; 2. 100 kDa filtrate; 3. 100 kDa retentate. B. WB probe streptavidin-IR label; lanes: 1. unprocessed sample; 2. 100 kDa filtrate; 3. 100 kDa retentate.

The aggregation of bP4 into high MW molecules presents a significant issue. We attempted to process bP4 with different strategies (urea, KCl, glycerol, and guanidine HCl) in order to solubilize the aggregates. In Figure 4, bP4 was treated with Trition X100 +0.5M urea, 0.5M Urea, 0.2M KCl, or 10% glycerol and then processed by ultrafiltration (100 kDa), and run on an SDS PAGE gel. Only treatment with urea and a non-ionic detergent was marginally successful. We then tried increasing the concentration of urea or using guanidine HCl in both the presence and absence of a non-ionic detergent (Figure 5). The results indicated that, although higher concentrations of urea in the presence of a non-ionic detergent was marginally successful, overall our strategies were not sufficient enough in sobulizing the high MW aggregates and allowing for further development of a multimeric bioscavenger.

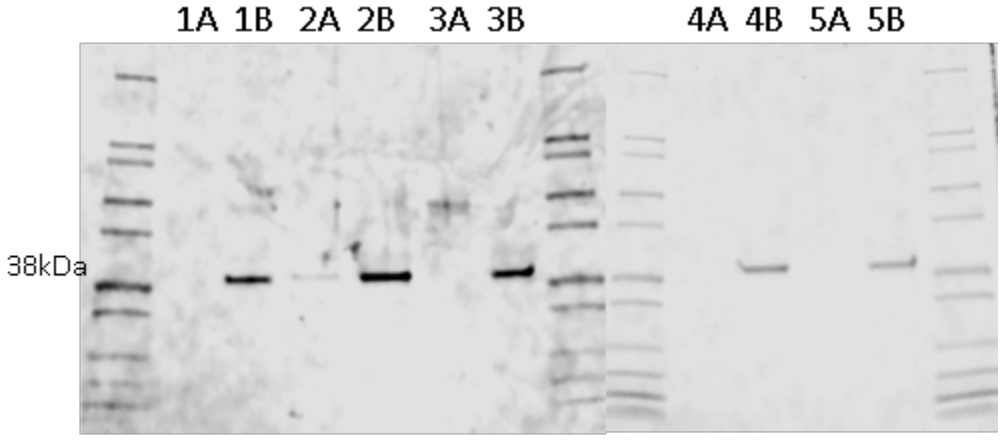

Figure 4. SDS PAGE bP4 solubility. A) 100k filtrate, B) 100K retentate; Treatments: 1) No treatment, 2) 0.2% Trition X100 + 0.5M Urea, 3) 0.5M Urea, 4) 0.2 KCl, 5) 10% Glycerol

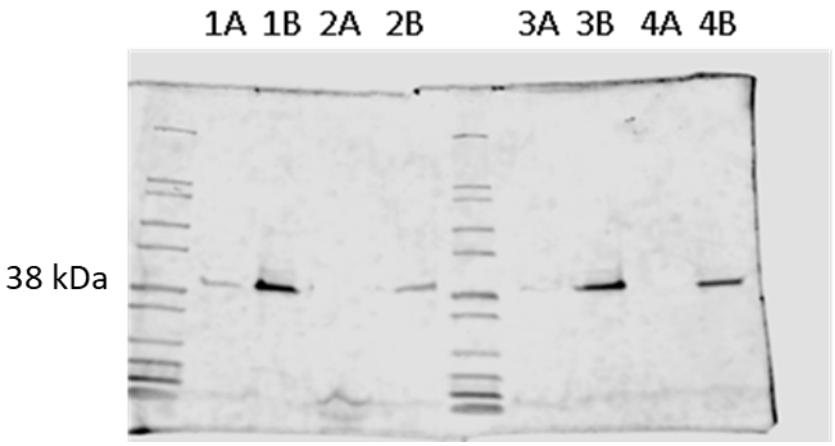

Figure 5. SDS PAGE bP4 solubility; A) 100k filtrate, B) 100K retentate; Treatments: 1) 0.2% Trition X100 + 1M Urea, 2) 1M Guanidine HCl, 3) 0.2 % Trition X100 + 1M Guanidine HCl, 4) No treatment

Conclusion

We have produced BAP-PON derivatives for the purpose of supporting a new strategy of creating bioscavenger multimeric complexes. In the course of this work we expressed proteins in *E. coli* and produced a BAP protein (P4) which retained similar activity to the PON1 enzyme. Though the activity was reduced when biotinylated, we believe that the catalytic efficiency would still be sufficient for use as a NA bioscavenger. The biggest issue with this platform is the unfortunate finding that sample processing has led to apparent protein aggregation. The issue of aggregation provides a challenge to the use of these products and future medical countermeasure development. The use of a peptide blocker could, in theory, rectify the issue of aggregation and yield active proteins. Alternatively, a different bioscavenger (i.e., organophosphorus hydrolase) with improved catalytic efficiency and lack of aggregate formation may be a better platform for this technology.

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