

1 *Research Article*

2 **Expression of Pokeweed Antiviral Protein Isoform S1** 3 **(PAP-S1) and of Ricin-A-Chain/PAP-S1 novel fusion** 4 **protein (RTA/PAP-S1) in *Escherichia coli* and their** 5 **comparative inhibition of protein synthesis *in vitro***

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12 **Abstract:** Fusion protein therapeutics engineering is advancing to meet the need for novel
13 medicine. Herein, we further characterize the development of novel RTA & PAP-S1 antiviral
14 fusion proteins. In brief, RTA/PAP-S1 and PAP-S1/RTA fusion proteins were produced in both cell
15 free and *E. coli in vivo* expression systems, purified by His-tag affinity chromatography, and
16 protein synthesis inhibitory activity assayed by comparison to the production of a control protein,
17 CalmL3. Results showed that the RTA/PAP-S1 fusion protein is amenable to standardized
18 production and purification and has both increased potency and less toxicity compared to either
19 RTA or PAP-S1 alone. Thus, this research highlights the developmental potential of novel fusion
20 proteins with reduced cytotoxic risk and increased potency.

21 **Keywords:** fusion proteins; protein therapeutics; ricin; pokeweed antiviral protein; protein
22 engineering; immunotoxins; ribosome-inactivating proteins.

23

24 **1. Introduction**

25 Pokeweed antiviral proteins (PAPs) are potent type I Ribosome Inactivating Proteins (RIPs)
26 expressed in several organs of the plant pokeweed (*Phytolacca americana*), as reviewed by
27 Domashevskiy and Goss [1]. They are chiefly secreted and bound within the plant cell wall matrix.
28 Here, they are known to function in defense against pathogens through the inhibition of both
29 prokaryotic and eukaryotic ribosomes and protein synthesis. Among the PAP gene family, different
30 genes are expressed in various tissues and at different stages of development in *Phytolacca*
31 *americana*. PAP, PAP II, PAP-S1, PAP-S2, and PAP-R are the forms that appear in spring leaves,
32 summer leaves, isoform S1 and S2 in seeds, and roots, respectively. The molecular weight ranges
33 from 29 kDa for PAP to 30 kDa for PAP-S's [2]. PAP-S1 has been identified as the most effective in
34 inhibiting protein synthesis *in vitro* [3]. PAPs possess antiviral activity on a wide range of plant and
35 human viruses; different forms of PAP expressed in transgenic plants leads to broad-spectrum
36 resistance to viral and fungal infections [4-5]. Relevant to the recent Zika epidemic, PAP is efficient
37 against Japanese encephalitis virus [6]. Antiviral activity is also present against HIV-1 [7], human
38 T-cell leukemia virus-1 (HTLV-1) [8], herpes simplex virus (HSV) [9], influenza [10], hepatitis B
39 virus (HBV) [11], and poliovirus [12]. Moreover, different forms of PAP have moderate cytotoxicity
40 to non-infected cells and, thus, offer unique opportunities for new applications in therapy and as a
41 protective protein against pathogens in transgenic plants.

42 Ricin is produced in the seeds of the castor oil plant, *Ricinus communis*, and is one of the most
43 potent type II RIPs, as reviewed by Lord et al [13]. It can efficiently deliver its A chain into the
44 cytosol of cells through the action of its B chain. The B chain serves as a

45 galactose/N-acetylgalactosamine binding domain (lectin) and is linked to the A chain via disulfide
46 bonds. After the ricin B chain binds complex carbohydrates on the surface of eukaryotic cells
47 containing either terminal N-acetylgalactosamine or beta-1,4-linked galactose residues, it is
48 endocytosed via clathrin-dependent as well as clathrin-independent mechanisms and is thereafter
49 delivered into the early endosomes. It is then transported to the Golgi apparatus by retrograde
50 transport to reach the endoplasmic reticulum (ER) where its disulfide bonds are cleaved by
51 thioredoxin reductases and disulfide isomerases. The median lethal dose (LD50) of ricin is around
52 22 micrograms per kilogram of body weight if the exposure is from injection or inhalation (1.78
53 milligram for an average adult). It is important to note that the ricin A chain (RTA) on its own has
54 less than 0.01% of the toxicity of the native lectin in a cell culture test system. It was furthermore
55 shown that RTA alone had no activity on non-infected and tobacco mosaic virus (TMV)-infected
56 tobacco protoplasts alike. Though there are currently no commercially available therapeutic
57 applications, RTA is extensively studied in the development of immunotoxins [14].

58 The therapeutic potential of PAP and RTA has been explored for over thirty years, though side
59 effects have limited clinical application. As evaluated by Benigni et al [15], while these proteins
60 have shown very low cytotoxicity to non-infected cells, PAP administration in mouse models has
61 resulted in hepatic, renal and gastrointestinal tract damage with an LD50 as low as 1.6mg/Kg.
62 Interestingly, RTA shows no toxicity even at high doses with similar half-life times. However, all
63 RIP's show immunosuppressive effects to various degrees. Other studies have described the
64 various dose-limiting side effects of these proteins when used as immunotoxins (i.e. vascular leak
65 syndrome, hemolytic uremic syndrome, and pluritis, among others) [16-17]. Excitingly, some
66 patients achieved complete or partial remission against Refractory B-Lineage Acute Lymphoblastic
67 Leukemia with sub-toxic dosages, for example.

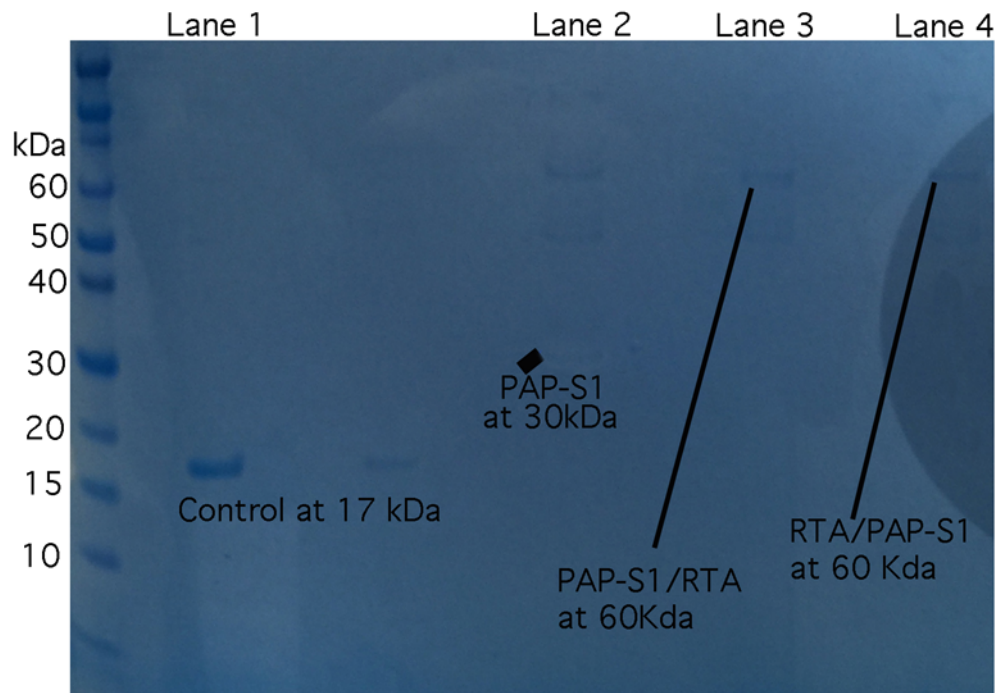
68 Fusion and hybrid PAP proteins have also been developed in pursuit of selectively targeting
69 infected cells and selectively recognizing viral components, though with limited success [18-19].
70 Indeed, the engineering of novel therapeutic fusion proteins with higher specificity, selectivity, and
71 potency with fewer side effects is a leading strategy in drug development.

72 Thus, this research furthers study of a previously created and functional novel fusion protein
73 between RTA and PAP-S1 [20]. Here, we describe the increased potency over PAP-S1 alone,
74 selectivity for infected cells, and reduced side effects associated with dosage. Additionally, we
75 describe the development of an adequate and scalable production system that enabled accurate
76 determination of PAP-S1 and RTA/PAP-S1 protein synthesis inhibition *in vitro*.

77 2. Results

78 2.1. Production and Purification of recombinant Proteins in Cell Free Expression System

79 The proteins were expressed and bands visible where expected (Figure 1). It is noted that
80 expression of the three recombinant proteins is very low compared to the control protein and, more
81 importantly, with a much lower purity; expression of PAP-S1 is the lowest. The low levels of
82 expression of the recombinant proteins were expected as the proteins are known to be toxic to
83 prokaryotic ribosomes. Total protein content and purity of each sample is shown in Table 1.



84

85 **Figure 1.** Recombinant proteins gel stained with Coomassie blue after His-Tag purification.

86 Lane 1: Control protein after His-Tag Purification. The band is clearly visible at 17kDa.

87 Lane 2: PAP-S1 recombinant protein at the 32kDa line.

88 Lane 3: PAP-S1/RTA recombinant protein at the 60kDa line.

89 Lane 4: RTA/PAP-S1 recombinant protein at the 60kDa line

90 *All other bands are due to proteins going through the His-Tag purification column from the
91 initial expression reaction.

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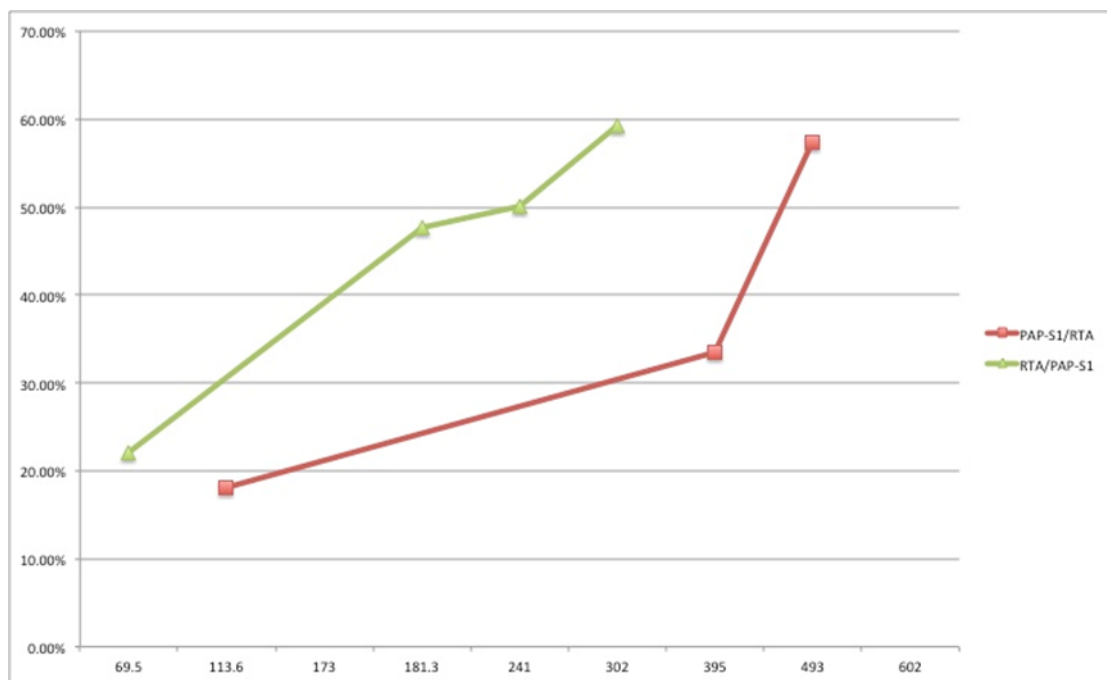
93 **Table 1.** Total protein content and purity. For each sample, protein content was determined using
94 Qubit™ 3.0 Fluorometry; purity was determined by GelAnalyzer 2010 using values from Figure 1S
95 (See Appendix 2 for details).

Protein	Total Produced	Reaction Volume	Purity	Final Product in 50uL
CalmL3 – control	5338ng	50uL	68%	106.8ng/uL
PAP-S1	4514ng	150uL	25.90%	90.3ng/uL
PAP-S1/RTA	5924ng	150uL	33.60%	118.5ng/uL
RTA/PAP-S1	3626ng	100uL	29.40%	72.5ng/uL

96 2.2. Inhibitory activity of recombinant proteins on *E. coli* protein synthesis

97 Two different prokaryotic kits, the *E. coli* S30 T7 High-Yield Protein Expression System and the
98 Expressway™ Mini Cell-Free Expression System, were used to control for variances in production
99 systems. The same control protein was produced, namely CalmL3. Various concentrations of

100 recombinant proteins were added to each reaction and the amount of CalmL3 protein produced
101 was compared to the amount of CalmL3 produced without the addition of any recombinant
102 protein. These values were plotted as percent protein inhibition compared to control versus
103 concentration (Figure 2). The IC₅₀ of PAP-S1/RTA was found to be around 460nM while the IC₅₀ of
104 RTA/PAP-S1 was around 241nM (based on Figure 6S and Figure 7S, see appendix 3 for details); the
105 IC₅₀ of PAP-S1 is known to be around 280nM [3]. Those initial results confirm RTA/PAP-S1 as
106 more potent than PAP-S1/RTA. This was expected as the C terminal is known to play a major role
107 in activity [23]. Those results also confirm that RTA/PAP-S1 is more potent than PAP-S1 alone.
108 RTA/PAP-S1 will thus be the subject of the rest of the paper. The increased activity of RTA/PAP-S1
109 compared to PAP-S1 alone can probably be explained by the fact that PAP-S1 and RTA do not dock
110 onto the ribosome at the same site. Indeed, it was found that after PAP-S1 partially depurinates the
111 *E. coli* ribosome, RTA is able to depurinate the same ribosome while RTA cannot depurinate an
112 intact *E. coli* ribosome on its own [19-26].



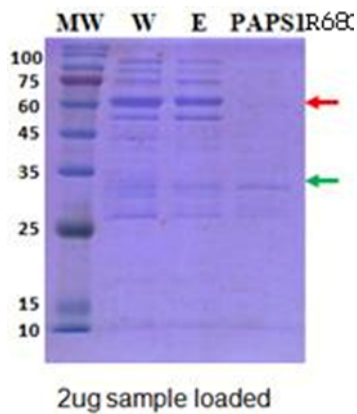
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114 **Figure 2.** Percent protein inhibition compared to control. The Y-axis represents percent
115 inhibition compared to control and the X-axis represents the concentration of each respective
116 protein in nM.

117 2.3. Production and Purification of recombinant Proteins in *E. coli* culture

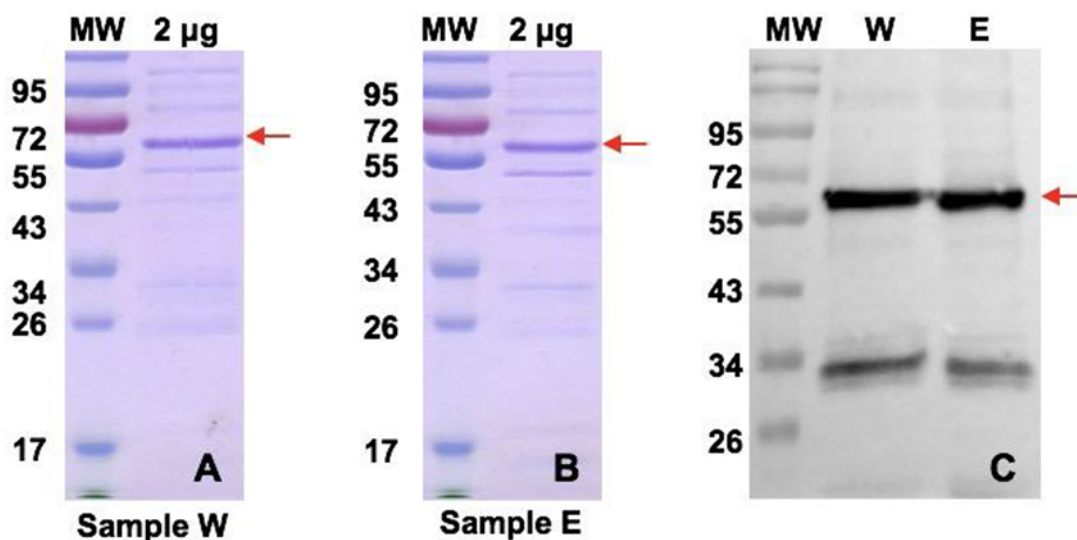
118 We were unable to produce the wild type proteins in either insect or yeast cell cultures. Thus,
119 we decided to produce a mutated form of PAP-S1 (PAP-S1R68G) with a native signal peptide. We
120 believed this would stabilize protein production but would greatly reduce activity in *E. coli* and
121 somewhat reduce activity in eukaryotic cells in *E. coli* cell culture [21, 24]. Native environment of
122 both PAP-S1R68G (purity of 60%) and RTA/PAP-S1R68G (purity of 55%) production are shown in
123 Figure 3.a (purity determined by GelAnalyzer2010). More than 400ug of each protein was
124 produced. However, as shown in Figure 3.b, 6-His tag purification process was very inefficient and
125 a large amount of protein was lost in the wash (kept for further analysis). This loss may have been
126 due to the 6-His tag being hidden by the protein in its final conformation. It is possible the double
127 band is due to protein degradation or unwanted recombination at the production site by *E. coli*
128 cells.

129 a)



130

131 b)



132

133 **Figure 3.** Protein Purification. a) Stained gel of RTA/PAP-S1R68G from third wash (sample W)
 134 and from pooled eluted fraction (sample E) at 60kDa (red arrow) and of PAP-S1R68G at 30kDa
 135 (green arrow), all from native environment, after buffer exchange. The purity of sample W was
 136 65%, of sample E 55%, and of PAP-S1R68G 60%. b) Close up and Western Blot of sample W and E.
 137 A double band is present and may be due to degradation of the protein or as a result of an
 138 unwanted recombination by *E. coli* cells.

139 For each sample in Table 2, the final concentration was determined using a Bradford protein
 140 assay; purity was determined using the GelAnalyzer 2010 (see appendix 4 for details). The yield
 141 was very low, and again, a lot of protein was lost in the washes as the 6-His tag purification does
 142 not appear to be the right system for those proteins.

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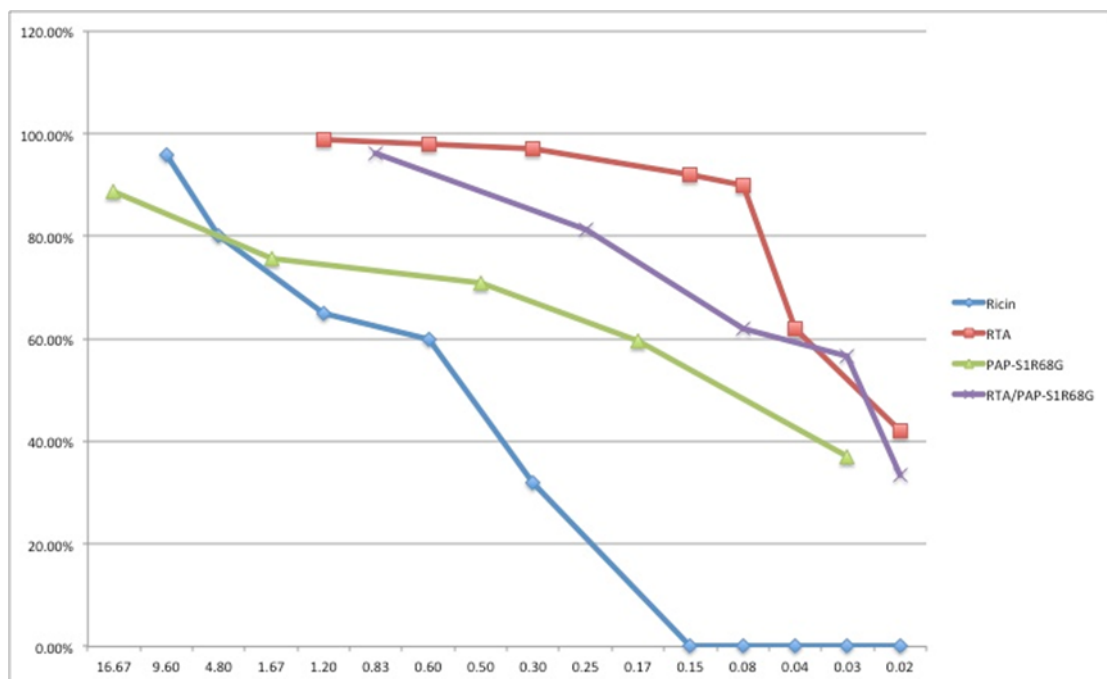
144 **Table 2.** Protein concentration and purity. For each sample, protein content was determined using a
 145 Bradford protein assay; purity was determined by GelAnalyzer 2010 as explained in appendix 4.

Native protein	Final concentration	Volume	Final product	Purity
PAP-S1R68G	0.23mg/ml	3.2ml	0.736mg	60%
RTA/PAP-S1R68G	0.53mg/ml	1.35ml	0.716mg	55%

(sample E)				
RTA/PAP-S1R68G	0.26mg/ml	4.8ml	1.25mg	65%
(sample W)				

146 2.4. Inhibitory activity of recombinant proteins in the Rabbit Reticulate Lysate TnT® system

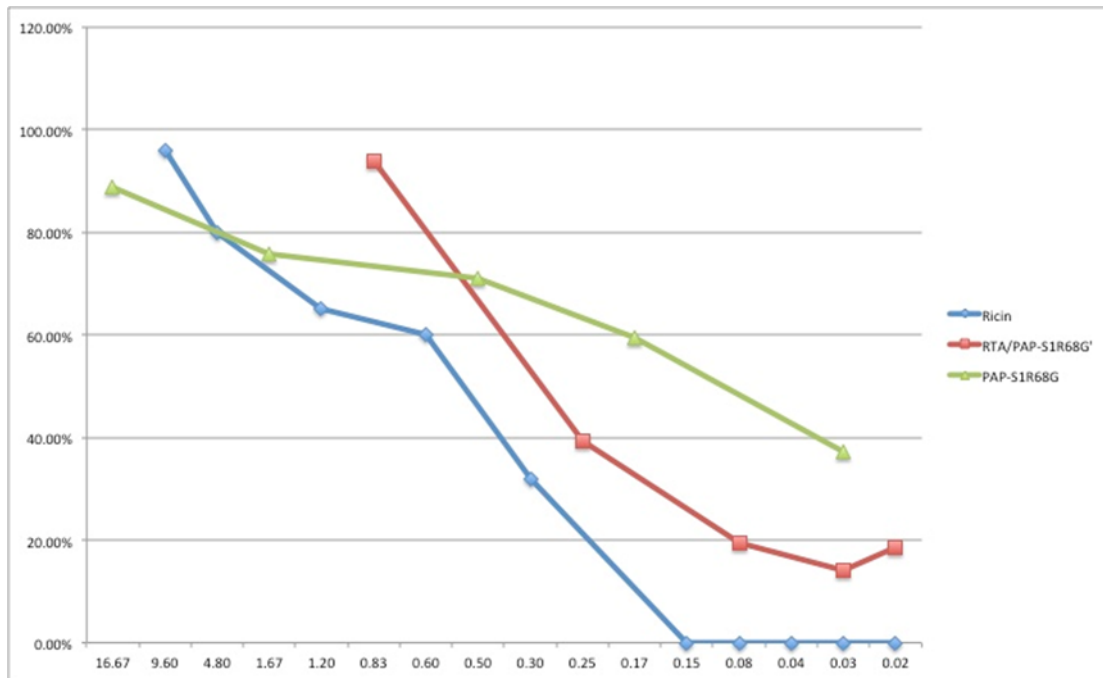
147 The inhibitory activity of PAP-S1R68G and RTA/PAP-S1R68G were determined using 5
148 different concentrations of PAP-S1R68G and RTA/PAP-S1R68G (sample E) on the Rabbit Reticulate
149 Lysate TnT® system using Luciferase as control. A Luciferase assay was used to determine
150 Luciferase expression levels using a luminometer. The comparative plot is shown in Figure 4 and
151 includes previous data on Ricin and RTA obtained similarly [22]. As can be observed,
152 RTA/PAP-S1R68G behaves more like RTA than PAP-S1R68G and has an IC₅₀ at 0.025nM (similar to
153 RTA 0.03nM) against 0.06nM for PAP-S1R68G. The total inhibition is attained at 0.83nM for
154 RTA/PAP-S1R68G while PAP-S1R68G barely reaches 90% at 16.67nM, possibly due to the single
155 point mutation (R68G). It is also interesting to note that PAP-S1R68G has about the same IC₅₀ as
156 PAP-S2 (0.07) but a much higher total inhibition point (around 1.2nM for PAP-S2) [2]. These results
157 not only show that RTA/PAP-S1R68G is at least twice as fast as PAP-S1R58G but also 16 times more
158 potent. It is comparable to native RTA and can thus be assumed that non-mutated RTA/PAP-S1 will
159 be even faster.



160

161 **Figure 4.** Comparative inhibitory activity. The Y-axis represents percent inhibition compared
162 to control and the X-axis represents the concentration of each respective protein in nM. Results
163 represent the average S.D. for two individual experiments.

164 The same inhibitory activity tests (Figure 5) were run under the same conditions for the
165 proteins from sample W in order to determine whether binding or protein type isolation issues
166 were causing the high amount of protein loss during washing. The results suggest behavior that is
167 more like wild type Ricin protein, but with an IC₅₀ of 0.3nM against 0.5nM for wild type Ricin.



168

169 **Figure 5.** Comparative inhibitory activity. The Y-axis represents percent inhibition compared to
170 control and the X-axis represents the concentration of each respective protein in nM. Results
171 represent the average S.D. for two individual experiments.

172 The existence of two conformations of the same protein is possible as this fusion protein is new
173 and it is difficult to predict exact conformation. Resequenced cDNA verified there were no acquired
174 plasmid mutations. A prediction software [25] showed high probability (score of 0.9131) of
175 existence of a di-sulfide bond between C173 and C539. This could explain the observed behavior
176 and the difficulty in using 6-His tag for affinity purification. However, this remains to be proven, as
177 this behavior was not observed in cell free expression system. Moreover, antibodies against PAP-S1
178 may solve the yield issue and any interaction with the 6-His tag itself.

179 3. Discussion

180 The fusion protein between Ricin A chain C terminus and PAP-S1 N terminus was observed to
181 be functional and active in both eukaryotic and prokaryotic cell free systems with a great increase in
182 both speed and potency compared to PAP-S1 alone. It was also observed that it was comparable in
183 activity to Ricin A chain in a eukaryotic system. It is the opinion of the authors that additional
184 research should be done in order to determine both the cytotoxicity and selectivity of RTA/PAP-S1
185 to PAP-S1 against a wide range of mammalian infectious diseases, including some types of cancers
186 and a wide range of plant infectious diseases. We expect the RTA/PAP-S1 fusion protein to be a
187 much more viable, potent, and less cytotoxic alternative to PAP-S1 alone for both agricultural and
188 therapeutic applications.

189 4. Materials and Methods

190 The PureLink® Genomic Plant DNA Purification Kit, HisPur™ Ni-NTA Spin Purification Kit,
191 0.2 m, Phire Plant Direct PCR Master Mix, PureLink™ Quick Gel Extraction and PCR Purification
192 Combo Kit, Phire Hot Start II DNA Polymerase, Expressway™ Mini Cell-Free Expression System
193 and NuPAGE™ 10% Bis-Tris Protein Gels, 1.5 mm, 15-well were purchased from Thermo Fisher
194 Scientific. PCR Oligonucleotides and overlap extension products were synthesized by Integrated
195 DNA Technologies. Rabbit Reticulate Lysate TnT® Quick Coupled Transcription/Translation
196 System, Luciferase Assay System and the *E. coli* S30 T7 High-Yield Protein Expression System were

197 purchased from Promega. cDNA coding for the PAPS1[R68G] protein and
198 Ricin-A-Chain/PAP-S1R68G was chemically synthesized with optimization for *E. coli* expression by
199 GenScript. The *E. coli* pT7 expression vector and *E. coli* strains BL21(DE3) were purchased from
200 Proteogenix. Total sample protein content was analyzed using Qubit™ 3.0 Fluorometer and
201 Bradford protein assay. Luciferase assay readings were achieved using a Perkin Elmer EnVision
202 Microplate Reader. Gel analyses were performed using a GelAnalyzer 2010.

203 4.1. *E. coli* cell free expression and *E. coli* protein synthesis inhibition

204 4.1.1. Design of the DNA sequences of the proteins for *E. coli* cell free expression system

205 The DNA sequenced was designed and isolated as described in our previous work [20]. In
206 short, fresh seeds of both *Ricinus communis* and *Phytolacca Americana* were purchased a local
207 supplier in Baltimore, MD and Ricin A Chain (RTA) and PAP-S1 (with native signal peptide)
208 isolated and PCR amplified. The 6-His tag was added to PAP-S1 at the C terminal. The RTA/PAP-S1
209 fusion protein was achieved through PCR extension, using the native RTA polylinker, between
210 RTA C terminal and PAP-S1 (without the signal peptide) N terminal with the 6-His tag at RTA N
211 terminal. The PAP-S1/RTA fusion protein was achieved through the same means but between
212 PAP-S1 C terminal and RTA N terminal with the 6-His tag at PAP-S1 N terminal.

213 4.1.2. *E. coli* cell free expression and purification

214 The PAP-S1, PAP-S1/RTA and RTA/PAP-S1 fusion proteins were produced using
215 Expressway™ Mini Cell-Free Expression System as previously described [20]. In short, Linear DNA
216 was used for all proteins in thrice the volume (150uL) for PAP-S1 and PAP-S1/RTA and twice the
217 volume for RTA/PAP-S1 using the T7 promoter System. The proteins were purified using the
218 HisPur™ Ni-NTA Spin Purification Kit, 0.2 m before being run on protein gels for confirmation.
219 The total protein content was determined using Qubit™ 3.0 Fluorometer. Gels were analyzed using
220 the GelAnalyzer2010.

221 4.1.3. Inhibition of protein synthesis in cell free *E. coli* system

222 Enzyme activity of the purified recombinant proteins was determined by the intensity of the
223 band on protein gel of a control against expression of the control without the recombinant proteins,
224 after protein purification using the HisPur™ Ni-NTA Spin Purification Kit, 0.2 m, as previously
225 described [20] using the *E. coli* S30 T7 High-Yield Protein Expression System. The control used was
226 the pEXP5-NT/CALML3 control vector with a DNA template expressing an N-terminally-tagged
227 human calmodulin-like 3 (CALML3) protein (under the T7 promoter). The concentration of
228 CALML3 was determined for increasing concentrations of recombinant PAP-S1 and fusion proteins
229 by measuring band intensity on a protein gel (Coomassie blue stained) by GelAnalyzer2010.

230 4.2. *E. coli* in vivo expression system and Rabbit Reticulate Lysate protein synthesis inhibition

231 4.2.1. Design of the DNA sequences of the proteins for *E. coli* in vivo expression system

232 The cDNA coding for a mutated version of PAP-S1 and RTA/PAP-S1, namely PAP-S1R68G
233 and RTA/PAP-S1R68G, were chemically synthesized with optimization for *E. coli* expression by
234 GenScript. The mutated form of PAP-S1 was used in order to reduce *E. coli* ribosome depuration
235 by PAP-S1 and RTA/PAP-S1 while safeguarding their Eukaryotic ribosome depuration activities
236 [21]. The native PAP-S1 signal peptide was kept for PAP-S1R68G with the addition of an *E. coli*
237 signal peptide of two amino acids at PAP-S1R68G and RTA/PAP-S1R68G N terminal and with the
238 6-His tag at PAP-S1R68G and RTA/PAP-S1R68G C terminal (see appendix 1 for details).

239 4.2.2. *E. coli* in vivo expression vector

240 The cDNA sequences described above were cloned in an *E. coli* pT7 expression vector using
241 the NcoI/XhoI cloning strategy (map of vectors in Appendix 1).

242 4.2.3. *E. coli* protein production

243 Optimal conditions for *E. coli* B121(DE3) and modified B121(DE3) were determined for
244 PAP-S1R68G and RTA/PAP-S1R68G respectively in small volumes before being scaled up to 1L
245 production culture (please contact the authors for more details). In short, bacteria starter were
246 obtained by incubation at 37°C and then followed by IPTG induction at specific temperatures and
247 incubation times. The bacteria were then harvested by centrifugation, followed by lysis. The
248 supernatant was collected after centrifugation for both proteins, the native proteins extracts.

249 4.2.4. *E. coli* protein purification

250 The purification of the native proteins extracts was achieved by affinity versus His-tag on
251 Ni-resin. The equilibration was done with a standard binding buffer. Wash and elution was
252 performed by imidazole shift. After purification, fractions of interest were pooled, concentrated,
253 and analyzed by SDS-PAGE. The final concentration was determined by Bradford protein assay.

254 4.2.5. Rabbit Reticulate Lysate protein synthesis inhibition

255 The inhibitory activity of PAP-S1[R68G] and RTA-PAP-S1[R68G] were tested by using the
256 Rabbit Reticulate Lysate TnT® Quick Coupled Transcription/Translation System and the Luciferase
257 Assay System. Briefly, each transcription/translation reaction run was performed according to the
258 instructions for use (IFU) in the presence of a T7 Luciferase reporter DNA, and the Luciferase
259 expression level was determined with a Perkin Elmer EnVision Microplate Reader.
260 Transcription/translation runs were done twice with and without addition of five different
261 concentrations of PAP-S1R68G and RTA-PAP-S1R68G in order to determine the inhibitory effect of
262 the proteins. PAP-S1R68G and RTA-PAP-S1R68G concentrations were adjusted by taking sample
263 purity into consideration.

264

265 **Conflicts of Interest:** "The authors declare no conflict of interest."

266

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