**Title of Project:** INHIBITION *OF XYLELLA FASTIDIOSA* POLYGALACTURONASE TO PRODUCE PIERCE'S DISEASE RESISTANT GRAPEVINES

**Sub-Title of Report:** Final Report for CDFA Agreement Number 12-0129

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**ABSTRACT**
Polygalacturonases (PG) (EC 3.2.1.15), catalyze the random hydrolysis of 1, 4-alpha-D-galactosiduronic linkages in pectate and other galacturonans. *Xylella fastidiosa* (Xf) possesses a single PG gene, *pglA* (PD1485) and Xf mutants deficient in the production of PG result in lost pathogenicity and a compromised ability to systemically infect grapevines. We have cloned the *pglA* gene into a number of protein expression vectors and a small amount of active recombinant PG has been recovered, unfortunately most of the protein expressed is found in inclusion bodies in an inactive form. The goal of this project is to use phage panning to identify peptides or single chain fragment variable antibody (scFv) libraries that can bind to and inhibit Xf PG. Once peptides or scFvs are discovered that can inhibit PG activity in vitro these peptides will be expressed in grapevine root stock to determine if the peptides can provide protection to the plant from PD.


**LAYPERSON SUMMARY OF RESEARCH PROJECT**

We have completed panning experiments against Av PG, Xf PG and Aa PG using a number of different phage libraries. The results show that one of the peptides derived from these experiments is able to inhibit Av PG activity in *in vitro* inhibition assays. This provides a proof of concept that phage panning is a viable method to isolate inhibitors of PG activity. We also tested if this peptide had any inhibitory effect on other bacterial and fungal PGs and which amino acids in the peptide are necessary for the PG inhibitory effects.

**INTRODUCTION**

Polygalacturonases (PG) have been shown to be virulence factors of a number of plant pathogenic bacteria including *Ralstonia solanacearum*, *Xanthomonas campestris*, and *Erwinia carotova* (Huang and Allen 2000; Dow et al. 1989; Lei et al. 1985). *Xylella fastidiosa* (Xf) possesses a single PG gene *pglA* (PD1485), and mutation of this gene results in lost pathogenicity and reduced ability to systemically infect grapevines (Roper et al. 2007). In order for Xf to systemically infect a grapevine it must break down the pit membranes that separate individual xylem elements. Pectic polymers determine the porosity of the pit membrane (Baron-Epel, et al. 1988; Buchanan et al. 2000) and Xf PG allows the bacterium to breakdown the pectin in these membranes in *Vitis vinifera* grapevines. The premise of this research is to identify a peptide that can be expressed in the xylem of a grapevine that can suppress Xf PG activity thus limiting the ability of Xf to spread systemically through grapevines and cause PD.

To accomplish this we will used phage display of a random heptapeptide library and a scFv antibody library attached to the coat protein gp38 of M13 phage in phage panning experiments using enzymatically inactive recombinant Xf PG or enzymatically active *Agrobacterium vitis* PG as the target. After 3 rounds of panning, phage that showed a high binding affinity for PG will be screened for their ability to inactivate PG activity *in vitro* in reducing sugar assays.

If a suitable inhibitory peptide is discovered it will be cloned into an agrobacterium binary vector and used to transform tobacco and grapevines by the UCD Plant Transformation Facility. These transgenic plants will then be inoculated with Xf and compared to non-transgenic plants in PD symptom progression. If significant disease inhibition is shown we will use these transgenic grapevines as rootstock to see if they can also provide resistance to grafted scions.

**OBJECTIVES**

**PLEASE NOTE: as requested by CDFA, this FINAL REPORT contains results obtained for Objective 1, which was apparently the only "official" objective funded for this granting period. In addition I also report activities and results on several additional experiments that were undertaken that complement the original overall goal of this project which is the title of the project: "Inhibition of *Xylella fastidiosa* polygalacturonase to produce Pierce's Disease resistant grapevines". I include these experiments under "Addendum 1 and 2".**

**Objective 1:** Isolate sufficient amounts of biologically active *Xylella fastidiosa* (Xf) and/or *Agrobacterium vitis* (Av) polygalacturonase (PG) enzyme to conduct phage panning and PG-inhibition assays.

**Addendum 1**: Isolate M13 phages that possess high binding affinities to Xf PG, as well as *Agrobacterium vitis* PG and *Aspergilus aculeatus* PG from a M13 random peptide or scFv antibody libraries.

**Addendum 2:** Determine if selected M13 phage and the gp38 M13 protein that mediates phage binding to Xf PG and/or surrogate Av PGs can inactivate PG activity *in vitro*.

## Summary of Results and Discussion:

### Objective 1: Isolate a sufficient amount of biologically active *Xylella fastidiosa* (Xf) or *Agrobacterium vitis* (Av) polygalacturonase (PG) enzyme to conduct phage panning and PG-inhibition assays.

Xf does not produce a detectable amount of PG when grown in biological media. Furthermore, attempts at expressing Xf PG in *E. coli*, yeast, and plant based viral expression systems did not produce active Xf PG. Because of this, Xf strains were engineered that will constitutively express the PG gene. The pBBR1MCS and pPROBE broad host range cloning vectors provide the Xf expression plasmid backbone and the constitutive nptII promoter was utilized to drive protein expression (Miller et al. 2000, Kovach et al. 1995). GFP reporter constructs made using this plasmid system stably expressed GFP in Xf under antibiotic selection. Additionally, the amount of GFP produced using this system is readily detectable on a Coomassie stained polyacrylamide gel (Figure 1). Xf PG-expressing constructs have been tested for the production of Xf PG and western blot analysis using polyclonal anti-Xf PG antibodies which showed that the constructs are producing Xf PG, as indicated by the presence of a 55kd band in the Xf PG over-expression strains that is not present in the Xf strain over-expressing GFP (Figure 1). Tandem mass spec analysis of Xf produced PG indicates that Xf PG is being processed in Xf and this processing is likely a major factor in the apparent size difference between *E. coli* produced recombinant PG and Xf produced PG.. We tested these strains for PG activity and there was some very low activity associated with some of the Xf PG containing fractions. However, the activity of these fractions was not sufficient to conduct in vitro inactivity assays and the plasmids generated seem to be somewhat unstable in Xf as GFP and PG expression drops off after repeated transfers on selective media. This fact has prevented the production of the large amounts of active Xf PG we need for the subsequent objectives in this project.

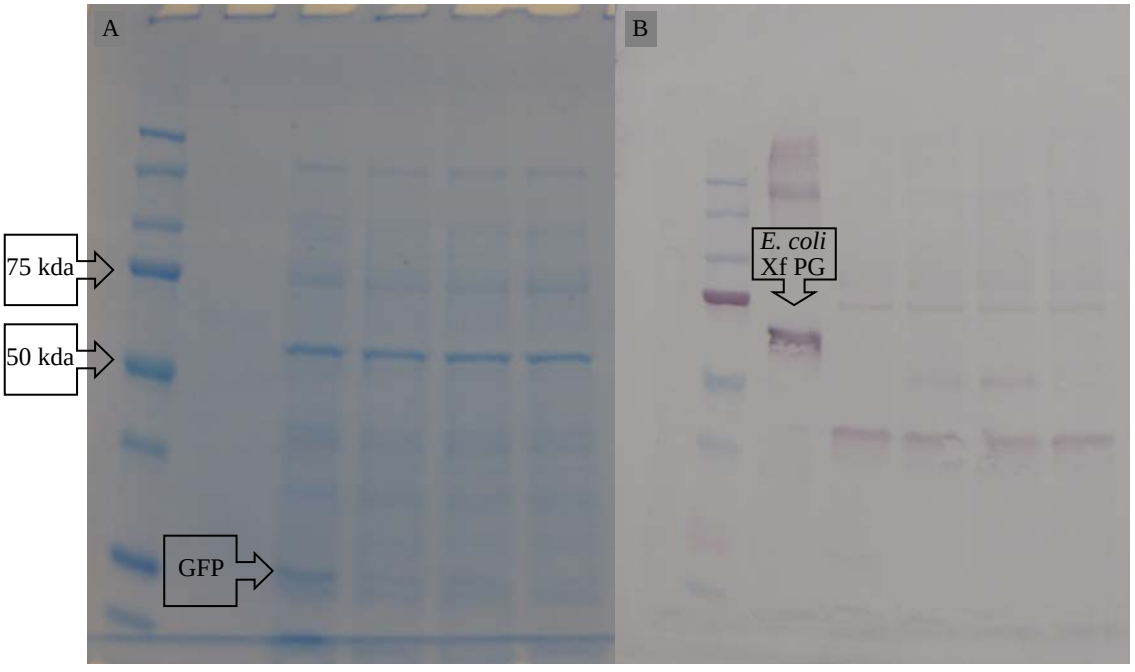


**Figure 1**. **A:** Coomassie stained polyacrylamide gel electrophoresis, **Lane 1**: Bio-rad dual color protein ladder, **lane 2**: Recombinant Xf PG produced in *E. coli*, **lane 3**: Xf over-expressing GFP, **lanes 4-6** Xf over-expressing XfPG **B:** Western blot analysis using polyclonal anti Xf PG antibodies, **Lane 1**: Bio-rad dual color protein ladder, **lane 2**: Recombinant Xf PG produced in *E. coli*, **lane 3**: Xf over expressing GFP, **lanes 4-6**: Xf over-expressing Xf PG. Right arrow denotes bands in Xf PG over-expressing Xf strains corresponding to predicted 55kda protein that are not present in GFP producing Xf strain.

*Agrobacterium vitis* (Av) is a plant pathogenic bacterium that causes crown gall disease in grapevines. Like Xf, Av also requires a PG in order to move from xylem element to xylem element. The Av PG gene has been previously cloned and shown to be active in *in vitro* activity assays (Herlache et al 1997). In addition, because the active sites of PGs are highly conserved and need to degrade the same substrates in the same host plant (*V. vinifera*); a peptide which inhibits Av PG may also inhibit Xf PG. Furthermore, an inhibitor of Av PG activity would also prove useful for California grape growers as a possible control method for crown gall disease of grapevines. For this reason we cloned the Av PG gene into an *E. coli* overexpression system to produce recombinant, enzymatically active Av PG for use in inhibition assays (**Figure 2**).

**Figure 2**

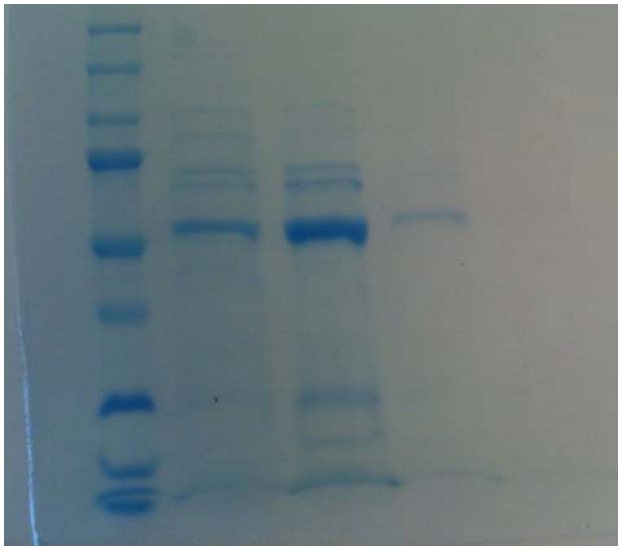

**Figure 2. Coomassie stained 10% SDS polyacrylamide gel containing His column purified recombinant Av PG elutions. Note distinct Av PG bands at the 55 kda predicted size. Lane 1: Protein Mass ladder, Lane 2: Elution 1, Lane3: Elution 2, Lane4: Elution 3, Lane 5: Elution 4.**

We assessed the activity of these protein fractions using two different assays. The first is the 2-cyanoacetamide reducing sugar assay, a spectrophotometric method which quantitatively measures the increase in reducing end accumulation due to PG enzymatic degradation of polygalacturonic acid (Gross 1982). The second is a cup plate diffusion assay (Figure 3) in which activity is represented by a colorimetric clearing around where the enzyme is introduced into a plate of agarose containing polygalacturonic acid (Taylor and Secor 1988).

Experiments showed recombinant Av PG is being produced in large amounts and is enzymatically active in cup plate assays (Figure 3).


**Figure 3. Ruthenium red stained cup plate assay showing Av PG activity. Note cleared region around Av PG well that is not present in negative control well indicating PG enzymatic activity.**

An additional issue further complicating this situation is the fact the Xf PG enzyme seems to be unique among all other described active polygalacturonase enzymes in that it has a different substrate binding amino acid motif. While Xf PG polygalacturonase contains all the catalytic amino acids for the hydrolysis of 1,4-alpha-D-galactosiduronic linkages it has very different substrate binding amino acids. It has been shown in previous research with *Aspergillus niger* polygalacturonase that mutation of this motif results in only 14% residual polygalacturonase activity. This information suggests that it could be likely that Xf PG will have a substantially lower activity than other polygalacturonases as well as a different manner of substrate binding, or perhaps preference for a different pectic substrate other than polygalacturonic acid.

Given the restraints described above, we conducted additional experiments that focused on two questions. First can we produce soluble Xf PG *in vitro* and second, do the altered amino acids in Xf PG result in a reduced enzyme activity, different degradation product sizes or different substrate specificity? We decided to address both of these questions through the creation of a protein chimera using the catalytic and substrate binding domains of Xf PG to replace the catalytic and substrate binding domains of an active polygalacturonase enzyme from a different prokaryotic plant pathogen. *Agrobacterium vitis* (Av). Av PG is the same size as Xf PG and likewise it is important in the virulence of Av to grapevines. Furthermore, a soluble, and more importantly an active form, of Av PG can be easily produced in sufficient quantities in recombinant *E. coli* expression systems.

The N terminal and C terminal regions of both *A. vitis* and Xf PG genes possess the most sequence variation. It follows that these highly variable regions are likely preventing recombinant Xf PG from being produced in a soluble form. If this is true it should follow that a chimera protein containing the variable N terminal and C terminal regions from the *A. vitis* polygalacturonase combined with the catalytic and substrate binding regions from the Xf PG could have an increased likelihood of being expressed in *E. coli* as a soluble protein. At the same time, such a chimera would allow us to assess the active site amino acids of Xf PG and determine if they can catalyze the degradation of polygalacturonase. Furthermore, we hypothesized that using two chimeras, one constituting the major amino acids involved in catalysis (AX1APG) and the other containing the catalytic amino acids as well as the substrate binding amino acids

(AX2APG) would allow us to determine if indeed these specific amino acids are biologically relevant for substrate binding (Figure 4). Moreover, if the Xf PG motif was mutated to the standard polygalacturonase motif used by all other known active polygalacturonases we would expect to see a more biologically active enzyme. If the chimeric Xf/Av PGs were active, these could function as surrogates for Xf PG in inhibition assays. Results showed that both chimeras are showing activity however the second chimera which contains more Xf PG sequence is less active (Figure 5).

**AX1APG:**


**AX2APG:**


**Figure 4.** Gene diagrams for each of the chimera constructs.

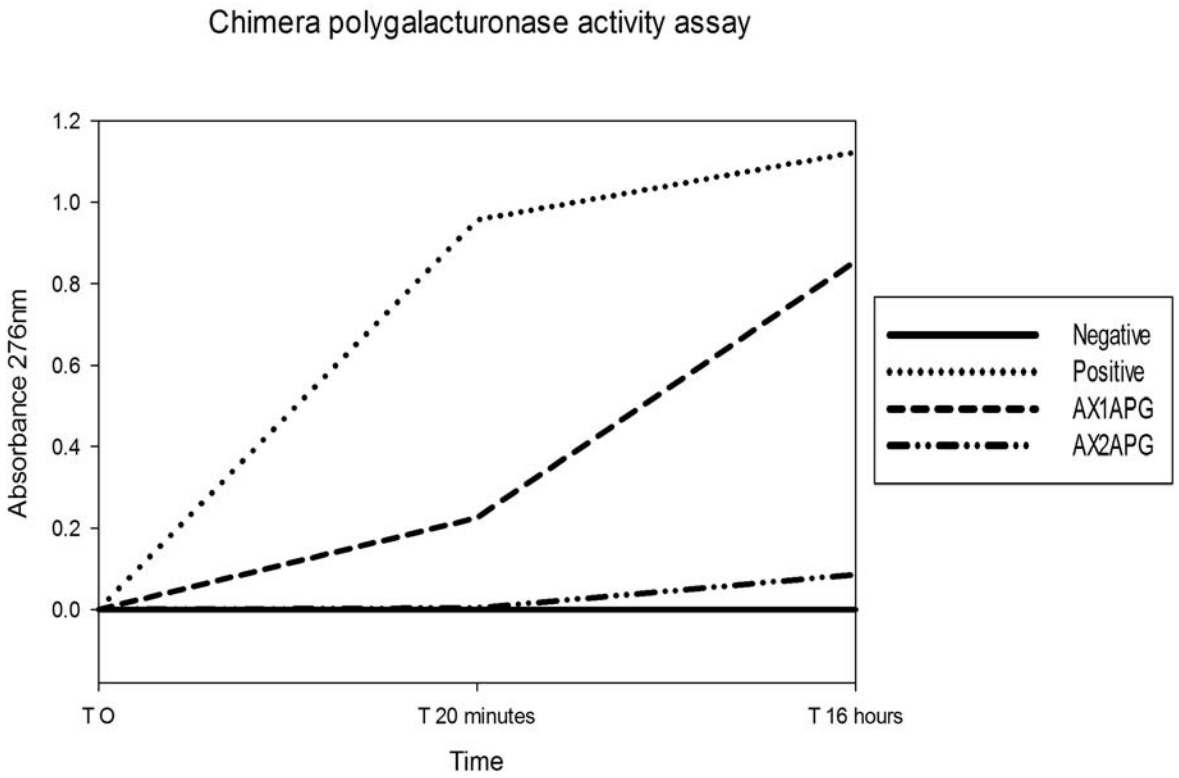


**Figure 5** Polygalacturonase activity assay showing that both polygalacturonase chimeras, AX1APG and AX2APG, are enzymatically active.

**Addendum 1: Isolate M13 phages that possess high binding affinities to Xf PG, as well as *Agrobacterium vitis* and *Aspergilus aculeatus*, from a M13 random peptide or scFv antibody libraries**

Previous phage panning experiments were conducted using smaller peptides constituting sections of the active site of Xf PG, FPLC purified recombinant Xf PG and *Aspergillus aculeatus* PG as surrogates did not provide us with PG inhibitory peptides. For this reason we cloned the Av PG gene into an *E. coli* overexpression system to produce recombinant Av PG to use in inhibition assays. Experiments showed recombinant Av PG is produced in large amounts and is enzymatically active in cup plate assays. Phage panning was carried out according to a standard protocol using Av PG as the target but instead of eluting with pH or trypsin, the phage were eluted with the PG substrate, polygalacturonic acid. This should provide us with phage that are interacting with the substrate binding cleft.
Twenty individual phages from each library (PhD 7 linear and PhD 7 circular (NEB)) were isolated from blue plaques after the final round of panning and single stranded phage DNA was extracted and sequenced to identify the peptide sequences. No clear consensus sequence was determined for all of the phages in either the linear or circular libraries, however 10 of the phages were found to contain portions of a common motif, and one of the phage sequences represented 25% of the linear peptide phage pool. Six peptides were chosen as candidates for use in the inhibition assays, peptides A-F and a phage ELISA was performed which confirmed specific binding to *A. vitis* polygalacturonase (Figure 6).

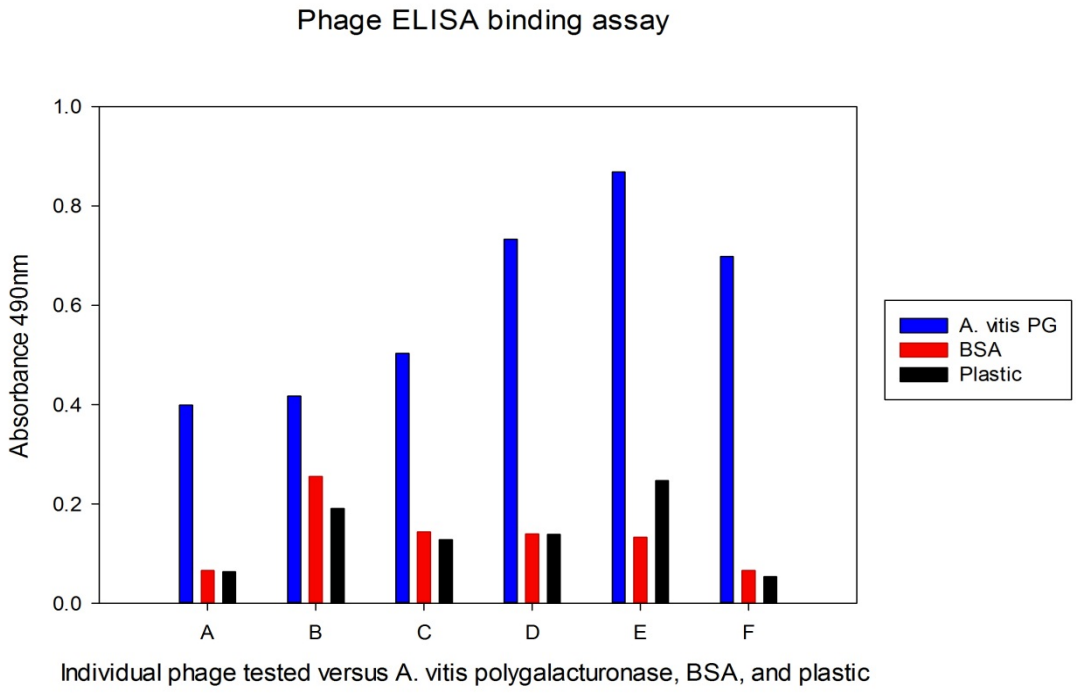


**Figure 6.** Monoclonal phage ELISA with *A. vitis* polygalacturonase, BSA, and plastic as the targets. Each of the phage screened has a higher binding affinity for *A. vitis* polygalacturonase than BSA or plastic.

**Addendum 2: Determine if selected M13 phage and the gp38 M13 protein that mediates phage binding to Xf PG and surrogate PGs can inactivate PG activity *in vitro*.**

All peptides (A-F) were synthesized with the C-terminal GGGS linker sequence included, as well as amidation of the C-terminus to negate the negative charge that a free C-terminus would generate. This negative charge would not have been present when the peptide linker was fused to the PIII phage protein. *A. vitis* polygalacturonase activity in the presence of each peptide was monitored and peptide A was the only peptide that showed an inhibitory effect on *A. vitis* polygalacturonase activity (Figure 7); peptide A was able to reduce enzyme activity approximately 20% compared to the positive control. This result does provide a proof of concept that phage panning can identify polygalacturonase inhibitory peptides.

**Figure 7**

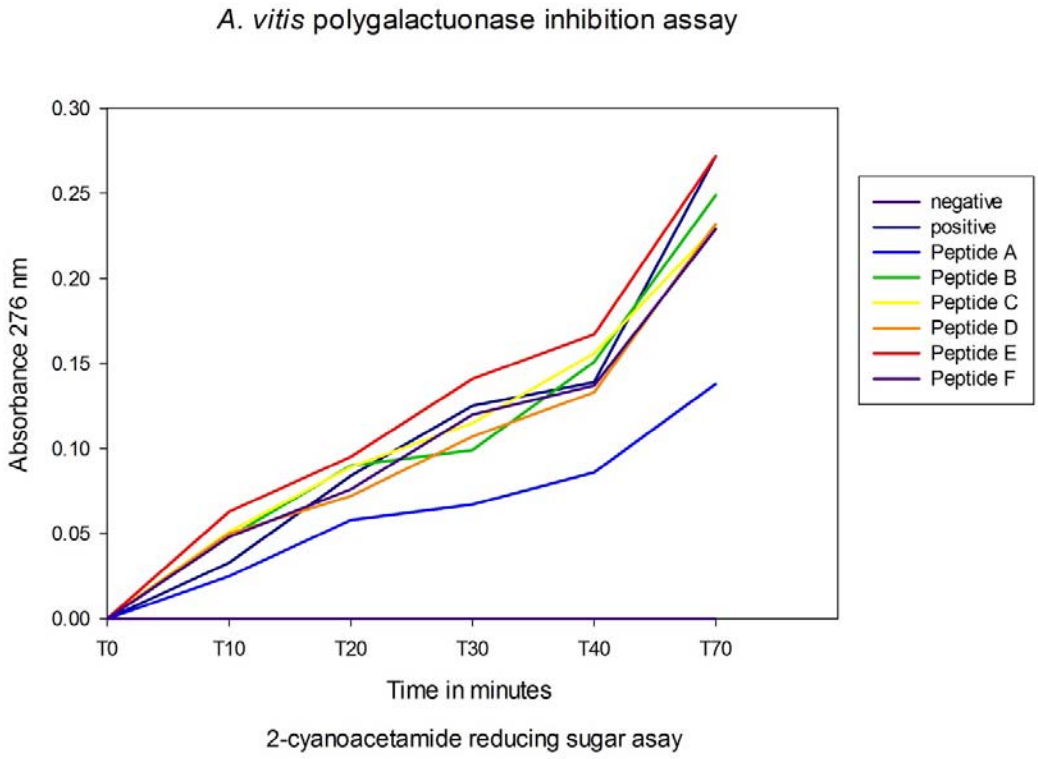


**Figure 3**. Av PG was incubated with each synthesized peptide for 30 minutes, this solution was then added to a solution of 0.05% polygalacturonic acid and the reaction progress was followed using the 2-cyanoacetamide reducing sugar assay. Peptide A shows the ability to inhibit the activity of AvPG when compared to the positive control and other peptides tested.

While not completely inhibitory under the conditions tested this does provide a proof of concept that phage panning can be used to select peptides that are inhibitory toward polygalacturonase enzymes. We are currently in the process of determining which amino acids are essential for this inhibitory effect. Understanding which amino acids are playing a role in the inhibition should

allow us to increase the inhibitory effect of this peptide or peptide derivatives. We are also testing peptide A against other PGs to determine if this inhibitory action is specific to Av PG or if it is able to inhibit other bacterial or fungal PGs.

## CONCLUSIONS

Despite a concerted effort over several years we were unable to express enzymatically active *X. fastidiosa* (Xf) polygalacturonase (PG) using a variety of *E. coli* expression vectors, a yeast protein expression system and a viral expression system that has successfully expressed many types of proteins in plants. All of the *E. coli* and yeast expression systems produced a lot of Xf PG but it was present in insoluble, enzymatically inactive inclusion bodies; enzymatically active Xf PG was to be used as a phage panning target to identify peptides or recombinant antibodies that could inactivate Xf PG. For this reason we evaluated, as a proof of concept, the use of peptides to inactivate a closely related PG from another grapevine bacterial pathogen, *Agrobacterium vitis (Av* PG).

Av PG was PCR amplified and cloned in an *E. coli* expression system and the product made was enzymatically active in both reducing sugar assays and a cup plate assay. Purified Av PG was used as a target in phage panning experiments and several peptides were identified that bound to Av PG protein. Six of the best candidate inhibition peptides were synthesized and tested in PG activity assays for their ability to affect the activity of Av PG. One of the 6 peptides that were tested lowered the activity of Av PG by approximately 20%, the other 5 peptides did not affect Av PG activity and these peptides most likely bound to regions of the Av PG protein that were not involved in the pectin hydrolysis or substrate binding. This result established proof of concept that phage panning can be used to identify peptides that have the ability to inhibit the activity Av PG. The utility of this peptide in blocking activity of Xf PG needs to be evaluated when a method is found to produce enzymatically active Xf PG.

Two chimeric proteins containing the hydrolysis or substrate binding site of Xf PG were produced using the soluble Av PG protein as a backbone with the Av PG active site replaced by the Xf PG active sites. One of the 2 chimeric proteins that were produced was capable of hydrolyzing galacturonase substrate showing that factor(s) involved with the secondary structure of *E. coli* cloned Xf PG was probably responsible for the inactivity of the numerous cloned Xf PG protein expressed in *E. coli* expression systems.

The inability to easily produce enzymatically active Xf PG greatly impacted the progress that was anticipated for the original project. Other scientists using a variety of other protein expression systems were likewise stymied in their ability to produce active Xf PG. This result is totally unexpected as enzymatically active PGs have been cloned and expressed from a number of plant pathogenic bacteria and fungi. We concur with the anaylsis of the National Academy evaluation of potential methods to develop resistance to PD would be to produce transgenic grapevines that blocked the active of Xf PG. Unfortunately were not able to conclusively identify a peptide that would inactivate Xf PG because we were not able to produce active Xf PG. Once active Xf PG is produced we believe it is quite feasible to identify peptide(s) that inhibit Xf PG based on our results using Av PG as a model. The delivery of inhibitory peptide(s) into the xylem of transgenic grapevine will be the second important goal of validating this potential cure for Pierce's disease.

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