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Maceration of plant tissue by fungi is inhibited by recombinant antipectinase antibodies

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Abstract

Polyclonal antiserum from mice immunized with extracellular proteins from *Rhizoctonia solani* inhibited pectinase and cellulase activities in cell free culture supernatants of *Rhizoctonia solani*. Spleen mRNA from these mice was used to construct a cDNA library from which antipectinase ScFv antibodies were isolated using phage display techniques. Soluble ScFv antibodies produced by individual clones in *Escherichia coli* inhibited polygalacturonase in the culture supernatants of a range of fungal pathogens, including ascomycetes, basidiomycetes, and oomycetes. The soluble antibodies also inhibited maceration of potato tissue by these pathogens.

Abbreviations: AG – Anastomosis group; PG – polygalacturonase; ScFv – Single chain antibody.

Introduction

Necrotrophic fungi infect plants and produce a repertoire of enzymes which degrade host cell walls. This leads to dissolution of the tissue and lysis of the cells. Chief among these enzymes are the cellulases and pectinases (Walton, 1994; Oliver and Osbourne, 1995; DeLorenzo et al., 1997). Pectin-degradation is carried out mainly by the polygalacturonases (PGs) which may have either an exo- or an endo-, mode of action (Cook et al., 1999). The endo-acting enzymes degrade pectin-releasing oligogalacturonides with 5–15 residues, whilst the exo-acting enzymes release single galacturonide residues from the ends of the pectin chains. Some PGs act in both an exo- and endo-modes (DeLorenzo et al., 1997). Another pectin-degrading enzyme produced by fungi is pectin lyase which can also act in either an endo- or an exo-mode of activity.

There is considerable evidence that pectinases and PG contribute to virulence in bacterial soft rot pathogens and necrotrophic plant pathogenic fungi. Pectic enzymes of the fungus *Colletotrichum lindemuthianum* interact differentially with the walls of resistant and susceptible host cultivars (Lafitte et al., 1993). The synthesis of PG-inhibiting proteins (PGIP) as a defence mechanism occurs more rapidly and to a greater degree in resistant than in sensitive lines (DeLorenzo et al., 1997). Purified pectic enzymes mimic the tissue-degradation pattern observed as a result of fungal infection (Bateman and Basham, 1976). The most compelling evidence for the involvement of pectic enzymes as virulence factors derives from studies with defined mutants of the bacterial wilt pathogen *Erwinia*, and disruption of pectic-enzyme genes in the bacteria *Agrobacterium tumefaciens* (Rodriguez-Valenzuela et al., 1991) and *Pseudomonas viridflavia* (Liao et al., 1988). These studies have shown that

mutating or disrupting one or more pectic enzyme genes leads to a dramatic reduction in virulence. This approach has proved successful with only one fungal species, *Botrytis cinerea* (tenHave et al., 1998). Attempts to reduce virulence/pathogenicity by disruption of pectic-enzyme genes in the fungal species *Fusarium* (Garcia-Maceira et al., 2000) *Penicillium* (Wagner et al., 2000), and *Cochliobolus* (Scott-Craig et al., 1998), proved unsuccessful. This is considered to be due to the production of multiple forms of pectic enzymes, some of which may only be produced *in planta* (Brookhauser et al., 1980; Kelemu and Collmer, 1993; Garcia-Maceira et al., 2000; Wagner et al., 2000).

An alternative approach would be to inhibit the activity of fungal pectinases in a plant by expressing genes for antipectinase antibodies in the plant. It is our hypothesis that antipolygalacturonase antibodies would inhibit all of the PG produced by the pathogen against which the antibodies are raised. This would overcome the limitation of targeting one gene at a time. Here we report the cloning of recombinant genes for two antipectinase ScFv antibodies from a phage display library, the production of the antibodies in bacterial culture, and the effect of the antibodies on PG and macerating activities of extracellular proteins from different fungal species.

Materials and methods

Fungal isolates

Rhizoctonia solani 11034 (AG 8; ZG1-1), a highly virulent isolate, was recovered from diseased wheat plants in a paddock at Newdegate, Western Australia (Yang, 1993). Isolates of *Ascochyta fabei*, *Fusarium solani*, *Botrytis cinerea*, *Colletotrichum gleosperoides* and *Sclerotinia sclerotiorum*, were obtained from Geoff Dwyer, Murdoch University, WA. *Phytophthora cinnamomi* was obtained from Mark Dobrowolski, Murdoch University, WA, and *Leptospaeria maculans* was obtained from Martin Barbetti, Agriculture WA. All isolates were maintained on potato dextrose agar (PDA) at 4 °C.

Production of fungal extracellular enzymes

Fungal isolates were inoculated into Petri dishes containing 20 ml liquid minimal medium with 1% citrus pectin (Sigma) added as a carbon source (Sweetingham et al., 1986). The dishes were incubated in the dark at 26 °C for 10 days without agitation. The culture fluid was collected by filtering through three layers of Whatman No 1 paper. The filtrate was further clarified by filtration through a 0.45-μm Millipore membrane filter size.

Inoculation of mice for production of antibodies

Extracellular enzymes from a culture of *R. solani* 11034 were concentrated as described by Matthew and Brooker, 1991, and the concentrate used for inoculation of mice. Six female BALB/c mice were immunized with 100 μg protein as measured by the Bradford Reagent (Biorad). Each mouse received five intraperitoneal injections over a 3-month period. For the first immunization, Freund's complete adjuvant (FCA, Sigma) was used, while incomplete adjuvant (FIA, Sigma) was used for subsequent immunizations. Five immunizations were carried out at 21-day intervals. Four days after the fifth injection, the spleens were harvested for construction of the phage display library.

Cloning of antipectinase antibody genes

Recombinant ScFv genes were cloned from a cDNA library constructed in the M13 phage display vector pCANTAB5 using the Recombinant Phage Antibody System (RPAS, Amersham). Messenger RNA was harvested from the spleens of immunized mice and used for the construction of a recombinant ScFv library in the phagemid display vector pCANTAB5E. ScFv antibodies are smaller versions of antibodies and consist of the variable regions of the heavy and light chain immunoglobulin genes joined by a flexible linker peptide (Bird et al., 1988). The constant regions are not represented. This vector contains both a plasmid origin of replication and an M13 single strand origin of replication. Splicing of the antibody insert into this vector creates a fusion protein in which the insert protein is fused to the gIII tail protein from phage M13. All procedures were carried out as described by the supplier.

Pectinase binding clones were isolated from the library by a single round of biopanning against immobilised extracellular proteins from *R. solani* 11034 grown in citrus pectin medium. Immobilisation was achieved by adding 5 ml of a 5 μg ml^{-1}

solution of extracellular proteins in PBS to a 25 ml tissue culture flask and incubating overnight at 37 °C. Subsequent steps in the biopanning procedure were carried out as recommended by the supplier of the RPAS kit (Amersham). Individual clones eluted from the immobilized enzymes were screened by ELISA test for binding to PG from *Aspergillus niger* (Sigma). Soluble antibodies were produced by infecting clones into the non-amber suppressing *E. coli* HB2151 (Amersham). Antibodies were either recovered from the culture supernatant, or from the periplasmic space (Harrison et al., 1996). The concentration of ScFv was determined by binding the soluble ScFv to a microtitre plate coated with extracellular fungal protein, and using the HRP conjugated anti-E-tag antibody to detect bound soluble ScFv. The amount of bound ScFv is proportional to the amount of HRP substrate cleaved.

DNA sequencing

DNA sequencing was carried out with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit on an ABI 373 XL sequencer with stretch upgrade. The sequences were aligned using the Global Pair Alignment programme (Myers and Miller, 1988). The primers used for sequencing were supplied by the manufacturer as part of the RPAS kit.

Inhibition of enzyme activity

Extracellular enzymes were dialyzed against 50 mM acetate buffer pH 5.5. The effect of recombinant antibodies on the activity of enzymes in the supernatant was determined by mixing the antibodies with the enzyme solution and incubating at room temperature for 10 min. The mixtures were centrifuged at 14,000 rpm for 5 min, and the supernatant used for determination of enzyme activity. The antibodies were either recombinant ScFvs produced in bacterial cultures, or the serum IgG fraction purified by affinity chromatography from immunized or pre-immunized mice (Richman et al., 1982). In the case of the bacterial ScFvs, the centrifugation step does not remove the enzyme–antibody complexes from solution (data not shown).

Polygalacturonase and cellulase activity were determined by measuring the amount of reducing sugar released from polygalacturonic acid or carboxymethylcellulose, respectively in a 30 min incubation at 25 °C (Wood and Bhat, 1988). The reaction in 50 mM acetate buffer pH 5.5 contained a final concentration of 1% polygalacturonic acid (Sigma). The results are the average of three replicated experiments.

Maceration activity was assayed by incubating five slices (8 × 1 mm) of potato tuber in 2 ml of a solution of enzyme at 25 °C for 15 h. The wet weight of the slices was measured at the start of the incubation. After the incubation, the macerated tissue was removed, the remaining tissue was blotted dry and the wet weight measured. Antibodies were added to the reaction mixture at a concentration of 1.3 mg ml^{-1}. The results are the average of three replicated experiments

Western blotting

Proteins (100 μg) from culture supernatant, or from periplasmic extract (prepared as described in the manual for the Recombinant Phage Antibody System, Amersham-Pharmacia) were separated by electrophoresis on SDS-polyacrylamide with a 5% stacking gel and a 12.5% separating gel. Electrophoresis was at 40 mA for 2.5 h. The protein sample was mixed with an equal volume of SDS gel-loading buffer (50 mM Tris–HCl pH 7.0, 2% SDS, 50% glycerol, 10% 2-mercaptoethanol, 0.1% bromophenol blue), incubated at 100 °C for 2 min and centrifuged 14,000 rpm for 1 min before electrophoresis.

Proteins in the gel were transferred to nitrocellulose using a BioRad Trans-Blot apparatus. Transfer was carried out overnight in 0.025 M phosphate buffer (pH 6.5) at 23 V. Probing and detection were carried out using the ECL Western Blotting Kit (Amersham) as described by the manufacturer. The secondary antiserum was HRP conjugated anti-E-tag antibody (Amersham-Pharmacia). This secondary antibody binds to a specific site, the E-tag that is part of the ScFv sequence.

Results

Sera from mice immunized with *R. solani* fungal extracellular enzymes showed a positive response to the fungal antigen in ELISA tests at 1:5000 dilution (data not shown). The antibody titre in-

creased significantly between the 1st and 4th inoculation. The response with preimmune serum, or serum from animals inoculated with media protein was much weaker indicating that there was significant production of antibodies directed against the fungal extracellular proteins. Further evidence for this came from the results of enzyme inhibition studies using the purified IgG fraction from the serum.

Cellulase, and a PG activities were completely inhibited by addition of the IgG fraction to the extracellular protein preparation, while preimmune serum had no effect (Figure 1).

Cloning of antipectinase antibody genes

Biopanning the library against an immobilised extracellular protein preparation from *R. solani* resulted in the isolation of 192 clones. Pectinase binding clones were identified by their ability to bind to a pectinase from *Aspergillus niger*. ELISA tests showed that all clones isolated from the biopanning showed strong binding to an extracellular protein preparation from *R. solani*, but that binding to the *A. niger* pectinase was highly variable (data not shown). We encountered a problem with stability of the antipectinase antibody clones. In many cases, progeny derived from clones would lose their insert. Two clones (B1 and B4) which appeared to be stable, and which showed strong binding to pectinase from *A. niger* were selected for further studies. The plasmids containing these cloned inserts are designated pVMB1 and pVMB4.

DNA sequencing showed that the clones were 83% similar in the heavy chain region, and 90% similar in the light chain region (data not shown). The GenBank accession numbers for these DNA sequences are ScFvB1, AY741402, and ScFvB4, AY741403. The deduced amino acid sequence confirmed the expected protein structure in which the heavy and light chain variable regions are connected by three consecutive $[(Gly)_4(Ser)_3]$. A comparison of the deduced amino acid sequence with those in the SwissPro protein database (BLAST search) confirmed that the sequences are antibodies.

Production of soluble antibodies in E. coli

To produce the soluble form of the antibodies for enzyme binding studies, the plasmids were transferred to the non amber suppressing strain, *E. coli* HB2151. In this strain, translation terminates at the amber codon between the ScFv sequence and the M13 gIII protein producing a soluble form of the antibody that is secreted into the medium. ELISA tests of the periplasmic extract, and the

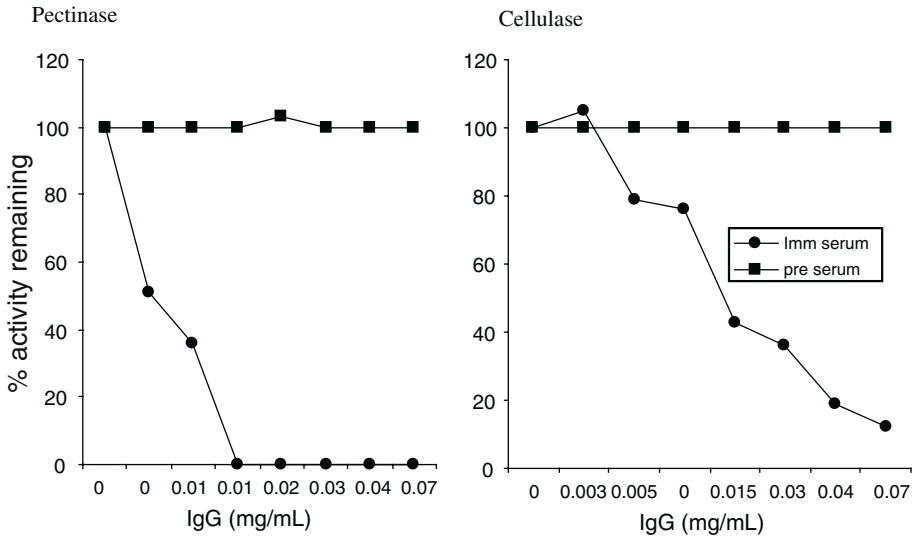

Figure 1. Inhibition of pectinase and cellulase of *R. solani* by mouse-IgG serum fraction. The IgG fraction was purified from mouse serum both before (pre-serum) and after (immune serum) inoculation with fungal extracellular proteins.

Table 1. Production of soluble ScFv antibodies in *E. coli*

Culture conditions	Culture volume (ml)	Incubation time (h)	Temp (°C)	Yield of soluble ScFv (mg 100 ml^{-1} culture)	
				Periplasm	Medium
250-ml flask	50	16	37	0	2
250-ml flask	50	16	30	1	9.5
Tube	10	16	30	0	1
250-ml flask	50	20	30	NT	22
250-ml flask	50	20	16	NT	15
250-ml flask	50	48	16	NT	34

The concentration of soluble antibodies was determined by binding of the soluble antibody to *R. solani* extracellular enzyme preparation in an ELISA test.
NT, not tested.

culture supernatant confirmed that the antibody produced by this strain did not accumulate in the periplasm but was secreted into the medium (Table 1). Western blotting also showed the presence of the ScFv in the culture supernatant and periplasmic extract (Figure 2). There were no cross reactive bands from the plasmid free host strain (Figure 2, lanes 1 and 2). Aeration was an important influence on the yield, as growing in a shake flask resulted in a higher yield compared to growth in a tube. Decreasing the temperature to 16 °C significantly increased the yield, although a longer incubation time of 48 h was required to achieve this.

ScFv B1 and B4 bound to extracellular proteins from a number of fungal pathogens (Figure 3). The number and size of these reactive proteins varied with the species. The banding pattern for *R. solani* consisted of a greater number of bands compared to the other species. Both *B. cinerea* and *S. sclerotiorum* had identical patterns consisting of two bands. One of these was common to *R. solani* and *F. solani*. The *A. niger* PG used for identification

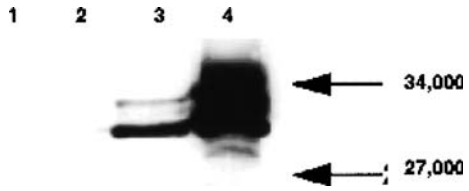

Figure 2. Production of soluble antibodies by bacterial cultures. ScFv's in bacterial culture supernatants or periplasmic extracts were detected by western blotting with HRP-conjugated, anti-E-tag antibody. Lane 1, *E. coli* HB2151 supernatant; Lane 2, *E. coli* HB2151 periplasmic extract; Lane 3, *E. coli* HB2151 pVMB1 culture supernatant; Lane 4, *E. coli* HB2151pVMB1 perplasmic extract.

of pectinase binding clones from the library consisted of a major band in the 20–27 KDa region, and a minor band in the high molecular weight range. A number of weak bands were observed in preparations from *A. fabei* and *C. gloeosporoides*. The ScFv's did not cross react with media proteins. The banding patterns observed for ScFvB4 were identical to those for ScFvB1 (Figure 3).

All of the bands observed with B4 corresponded to bands obtained with B1. Some of the fainter bands in the B1 patterns were not evident in the B4 patterns, e.g., the *C. gloeosporoides* and some of the *R. solani* smaller bands. There were no bands specific to B4.

Effect of soluble antibodies on fungal polygalacturonase activity

Both antibodies inhibited the activity of PG from a variety of fungi but to different extents (Figure 4). In general B1 was less inhibitory than B4, however this trend was reversed with the enzyme from *A. niger*. The PGs from *R. solani*, *A. niger*, and *S. sclerotiorum* were the most sensitive to B1 and showed significant inhibition (30–40%) at low concentrations (0.13 mg ml^{-1}). With the exception of the *A. niger* PG, further increases in the concentration of B1 did not result in further inhibition. Of the other PGs inhibited by B1, those from *F. solani* and *P. cinnamomi* required ten to twenty fold higher concentrations of antibody for significant inhibition. The PGs from *B. cinerea*, *A. fabei*, *L. maculans* and *C. gloeosporoides* were not inhibited by B1.

B4 was more inhibitory than B1 with the exception of the *A. niger* PG (Figure 4). The *R. solani*

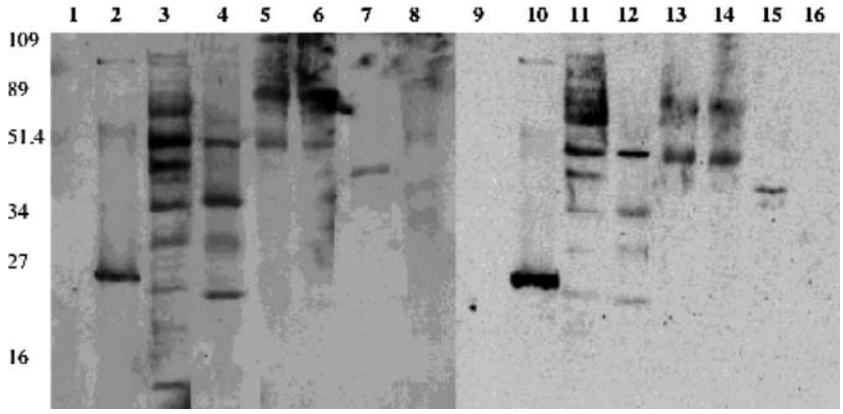

Figure 3. Binding of recombinant ScFvs to extracellular proteins from different fungal species. Western blots of proteins from culture supernatants were probed with the bacterial ScFvs B1 (lanes 1–8) and B4 (Lanes 9–16). The secondary antibody was HRP-conjugated, anti-E-tag antibody. Lanes 1 and 9, medium protein; Lanes 2 and 10, *A. niger* pectinase (Sigma); Lanes 3 and 11, *R. solani*; Lanes 4 and 12 *F. solani*; Lanes 5 and 13, *B. cinerea*; Lanes 6 and 14, *S. scleortiorum*; Lanes 7 and 15, *A. fabei*; Lanes 8 and 16, *C. gloeosporoides*.

enzyme was very sensitive to the addition of B4, being completely inhibited at low concentrations. At higher concentrations, B4 also completely inhibited the PGs from *F. solani*, *B. cinerea, S. sclerotiorum* and *P. cinnamomi*. PG from *A. fabei*, and *C. gloeosporoides*, were insensitive the B4, whilst the *L. maculans* enzyme was only slightly inhibited.

With the exception of *F. solani*, addition of a mixture of both antibodies did not give greater inhibition than observed with B4 alone. With *F. solani,* complete inhibition was obtained at a ten fold lower concentration of antibody compared to the amount of single antibody required. In two cases (*B. cinerea* and *S. sclerotiorum*) the inhibition curve obtained with the mixture was intermediate between that observed with the B1 and B4 single antibodies.

Inhibition of macerase activity

Infection of plant tissue by necrotrophic fungi leads to maceration of the tissue. This is brought about by the action of the extracellular enzymes, mainly the PG secreted by the fungus (Caprari et al., 1996). We therefore tested the effect of the recombinant antibodies on maceration of plant tissue by fungal enzymes.

With the exception of *A. fabei,* enzymes from five fungal species caused significant maceration of the tissue (Figure 5). *Colletotrichum gloeosporoides* was the most efficient resulting in 100% maceration, while the others varied between 20% (*B. cinerea*) and 50% (*S. sclerotiorum*). The maceration activity of *R. solani*, *F. solani, B. cinerea* and *S. sclerotiorum* was inhibited by either antibody alone, as well as the mixture of antibodies. Maceration activity of the *R. solani* enzyme was most sensitive to the presence of the antibody. The maceration activity of *C. gloeosporoides* was not affected by the antibodies.

Discussion

The aim of this research was to clone genes for recombinant antibodies which bind to and inhibit the activity of fungal pectinases. Two clones were isolated from a mouse cDNA library constructed in a phage display vector on the basis of their affinity for *A. niger* PG. Soluble ScFv antibodies produced by expression of these genes in *E. coli* inhibited pectinase and maceration activity from a variety of fungal pathogens including ascomycetes, basidiomycetes and oomycetes. The ability to bind to pectinases from such a diverse range of fungal species suggests conservation of structural features in pectinases. This is supported by comparative analysis of sequences and crystal structures of PGs from a range of bacterial and fungal pathogens (Pickersgill et al., 1998; Cho et al., 2001). Four highly conserved sequences on the surface of the molecule have been identified.

R. solani synthesized a more complex mixture of pectinases compared to the other species tested.

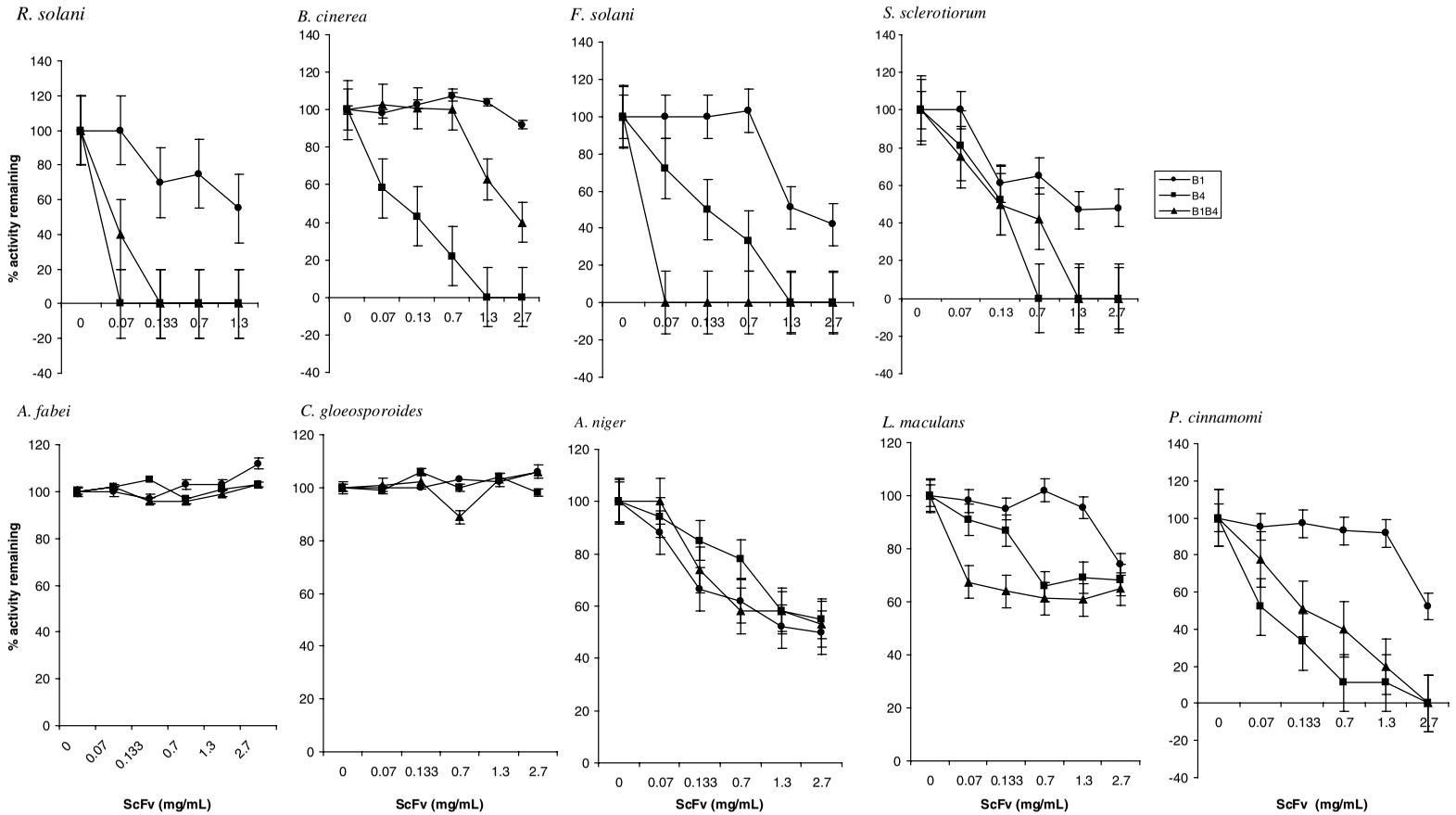

Figure 4. Inhibition of fungal polygalacturonase activity by ScFv B1 and B4. Increasing amounts of bacterial ScFv were added to a constant amount of enzyme. Activity was measured by the release of reducing sugars from polygalacturonic acid.

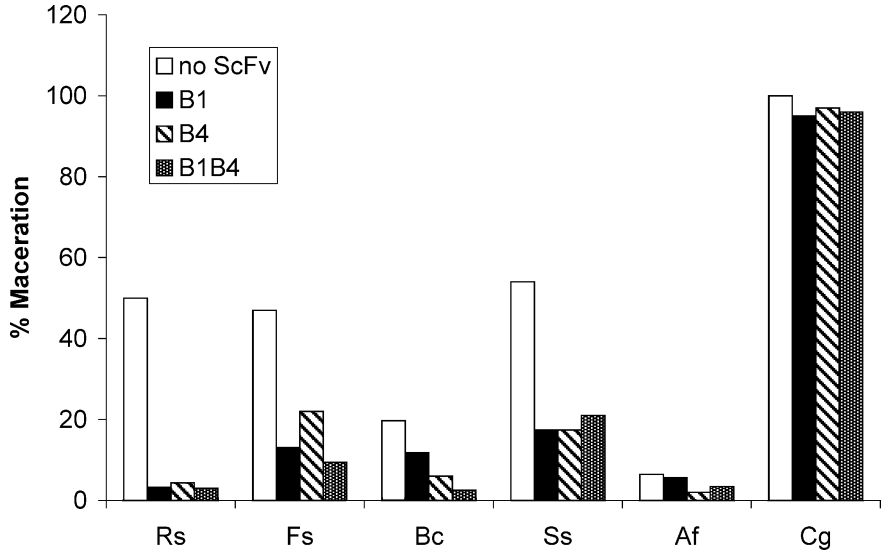

Figure 5. Inhibition of fungal maceration activity by ScFv B1 and B4. Potato slices were incubated for 15 h in 2 ml of enzyme solution containing no antibodies (no ScFv), or 1.3 mg ml^{-1} antibodies. Rs, *R. solani*; FS, *F. solani*; Bc, *B. cinerea*; Ss, *S. sclerotiorum*; Af, *A. faba*; Cg, *C. gloeosporoides*.

The production of multiple forms of pectinases by *R. solani* has been described previously (Scala et al., 1980; Marcus et al., 1986; O'Brien and Zamani, 2003). Factors which contribute to the complexity of fungal pectinases are glycosylation (Prusky et al., 1989; Caprari et al., 1996; Garcia-Maceira et al., 2000), and a multiplicity of genes (Kelemu and Collmer, 1993; Fraissinet-Tachet et al., 1996; DiPietro and Roncero, 1998).

Expression of the cloned ScFv gene in amber suppressor strains of *E. coli* results in the production of a fusion protein between ScFv and the gIII protein of phage M13. However, the fusion protein is only produced in amber suppressor strains of *E. coli* which contains an amber codon just 3′ to the ScFv coding region. In non-suppressor strains, translation terminates at the amber codon releasing the soluble antibody which is secreted. We have noticed that the B1 and the B4 plasmids were unstable in the non-suppressor *E. coli* strain supplied with the kit (data not shown). When a number of parallel cultures were set up from colonies on the same plate, only a small number of these were capable of synthesizing soluble antibodies after overnight growth. This phenomenon has been described by other researchers and is ascribed to blocking of the secretory system by 'misfolded' antibody molecules (Malloy et al., 1995; Rippmann et al., 1998; Susi et al., 1998; Fernandez et al., 2000). The misfolded molecules are thought to aggregate in the form of inclusion bodies which cause jamming of the *sec* mechanism, induction of periplasmic proteases, and enhanced outer membrane permeability all of which contribute to the formation of aggregates of the ScFv. Rippmann et al. (1998) studied the expression of a variety of ScFvs in *E. coli* and observed that induction of ScFv expression was inhibitory to growth, and in some cases continued induction led to cell lysis. The effects of ScFv expression depended on the amino acid sequence of the protein.

We found that lowering the temperature of incubation reduced the toxic effects of ScFv expression and increased the levels secreted into the culture medium. This effect of lower incubation temperature has been reported previously (Susi et al., 1998). The interpretation is that misfolding is reduced at 16 °C and thus, more of the correctly folded protein is released into the medium without blocking of the *sec* mechanism. Similar results were reported by Malloy et al. (1995) who found significantly greater affinity of the bacterial antibody for the antigen when the incubation temperature was reduced from 37 to 25 °C.

Binding of the antibodies inhibited the activity of PG, a principal component of the pectic enzyme

repertoire. With the exception of the *A. niger* PG, B4 tended to be more inhibitory than B1. The effects of treating the enzyme with the mixture of antibodies varied. In some cases (*R. solani*, *B. cinerea*, *S. sclerotiorum* and *P. cinnamomi*) the level of inhibition was intermediate between the levels obtained with the single antibodies. This suggests that in these enzymes the antibodies bind to a single site, or to two adjacent sites and that the binding of one prevents binding of the other. In reactions containing the mixed antibodies some molecules will be bound by B1 and others by B4. Exceptions to this were the *F. solani* and *L. maculans* enzymes where greater inhibition was observed with the mixture than with either of the single antibodies. In the case of these enzymes it is possible that both antibodies can bind at the same time.

Degradation of the pectin layer of the cell wall leads to tissue maceration. There is evidence pointing to the involvement of PG as the macerating component. Caprari et al. (1996) carried out site-specific mutagenesis of a *F. moniliforme* PG gene and found that mutations that abolished PG activity also abolished maceration activity. Both of the antibodies inhibited the maceration activity of the fungi tested with the exceptions of *C. gloeosporoides* and *A. fabei*, a pattern which mirrors the effects on PG activity.

In conclusion, this study describes the isolation of recombinant genes for antipectinase ScFv antibodies from a cDNA library. The ScFv antibodies should be very useful in investigating the role of pectinases in the virulence of phytopathogenic fungi. The genes for these ScFv antibodies should be very useful for engineering plants for resistance to fungal pathogens.

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