DISCOVERY AND CHARACTERIZATION OF A CLASS OF FUNGAL ENDOGLUCANASE INHIBITOR PROTEINS FROM HIGHER PLANTS

by

QIANG QIN

(Under the direction of William S. York and Alan G. Darvill)

ABSTRACT

Microbial pathogens secrete an array of enzymes that cleave polysaccharides in the cell walls of their plant hosts. These enzymes include *endo*glucanases, polygalacturonase, pectin lyases, xylanases, and various glycosidases. Many of these cell-wall degrading enzymes are inhibited by previously identified and characterized plant proteins, such as polygalacturonase inhibiting protein (PGIP), xylanase inhibiting protein (XIP), and pectin lyase inhibiting protein (PNLIP).

This dissertation describes the identification and characterization of a plant-derived xyloglucan-specific *endo*glucanase inhibitor protein (XEGIP) from suspension-cultured tomato (*Lycopersicon esculentum*) cells. Previously, no *endo*glucanase inhibiting proteins have been identified from any plant source, although *endo*glucanase substrates (cellulose and hemicelluloses) are major components of the plant cell wall. XEGIP inhibits a xyloglucan-specific *endo*glucanase (XEG) from *Aspergillus aculeatu*s by forming a 1:1 protein:protein complex with a K_i of approximately 0.5 nM. XEGIP also inhibits a previously unknown xyloglucan-specific *endo*glucanase (CfXEG) secreted by a fungal pathogen of tomato (*Cladosporium fulvum*). CfXEG was purified from the culture medium of *C. fulvum* grown on xyloglucan-rich tamarind seed powder as the sole carbon source. The cDNA encoding XEGIP was cloned and sequenced. These results were used to perform a database analysis, which revealed that XEGIP-like proteins are widely distributed in the plant kingdom. XEGIP homologs include EDGP, a carrot protein that had previously been implicated in disease resistance, although its precise function was unknown. Based on its homology to XEGIP, EDGP was isolated and found to inhibit XEG.

Sequence alignment of XEGIP with TAXI (*Triticum aestivum* xylanase inhibitor), a recently characterized wheat protein, suggests that both proteins belong to a protein super family, hemicellulase inhibitor proteins (HIPs). Interestingly, Family 12 *endo*glucanases and Family 11 xylanases, which are the known HIP ligands, have very similar 3-D structures even though their sequence similarities are low. Based on their *in vitro* activity, *in vivo* localization, and enhanced expression in response to wounding and microbial infection, HIPs are proposed to function as disease resistance factors. In order to facilitate the evaluation of this hypothesis, an immunoaffinity chromatography based method was developed to screen for the binding of HIPs to their ligands. Further

examination of the roles of HIPs in *vivo* may shed light on the mechanisms by which plants limit colonization by pathogenic microbes.

INDEX WORDS: Cell wall, *Endo*glucanase, Inhibitor, Protein-protein interaction, Xyloglucan, Hydrolase

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DEDICATION

献给逝去的母亲

To the memory of my mother

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CHAPTER 1

INTRODUCTION AND LITEREATURE REVIEW

Introduction

This dissertation describes the discovery, characterization, and cloning of the first proteinacious β-1,4-*endo*glucanase inhibitor (XEGIP) from plants or any other source (Chapter 2). XEGIP is a basic, 51 kDa protein purified from suspension-cultured tomato and shown to inhibit the hydrolytic activity of a xyloglucan-specific *end*oglucanase (XEG) from the fungus *Aspergillus aculeatu*s. The cDNA encoding XEGIP was cloned and sequenced. The *XEGIP* sequence shares similarity to ESTs from a broad range of plant species, suggesting that *XEGIP*-like genes are widely distributed in the plant kingdom.

In addition to XEGIP-like proteins, other plant proteins that inhibit cell wall degrading enzymes (CWDEs) from microbes have been previously characterized. Interestingly, plants also secrete enzymes (e.g. β-1,3-*endo*glucanases) to degrade the fungal cell wall. The fungus counteracts these enzymes by producing glucanase inhibitor proteins (GIPs). The current knowledge of the interactions between CWDEs and their inhibitors are reviewed in this chapter. The evolution of cell-wall hydrolases and their inhibitors can be considered as an ongoing arms race between plants and their pathogens: cell wall hydrolyzing enzymes are acquired by the attacking species while the corresponding inhibitor proteins co-evolve in the species whose walls are being attacked. XEGIP and XEGIP-like proteins are the most recently discovered weapons of this type in the plant's arsenal to resist invasion by pathogenic microbes.

Literature review

In plants, the cuticle and cell wall constitute the first line of defense against microbial attack. Primary cell walls are composed of a complex network of carbohydrates, including cellulose, hemicellulose (e.g. xyloglucan in dicotyledons and arabinoxylan in graminaceous monocotyledons), and pectins (Carpita and Gibeaut, 1993). Pathogens produce an array of cell-wall degrading enzymes (CWDEs) (Walton, 1994; Esquerre-Tugaye et al., 2000) that facilitate penetration of the cell wall and its use as a carbon source. These enzymes include polygalacturonases (*endo* and *exo* mode), pectin lyases, pectate lyases, pectin esterases, cellulases, cellobiohydrolases, hemicellulases (e.g. xylanases that can digest xylans and *endo*-β-1,4-glucanases that can digest xyloglucans), and a variety of *exo*-glycosidases (e.g. glucosidase, xylosidase etc.). The roles of these CWDEs in pathogenesis have been extensively studied. Due to the functional redundancy of isoenzymes in microbes, disruption of one or more CWDE genes in a pathogen rarely leads to a loss of virulence. Nevertheless, gene disruption experiments have provided convincing evidence that CWDEs are involved in pathogenicity.

Plants counteract CWDEs by synthesizing extracellular proteins that inhibit or modify their enzyme activities. Prior to the results described in this dissertation, four classes of such proteinacious inhibitors have been reported: polygalacturonase inhibitor proteins (PGIP); pectate lyase inhibitor proteins (PNLIP); pectin methylesterase inhibitor proteins (PMEIP) and xylanase inhibitors (XIP). This section reviews the current

literature on these CDWEs, their roles in pathogenicity, and their inhibition by plant proteins.

Cellulase and related β-1,4-endoglucanases

Cellulases belong to a group of microbial enzymes that synergistically hydrolyze cellulose to glucose. These cellulolytic enzymes include: (1) *exo*-β-1,4-glucan cellobiohydrolases, which hydrolyze cellulose, releasing cellobiose from the nonreducing end; (2) *endo*-β-1,4-glucanases, which hydrolyze internal glycosidic bonds of cellulose and xyloglucan to form cello-oligosaccharides xyloglucan oligosaccharides respectively; (3) β-glucosidases, which cleave cellodextrins into glucose. Cellulose and xyloglucan, the two major *endo*glucanase substrates in dicotyledonous plants, form a major load bearing network in the cell well and therefore are presumably targets of microbial attack. To date, only one xyloglucan-specific *endo*glucanase has been previously identified from a microbial source and characterized (Pauly et al., 1999). This dissertation, describes another xyloglucan-specific *endo*glucanase from the pathogenic fungus, *Cladosporium fulvum* (Chapter 4).

Cellulases have been detected in several phytopathogenic microbes (references in (De Lorenzo et al., 1997). However, the roles of these enzymes in pathogenesis, especially by fungal pathogens, are not fully understood. For example, two cellulase genes have been characterized in the maize fungal pathogen *Cochliobolus carbonum*. The disruption of each of these genes individually has no apparent effect on the pathogenicity of the corresponding mutant (Sposato et al., 1995; Ahn et al., 2001). However, symbiotic colonization of lettuce roots by the mycorrhizal fungus *Glomus fasciculatum* is correlated

to the production of *endo*glucanase by the symbiont (Garcia-Garrido et al., 1992). Cellulases have been implicated in pathogenicity of several bacteria, including *Ralstonia solanacearum* (Roberts et al., 1988; Denny et al., 1990; Kang et al., 1994; Saile et al., 1997) and *Clavibacter michiganensis* (Jahr et al., 2000), which cause tomato wilting disease. That is, infection by an *endo*glucanase mutant of *R. solanacearum* led to less severe wilt symptoms and reduced stem colonization compared to wild-type strains (Saile et al., 1997). A plasmid containing an *endo*glucanase gene confers wilt induction capability upon nonvirulent strains of *C. michiganensis* (Jahr et al., 2000). A gene contributing to the major *endo*glucanase activity of culture medium of the pathogenic bacterium *Xanthomonas campestris* pv. c*ampestris* was found to have minor role in the early stages of its infection on turnip and radish, as progression of visible disease symptoms is slower in a strain in which this gene was mutated (Gough et al., 1988). In addition, cellulase CelV mutants of *Erwinia carotovora* exhibited delayed maceration of plant tissues, suggesting reduced virulence (Walker et al., 1994; Mae et al., 1995) and further supporting the involvement of *endo*glucanases in pathogenesis.

Xylanase

Endo-β-1,4-xylanases have been isolated and characterized from many pathogenic and non-pathogenic fungi. Most xylanases studied to date can be categorized into two glycosyl hydrolase families according to their sequence similarity (http://afmb.cnrs-mrs.fr/CAZY/). Family 10 xylanases have molecular weights greater than 30 kDa and a catalytic domain consisting of an eightfold β/α barrel (Harris et al., 1996; Natesh et al.,

2003). Family 11 xylanases have molecular weights that are less than 30 kDa and a catalytic domain with an all β-strand sandwich fold structure (Himmel et al., 1997).

In addition to their ability to digest xylan in plant cell wall, some xylanases, especially those of family 11, are elicitors of a variety of plant defense reactions. For example, when a fungal β-1,4-*endo*xylanase isolated from *Trichoderma viride* (Dean et al., 1989; Fuchs et al., 1989; Dean and Anderson, 1991) was applied to tobacco or tomato leaves, the plants responded by synthesizing ethylene, phytoalexins, and pathogenesis-related proteins, eventually leading to hypersensitive necrosis (Bailey et al., 1990; Lotan and Fluhr, 1990; Bailey et al., 1991; Bailey et al., 1992, 1993). Such elicitor activity is cultivar-specific: this particular xylanase can induce defense responses in *Nicotiana tabacum* cv Xanthi but not in *N. tabacum* cv Hicks (Sharon et al., 1993).

Induction of defense response by xylanases is unlikely to be mediated by xylan oligosaccharin elicitors released by the hydrolytic activity of these enzymes. Experimental results did not support the presence of any heat-stable elicitors (Fuchs et al., 1989) and no xylan oligosaccharides were detected (Dean et al., 1991) in infected plants. Sharon et al. (1993) performed experiments involving xylanase treatment of cell-wall free protoplasts and concluded that the response of *N.tacacum* cv Xanthi to xylanase is not promoted by the cell wall, which is not required for this response. These authors proposed that the ability of xylanase to elicit defense responses is independent of its enzymatic activity. This hypothesis has been confirmed by two independent groups

(Enkerli et al., 1999; Furman-Matarasso et al., 1999) who performed site-directed mutagenesis of xylanase. Mutations of amino acids at the catalytic center of xylanase either significantly decrease or completely abrogate the enzymatic activity, but do not decrease the protein's ability to induce ethylene biosynthesis in suspension-cultured tomato cells and hypersensitive necrosis in leaves of tomato and tobacco plants. These mutation experiments suggest that plants are able to perceive the presence of the pathogen through direct recognition of the xylanase. Recently, an internal pentapeptide sequence of the xylanase has been mapped to be the elicitation active site (Rotblat et al., 2002). Substitution of this peptide sequence inhibited the elicitation activity but not the enzyme activity of the xylanase protein, further demonstrating that elicitation activity is independent of its enzyme activity.

The xylanase is presumably recognized by receptors on the surface of the plant cell. In fact, a high-affinity binding site for ethylene-inducing xylanase on *Nicotiana tabacum* membranes has been identified (Hanania and Avni, 1997). The binding of fluorescently labeled xylanase to the plasma membrane was observed when the xylanase responsive cultivar *N. tacacum* cv Xanthi was tested but not when the non-responsive cultivar *N. tabacum* cv Hicks was tested. The binding is specific and saturable, with a dissociation constant of 6.2 nM. Chemical cross-linking of the xylanase to microsomal membranes from responsive plants revealed a 66 kDa protein complex. It was proposed that the binding protein is the receptor that directly perceives the xylanase elicitors, inducing a signal transduction cascade that leads to defense responses (Hanania and Avni, 1997).

In some cases, disruption of the xylanase genes of a pathogen did not affect its pathogenicity. For example, a triple mutant of maize fungal pathogen *Cochliobolus carbonum* was obtained by crossing three mutants, each of which contained a mutation in a different xylanase gene. The triple mutant retains full pathogenicity compared to the wild type (Apel et al., 1993). A double xylanase mutant of rice blast fungus *Magaporthe grisea* shows no reduced pathogenicity, apparently because it expresses previously undetected xylanase isozymes (Wu et al., 1997). Similarly, targeted inactivation of two xylanase genes (one family 10 and one family 11) of tomato vascular wilt fungus *Fusarium oxysporum f*. sp. *Lycopersici* has no detectable effect on its virulence (Gomez-Gomez et al., 2001; Gomez-Gomez et al., 2002). A mutant strain of the bacterial pathogen *Xanthomonas oryzae* pv *oryzae* (*Xoo*) that is unable to secrete xylanase is also avirulent (Ray et al., 2000). However, loss of pathogenicity cannot be exclusively attributed to the lack of extracellular xylanases since this mutant was deficient in a general secretary pathway, preventing other extracellular proteins that are potentially important in pathogenicity from being secreted.

Xylanase inhibitor proteins

Xylanase inhibitors have been detected in wheat (*Triticum aestivum*) flour (Debyser et al., 1997; Rouau and Surget, 1998; Debyser et al., 1999)) and purified (McLauchlan et al., 1999; Gebruers et al., 2001). One xylanase inhibitor, XIP-I, is a 30 kDa protein with a pI of 8.7-8.9. XIP-I was cloned and the functional recombinant gene product was obtained by heterologous expression in *E. coli* (Elliott et al., 2002; Furniss et

al., 2002). XIP-I exhibits highest similarity to sequences annotated as class III chitinases, but it has no detectable chitinase activity. XIP-I inhibits both family 10 and 11 xylanases from fungi but does not inhibit any bacterial xylanases tested. The binding affinity and pH range of interaction varies, depending on the xylanase tested. Another xylanase inhibitor protein, *Triticum aestivum* xylanase inhibitor (TAXI) was also purified from wheat flour (Debyser et al., 1997; Debyser et al., 1999). Recently TAXI has been found to contain two different components, TAXI-I and TAXI-II (Gebruers et al., 2001). Each protein has a molecular weight about 40 kDa and occurs in two forms: one is proteolytically processed while the other is not. The proteolytically processed forms can dissociate into two fragments under reducing conditions, as demonstrated by SDS-PAGE. The non-proteolytically processed forms are active while the activity of the processed forms have not been determined. TAXI-I and II have different pIs and each has a different xylanase-specificity. TAXI-I inhibits xylanase from *A. niger* and *B. subtilis* (both are family 11 xylanases) whereas TAXI-II only inhibits *B. subtilis* xylanase. Neither protein inhibits a family 10 xylanase from *A. aculeatus*.

Polygalacturonase

Homogalacturonan is a major pectic polysaccharide in plant primary cell walls. It is composed of 4-linked α-D-galactosyluronic acid residues, many of which are methylesterified. When phytopathogenic fungi are cultured on isolated plant cell wall materials, the first cell wall degrading enzyme the fungi synthesize is polygalacturonase (PG) (Karr and Albersheim, 1970; English et al., 1972; Jones et al., 1972). Extensive studies have shown that polygalacturonase can induce a wide variety of plant defense

reactions, including necrosis and induction of the synthesis of phytoalexins, β-1,3-glucanases, and chitinases (Hahn et al., 1989). There is considerable evidence showing that PG may function as an elicitor through its enzymatic activity. *In vitro*, PG digests the homogalacturonan in plant cell walls, generating oligogalacturonides fragments. Such oligogalacturonides are actually a class of well-characterized oligosaccharins (Darvill et al., 1992), which can themselves induce plant defense reactions. The ability of oligogalacturonides to induce a defense response is size dependent (Hahn et al., 1981; Nothnagel et al., 1983; Jin and West, 1984). That is, a degree of polymerization (DP) between 10 and 15 is generally required for biologically activity of the oligogalacturonides. Smaller oligogalacturonides are not effective elicitors. Although it has been clearly demonstrated that these oligogalacturonides *per se* can induce defense reactions, the existence of such hydrolyzed cell wall fragments during fungal infection has not been demonstrated *in vivo.*

The roles that polygalacturonase genes play in pathogenesis have been extensively studied. An *endo*polygalacturonase (*endo*PG) was found to be expressed during the fungal infection of maize plants by *Fusarium moniliforme*. Different strains of *F. moniliforme* express a different number of *endo*PG isoforms (Daroda et al., 2001). An *endo*polygalacturonase gene (*CLPG1*) of *Colletotrichum lindemuthianum* is expressed during both saprophytic growth and plant infection while a distinct but homologous (61% identical) gene (*CLPG2*) is expressed only under certain conditions when growing in axenic culture (Centis et al., 1997). Further studies indicated that *CLPG2* is expressed at

the early stages of germination of the conidia and during appressorium formation both *in vitro* and on the host plant (Dumas et al., 1999).

Several experiments support the idea that PGs are important pathogenic factors. Targeted disruption of a polygalacturonase gene in a pathogenic strain of *Aspergillus flavus* caused a significant reduction in aggressiveness. In contrast, expressing of the cloned gene in a less pathogenic strain previously lacking the gene increased lesion size (Shieh et al., 1997). Recently, it was found that a double mutant of the cereal pathogen *Claviceps purpurea* lacking two *endo*polygalacturonase genes (*cppg1/cppg2*) is almost nonpathogenic on rye, even though the vegetative properties of the fungus are not affected. Complementation of the mutants with wild-type copies of both *cppg1* and *cppg2* fully restored pathogenicity, demonstrating that the *endo*polygalacturonases encoded by *cppg1* and *cppg2* represent pathogenicity factors (Oeser et al., 2002). An *endo*polygalacturonase gene *Bcpg1* from fungus *Botrytis cinerea* is expressed during infection of tomato leaves. Although the *bcpg1* mutant is still pathogenic and displays similar ability to penetrate the leaf epidermis, a significant decrease in the lesion size beyond the inoculation spot was observed (ten Have et al., 2001). Two *endo*PG genes from two morphologically indistinguishable citrus pathogens of the genus *Alternaria*, *A. citri* and *A. alternate*, share 99.6% sequence identity. However, the *endo*PG mutant of *A. citri* was significantly reduced in its ability to cause black rot symptoms on citrus whereas disruption of the *endo*PG gene in *A. alternate* did not change its ability to cause brown spot on citrus, showing the roles of *endo*PG differ even in closely related fungal pathogens (Isshiki et al., 2001).

Disruption of PG genes in some fungi showed no effect on their pathogenicity. Naturally occurring *Fusarium oxysporum* f. sp. *melonis* isolates deficient in an *endo*polygalacturonase gene (*pg1*) were transformed with the cloned *pg1* gene. The transformants showed certain growth advantages over wild-types when grown on pectin. However, the virulence of these transformants did not increase (Di Pietro and Roncero, 1998). A double mutant of *Cochliobolus carbonum* lacking two major extracellular polygalacuronases exhibits less than 1% of the PG activity of the wide-type, yet maintains its full virulence and ability to grow normally with pectin as sole carbon source (Scott-Craig et al., 1998). The lack of a pathogenesis phenotype in this mutant may be due to residual pectin-degrading activity.

Ralstonia solanacearum causes bacterial wilt in many crop plants. Both *endo*PG and *exo*PG appear to be required for rapid colonization and full virulence on tomato plants. Mutants lacking *PehA* (an *endo*-PG), or *PehB* (an *exo*-PG), or both *PehA* and *PehB* are all less virulent than the wild-type strain on wounded eggplants (Huang and Allen, 2000).

Polygalacturonase-inhibiting protein (PGIP)

Polygalacturonase-inhibiting proteins (PGIPs) (See recent reviews:(De Lorenzo et al., 2001; Federici et al., 2001; De Lorenzo and Ferrari, 2002) were discovered more than 30 years ago (Albersheim and Anderson, 1971). Several of those plant proteins have been

purified and their genes cloned (Toubart et al., 1992). All dicotyledonous plants examined so far have at least one PGIP that has a broad specificity against fungal PGs. PGIP forms a complex with PG *via* a specific, reversible, and saturable process (reviewed by (Hahn et al., 1989). Binding is accompanied by a slowing or modification of PG activity (Cervone et al., 1987a; Cervone et al., 1987b). This change in PG activity increases the lifetime of biological active oligogalacturonides, which otherwise would be degraded into smaller inactive oligomers. Therefore, PGIP increases the local concentration of biologically active oligogalacturonides, enhancing the plant's ability to detect and respond to the pathogen. Furthermore, processive PGs with an *endo/exo* cleavage mode produce small inactive oligogalacturonides more efficiently than larger, active oligogalacturonides. Therefore, plants have evolved PGIPs that preferentially inhibit processive PGs over non-processive PGs, which can efficiently produce biologically active oligogalacturonides (Cook et al., 1999).

Expression of PGIPs is induced by pathogen infection (Favaron et al., 1994; Nuss et al., 1996; Mahalingam et al., 1999; Yao et al., 1999), salicylic acid, elicitors, and wounding (Bergmann et al., 1994; Yao et al., 1999). Different members of the PGIP gene family are differentially expressed in response to stress stimuli, suggesting the redundancy of PGIP genes may originate from the requirement for distinct responses to different challenges. The ability of PGIP to play a role of in plant disease defense has been demonstrated by overexpression of pear *PGIP* gene in tomato plants (Powell et al., 2000). The transgenic tomato displayed reduced symptoms upon infection by *Botrytis*

cinerea. Similar results have been observed in *Arabidopsis* and tobacco (De Lorenzo and Ferrari, 2002)

PGIPs belong to a large family of leucine-rich repeat (LRR) proteins (Jones, 1996). Members of the LRR protein family share a structural motif that is thought to be responsible for protein-protein interactions and have many different functions such as hormone–receptor interactions, enzyme inhibition, cell adhesion and cellular trafficking (Kobe and Kajava, 2001). It has been shown that a single amino acid substitution in the LRR motif changed the recognition specificity of PGIP (Leckie et al., 1999). All but 8 of the amino acids are identical in PGIP-1 and PGIP-2 from *Phaseolus vulgaris* L. Nevertheless, PGIP-1 is unable to interact with PG from *Fusarium moniliforme* but is able to interact with PG from *Aspergillus niger*. PGIP-2 can interact with both PGs. A single mutation (K253Q), endows PGIP-1 with the ability to interact with *F.moniliforme* PG.

Pectate/Pectin Lyase

Pectin lyases (PLs) degrade highly methylesterified homogalacturonan, while pectate lyases (PNLs) degrade homogalacturonan with a low degree of methyl esterification. Pectate/pectin lyases cleave homogalacturonan backbones by β-elimination, which results in the formation of a 4,5-unsaturated residue at the nonreducing end. Like polygalacturonases, the role of pectate/pectin lyases in pathogenicity varies from microbe to microbe. When two inducible pectate lyase genes of the pea plant pathogen *Nectria hematococca* were simultaneously mutated, a drastic

reduction in virulence was observed: the double gene disruptant produced very few mild lesions and did not penetrate deeply into the tissue of pea epicotyl segments. Single gene-disrupted mutants and wild type pathogen produced significant lesions and penetrated extensively and deeply into the host tissue (Rogers et al., 2000). Supplementation of either of the two gene products, purified pectate lyases, to the double mutant rescued its virulence. This demonstrated that at least some cell wall degrading enzymes, pectate lyases in this case, indeed can act as virulence factors. Similarly, disruption of the pectate lyase gene *pelB* in *Colletotrichum gloeosporioides* reduced its virulence on avocado fruit, reducing the decay diameter by 36-45% compared with the wild type (Yakoby et al., 2001). However, inactivation of pectin lyase gene in the plant fungal pathogen *Glomerella cingulata* had no effect on its pathogenicity (Bowen et al., 1995).

In the bacterium *Pseudomonas viridiflava*, disruption of its pectate lyase gene destroyed its ability to induce soft rot in plants (Liao et al., 1988). Initially, disruption of four pectate lyase genes, an *exo*-poly-α-D-galacturonosidase gene, and an *exo*polygalacturonate lyase in *Erwinia chrysanthemi* showed no significant effect on its ability to macerate plant tissues (Ried and Collmer, 1987). Further studies led to the discovery a new set of pectate lyase isozymes that were only induced *in planta* (Kelemu and Collmer, 1993). The existence of so many isozymes of pectic lyases may contribute to the wide host range of *E. chrysanthemi* (Beaulieu et al., 1993).

Pectin lyase inhibitor protein

Sugar beet produces a protein (PNLIP) that inhibits a pectin lyase from *Rhizoctonia solani*, which is a pathogen of sugar beet (Bugbee, 1993). PNLIP is a 57.5 kDa protein with a pI of 9.9. Extracts from *R. solani* infected tissue have greater PNLIP activity than those from healthy tissue, suggesting that PNLIP has a role in the response of sugar beet to *R. solani*. The inhibitor was equally effective against PNL from *Phoma betae* but was less effective against PNL from *Aspergillus japonicus*. The sequence of this protein has not been determined to date.

Pectin methylesterase (PME) and its inhibitor protein

A pectin methylesterase (PME) mutant of *Erwinia chrysanthemi* 3937 was clearly shown to be noninvasive on inoculated *Saintpaulia ionantha* plants (Boccara and Chatain, 1989). Although an active PME gene is required for growth of the bacterial pathogen *Ralstonia solanacearum* on methylated pectin as carbon source, a mutant in which the PME gene is disrupted, is as virulent as the wild-type strain (Tans-Kersten et al., 1998)

A kiwi fruit protein (Camardella et al., 2000) with homology to plant invertase inhibitors has been reported to inhibit only plant-originated pectin methylesterase by forming a 1:1 complex (Balestrieri et al., 1990; Giovane et al., 1995). It has been

hypothesized that this PME inhibitor may play a physiological role in the plant, possibly in the regulation of the fruit ripening process (Camardella et al., 2000).

Glycosidase

The function of microbial glycosidases in pathogenicity has not been widely studied. Mutation of a xylosidase gene in *Cochliobolus carbonum* decreased the total xylosidase activity in the culture filtrate to 25% of wild-type levels, but the mutant retained its pathogenicity on maize (Wegener et al., 1999). However, since the fungus has at least one additional xylosidase gene, the role of xylosidases in pathogenicity cannot be excluded.

Plant-derived fungal cell wall degrading enzymes and their microbial inhibitor proteins

Most inhibitors of CWDEs discovered so far are produced by plants in order to defend themselves against microbial enzymes. However these roles are reversed in at least one case: plants produce β-1,3-*endo*glucanases (EGases) to degrade fungal cell walls which are counteracted by fungal glucanase inhibitor proteins (GIPs) (Albersheim and Valent, 1974; Ham et al., 1995; Ham et al., 1997; Rose et al., 2002). β-glucan oligosaccharide elicitors, which induce numerous plant defense responses (Cote and Hahn, 1994; Shibuya and Minami, 2001) are released when β-1,3-glucans in the fungal cell wall are degraded by plant β-1,3-*endo*glucanases. The pathogen increases its ability to colonize the plant by producing a family of proteins that inhibit plant EGases. For

example, a glucanase inhibitor protein (GIP-1), has been purified from the oomycete pathogen of soybeans, *Phytophthora sojae* (Ham *et al.*, 1997), cloned, and characterized (Rose *et al.*, 2002). GIP-1, which binds with high affinity to a soybean EGase, is homologous to serine proteases but lacks the catalytic triad required for protease activity.

Conclusion

In conclusion, plants produce a wide range of proteins that bind to cell wall degrading enzymes secreted by microbial pathogens. Numerous studies implicate these proteins in the plant's response to pathogenic attack, although different mechanisms, including direct recognition of fungal proteins and prevention of oligosaccharin degradation by the fungal enzyme, have been proposed. Conversely, plants produce enzymes to degrade the cell walls of invading microbes, which synthesize inhibitor proteins to prevent their cell walls from being hydrolyzed. This dissertation describes XEGIP-like proteins, a new family of inhibitor proteins in the plant's arsenal in this on-going arms race.

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CHAPTER 2

CHARACTERIZATION OF A TOMATO PROTEIN THAT INHIBITS A XYLOGLUCAN-SPECIFIC ENDOGLUCANASE[1]

[1]Qin, Q, C. W. Bergmann, J. K.C. Rose, M. Saladie, V.S. K. Kollia, P. Albersheim, A. G. Darvill and W. S. York. 2003. *Plant Journal*. In press. Reproduced with permission from the publisher.

Summary

A basic, 51 kDa protein was purified from suspension-cultured tomato and shown to inhibit the hydrolytic activity of a xyloglucan-specific *endo*glucanase (XEG) from the fungus *Aspergillus aculeatus*. The tomato (*Lycopersicon esculentum*) protein, termed XEG inhibitor protein (XEGIP), inhibits XEG activity by forming a 1:1 protein:protein complex with a $K_i \approx 0.5$ nM. To our knowledge, XEGIP is the first reported proteinaceous inhibitor of any *endo*-β-1,4-glucanase, including the cellulases. The cDNA encoding XEGIP was cloned and sequenced. Database analysis revealed homology with carrot extracellular dermal glycoprotein (EDGP), which has a putative role in plant defense. XEGIP also has sequence similarity to ESTs from a broad range of plant species, suggesting that *XEGIP*-like genes are widely distributed in the plant kingdom. Although Southern analysis detected only a single *XEGIP* gene in tomato, at least 5 other *XEGIP*-like tomato sequences have been identified. Similar small families of XEGIP-like sequences are present in other plants, including *Arabidopsis.* XEGIP also has some sequence similarity to two previously characterized proteins, basic globulin 7S protein from soybean and conglutin γ from lupin. Several amino acids in the XEGIP sequence, notably 8 of the 12 cysteines, are generally conserved in all of the XEGIP-like proteins we have encountered, suggesting a fundamental structural similarity. Northern analysis revealed that XEGIP is widely expressed in tomato vegetative tissues and is present in expanding and maturing fruit, but is down regulated during ripening.

Introduction

Cell walls play an important role in the plant's ability to defend themselves against fungal pathogens (Côté and Hahn, 1994; Howard, 1997). The main components of primary cell walls are members of two polysaccharide networks, one consisting of cellulose and hemicellulose, and the other consisting of pectic polysaccharides. Most plant pathogens secrete a mixture of enzymes that can hydrolyze the polysaccharides in the primary cell wall (Walton, 1994). These enzymes include various polygalacturonases, pectin methyl esterases, pectin/pectate lyases, acetyl esterases, xylanases and a variety of *endo*glucanase that cleave cellulose, xyloglucan, and other glucans.

Plants synthesize a variety of proteins that inhibit the cell wall-degrading enzymes secreted by pathogens. The best characterized of these proteins are polygalacturonase-inhibiting proteins (PGIPs), which were first described more than thirty years ago (Albersheim and Anderson, 1971). PGIPs act by forming specific, reversible, and saturable complexes with microbial polygalacturonases (PGs), which cleave unesterified homogalacturonan (poly-α-1,4-linked-D-galactosyluronic acid), the major pectic polysaccharide in primary cell walls (De Lorenzo *et al.*, 2001; Stotz *et al.*, 2000). The limitation of fungal colonization in transgenic tomato plants expressing pear PGIP is direct evidence of PGIP's role in defense (Powell *et al.*, 2000). Sugar beet produces a protein (PNLIP) that inhibits a pectin lyase from *Rhizoctonia solani*, which is a pathogen of sugar beet (Bugbee, 1993). Xylanase inhibitors have been detected in wheat flour (Debyser *et al.*, 1999; Rouau and Surget, 1998), purified (McLauchlan *et al.*, 1999), and characterized (Gebruers *et al*., 2001; Elliott *et al*., 2002). A kiwi fruit protein (Camardella

et al., 2000) with homology to plant invertase inhibitors has been reported to inhibit tomato pectin methylesterase by forming a 1:1 complex (Balestrieri *et al.*, 1990; Giovane *et al.*, 1995).

Plants also produce enzymes that are capable of degrading the cell walls of pathogens. For example, the plant enzyme *endo*-β-1,3-glucanase (EGase) degrades β-1,3/1,6-glucans in fungal cell walls, releasing β-glucan oligosaccharide elicitors, which induce numerous plant defense responses (Côté and Hahn, 1994; Shibuya and Minami, 2001). Some plant pathogens have recently been shown to synthesize a family of proteins that inhibit plant EGases. For example, a glucanase inhibitor protein (GIP-1), has been purified from the oomycete pathogen of soybeans, *Phytophthora sojae* (*Psg*) (Ham *et al.*, 1997), cloned, and characterized (Rose *et al.*, 2002). GIP-1, which binds with high affinity to a soybean EGase, is homologous to serine proteases but lacks the catalytic triad required for protease activity.

Here we describe the identification and molecular characterization of XEGIP, the first plant protein that has been shown to inhibit an *endo*glucanase. *Endo*glucanase inhibition by XEGIP represents a new class of protein-protein interactions whose discovery may have important implications with regard to plant pathogenesis and plant-microbe co-evolution.

Results

Identification and isolation of XEGIP from tomato-cell culture

A protein (XEGIP) that inhibits the hydrolytic activity of a xyloglucan-specific β-1,4-*endo*glucanase (XEG) isolated from *A. aculeatus* (Pauly *et al.*, 1999) was detected in the culture medium of suspension-cultured tomato cells. Filtered culture medium was mixed with 4 volumes of ethanol, and the resulting precipitate (containing xyloglucan) was resuspended in water, dialyzed, and lyophilized. When the xyloglucan-enriched material was treated with XEG, none of the expected oligosaccharide products were detected by MALDI-TOF-MS analysis of the reaction mixture, even after extensive treatment with an excess of XEG. The absence of XEG-generated oligosaccharides was confirmed by a colorimetric assay using *p*-hydroxy-benzoic acid hydrazide (Lever, 1972) to detect the reducing ends of any oligosaccharides produced by the XEG. In contrast, the digestion proceeded to completion and reducing oligosaccharides were easily detected when the crude xyloglucan preparation was boiled for 5 minutes before adding the XEG. The heat-labile nature of the inhibitory factor suggested that it is a protein.

A protein whose presence is correlated with the inhibitory activity was purified from the ethanol-precipitated material by ion-exchange and size-exclusion chromatography (Figure 2.1, Lanes 1-4, described in **Experimental Procedures**). The molecular weight of the purified protein is approximately 50 kDa as determined by SDS-PAGE (Figure 2.1**,** Lane 4). MALDI-TOF-MS established that the protein has a molecular weight of 50853 Da (data not shown). The amount of this protein in the tomato cell culture medium appears to depend on culture conditions, constituting approximately

2% of the ethanol precipitated protein from one culture, but up to 10% of the protein in other cultures, as estimated by SDS-PAGE.

Various amounts of the purified 51 kDa protein were mixed with XEG, and the resulting mixtures were added to xyloglucan solutions. Aliquots of each reaction mixture were taken after 10 min, 30 min, and 24 hr, and the extent of xyloglucan digestion was determined using the reducing sugar assay. The results (Figure 2.2) showed that the XEG-catalyzed reaction is completely inhibited in the presence of an excess of the purified 51 kDa protein, even when the incubation time is extended to 24 hours. Accordingly, the 51 kDa protein was named xyloglucan-specific *endo*glucanase inhibitor protein (XEGIP).

Stoichiometry of the XEGIP-XEG interaction

XEGIP inhibits the activity of XEG by binding to the enzyme. Binding was first detected as a shift in the retention time of XEG and XEGIP during size-exclusion chromatography (SEC) on Superdex-75 (Figure 2.3). A constant amount of XEG was mixed with increasing amounts of XEGIP, and the resulting mixtures were subjected to SEC. Increasing the XEGIP concentration resulted in the appearance and growth of a higher molecular mass peak and the disappearance of the XEG peak. The newly formed peak was collected and analyzed by SDS-PAGE (data not shown). The resulting gels consistently contained two bands, corresponding to XEG and XEGIP, irrespective of the length of time that the enzyme and inhibitor were preincubated before they were subjected to SEC. The molar extinction coefficients (ε) for XEG and XEGIP were determined (see **Experimental procedures**) allowing the XEGIP:XEG ratio to be measured for each mixture applied to the column. Ideally, if a 1:1 complex forms with a dissociation constant

(K_d) that is significantly smaller than the concentration of XEG, the XEG peak should disappear just when the XEGIP:XEG ratio in the mixture is 1:1. When the XEGIP:XEG ratio is greater than 1, a (free) XEGIP peak should be visible as a shoulder on the XEG-XEGIP complex peak. Within the accuracy of our estimations of ε for XEG and XEGIP, these are the results obtained (Figure 2.3). Taken together, the appearance of a high-molecular weight complex peak, the disappearance of the XEG peaks when the XEGIP:XEG ratio is greater than or equal to 1, and the absence of detectable proteolytic fragments support the hypothesis that XEGIP inhibits XEG by binding to XEG to form a 1:1 complex and that XEGIP does not proteolytically degrade XEG.

Additional evidence for the binding of XEGIP and XEG was obtained using an affinity chromatography matrix consisting of XEG immobilized on CNBr-activated Sepharose. Neutral and anionic polysaccharides and proteins were removed from the ethanol-precipitated materials from tomato cell culture media by cation exchange chromatography. XEGIP and other cationic molecules that had bound to the ion-exchange matrix (Figure 2.4, lane 1) were eluted with a high salt buffer and applied to the XEG column. Material that bound to the XEG column was eluted with 2M imidazole/HCl, pH 7, and analyzed by SDS-PAGE (Figure 2.4, lane 4), revealing a single protein, which was identified as XEGIP by its electrophoretic mobility and whose inhibitory activity was recovered upon removal of the imidazole by dialysis. The immobilized XEG in the regenerated affinity column retains its ability to digest xyloglucan and can be reused to bind XEGIP (data not shown), thus confirming that the inhibition is not due to proteolysis.

Kinetic studies of XEGIP-XEG binding

An attempt was made to determine the dissociation constant (K_d) for the interaction of XEGIP with XEG by surface plasmon resonance (SPR) using a BiaCore® apparatus. Toward this goal, purified XEGIP was covalently immobilized on a BiaCore® sensor chip. The sensogram obtained by passing soluble XEG over the XEGIP-coated chip indicated that the interaction of XEG with XEGIP was essentially irreversible under the experimental conditions used. Thus, the value of K_d could not be obtained by this method.

Most standard methods for determining the inhibition constant K_i are based on the assumption that formation of the enzyme-inhibitor complex does not significantly decrease the concentration [I] of the free inhibitor relative to the total concentration $[I]_t$ of added inhibitor (Segel, 1975). That is, [I] is assumed to be equal to $[I]_t$. However, this is not the case when XEGIP is mixed with XEG that is at a concentration normally used to determine enzyme activity. As illustrated in Figure 2.3, when the XEGIP:XEG ratio is less than 1, essentially all of the added XEGIP forms a complex with the XEG, and [I] is much less than $[I]_t$. Free XEGIP cannot be detected until the XEGIP:XEG ratio is greater than 1, at which time the XEG is completely saturated and the rate of the XEG-catalyzed reaction is zero. A graphical method specifically designed to determine the association constants of such tightly bound inhibitors has been proposed by Dixon (1972) and described by Segel (1975). This analysis is carried out at low protein concentrations such that enzyme-inhibitor interactions are minimized and the equilibrium between complex formation and disassociation can be characterized, that is, concentrations where more than half of the inhibitor is free and the amount of free enzyme is sufficient to detect its activity.

In the case of the XEGIP-XEG interaction, this corresponds to an XEG concentration of approximately 1 nM. As illustrated in Figure 2.5, K_i for the interaction of XEG and XEGIP was determined by this method to be 0.5 nM.

Specificity of the XEGIP-XEG interaction

XEGIP did not show any capacity to inhibit other plant cell wall degrading enzymes tested. For example, fungal polygalacturonases (Cook *et al.*, 1999), an *endo*glucanase (Megazyme, Wicklow, Ireland) from *Trichoderma longibrachiatum* that cleaves xyloglucan and other β-1,4-linked glucans, and a xyloglucan *endo*transglycosylase (LeXET2) that specifically uses xyloglucan as a substrate (Catalá *et al.*, 2001), are not inhibited by XEGIP.

Cloning of XEGIP cDNA

Peptide sequences of tryptic fragments of XEGIP were determined by the Edman procedure (see **Experimental procedures**) and used to search the publicly available databases. Several homologous gene sequences were identified. Degenerate primers designed on the basis of well-conserved regions of these homologous sequences were used to generate several PCR products, which were cloned and sequenced (see **Experimental procedures**). Short nucleotide sequences within one of the PCR products corresponded to data obtained by Edman sequencing. Searches of the TIGR Gene Indices database (http://www.tigr.org/tdb/tgi.shtml) with this cloned fragment revealed that it overlaps one Tentative Consensus sequence (TC85129) and one EST singleton (EST 258116 Accession number AI 777151). A new set of gene specific primers was designed accordingly and used to clone the full-length cDNA of *XEGIP*. The cDNA sequence was deposited in

GeneBank (Accession number AY 155579). Its deduced amino acid sequence is shown in Figure 2.6.

The deduced amino acid sequence of the *XEGIP* gene product agrees closely with the results of quantitative amino acid analyses of purified XEGIP (data not shown) and with the results of mass spectral analysis of tryptic fragments of XEGIP. That is, the measured molecular weights of all 18 major peptide fragments detected by MALDI-TOF analysis of trypsin-treated XEGIP matched (within 0.1%) the theoretical values predicted for tryptic peptides generated *in silico* (data not shown). Eight of the trypsin-generated XEGIP peptides were also isolated by RP-HPLC and sequenced by MS-MS. All of the resulting peptide sequences (marked in Figure 2.6) matched the deduced amino acid sequence of XEGIP.

The deduced protein sequence has a putative signal peptide (22 AA) predicted by SignalP program (http://www.cbs.dtu.dk/services/SignalP/). The putative cleavage site is marked by an arrowhead in Figure 2.6. The predicted mature XEGIP protein has 415 amino acids and a molecular weight of 44229.74 Da. The mature protein also has five potential N-glycosylation sites, which are likely to account for some or all of the 6625 Da difference between the predicted molecular weight and the molecular weight measured by MALDI-MS (50,853 Da). Indeed, XEGIP binds to a Con-A lectin column and is eluted by methyl-α-D-mannoside (data not shown), confirming that XEGIP is a glycoprotein. EDGP, the carrot homologue of XEGIP (see below) is also reported to be glycosylated (Satoh and Fujii, 1988).

XEGIP has 62% amino acid sequence identity to extracellular dermal glycoprotein (EDGP) from carrot (Satoh *et al.*, 1992) and homology to multiple EST sequences from tomato and diverse plant species including soybean, *Lotus*, carrot, cotton, maize, rice, sorghum, *Medicago*, and *Arabidopsis.* (For each of these genes, the accession number and homology to the XEGIP sequence is listed at the end of this article.) In addition, XEGIP shares some conserved residues with basic globulin 7S protein (Bg7S) from soybean (35% identity) (Kagawa and Hirano, 1989), conglutin γ (Cγ) from *Lupinus albus* (35% identity), and some less related sequences from tomato and *Arabidopsis* (Figure 2.6). Eight of the twelve cysteines in XEGIP are strictly conserved in all of the XEGIP-like proteins illustrated in Figure 2.6, and all twelve of these cysteines are conserved in most of these proteins, suggesting similarities in their three-dimensional structures.

Gel blot analyses

Genomic DNA analysis identified a single *XEGIP* gene in tomato, regardless of whether a full- or partial-length probe was used (Figure 2.7), indicating that *XEGIP* is present as a single copy in tomato. However, five other XEGIP-related genes from tomato were identified in the databases. These genes encode proteins whose amino-acid sequences are from 24% (partial sequence for Tomato-2) to 51% (XEGIP-5) identical to XEGIP (Figure 2.6), which presumably are sufficiently divergent to preclude cross-hybridization in the Southern analysis. The two most closely related genes in *Arabidopsis (Arabidopsis-1* and *2)*, share 58% and 63% amino acid sequence identity with XEGIP, respectively, and share 89% identity with each other (Figure 2.6).

Northern analysis revealed that XEGIP mRNA was expressed in all vegetative tissues examined, with lower expression levels in young healthy leaves (Figure 2.8A). XEGIP mRNA abundance increased during fruit expansion, peaked immediately prior to the onset of ripening at the mature green stage, and declined as ripening progressed (Figure 2.8B).

Discussion

A plant protein that inhibits a xyloglucan-specific fungal *endo*glucanase (XEG) was purified from suspension-cultured tomato cells and termed XEG inhibitor protein (XEGIP). XEGIP, the first protein from plants or any other source that has been shown to inhibit an *endo*-β-1,4-glucanase, shows no detectable proteolytic activity and inhibits XEG *via* a strong 1:1 interaction with a K_i of approximately 5×10^{-10}M. The XEG-XEGIP complex has no detectable *endo*glucanase activity, but active XEG and XEGIP can be regenerated upon dissociation of the immobilized complex with 2M imidazole buffer. The molecular basis of its interaction with XEG is yet to be determined, as XEGIP does not contain any recognizable protein-protein interaction motifs, such as leucine-rich repeats. XEGIP appears to represent the newest class of plant-derived proteins that inhibit microbial enzymes that degrade plant cell walls. *Endo*glucanases, which constitute a major class of these microbial enzymes, hydrolyze cellulose and xyloglucan and are implicated in pathogenicity. For example, *endo*glucanases of *Ralstonia solanacearum* (Denny *et al.*, 1990; Kang *et al.*, 1994; Roberts *et al.*, 1988; Saile *et al.*, 1997) and *Clavibacter michiganensis* (Jahr *et al.*, 2000) are believed to be involved in pathogenesis of tomato wilting disease. Cellulase (*endo*glucanase) CelV mutants of *Erwinia carotovora* exhibited delayed maceration of plant tissues, suggesting reduced virulence (Mae *et al.*,

1995; Walker *et al.*, 1994) and further indicating the involvement of *endo*glucanases in pathogenesis. In *Aspergillus aculeatus*, seven *endo*glucanases, including cellulase and XEG, have been identified (de Vries and Visser, 2001). Although *A. aculeatus* is a saprophyte and not considered to be a tomato pathogen, the spectrum of plant cell wall hydrolases that it secretes is likely to be similar to those of pathogenic fungi and bacteria. For example, the maize pathogen *Cochliobolus carbonum* secrets MLG2, a mixed-linked (β-1,3-β-1,4) glucanase into its culture medium. MLG2 and XEG both belong to Family 12 of the glycosyl hydrolases (Coutinho and Henrissat, 1999; Kim *et al.*, 2001; Goedegebuur *et al.*, 2002; Yuan *et al.*, 2001), which include cellulases, *endo*-β-1,4-glucanases, and β-1,3-β-1,4-glucanase from fungi and bacteria. It remains to be seen whether the ability to interact with Family 12 enzymes is a general characteristic of XEGIP-related proteins. It is possible that XEGIP-related proteins have evolved to counteract cellulolytic and hemicellulolytic enzymes produced by plant pathogens, analogous to the inhibition of pathogenic polygalacturonases by plant PGIPs.

Alternatively, XEGIP may play a role in regulating endogenous plant enzymes, thereby affecting the modification and reorganization of cell walls during growth and development. However, we have demonstrated that the tomato xyloglucan *endo*transglucosylase LeXET2 (Catalá *et al.*, 2001) is not inhibited by XEGIP. Except those that are closely related to the xyloglucan *endo*transglucosylases, all known plant *endo*-β-1,4-glucanases belong to Family 9 (Henrissat and Bairoch, 1996), and have an inverting rather than retaining mechanism of action. None of the Family 9 enzymes have been tested for inhibition by XEGIP.

EDGP, the XEGIP homolog in carrot, is a 57 KDa protein that is expressed at high level in the dermal tissues of roots, petioles, and leaves, as well as in developing seeds. EDGP has been proposed to be involved in pathogen resistance due to its localization in dermal tissues and expression in response to wounding (Satoh *et al.*, 1992). However, its function has not been unambiguously established *in vivo* or *in vitro*. The sequence homology between EDGP and XEGIP (62% identity) suggests that they have similar functions; indeed, the ethanol precipitate of carrot cell culture medium contains a heat-sensitive component that inhibits XEG. A protein was purified (95% homogeneity) from the precipitated material and identified as EDGP by its apparent molecular weight (SDS-PAGE) and tryptic peptide-mass fingerprinting (data not shown). Peak shift experiments such as those shown in Figure 2.3 confirmed that EDGP binds to XEG (data not shown).

XEGIP and EDGP are both secreted into the medium of suspension-cultured cells that were derived from callus, which normally grows in response to wounding a plant tissue. As pointed out by Satoh *et al.* (1992), plant proteins that are typically expressed in response to wounding are also detected in suspension-cultured cells. These include invertase (Lauriere *et al.*, 1988), hydroxyproline-rich glycoproteins (Brownleader and Dey, 1993; Esaka *et al.*, 1992; Hirsinger *et al.*, 1997; Lamport, 2001; Lamport and Northcote, 1960), chitinase (Arie *et al.*, 2000; Kunze *et al.*, 1998; Wojtaszek *et al.*, 1998), β-1,3-glucanase (Kunze *et al.*, 1998), and peroxidase (Breda *et al.*, 1993; Schnabelrauch *et al.*, 1996). Thus, the high expression of XEGIP-related proteins in cultured cells is consistent with a possible role in the plant's response to stress.

An *Ageratum conyzoide*s gene similar to EDGP (the carrot homolog of XEGIP) that is strongly up-regulated when the plant is infected by *Agrobacterium tumefaciens,* has been detected by cDNA-amplified fragment length polymorphism (AFLP) studies (Ditt *et al.*, 2001). The 73 amino-acid sequence coded by the gene fragment is homologous to both XEGIP and EDGP. Furthermore, the N-terminal sequence (28 amino acids) reported for a 46 kDa cotton seed protein with antifungal activity (Chung *et al.*, 1997) is similar to a sequence within XEGIP (50% identity). Taken together, these observations suggest that XEGIP-related proteins may be involved in the plant's defense against pathogenic attack.

Basic 7S globulin (Bg7S) from soybean seeds and conglutin γ (Cγ) from *Lupinus albus* seeds (Blagrove *et al.*, 1980; Blagrove and Gillespie, 1975; Elleman, 1977) also have amino acid residues that are conserved with XEGIP (Figure 2.6), even though amino acid sequences of these proteins are only 35% identical to those of XEGIP and EDGP, suggesting a remote evolutionary relationship. The cysteine residues are especially well conserved, suggesting that the three-dimensional structures of these proteins may have features in common with XEGIP.

Proteins that are closely related to Bg7S and Cγ are widely present in the seeds of leguminous plants (Kagawa *et al.*, 1987) and have long been considered as storage proteins (Blagrove *et al.*, 1980; Kolivas and Gayler, 1993). However, recent research suggests that these proteins may have additional physiological roles. When soybean or *Lupinus albus* seeds are immersed in 50-60 °C water, large amounts of Bg7S and Cγ, respectively, are released along with other proteins (Kagawa *et al.*, 1987). The Cγ was found to be newly synthesized rather than constitutive (Duranti *et al.*, 1994), suggesting a potential role in

stress response. Bg7S binds insulin and insulin-like growth factors (Komatsu and Hirano, 1991), while Cγ was observed to have lectin-like activity (Duranti *et al.*, 1995). Thus, it appears that two common characteristics of XEGIP-related proteins are their capacity to bind to specific proteins or peptides and their enhanced expression when the plant is exposed to a stressful stimulus such as heat-shock, wounding, or infection.

In summary, we suggest that XEGIP may be a plant defense protein that functions as an inhibitor of a microbial Family 12 glycanase, and as such, XEGIP may play an important protective role by preventing such glycanases from degrading the plant cell wall. Alternatively, XEGIPs and related proteins may play a general role in protecting plants against biotic or abiotic stresses, as suggested by their localization and expression upon wounding, pathogen infection, and heat shock. Further evidence is required to establish the biological function of XEGIP and XEGIP-related proteins *in vivo*.

Experimental procedures

Chemicals, reagents, substrates and enzymes

Buffer salts, acids, and bases were obtained from J.T. Baker (Philipsburgs, NJ, USA). Organic solvents were from Fisher Scientific (Pittsburgh, PA, USA) and other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA). All chromatography columns and matrices used for protein purification were bought from Amersham Biosciences (Piscataway, NJ, USA).

Xyloglucan-specific *endo*glucanase (XEG) from *Aspergillus aculeatus* was generously provided by Novozymes (Copenhagen, Denmark) and was purified as described (Pauly *et al.*, 1999).

Tomato-cell culture

Tomato (*Lycopersicon esculentum* 'Bonnie Best') cell suspension cultures were generated and maintained as described (Smith *et al.*, 1984).

Enzyme inhibition assay

Varying amounts of column fractions (2-50 μL depending on the XEGIP content) were mixed with a defined amount of XEG before adding 0.5-1.0 mL of 4 mg/mL of purified tamarind xyloglucan in 20 mM NaOAc buffer, pH 5.3. The reaction was incubated at room temperature and aliquots were analyzed by a colorimetric assay (Lever, 1972) at different time points to determine the amount of reducing sugar generated by the reaction. The tamarind xyloglucan was reduced with $NaBH_4$ in advance to minimize background in the reducing sugar assay.

The inhibition constant (K_i) was obtained by the graphical method for determining binding constants for tightly bound inhibitors (Dixon, 1972; Segel, 1975), as illustrated in Figure 2.5. The initial rates of catalysis were determined as described above except that purified XEG and XEGIP were used and the concentrations of the two proteins were kept sufficiently low so that both XEG and XEGIP remained partially free in the assay solution. (That is, both $[Complex] < [XEGIP]_{tot}$ and $[Complex] < [XEG]_{tot}$.)

Purification of XEGIP

Culture medium from 4L of 7-day old cells was filtered through two layers of cheesecloth. Ethanol (95%, 4 volumes) was added to the filtrate to precipitate proteins and polysaccharides. After keeping the solution at 4 °C overnight, the ethanol-precipitated material was collected by decanting and centrifugation. The precipitate was resuspended in water (50 mL), stirred for several hours at 4 °C, any remaining insoluble material was removed by a second centrifugation, and the resulting supernatant was analyzed by SDS-PAGE (Figure 2.1, lane 1). The supernatant was concentrated at 30 °C, dialyzed overnight at 4 °C against 20 mM NaOAc, pH 5.2-5.4. The retentate was adjusted to pH ~7.2 by adding 1 M imidazole (free base) and was applied to a Q-Sepharose anion exchange column to remove pectins and anionic proteins. An aliquot of the flow-through fraction was analyzed by SDS-PAGE (Figure 2.1, lane 2) and the remainder was dialyzed against 20 mM NaOAc pH 5.2 buffer and then loaded on a HiTrap-S cation exchange column equilibrated in the same buffer. The cation-exchange column was washed with loading buffer to elute neutral polysaccharides, which were collected for structural analysis. The HiTrap-S column was then eluted with a pH and salt gradient (eluent A, 20 mM NaOAc pH 5.2; eluent B, 20 mM HEPES pH 7.4 containing 2 M NaCl; 0-100% B in 60 min at 0.5 mL/min). Fractions (0.5 mL) were collected and assayed for XEG-inhibition. Active fractions (Figure 2.1, lane 3) were pooled, concentrated, applied to a Superdex 75 SEC column and then eluted with 20 mM NaOAc, pH 5.2, containing 0.3 M NaCl. Fractions with inhibitory activity against XEG were pooled. SDS-PAGE of the purified inhibitor protein resulted in a single band (Figure 2.1**,** lane 4).

Internal peptide sequencing by Edman degradation

Internal peptide sequencing by Edman degradation of tryptic fragments of XEGIP was performed by Michigan State University Macromolecular Structure, Sequencing and Synthesis Facility.

Amino acid assay and protein quantitation

Quantitative amino acid analyses (AAA) were conducted by Harvard Microchemistry Facility. The amount of alanine or phenylalanine in standard XEG and XEGIP samples along with the deduced amino acid sequences of the proteins were used to accurately determine protein concentration in these samples. This allowed extinction coefficients (ε) of XEG and XEGIP (82,123 and 35,867 M^{-1} cm^{-1}, respectively) to be obtained by measuring the absorbance of these samples at 280 nm in 50 mM NH_4COOH, pH 6.3. Absorption at 280 nm was used to quantify the purified proteins used in kinetic studies. Otherwise, protein concentrations were determined by the Bradford assay (Bradford, 1976).

Trypsin digestion

Purified XEGIP (~400 μg) was dissolved in 50 μL of reducing buffer (8M urea, 50 mM Tris-HCl, pH 7.5) and 5 μl of 0.5 M DTT was added. The mixture was incubated at 50 °C for 15 min. Iodoacetamide (5 μL of 0.1 M in reducing buffer, freshly made) was then added and the reaction was incubated in the dark for 15 min. A Microcon 3K filter (Millipore, Bedford, MA, USA) was used to replace the reducing buffer with 50 mM NH_4HCO_3, pH ~8.0, final volume approximately 150 μL. After adding 5 μL trypsin

(Sigma sequencing grade 1.0 μg/μL), the reaction was incubated at 37 °C in a water bath. The progress of the reaction was monitored by MALDI-MS until no further change was observed. The resulting peptides were vacuum dried and dissolved in 200 μL 0.1% TFA for HPLC.

HPLC separation of trypsin-generated peptides

Peptides were separated on a Phenomenex Prodigy ODS (5 μm particles, 250 × 2.0 mm, 100 Å) or a Supelco Discovery C-18 (5 μm particles, 150 × 2.1 mm) column. Peptides were eluted from the columns at a flow rate of 0.2 mL/min by the following gradient: (0-10% buffer B over 10 min., then 10-60% buffer B over 90 min., then 60-100% buffer B over 20 min., Buffer A: 0.1% TFA (Sequanal Grade, Pierce, Rockford, IL, USA) in water. Buffer B: 0.085% TFA in 80% acetonitrile.) Peptides were detected by monitoring the absorbance at 214 nm. Fractions were manually collected and assayed by MALDI-MS. Selected pure peptides were subject to MS-MS sequencing.

Mass spectrometry

MALDI-TOF mass spectrometry of xyloglucan oligosaccharides was performed as previously described (Pauly *et al.*, 2001). MALDI-TOF mass spectrometry of peptides and proteins was performed according to standard procedures (Jiménez, 1995). Mass spectra of peptides were also recorded with a Q-TOF hybrid mass spectrometer (Q-TOF2, Micromass, UK) equipped with an electrospray source (Z-spray) operated in the positive mode. The HPLC separated tryptic peptides were reconstituted in 50% methanol with 1% formic acid and infused into the Q-TOF mass spectrometer with a syringe pump (Harvard

Apparatus Cambridge, MA, USA) at a flow rate of 5μL/min. A potential of 3kV was applied to the capillary, and nitrogen was employed as both the drying and nebulization gas. [Glu]-Fibrinopeptide B was used as the calibration standard in the positive mode. For MS analysis, quadrupole Q1 was operated in the RF-only mode with all ions transmitted into the pusher region of the TOF analyzer and the MS spectrum was recorded from m/z 400-2000 with a 1-s integration time. For MS/MS spectra, the transmission window of quadrupole Q1 was set up to about 3 mass units and the selected precursor ions were allowed to fragment in the hexapole collision cell. The collision energies (40 - 55 eV) were optimized for maximized product ion yield and argon was used as collision gas. The MS/MS data were integrated over a period of 1- 2 minutes for each precursor ion.

Cloning the XEGIP cDNA

XEGIP amino acid sequence fragments, derived from Edman sequencing of the native protein, were used to search public databases and several hits were aligned using DNASIS (Hitachi Software, San Francisco, CA, USA). Conserved regions near 5' and 3' ends were used to design degenerate primers (Sense: 5'-TGG GTI GAY TGY GAY MAR RRI TA-3'; antisense: 5'-TCI ADY TGR WRI CCI CCD ATI ACI A-3'). First strand cDNAs were obtained by reverse transcription (M-MMLV reverse transcriptase; Invitrogen, Carlsbad, CA, USA) of mRNA extracted from suspension-cultured tomato cells (Plant RNeasy Kit, Qiagen, Valencia, CA, USA) and were used as PCR templates. PCR was performed using *Taq* polymerase (Fisher, Pittsburgh, PA, USA) on a Bio-Rad iCycler thermal cycler under the following conditions: 95 °C 4 min, 40 cycles (95 °C 30 s, 43-50 °C 50 s, 72 °C 1 min), 72 °C 7 min. PCR products were analyzed by electrophoresis

on 1% agarose gels. Bands with the expected size were recovered with the Qiagen gel extraction kit. The purified fragments were cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and competent TOP 10 cells provided with the kit were transformed with the vector according to manufacturer's instructions. Cell colonies that grew on ampicillin plates were selected and amplified. Plasmids were purified using the Qiagen Miniprep kit, digested with *EcolRI* and analyzed by electrophoresis. Those with an insert of the predicted size were sequenced at the Molecular Genetics Instrumentation Facilities of the University of Georgia.

One fragment cloned by the above method showed sequence overlap with one Tentative Consensus sequence (TC85129) and one EST singleton (EST258116) in the TIGR Gene Indices database (Quackenbush *et al.*, 2000; Quackenbush *et al.*, 2001). Assembly of these three sequences *in silico* resulted in a sequence that contained a full-length open reading frame. New sets of primers were synthesized accordingly to clone the full-length cDNA (Sense: 5'-CGT GCC GAT TAA ATC ATG GCT TCT-3'; antisense: 5'-ATT CAA TAC ATG AAT TAA AAC AAC-3'). The same templates were used as described above. PCR was performed with the high fidelity *Taq* polymerase AccuTaq (Sigma, St Louis, MO, USA) with the following conditions: 95 °C 4min, 40 cycles (95 °C 30 s, 48 °C 50 s, 72 °C 1 min), 72 °C 7 min. The resulting PCR fragments were cloned and sequenced as before.

DNA gel blot analysis

Genomic DNA was extracted from young tomato leaves (cv. Ailsa Craig) as described in (Murray and Thompson, 1980) and 10 μg aliquots were digested with the

appropriate restriction enzymes, fractionated on agarose gels and transferred to nylon membranes as previously described (Rose *et al.*, 1997). The membranes were hybridized at 42 °C in 50% formamide, 6 X SSPE, 0.5 % SDS, 5 X Denhardt's solution and 100 mg mL^{-1} sonicated salmon sperm DNA, with radiolabeled DNA probes corresponding to either the full-length XEGIP cDNA, or an 819 bp DNA fragment obtained by digestion of the XEGIP cDNA with *Pvu II*. The probes were synthesized with the Ready-To-Go DNA Labelling Beads (-dCTP) Kit (Amersham Biosciences, Piscataway, NJ, USA) using ^{32}P (dCTP), and purified with ProbeQant G-50 Micro Columns (Amersham Biosciences). Following hybridization, the membranes were washed three times in 5 X SSC, 1 % (w/v) SDS at 42 °C for 15 min followed by three washes in 0.2 X SSC; 0.5% SDS at 65°C for 20 min, and then exposed to film.

RNA gel blot analysis

RNA was extracted from tomato vegetative tissues and from a series of fruit developmental stages (ovaries, expanding stages I-III, immature green, mature green, breaker, turning, pink, light red and red ripe) as outlined in Rose *et al.* (1996) and Catalá *et al.* (2000). Total RNA (15 μg per lane) samples were subjected to electrophoresis on 1.2% agarose, 10% formaldehyde gels and transferred to Hybond-N membrane (Amersham Biosciences) as previously described (Rose *et al.*, 1997). Blots were hybridized and washed as described for the DNA blot analysis, but with the high stringency washes at 65°C in 0.5 X SSC, 0.5% SDS.

Accession numbers

The accession number and homology (percent amino acid sequence identity to XEGIP) for each gene and protein described in this article is given below. AY155579 (Tomato XEGIP, 100%); D14550 (Carrot EDGP, 61%); AJ297490 (Conglutin γ from *Lupinus albus*, 32%); P13917 (Soybean Bg7S, 32%); AAK59531 (Arabidopsis-1, 62%); AAC72120 (Arabidopsis-2, 57%); BAB89707 (Rice-1, 27%), BAB89708 (Rice-2, 25%); BAB89703 (Rice-3, 25%); BAB89705 (Rice-4, 27%); BAB89709 (Rice-5, 29%). The TIGR Gene Indices Tentative Consensus numbers and homologies are TC1987 (Lotus-1,46%); TC2190 (Lotus-2, 37%); TC43357 (Medicago-1, 63%), TC43356 (Medicago-2, 58%); TC43854 (Medicago-3, 34%), TC40118 (Medicago-4, 29%); TC44724 (Medicago-5, 40%); TC51626 (Medicago-6, 33%); TC51629 (Medicago-7, 33%); TC24971 (Potato-1, 90%); TC80165 (Soybean-1, 33%); TC80166 (Soybean-2, 62%); TC110649 (Soybean-3, 63%); TC100743 (Soybean-4, 33%), TC102507 (Tomato-2, 22%); TC99653 (Tomato-3, 39%); TC108379 (Tomato-4, 36%); TC103252 (Tomato-5, 51%); TC102514 (Tomato-6, 35%).

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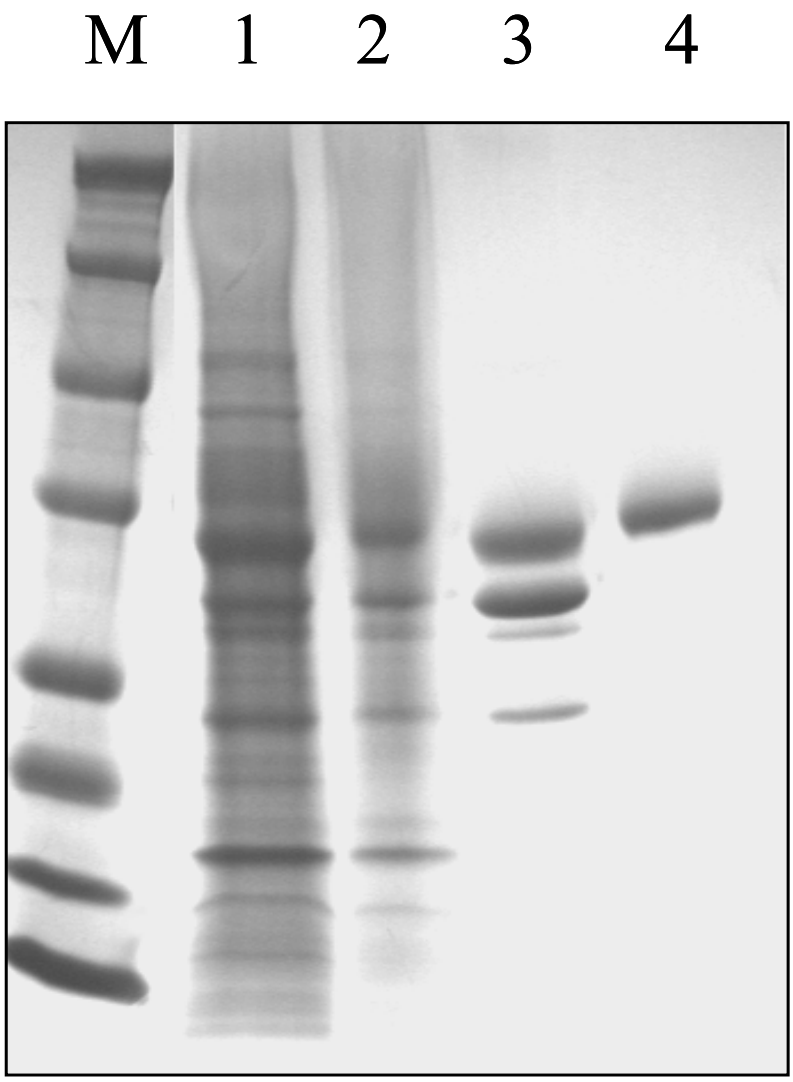


Figure 2.1. SDS-PAGE of fractions obtained during purification of XEGIP (see **Experimental Procedures** for details). Lane M, molecular weight standards (208 kDa, 115 kDa, 79 kDa, 49.5 kDa, 35 kDa, 28 kDa, 20 kDa, 7 kDa). Lane 1, ethanol precipitated material from the medium of suspension-cultured tomato cells. Lane 2, non-bound fraction from the Q-Sepharose anion-exchange column. Lane 3, XEGIP-active fractions that were eluted from the HiTrap-S cation-exchange column during a pH and salt gradient. Lane 4, XEGIP-active fraction from the Superdex-75 column.

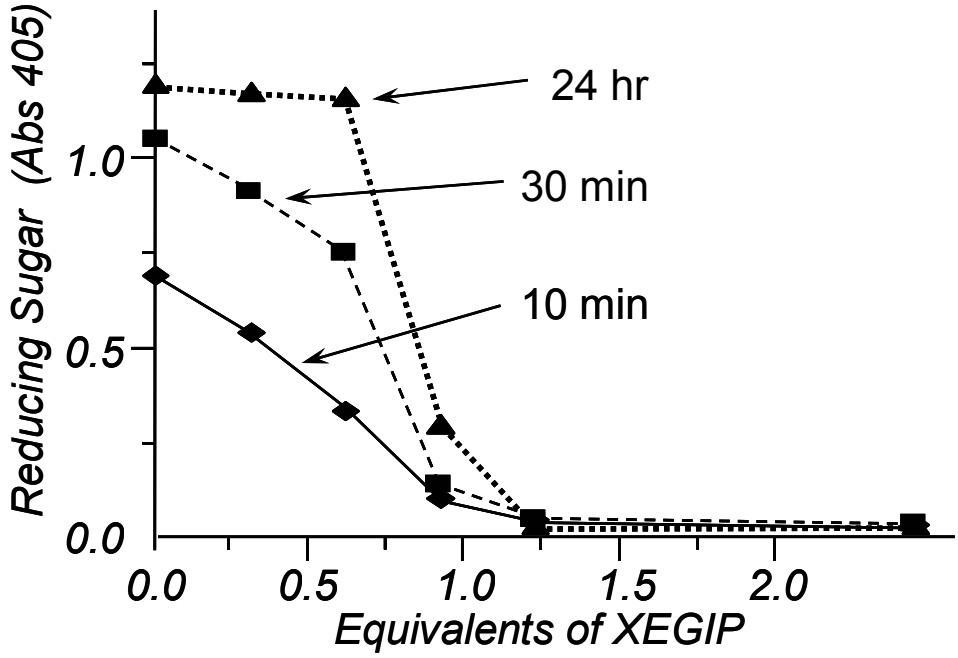


Figure 2.2. Inhibition of XEG activity by increasing amounts of XEGIP. The response of the PAHBAH assay for reducing sugars (see **Experimental Procedures**), which corresponds to the amount of the hydrolysis product generated by XEG, is plotted *vs.* the amount of XEGIP. The linear range of the assay extends to an absorption of about 0.75. Data points with higher absorption values underestimate the amount of reaction product. The amount of XEGIP required to completely inhibit XEG activity is independent of the reaction time. When slightly less than one equivalent of XEGIP is added, significant XEG-catalyzed hydrolysis is detected when the reaction time is extended, consistent with a non-proteolytic mechanism of inhibition.

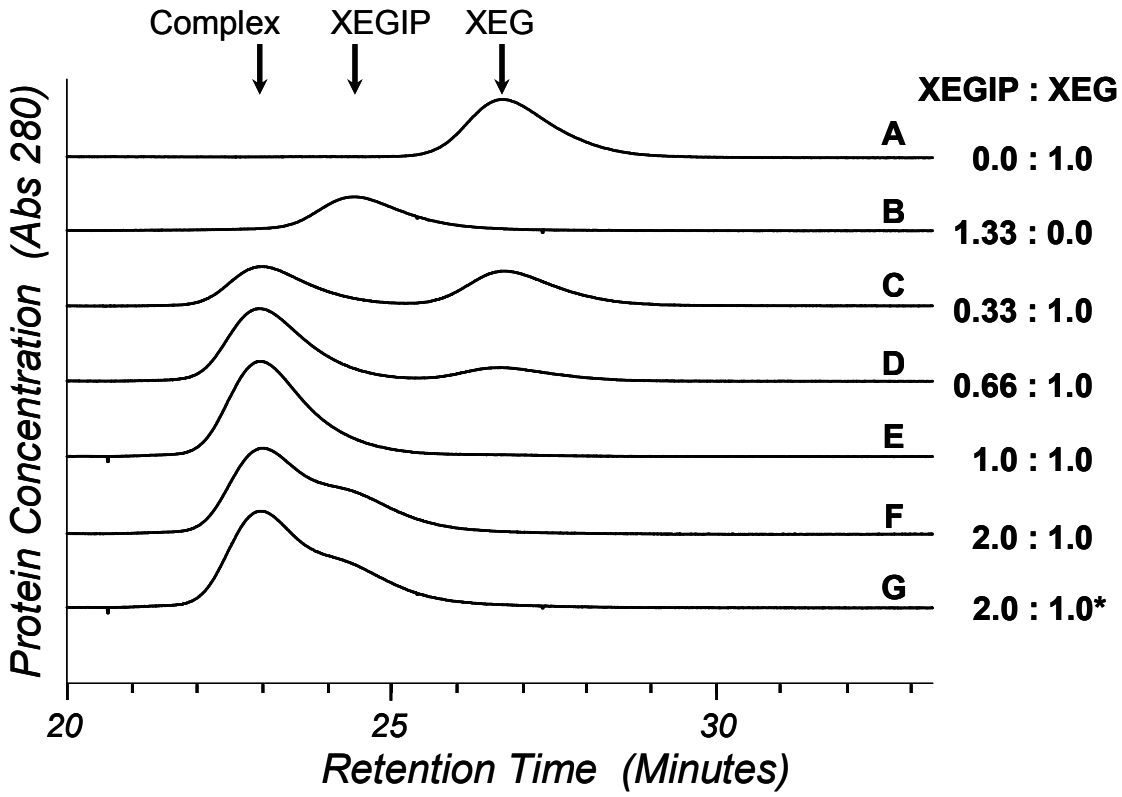


Figure 2.3. Interaction of XEG and XEGIP as determined by SEC on Superdex-75. Pure XEG (A), pure XEGIP (B) and mixtures of the two with varying ratios (C-F) were chromatographed. Increasing the XEGIP:XEG ratio resulted in a decrease in the concentration of free XEG and the appearance of an XEG-XEGIP complex (C-F). When the XEGIP:XEG ratio reached 1:1, the XEG peak completely disappeared (E). When sufficient XEGIP was added, the XEGIP:XEG ratio became greater than 1 (F) and a free XEGIP peak appeared as a shoulder on the XEG-XEGIP complex peak. Profile G is a computer-generated simulation (*) obtained by linear combination of profiles B (free XEGIP) and E (the saturated complex), taking into account the absolute amount of protein used to obtain each of these profiles (0.75×B+1.0×E). This simulated profile should be identical to profile F if XEG and XEGIP combine to form a 1:1 complex. The agreement of profiles F and G confirms the identification of free XEGIP as a shoulder on the complex peak and supports the hypothesis of a tightly bound 1:1 complex.

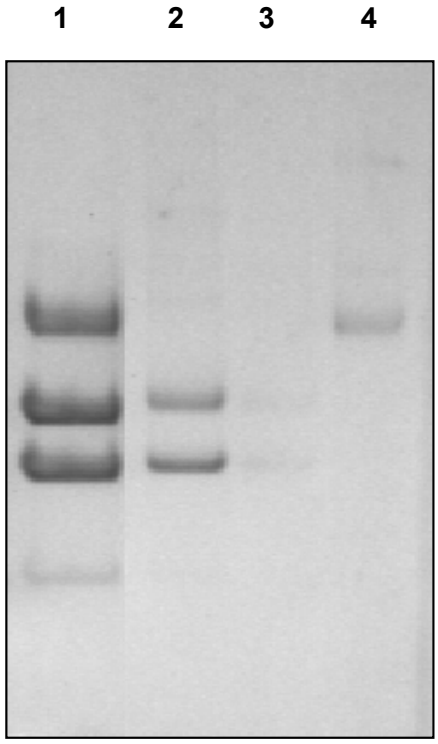


Figure 2.4. SDS-PAGE of affinity chromatography fractions. Lane 1, cationic proteins prepared by applying the material obtained by ethanol precipitation of tomato-cell culture medium (see **Experimental Procedures**) to a Hi-Trap S cation-exchange column, washing the column with starting buffer, and then eluting with starting buffer containing 2M NaCl. Lane 2, non-bound fraction obtained by applying cationic proteins (shown in Lane 1) to an XEG column and washing with starting buffer. Lane 3, proteins that were eluted from the loaded XEG column by starting buffer containing 2 M NaCl. Lane 4, XEG-bound proteins that were eluted from the XEG column with 2M imidazole, pH 7.

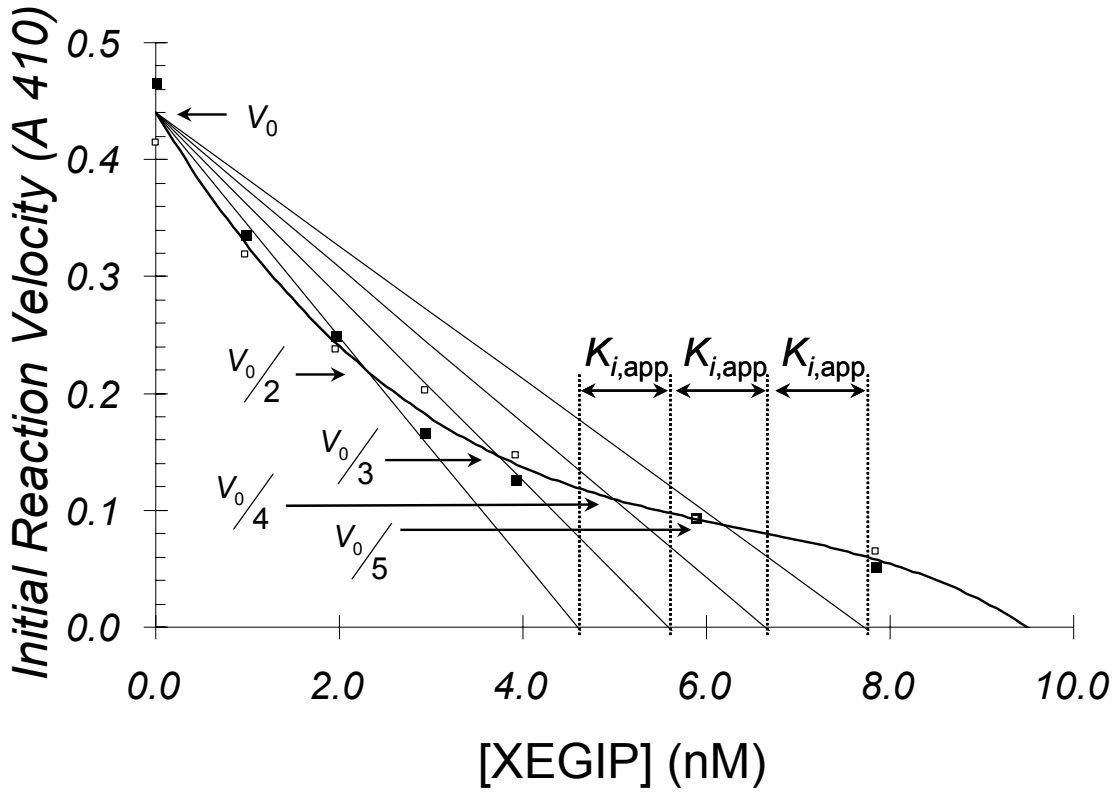
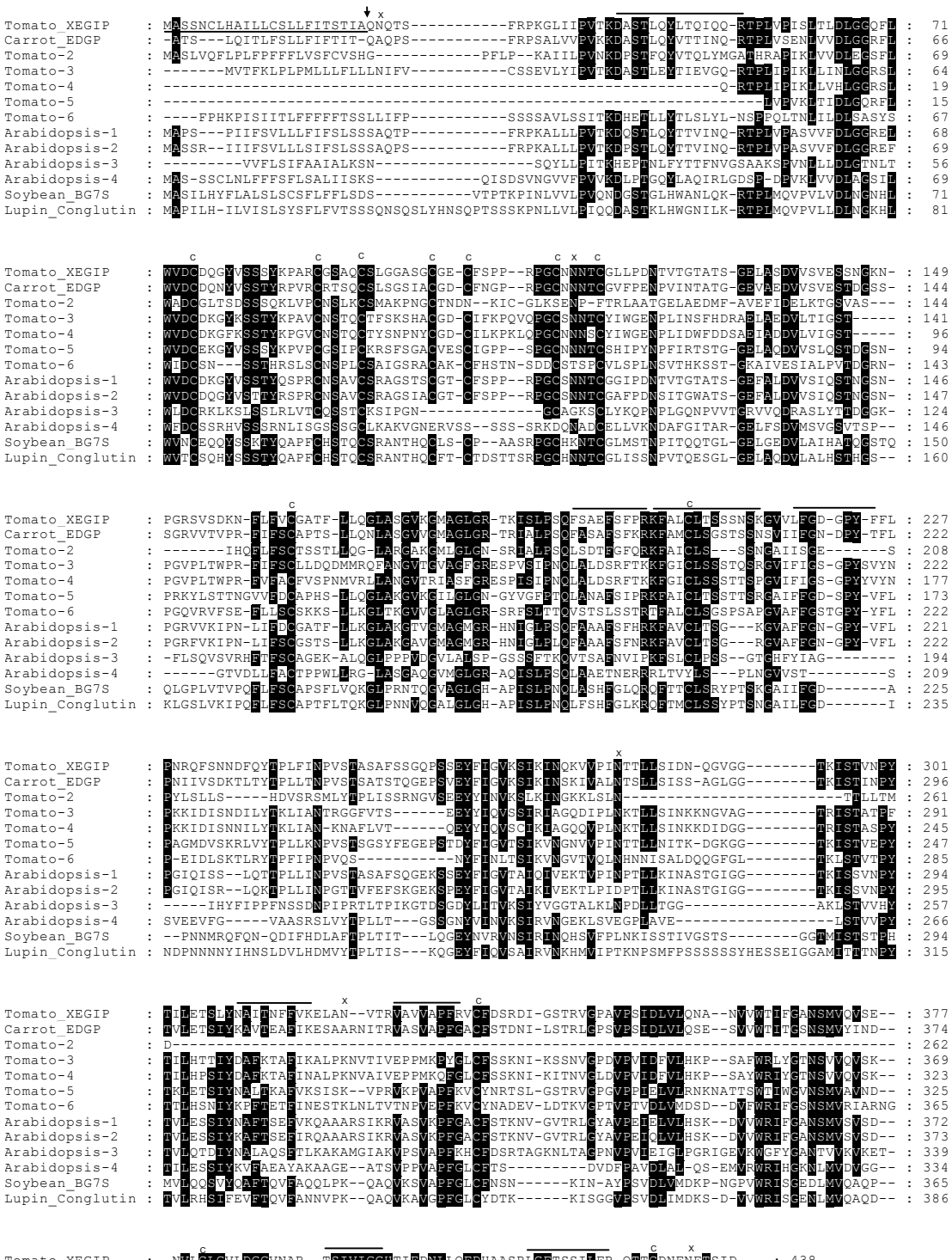


Figure 2.5. Determination of *K*i for the interaction of XEG with XEGIP (Segel, 1975). The initial rate of the XEG-catalyzed depolymerization of tamarind xyloglucan (4 mg/mL) was determined by the reducing sugar assay (*A* 410 nm) and plotted *vs.* the concentration of XEGIP. Straight lines are drawn connecting the intersection of the resulting curve with the *y*-axis (i.e., at *V*0) to points along the curve whose *y* value equals *V*0/*n*, where *n* is an integer (2, 3, 4, 5, etc.) These diagonal lines are extended so as to cross the *x*-axis, and the distance between the *x*-intercepts corresponds to the value of *K*i,app = *K*i(1 + [S]/*K*m). The data shown indicates that *K*i,app for the XEGIP:XEG interaction is 1.0 nM. Solving for *K*i = *K*i,app / (1 + [S]/*K*m) and using the previously determined value of 3.6 mg/mL for *K*m of tamarind xyloglucan as a substrate for XEG (Pauly *et al.*, 1999) resulted in a *K*i value of 0.5 nM.

Figure 2.6. Alignment of the amino acid sequence of XEGIP with those of structurally-related proteins: BG7S - basic globulin 7S protein from soybean; EDGP - extracellular dermal glycoprotein from carrot; Conglutin - conglutin γ protein from *Lupinus albus*. Protein sequences deduced from tomato and *Arabidopsis* genes are also included. ClustalW (http://www.ebi.ac.uk/clustalw/) and GeneDoc (http://www.psc.edu/biomed/genedoc/) were used, respectively, to align and present the sequences. Identical residues are highlighted in black. Conserved cysteines are marked with C. The predicted signal peptide of XEGIP is underlined and the cleavage site is marked by an arrow. Potential N-glycosylation sites are marked with X. Lines above XEGIP sequence indicate the tryptic fragments whose sequences were verified by Q-TOF mass spectroscopy.



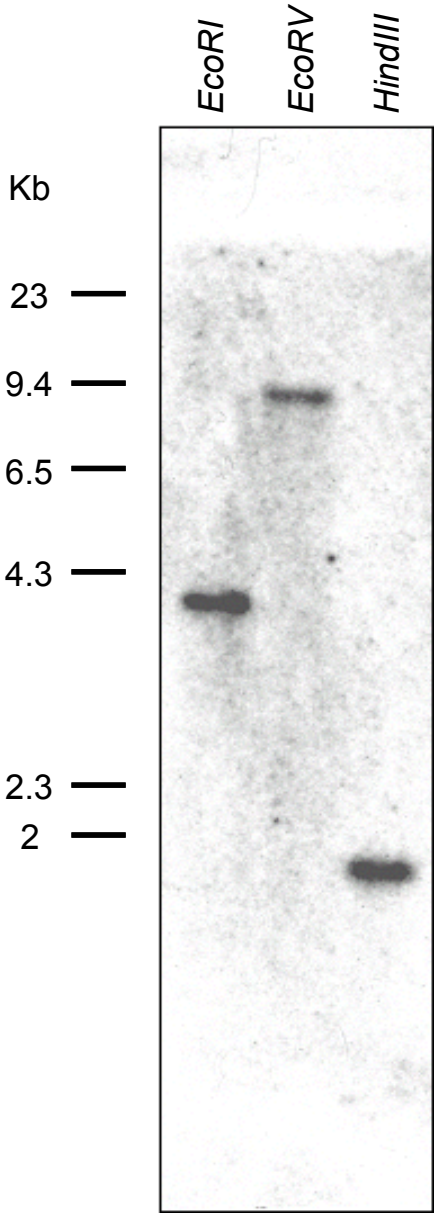


Figure 2.7. Genomic DNA analysis of *XEGIP*. Genomic DNA (10 μg per lane) was digested with the indicated restriction enzymes and the DNA gel blot hybridized with the full-length *XEGIP* cDNA probe and washed with 0.2 x SSC at 65 °C. Molecular weight markers are indicated in kb.

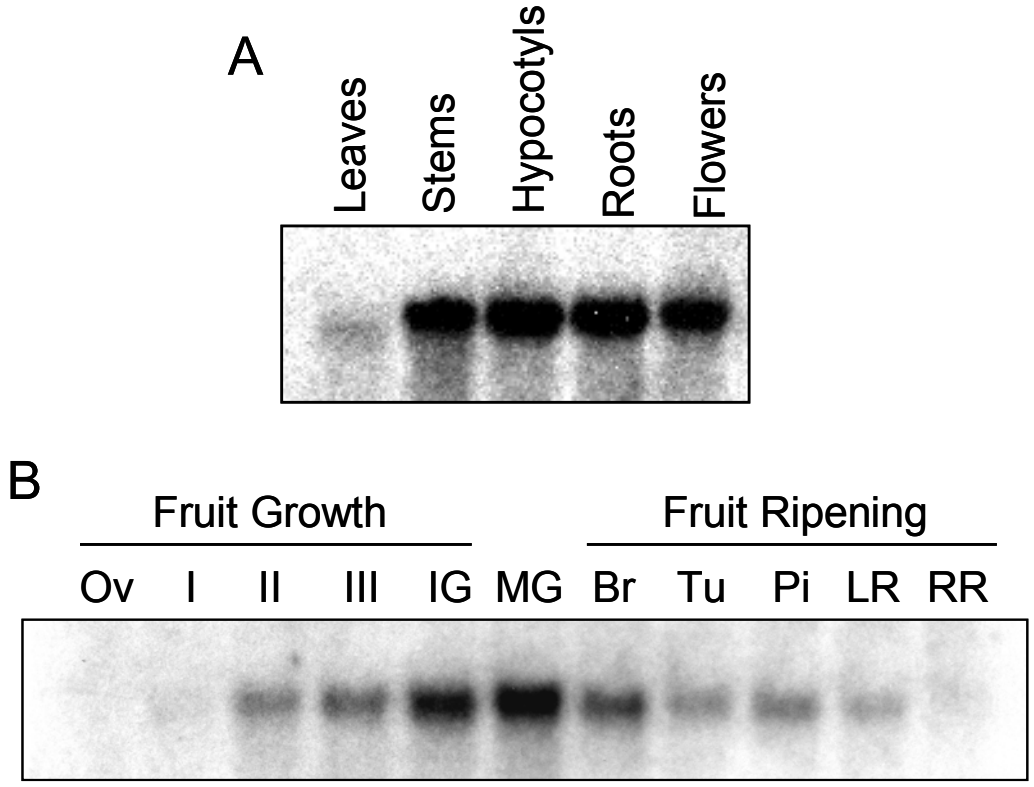


Figure 2.8. Analysis of *XEGIP* mRNA abundance in tomato tissues. Total RNA gel blot analysis (15 μg RNA per lane) of *XEGIP* expression in (A) vegetative tissues and flowers; (B) tomato fruit spanning a development series. (Ov, ovary; I-III, expansion stages I-III; IG, immature green, MG, mature green; Br, breaker; Tu, turning; Pi, pink; LR, light red; RR, red ripe).

CHAPTER 3

BIOCHEMICAL PROPERTIES OF XEGIP AND XEGIP-LIKE PROTIENS: HETEROLOGOUS EXPRESSION OF XEGIP, AN IMMUNOAFFINITY BASED SCREENING METHOD FOR IDENTIFYING PROTEINS THAT INTERACT WITH XEGIP AND A CARROT ORTHOLOG OF XEGIP

Introduction

This chapter addresses three topics. The first topic describes the heterologous expression of recombinant XEGIP in *Escherichia coli.* XEGIP represents the most recently discovered class of plant proteins that inhibit microbial cell wall degrading enzymes (Chapter 2). The interactions of such cell wall degrading enzymes with their inhibitors are determined by the molecular structures of these proteins. For example, the interactions of polygalacturonase-inhibiting proteins (PGIPs) with polygalacturonases (PGs) show molecular specificity: PGIPs from different sources vary in their inhibitory ability against a single PG while a single PGIP differs in its ability to inhibit different PGs. Studies have identified specific amino acid residues that contribute to PG binding affinity and ligand specificity (Leckie et al., 1999), demonstrating that structural variation of PGIP affects the PG-PGIP interaction. Such structure-function relationships may also exist in the interaction of XEGIP with *endo*glucanases. An efficient and effective heterologous expression system capable of generating large amounts of soluble XEGIP protein would facilitate similar structure-function relationship studies of XEGIP-like proteins. *Escherichia coli* has been used extensively for heterologous expression of recombinant proteins, as *E. coli* grows rapidly to high density on low cost substrates, the genetics are well characterized, and large numbers of cloning vectors and strains are commercially available. Attempts to use *E. coli* to express XEGIP, purification of the recombinant proteins, and efforts to renature these proteins are described.

The second topic of this chapter describes the development of an immunoaffinity based technique to screen XEGIP-interacting proteins in microbial cell wall degrading enzyme mixtures. The interactions of XEGIP-like proteins with XEG-like proteins may widely exist in plant-microbe interactions. It is necessary to identify more interacting pairs to further study the roles played by these proteins. A simple screening method was developed and was used successfully to identify a XEGIP-binding Family 12 *endo*glucanase in *Aspergillus oryzae*. The method described may also be applied to screen for other protein-protein interactions.

The third topic describes the purification of an XEG inhibitor protein from carrot culture medium and its identification as EDGP, which has been proposed as a plant disease resistance related gene because of its localization *in vivo* (dermal tissues) and its increased mRNA level upon wounding (Satoh and Fujii, 1988; Satoh et al., 1992). EDGP shares 62% sequence identity with XEGIP and has been hypothesized to have the same XEG-inhibiting activity as XEGIP (Chapter 2). Preliminary experiments indicate that ethanol precipitated material from the medium of suspension-cultured carrot cells contains a heat-sensitive inhibitor of XEG (Chapter 2). The results in this chapter confirmed that, like XEGIP, purified EDGP inhibits XEG by forming a complex with XEG.

Table 3.1. Constructions of XEGIP expression vectors

Constructs	Primers	Expected recombinant protein constructs
1	Forward: qq22 Reverse: qq23	w/ thioredoxin, w/ his
2	Forward:qq24 Reverse: qq23	w/o thio, w/ his
3	Forward:qq24 Reverse: qq25	w/o thioredoxin, w/o his
4	Forward: qq22 Reverse: qq25	w/ thioredoxin, w/o his tag

Results

Only constructs with a thioredoxin leading sequence are effective in Thio-XEGIP fusion protein expression

Four different XEGIP-containing pBAD/Thio expression vectors (Figure 3.1 and Table 3.1) were constructed and used to transform TOP 10 *E. coli*. cells. Protein expression in the resulting *E. coli*. strains was induced by addition of arabinose, and cells were harvested by centrifugation. Total proteins (extracted from intact cells directly by Laemmli buffer), soluble (solublized in lysis buffer that disrupts cells) and insoluble protein (insoluble in lysis buffer but soluble in Laemmli buffer) were analyzed by SDS-PAGE and Western blotting assays (Figures 3.2 and 3.3). Without induction, there was no detectable XEGIP expression due to tight control of the *ara*BAD promoter (Figure 3.2, lane 4). After arabinose induction, cells transformed with constructs 1 and 4 were able to express the recombinant XEGIP. However, the expressed proteins were aggregated into inclusion bodies (Figures 3.3). Polyclonal antibodies against native XEGIP were used to detect expressed recombinant XEGIP in Western blots (Figure 3.2B). No XEGIP was

detected in constructs 2 and 3, which lack a thioredoxin leading sequence. Antibodies that recognize thioredoxin and polyhistidine tags were also used for Western blotting (Figure 3.2C, D), confirming the expression of full length Thio-XEGIP fusion protein by cells transformed with construct 1(Figure 3.2D, lane 3). Some anti-XEGIP and anti-thioredoxin positive bands with a molecular weight that is lower than predicted for the Thio-XEGIP fusion protein were also detected in the Western blots (Figure 3.2 B and C), suggesting incomplete protein synthesis or protein degradation.

Purification of recombinant His tagged protein

Inclusion bodies containing recombinant Thio-XEGIP fusion protein (Figure 3.3, Lane 1) were first isolated using a nonionic detergent based bacterial cell lysis reagent (CelLytic™, Sigma) and then solublized with 8M urea and applied to Ni-chelating column. The polyhistidine tag in construct 1 allowed the recombinant Thio-XEGIP to be purified by immobilized metal chelating affinity chromatography (Figure 3.4). On-column refolding of the recombinant protein was attempted and followed by elution with an imidazole gradient. However, the eluted protein immediately began to precipitate, suggesting that the on column refolding was not effective. Refolding by rapid dilution of urea solublized protein in refolding buffer (see **Methods**) also failed.

Immunoaffinity chromatography

An immunoaffinity chromatography based method (Figure 3.5) was developed to identify XEGIP interacting enzymes (proteins) in mixtures of cell wall hydrolases. Polyclonal antibodies against XEGIP were covalently immobilized on gel matrix (see **Methods**). Purified XEGIP was incubated with protein(s) to be screened for their ability to form a complex with XEGIP. The mixture was applied to the immunoaffinity column, allowing free XEGIP and XEGIP-containing protein complexes to bind to the antibodies. Unbound and non-specifically bound proteins were eluted with TBS buffer. The bound proteins were then eluted with low pH (2.8) buffer and assayed by SDS-PAGE. As a negative control, each protein mixture to be screened was subjected to the same procedure without adding XEGIP. XEG and XEGIP were detected in the low pH eluant when a mixture of purified XEGIP and crude XEG were subjected to the affinity chromatography procedure (Figure 3.6A and B, Lane 3), but no XEG was detected in the low pH eluant when XEG alone was applied to the affinity column (Figure 3.6A and B, Lane 2). This result indicates that the immobilized polyclonal anti-XEGIP antibodies retain their ability to selectively bind the XEG-XEGIP complex.

Previous experiments, using immobilized XEGIP as an affinity matrix, suggested that Sanzyme®, a commercial mixture of plant cell wall degrading enzymes, contains at least one protein that binds to XEGIP (data not shown). The immunoaffinity method described above was used to test this hypothesis. Purified XEGIP was incubated with Sanzyme® and then the mixture was applied to the immunoaffinity column. Coomassie

blue stained SDS-PAGE of the eluted complex did not reveal any interacting band (Figure 3.6A, Lane 5). However, after proteins in the gel were visualized by silver staining, an additional band appeared in the sample lane (Figure 3.6B, Lane 5) compared to the control lane (Figure 3.6B, Lane 4), suggesting the presence of a very low abundance ~25 kDa protein that interacts with XEGIP. Accordingly, in another experiment, approximately 100 times as much Sanzyme® was mixed with the same amount of XEGIP before application to the immunoaffinity column, which was then washed thoroughly to remove any nonspecifically bound protein. Coomassie blue staining of the SDS-PAGE gel of the fraction subsequently eluted with low pH buffer indicated that a 25 kDa protein in the Sanzyme® preparation specifically binds to XEGIP (Figure 3.6C, Lane 1).

Identification of the interacting protein in Sanzyme® mixture

The XEGIP-interacting protein was cut out of the gel, digested with trypsin, and sent for MS-MS sequencing. The sequence of one of the tryptic fragments, VASSTTEDFSADL(or I), is nearly identical to a peptide sequence, VASSTTESFSADL, from another Family 12 enzyme found in *Emericella desertoruma* (Goedegebuur et al., 2002) and closely related to a sequence in XEG (VADSTTESFSGDL). These data suggest that a Family 12 carbohydrate hydrolase, closely related to XEG, is present in Sanzyme®, and that this hydrolase forms a complex with XEGIP. This indicates that XEG-like protein also exists in *Aspergillus oryzae* and can form a complex with XEGIP.

Purification of EDGP and its identification by peptide fingerprinting

In preliminary studies, ethanol precipitated materials from carrot suspension culture contain factors that can inhibit XEG. It has been reported that a large amount of EDGP, an epidermal glycoprotein, is present in carrot cell culture (Satoh et al., 1986; Satoh and Fujii, 1988; Satoh et al., 1992). EDGP shares 62% sequence identity with XEGIP, so the inhibitory factor in carrot medium is likely to be EDGP. To test this hypothesis, a suspension-culture of carrot cells was established, starting with callus from hypocotyl tissue. The same procedure used to isolate XEGIP from tomato cell suspension culture (Chapter 2) was applied to purify the inhibitory factor from culture medium harvested ten days after inoculation. This involved cation exchange (Figure 3.7) and size exclusion chromatography (SEC) (Figure 3.8). SDS-PAGE analysis (Figure 3.9, lane 3) revealed one major protein and a very minor protein in the SEC fractions with XEG-inhibiting activity. The major band has an apparent molecular weight of ~55 KDa, in good agreement with the reported molecular weight of EDGP (57 KDa). The major protein band was cut out of the gel and digested with trypsin. The molecular weights of the resulting tryptic peptides are in good agreement with those of *in silico* generated peptides of EDGP (Figure 3.10).

EDGP also binds to XEG to form a complex

An XEG shift experiment using size exclusion chromatography was conducted as described (Chapter 2). Results showed that like XEGIP, EDGP inhibits the enzyme

activity by forming a complex with XEG (Figure 3.11). That is, when XEG and EDGP were mixed and applied to the SEC column, a new peak, corresponding to the XEG-EDGP complex, was observed and a peaks corresponding to free XEG and EDGP were diminished in size.

Discussion

Heterologous XEGIP expression in E. coli

E. coli transformed with the expression vector constructs used in this research failed to express XEGIP in an active, soluble form. Two constructs that lack a thioredoxin leading sequence did not lead to expression of XEGIP at any level. Constructs with a thioredoxin fusion tag lead to expression of an insoluble Thio-XEGIP fusion protein which was found in inclusion bodies. Thioredoxin is a highly soluble 11.7 kDa protein found in yeast, plants, mammals and bacteria. When over expressed in *E. coli*, thioredoxin can accumulate up to 40% of total cellular proteins and still remains soluble. Fusion to thioredoxin fusion protein has been widely used to increase translation efficiency and solubility of eukaryotic proteins expressed in *E. coli* (LaVallie et al., 1993). In our studies, the thioredoxin tag did not prevent the formation of inclusion bodies but did increase the expression efficiency of the XEGIP gene. Apparently, the thioredoxin leader sequence may help in: 1) initiation of transcription of the fusion gene, 2) initiation of translation of the gene, and/or, 3) stabilizing and preventing degradation of the translation product (LaVallie et al., 1993).

The difficulty encountered in heterologous expression of XEGIP is not unusual. Such cell wall hydrolyzing enzyme inhibitors have been notoriously difficult to actively express in *E. coli*. or yeast (De Lorenzo et al., 2001). Only one inhibitory protein, xylanase inhibitor protein (XIP) has been expressed actively in *E. coli* (Elliott et al., 2002). Active PGIP has been successfully expressed only in transgenic plants.

The difficulty in expressing active XEGIP may arise from the presence of 12 cysteines in the mature protein, resulting in up to 6 disulfide bonds in properly folded, active protein. However, appropriate conditions for the formation of these disulfide bonds may not occur in the reducing environment of the *E. coli*. cytoplasm. Such disulfide bonds are often important to maintain specific three dimensional structures that are necessary for a protein's activity.

EDGP

It has been proposed that EDGP is a defense related protein because of its abundance in tissue culture, dermal localization and increased mRNA level upon wounding (Satoh et al., 1992). However, the molecular function has not previously been established for EDGP. Since EDGP shares 62% identity with XEGIP, it is not unexpected that EDGP is also able to inhibit XEG, as demonstrated here. XEGIP-like proteins, such as EDGP, are widely distributed in the plant kingdom. The ability of EDGP to inhibit XEG is consistent with the hypothesis that XEGIP-like proteins may play a role in plant

defense against pathogens by inhibiting microbial enzymes that hydrolyze components of the plant cell wall.

Screening unknown interacting proteins of XEGIP by immunoaffinity chromatography

A method to screen for and purify proteins that interact with XEGIP was developed based on immunoaffinity chromatography. When applied to Sanzyme®, a commercial enzyme mixture prepared from culture filtrates of *Aspergillus oryzae*, the method resulted in the identification of a distinct Family 12 cell wall hydrolase that interacts with XEGIP. Since this enzyme constitutes a very minor amount of the mixture, the initial comparison of the sample with the control showed a significant but very weak band. To solve this problem, XEGIP was mixed with large amount of Sanzyme® before application onto the antibody column. The Family 12 enzyme was affinity purified by binding to XEGIP to form a complex which bound to the immobilized antibody. This enrichment process made it possible to unambiguously identify the XEGIP-interacting protein by SDS-PAGE (Figure 3.6C). This immunoaffinity based screening method provides not only an effective way to detect proteins that bind to XEGIP, but also a simple method to purify them. In the process, neither of the two proteins involved is chemically modified so they can interact in their native forms. The complex formed can be eluted from immobilized antibodies and further studied. The immobilized antibody column can be reconditioned and reused.

Materials and Methods

Expression vector constructs

The expression vectors were constructed as shown in Figure 3.1 and Table 3.1. Primers used in PCR were:

qq22, CAAAATCAAACTTCATTCCGTCCCAAAGGC;

qq23, AGCAATTGAAGTGAAATTAAAATTGGCACA;

qq24, ATACCATGGCTCAAAATCAAACTTCATTCC;

qq25, ATTCAATACATGAATTAAAACAAC.

PCRs were performed on a Bio-Rad iCycler thermal cycler under the following conditions: 95 °C 4 min, 30 cycles (95 °C 30 s, 50 °C 50 s, 72 °C 1 min), 72 °C 7 min. The reactants include 5 µl of a 10 uM solution of each primer (forward and reverse), 5 µl 10X PCR buffer, 1µl of 10 mM dNTP, 21 µl H_2O, 0.5 µl AccuTaq polymerase (Sigma), and 12.5 µl tomato cDNA template (total 50µl) prepared as described in Chapter 2. PCR products were analyzed by electrophoresis on 1% agarose gels. Bands with the expected size were recovered with the Qiagen gel extraction kit and were ligated into the pBAD/TOPO® ThioFusion™ vector. (TOPO cloning reaction, Invitrogen, Carlsbad, CA; see manufacturer manual for details) The resulting vectors were used to transform chemically competent *E.coli*. TOP 10 cells. Transformed cells were grown overnight on ampicilin plates. Colonies were picked and grown in LB medium overnight; cracking gel

analysis was used to decide which colonies contained the correctt inserts. For constructs 1 and 4, restriction enzyme *Acc* I was used to cut the plasmid to determine the direction of insertion (desired insertion direction should generate 3622 nt, 2271 nt fragments; the opposite direction generates two 2900 fragments). Plasmids with inserts in the desired direction were sent for sequencing and the corresponding TOP 10 cell lines with correct constructs were used for subsequent expression experiments. For insertional direction determination of plasmids 2 and 3, *Nco* I was used (desired direction, 369, 5524; opposite direction, 1808, 4085). The restriction digestion conditions were: for 4 µl plasmid DNA, 0.5 µl *Acc* I (or *Nco* I) was added to 2 µl NEB buffer 4 with 13.5 µl H_2O.

To construct expression vector 2 and 3, the corresponding plasmids with correct insertional direction were digested with *Nco* I as described above and the restricted fragments were extracted (Qiagen Gel Extraction), and religated at *Nco* I sites. This process removed the thioredoxin gene sequence engineered in the commercial vector. To 8 µl of recovered linear restriction fragment, 2 µl 5X ligase buffer and 2 µl T4 ligase (NEB) were added. The ligation reaction was incubated at 15 C° overnight. Two µl of ligated plasmid were used to transform TOP 10 cells. Plasmids were prepared and screened as described above and cell lines with correct plasmids were used in protein expression experiments.

Induction of expression by arabinose

The transformed TOP 10 *E.coli*. cells were grown in LB medium containing 100 μg/ml ampicillin to OD ~0.5 at 37 C°. Protein expression was induced by adding 0.2% arabinose solution (w/v %) to a final concentration of 0.002%. The cells were harvested 4 hours after induction. *E. coli*. cells were disrupted by CellLytic^{TM} B bacterial cell lysis extraction reagent (Sigma) and both soluble and insoluble proteins (solubilized in Laemmli sample buffer) were subjected to SDS-PAGE assay and Western blotting using anti-XEGIP, anti-thioredoxin, and anti-his tag antibodies respectively.

Purification and attempt to renature recombinant Thio-XEGIP

Inclusion bodies from 100 ml harvested *E. coli*. cells were isolated using B-CLEAR reagents (Sigma) according to the manufacturer's instructions. The inclusion bodies were then resuspended in 6 ml binding buffer (6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0) and stirred for 30-60 min. at room temperature. Insoluble materials were removed by centrifugation and filtration. The supernatant was applied to an equilibrated Ni^{2+}-loaded HiTrap Chelating HP column (Amersherm Bioscience). The column was washed with 10 ml wash buffer (6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0) to remove any nonspecific binding. On column refolding of the bound Thio-XEGIP fusion protein was attempted by the use of a linear 6-0 M urea gradient, starting with the wash buffer and finishing with the refolding buffer (20 mM

Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0) over 60 min at a flow rate of 0.5 ml/min. The column was then eluted with elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 500 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0) gradient (0-100% in 20 min at a flow rate of 0.5 ml/min). Precipitation occurred quickly in the eluted fractions, suggesting the refolding process was not successful.

The precipitates were redisolved into 8M urea and rapidly diluted 10 times with another refolding buffer (50 mM Tris/1mM EDTA/2M non-detergent sulfobetaine (NDSB-201, Calbiochem, La Jolla, CA) /1 mM glutathione (reduced)/0.8 mM gluthathione (oxidized) by pippeting the denatured protein into the refolding buffer with constant stirring. The refolding was allowed to proceed for 24 hours at 4 C°. The solution was dialyzed against 50 mM Tris/1 mM EDTA to remove urea and NDSB, the solution was then concentrated and centrifuged, and both the supernatant and the precipitate were analyzed by SDS-PAGE. Results showed the recombinant Thio-XEGIP existed only in the precipitate, indicating that the refolding was not successful (data not shown).

Preparation of immunoaffinity column

Purified tomato XEGIP was used to raise polyclonal antibodies according to standard procedures (Coligan, 1995) at University of Georgia Monoclonal Facility. Immobilized anti-XEGIP polyclonal antibody column was made with Seize™ Primary Immnoprecipitation Kit (Pierce, Rockford, IL). The mouse serum was diluted with PBS to ~ 1mg/ml (Bradford assay). About 500 µl of AminoLink Plus Coupling Gel (50 %

slurry) was added to a microspin column inside a microfuge tube. The gel was washed twice with 0.4 ml Coupling Buffer (0.1 M sodium phophate, 0.15 M NaCl, pH 7.2) by gently resuspending the gel and then centrifuging at 80 x g (1000 RPM). About 500 µl diluted serum followed by 5 µl 5 M sodium cyanoborohydride were added to the gel. The reactants were mixed with the gel by gently inverting and the suspension was incubated overnight on an end-over-end rocker. Any free uncoupled antibody was removed by centrifugation and the gel was washed with coupling buffer. Then the gel was washed with 0.4 ml of quenching buffer (1M Tris HCl, pH 7.4) and another 0.4 ml of quenching buffer was added, followed by 4 µl of 5 M sodium cyanoborohydride. The quenching reaction was incubated for 30 min. The gel was thoroughly washed with 1M NaCl and then with binding buffer (modified Dulbecco's PBS buffer, 0.14 M NaCl, 0.008 M Na_2PO_4, 0.002 M K_3PO_4, and 0.01 M KCl, pH 7.4). The gel was stored in binding buffer until use.

Capture of protein-protein complex by immunoaffinity chromatography

Purified tomato XEGIP and protein mixtures to be screened (commercial cell wall hydrolyzing enzymes, pathogen protein extracts etc.) were dissolved or buffer-exchanged into binding buffer (Dulbecco's PBS). Approximately 2-5 µg XEGIP was added to ~0.5 ml of each protein mixture (1-5 mg/ml) respectively and incubated at room temperature for 5 min. The mixture was then applied to the immunoaffinity gel slurry and incubated for 1 hr on a rocker at room temperature. The gel was then thoroughly washed with TBS buffer (0.025 M Tris, 0.15 M NaCl, pH 7.2) to remove unbound proteins. The protein-

XEGIP complex or XEGIP bound to the antibody gel was eluted with ~ 200 µl elution buffer (pH 2.8) provided in the kit. The elution was repeated 3 times, and eluted proteins were collected in separate tubes, and immediately neutralized with 15 µl 1 M Tris, pH 9.5 buffer to prevent possible denaturation due to low pH. The immunoaffinity gel was exchanged into TBS buffer immediately after the final elution. After two additional TBS washes, 0.4 ml of binding buffer was applied to the column for storage. The protein mixtures to be screened were also subjected to the same process (without incubation with XEGIP), and the fractions eluted at low pH were used as controls (e.g. Figure 3.6A and B, Lane 4).

Identification of XEGIP-interacting components

The low pH eluted fractions (including controls) were desalted by dialysis and analyzed by SDS-PAGE. Bands of interest (which appeared in samples pre-incubated with XEGIP but were absent in the control) were cut out and digested by trypsin as follows: the cut band (5-10 mm^2) was put into a 0.5 ml centrifuge tube pierced with a syringe needle to make a hole at the bottom. The small tube was put inside a 1.5 ml centrifuge tube and centrifuged at 18,000 g until all the gel has passed through the hole into the larger tube as fine particles. The gel particles were destained by 50% acetonitrile with 0.1% TFA and dried under vacuum. Trypsin solution (16 ng/µl, 5-10 µl) in 50 mM NH_4HCO_3, pH 8.3 buffer was added, sufficient to cover the gel, and the reaction was incubated overnight at room temperature. The resulting peptides were extracted with ~10

µl buffer for 30 min with occasional 30 sec ultrasonic treatment, and analyzed by MALDI-MS and MS-MS sequencing.

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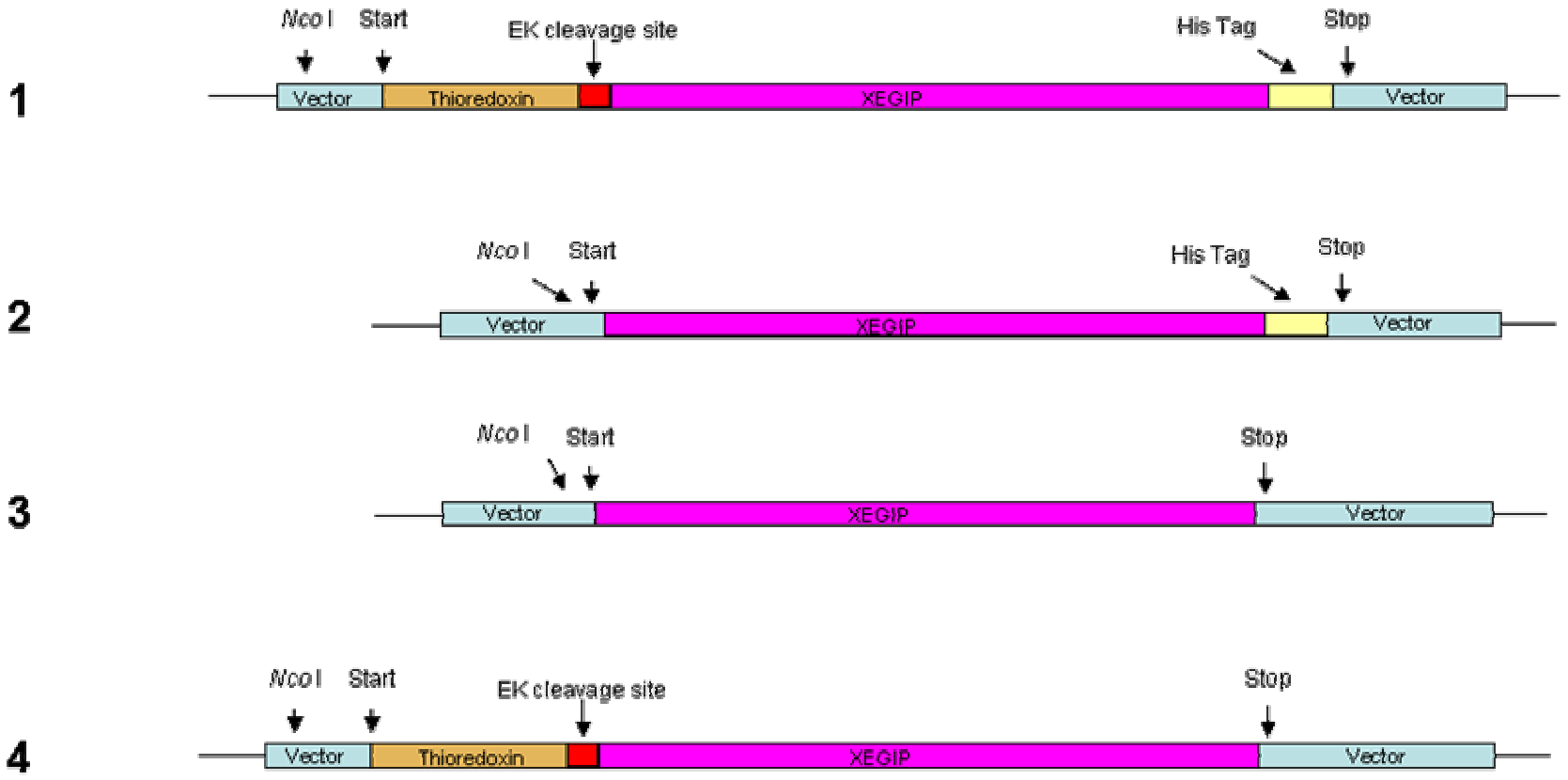
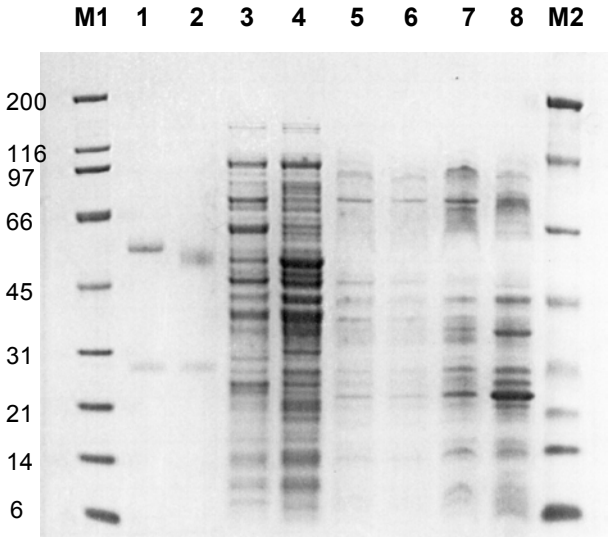


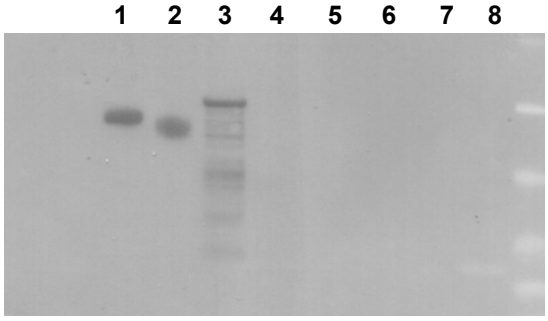
Figure 3.1 Construction of XEGIP expression vectors

Figure 3.2. SDS-PAGE (A) of proteins extracted from *E. coli*. containing XEGIP expression vectors and Western blots using antibody against XEGIP (B), Thioredoxin (C), and polyHis tag (D). Lane 1, mixture of XEG, XEGIP with reducing SDS-PAGE loading buffer; Lane 2, mixture of XEG, XEGIP with non-reducing loading buffer; Lane 3, Total proteins (extracted by Laemmli buffer) from construct 1 transformed *E. coli.* cells after arabinose induction; Lane 4, as lane 3, but before arabinose induction; Lane 5-7, Soluble proteins of construct 1 transformed E. coli. cells after 0.002%, 0.02% and 0.2% arabinose induction; Lane 8, Soluble proteins of construct 1 transformed *E. coli*. cells without induction

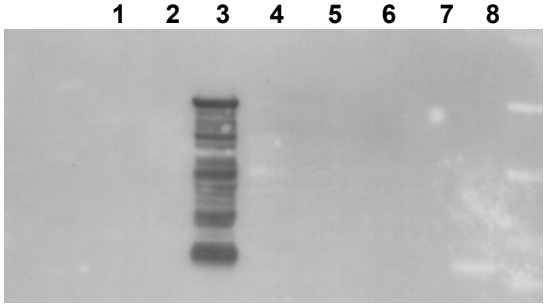
A. SDS-PAGE



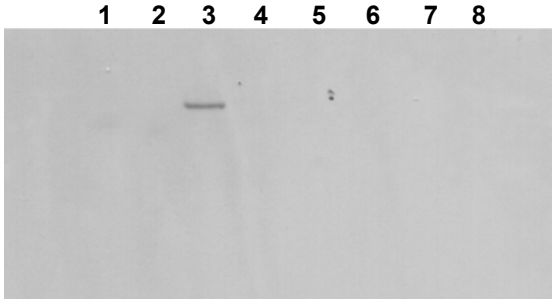
B. Anti-XEGIP



C. Anti-Thio



D. Anti-His tag



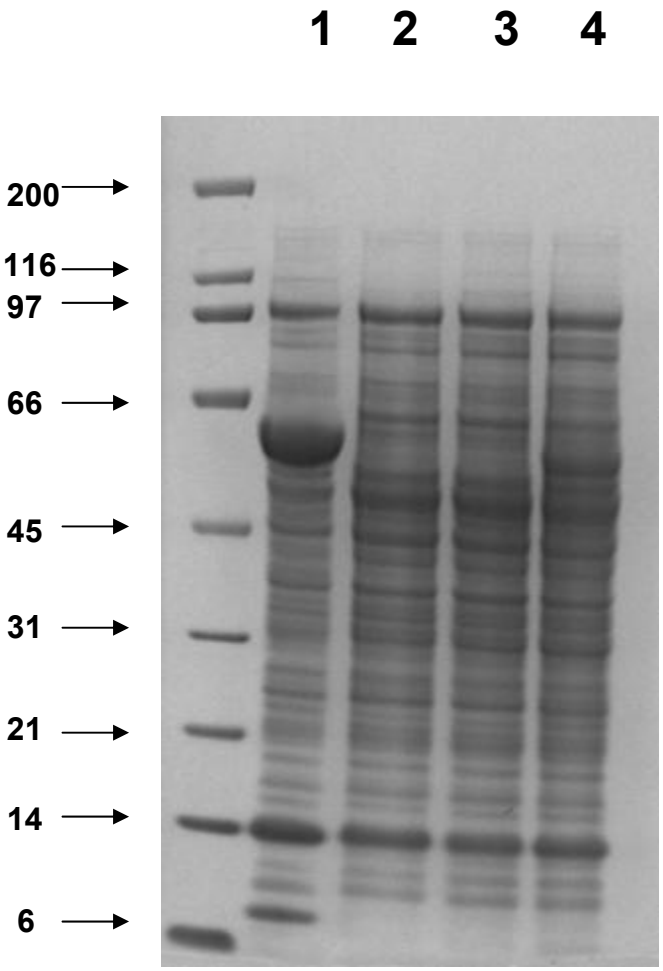


Figure 3.3. SDS-PAGE (Lane 1-4) of proteins recovered from inclusion bodies of *E. coli* transformed with XEGIP expression vectors 1-4, respectively.

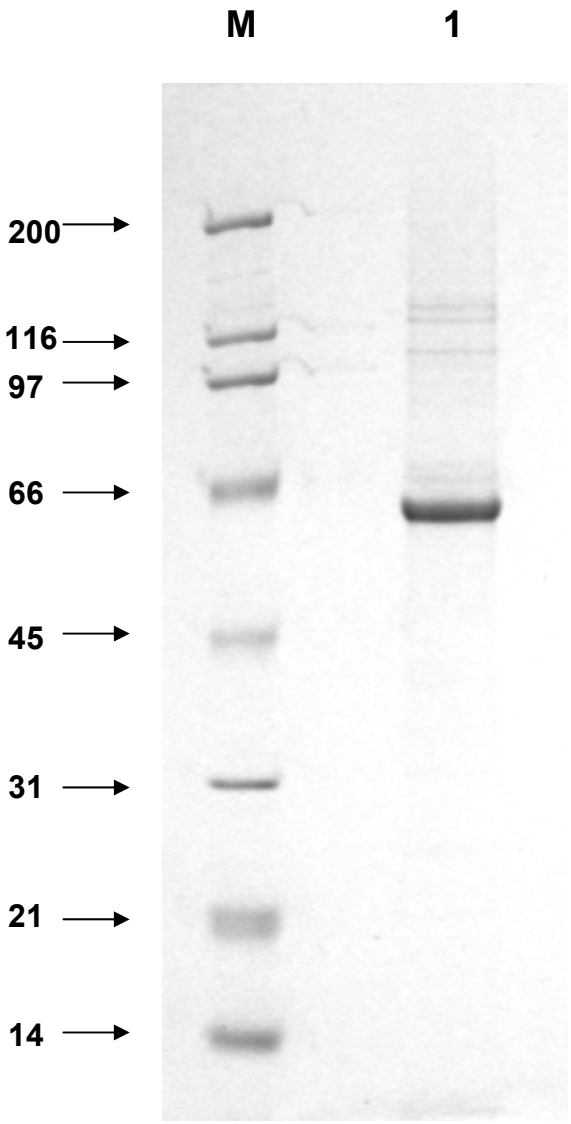


Figure 3.4.Metal chelating affinity purified recombinant Thio-XEGIP expressed in *E. coli*. cells transformed with expression vector 1 (Lane 1). Lane M contains molecular weight markers.

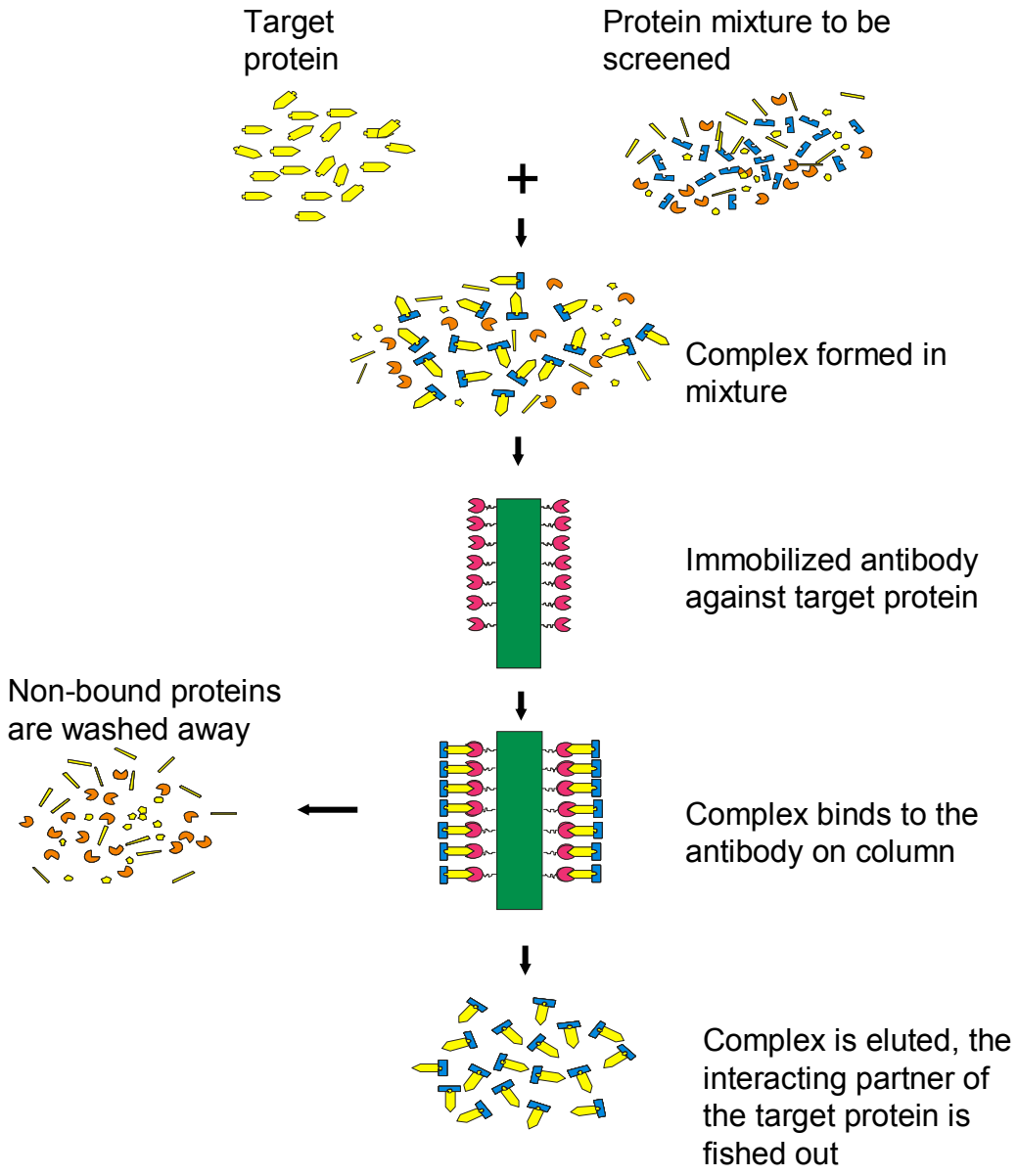
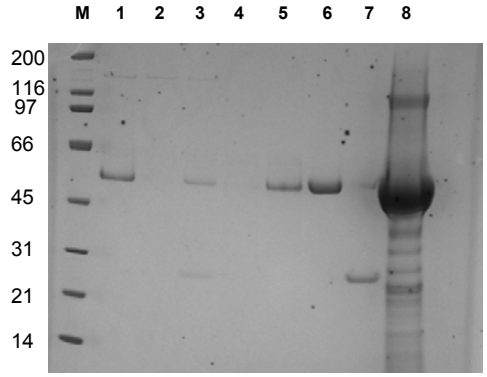


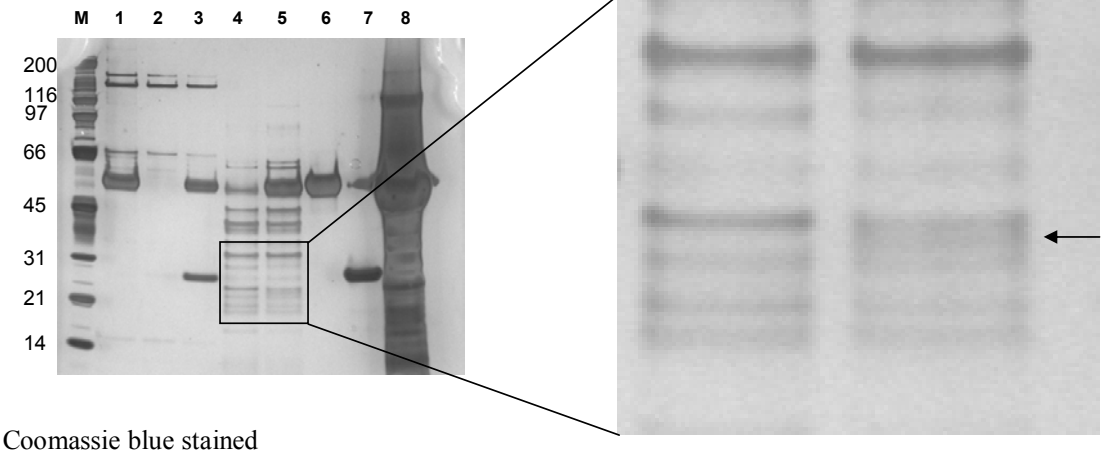
Figure 3.5. Immunology based screening method for protein-protein interactions

Figure 3.6. Coomassie blue stained (A) and silver stained (B) SDS-PAGE of low pH eluted proteins from anti-XEGIP immunoaffinity column. Lane 1-5 are proteins eluted from the column after application of purified XEGIP(1), crude XEG(2), mixture of XEGIP and crude XEG (3), Sanzyme (4), mixture of Sanzyme and purified XEGIP (5). Lane 6, 7, 8 are standards of purified XEGIP, XEG, and Sanzyme respectively. Lane 1 on C is the low pH eluted proteins after mixing the same amount of purified XEGIP with ~100 times as much Sanzyme than used in the experiment shown in Lane 5 of A and B. Lane 5 of B (see blowup) and lane 1 in C reveal an XEGIP-interacting protein (~ 25 KDa, indicated by red arrows) in the Sanzyme mixture.

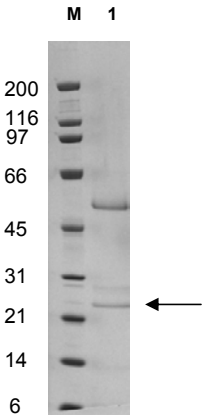
A. Coomassie blue stained



B. Silver stained



C. Coomassie blue stained



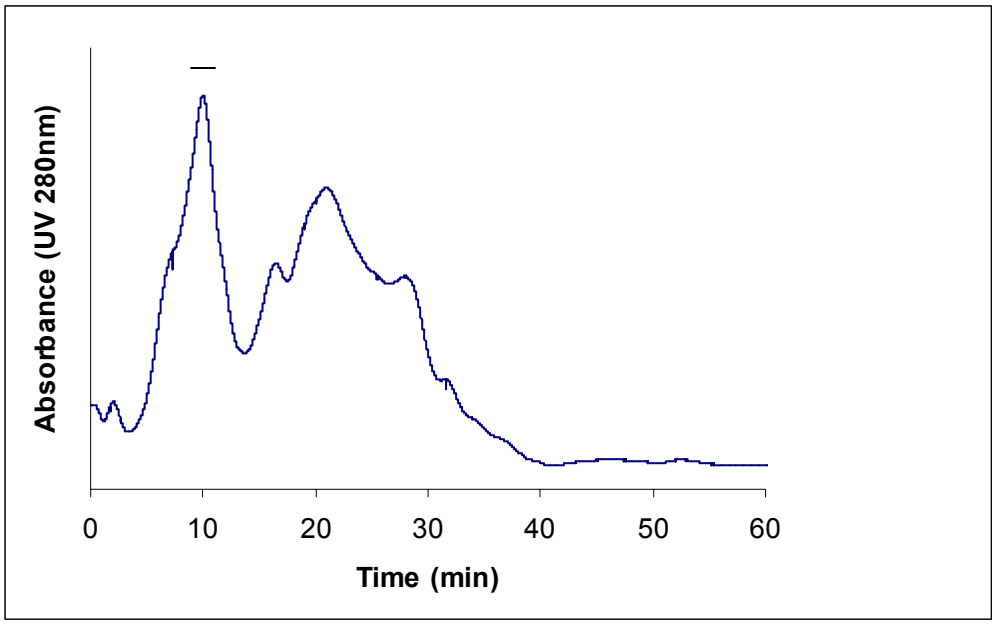


Figure 3.7. Cation exchange (HiTrap-S) chromatography profile of proteins in the medium of suspension-cultured carrot cells. XEG-inhibiting fractions are marked with a bar.

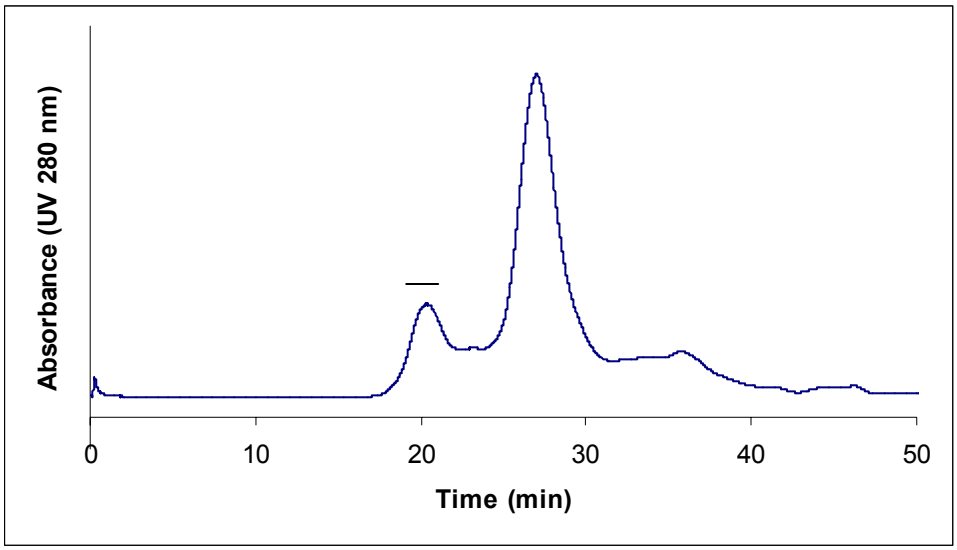


Figure 3.8. Size exclusion chromatography (Superdex 75) of carrot cell proteins previously enriched in XEG-inhibiting proteins by cation exchange chromatography. XEG-inhibiting fractions are marked with a bar.

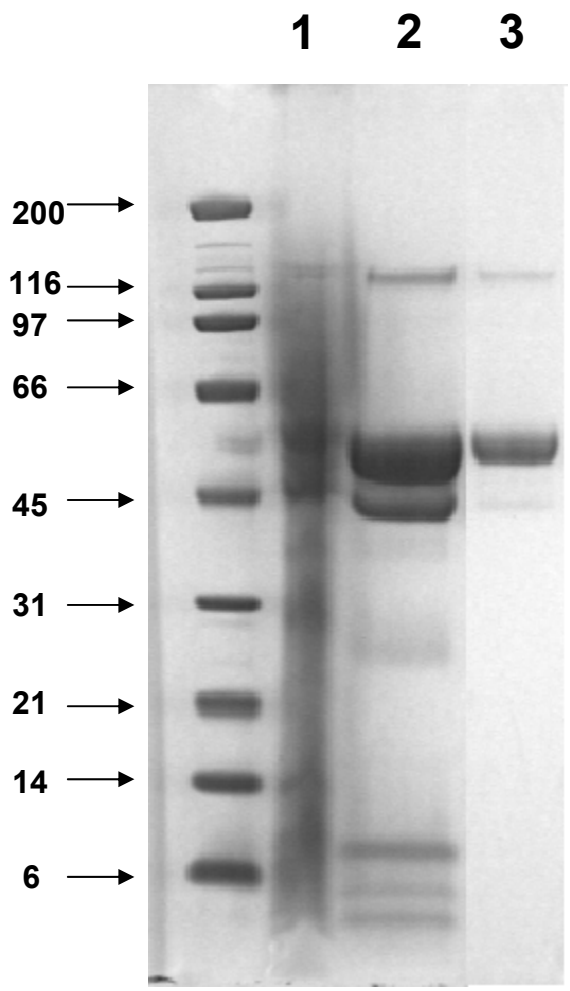


Figure 3.9. SDS-PAGE of fractions obtained during purification of EDGP. Lane 1, ethanol precipitated material from the medium of suspension-cultured carrot cells. Lane 2, XEG inhibiting fractions that were eluted from the HiTrap-S cation-exchange column during a pH and salt gradient. Lane 3, XEG inhibiting fractions from the Superdex-75 column.

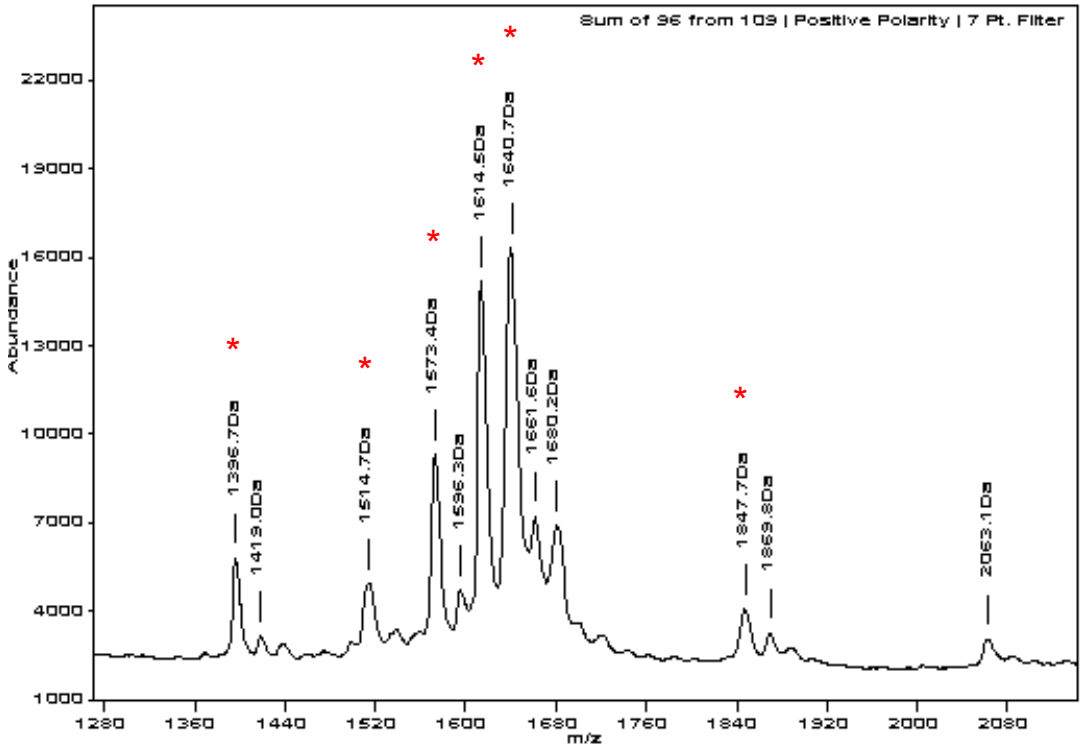


Figure 3.10. MALDI-MS of tryptic peptides of a carrot protein that inhibits XEG. Peaks with molecular weights matching those of EDGP peptides generated *in silico* are marked with a star.

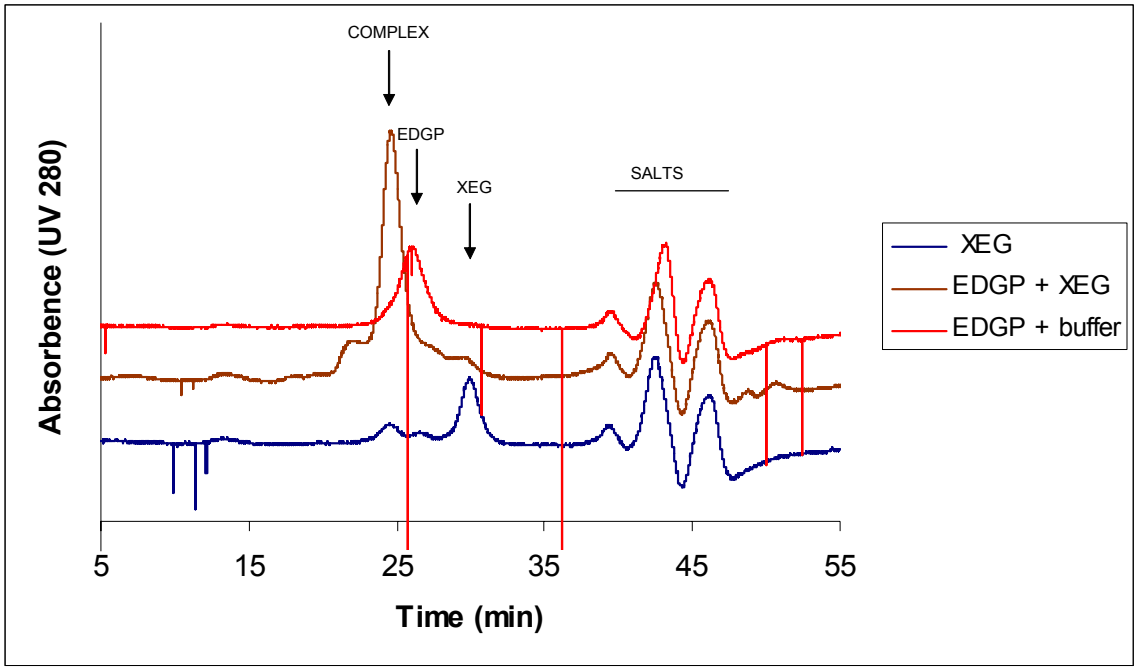


Figure 3.11. Interaction of XEG and EDGP as determined by SEC on Superdex-75.

CHAPTER 4

TOMATO EXTRACELLULAR PROTEIN XEGIP INHIBITS A XYLOGLUCANASE OF (HEMI)BIOTROPIC PATHOGEN *CLADOSPORIUM FULVUM* [1]

[1] Qin, Q., R. P. Clay, C. W. Bergmann, A. G. Darvill and W. S. York. 2003. To be submitted.

Summary

A xyloglucan-specific *endo*glucanase from the tomato pathogen *Cladosporium fulvum* (syn. *Fulvia fulva*) was detected, purified, and found to be inhibited by a tomato extracellular protein XEGIP *in vitro*. The *endo*glucanase activity in the culture medium of *C. fulvum* was tested using different polysaccharide substrates. Results showed that the specificity of the fungal *endo*glucanases depends on the carbon source on which the fungus was cultured. When the fungus is grown using carboxymethycellulose (CMC) as its sole carbon source, it produces enzymes (HE-cellulase) capable of hydrolyzing CMC and hydroxyethylcellulose (HE-cellulose) but incapable of hydrolyzing xyloglucan. In contrast, when *C. fulvum* is grown using xyloglucan-rich tamarind seed powder, it secretes an enzyme (i.e. XGase) that can digest xyloglucan but not HE-cellulose. XEGIP can significantly inhibit the XGase activity but not HE-cellulase activity. This is the first report that a plant protein inhibits a fungal *endo*glucanase from a plant pathogen. As XEGIP-like proteins are widely distributed in the plant kingdom and numerous *endo*glucanases have been found in microbes, such inhibition may play an important role in plant disease resistance.

Introduction

The plant cuticle and cell wall constitute the first line of defense against microbial attack. Plant pathogens have developed a variety of strategies to break this barrier, both mechanically and enzymatically. Primary cell walls comprise a complex network of polysaccharides, including pectins, cellulose, hemicellulose (e.g. xyloglucan in dicots and xylan in monocots), and pectins (Carpita and Gibeaut, 1993). To penetrate the cell wall and presumably use it as a carbon source, pathogens produce an array of cell-wall degrading enzymes (CWDEs) to hydrolyze plant cell walls (Walton, 1994; Esquerre-Tugaye et al., 2000). These enzymes include polygalacturonase (*endo* and *exo*), pectin lyase, pectate lyase, pectin methyl esterase, cellulase (*endo* and *exo*), cellobiohydrolase, hemicellulase (e.g. xylanase and *endo*-β-1,4-glucanase that can digest xyloglucan) and a variety of glycosidases (e.g. glucosidase, xylosidase etc.). Many of genes encoding CWDEs in plant pathogens have been disrupted alone or in combination to test their roles in pathogenesis. In some cases the pathogenicity of the microbe was not affected while some resulted in reduced virulence (especially in multiple gene disruption experiments, see Chapter 1). To date no single CWDE gene disruption has resulted complete loss of pathogenicity. This may be explained by the functional redundancy of enzymes that precludes loss of function when a single gene is disrupted.

Plants appear to have evolved at least one strategy to counteract the actions of microbial CWDEs, that is, the production of proteins that inhibit CWDEs. Such inhibitory proteins have been found in many plant species. Among these, polygalacturonase-inhibiting proteins (PGIPs) are the most widely studied (De Lorenzo et

al., 2001). PGIPs inhibit the hydrolyzing activity of microbial polygalacturonases (PGs), which cleave unesterified homogalacturonan (poly-α-1,4-linked-D-galactosyluronic acid), the major pectic polysaccharide in primary cell walls. The direct role of PGIP in disease defense has been demonstrated by limitation of fungal colonization in transgenic tomato plants that express pear PGIP (Powell et al., 2000). The interaction of PGIPs and PGs shows certain specificity: PGIPs from different sources vary in their inhibitory ability against a single PG while a single PGIP differs in its ability to inhibit different PGs. Studies have identified specific amino acid residues that contribute to PG binding affinity and ligand specificity (Leckie et al., 1999).

Other CWDE inhibitory proteins have been identified include a sugar beet protein (PNLIP) that inhibits a pectin lyase from *Rhizoctonia solani*, which is a pathogen of sugar beet (Bugbee, 1993), xylanase inhibitors that have been detected in wheat flour (Rouau and Surget, 1998; Debyser et al., 1999) and purified (McLauchlan et al., 1999) and a kiwi fruit protein (Camardella et al., 2000) that inhibits tomato pectin methylesterase by forming a 1:1 complex (Balestrieri et al., 1990; Giovane et al., 1995).

Cellulose and xyloglucan constitute a major load-bearing network of plant cell wall. Microbes synthesize a variety of enzymes, e.g. cellulases and other *endo*- *exo*-glucanases, to hydrolyze these polysaccharides. Some of these enzymes have been implicated in pathogenesis. For example, cellulase (*endo*glucanase) CelV mutants of *Erwinia carotovora* exhibited delayed maceration of plant tissues, suggesting reduced virulence (Walker et al., 1994; Mae et al., 1995). However, *endo*glucanase inhibiting proteins have only recently been identified in plants. The first of these was isolated from suspension-cultured tomato cells. This protein, termed XEGIP, inhibits a xyloglucan-

specific fungal *endo*glucanase (XEG) from *Aspergillus aculeatus*. XEGIP inhibits the hydrolytic activity of XEG by binding to XEG with a $K_i \approx 0.5$ nM (Chapter 2). Proteins that are homologous to XEGIP are widely present in the plant kingdom. It has been proposed that XEGIP-like proteins may be involved in plant disease resistance because of their localization, the observed *in vitro* function of XEGIP (inhibition of cell wall hydrolyzing enzyme), and the expression patterns of XEGIP-like proteins under certain stimuli (Chapter 2). However, the source of XEG, *A. aculeatus* (Pauly et al., 1999), is a saprophyte and not considered to be a plant pathogen, and a plant protein that inhibits an enzyme from a non-pathogenic fungus may not be related to plant disease resistance. Therefore, we have hypothesized that XEG-like *endo*glucanases are produced by other microbes, especially plant pathogens. The purpose of this study is to determine whether a tomato pathogen expresses XEG-like enzymes and if the corresponding enzyme activity is inhibited by XEGIP.

The fungus *Cladosporium fulvum* causes tomato leaf mould disease. Most tomato cultivars are resistant to this pathogen, but cultivars grown in the greenhouse are often susceptible (Joosten and de Wit, 1999). *C. fulvum* is often considered as a hemibiotroph. After germination of the conidia, thin hyphae develop and penetrate the plant through stomata. In resistant species (incompatible), the growing hyphae induce numerous defense reactions including a hypersensitive response, therefore preventing further pathogen proliferation. In susceptible species (compatible), the hyphae grow thicker and colonize the apoplastic space between plant cells. A relatively long time after inoculation (8-14 days), when the sporulation is extensive, symptoms develop and a destructive necrotrophic phase begins. The tomato-*Cladosporium fulvum* interaction has been studied

extensively as a model system for plant-pathogen gene-for-gene relationships (de Wit et al., 1994; de Wit and Joosten, 1999; Joosten and de Wit, 1999). Many pairs of plant resistance gene and fungal avirulence genes have been identified. Two extracellular proteins secreted by *C. fulvum* have been identified as virulence factors (Van den Ackerveken et al., 1993; Wubben et al., 1994). However, few studies have been done on the cell wall hydrolyzing enzymes secreted by *C. fulvum* and their roles in pathogenesis. This chapter decribes the specificity of cell wall hydrolyzing activities in *C. fulvum* suspension culture medium grown on different carbon sources and the inhibition of one *C. fulvum* enzyme by the tomato protein XEGIP.

Results

Enzyme activities of C. fulvum culture medium grown on different carbon sources

C. fulvum was grown in liquid culture containing Gamborg's B-5 basal medium with minimal organics (Sigma G5893, Sigma) and various carbohydrates as the sole carbon source at a concentration of 1% w/v. These include glucose, carboxymethylcellulose (CMC), powdered cellulose, and tamarind seed powder, which is rich in xyloglucan, but which also contains other components. Ten days after inoculation, the culture medium was tested for *endo*glucanases activity using soluble Ostazin Brillant Red-Hydroxyethyl-cellulose (ORB-HE-cellulose, Sigma, St. Louis, MO) and an insoluble blue Azurine-crosslinked tamarind xyloglucan (AZCL-xyloglucan, Megazyme, Wicklow, Ireland) as substrates. When the fungus was grown on tamarind seed powder, it secreted

extracellular enzyme(s) (i.e. XGase) capable of digesting xyloglucan but did not secrete enzymes that hydrolyze AZCL-HE-cellulose (i.e. HE-cellulase). In contrast, HE-cellulase activity but no XGase was detected in the culture grown on CMC. None of these activities were detected in the medium of fungus grown on glucose, apparently because of metabolite repression (Figure 4.1). *C. fulvum* did not grow in medium with cellulose powder as the sole carbon source.

The XGase activity can be significantly inhibited by the tomato extracellular protein XEGIP

The tomato protein XEGIP was purified and tested for its ability to inhibit two distinct types of *endo*glucanase activity secreted by *C. fulvum* into the culture medium. XGase activity was determined using AZCL-xyloglucan as a substrate and "HE-cellulase" activity was determined using HE-cellulose as a substrate. XEGIP inhibited approximately 70 % of the XGase activity in *C. fulvum* culture medium obtained using tamarind seed powder as a carbon source (Figure 4.2A). However, XEGIP did not inhibit the HE-cellulase activity present in the culture medium obtained using CMC as a carbon source (Figure 4.2B).

The culture medium contains xyloglucan fragments

XGase was also detected when *C. fulvam* was grown on a mixture of xyloglucan purified from tamarind seed powder and from the culture medium of suspension-cultured

tomato cells, as the sole carbon source. This XGase partially depolymerizes xyloglucan in the medium, generating oligosaccharide fragments, which were separated from the culture medium and identified by MALDI-MS (Figure 4.3). Ions in the MALDI spectra had m/z values consistent with the presence of the well known oligosaccharides fragments XXXG, XXLG, XLXG, and XLLG from tamarind xyloglucan, along with other xyloglucan oligosaccharides from tomato (Jia et al., 2003).

Purification of XGase activity from C. fulvum (CfXEG) from culture medium grown on tamarind seed powder

Culture medium of *C. fulvum* grown on tamarind seed powder was harvested 10 days after inoculation. The medium (pH ~ 7.6) was filtered and applied directly to ion exchange columns. It was interesting that at this pH, the XGase activity (referred as CfXEG) did not bind to either anion or cation exchange columns (Figure 4.4), suggesting its pI is around 7.6. However, another attempt to bind the activity to these columns at pH 5.0 also failed (data not shown). After screening other conditions, it was found that the CfXEG binds to a cation exchange column (Hi-Trap SP) at pH 3.4. This unusual behavior of CfXEG on ion exchange columns greatly simplifies the purification process. CfXEG was thus prepared by sequentially passing the filtered culture medium through both anion and cation column at pH 7.6. The pH of flow-through was then adjusted to 3.4 and this solution was applied to a cation exchange column. CfXEG was eluted from the cation exchange column with a salt gradient (Figure 4.5). CfXEG containing fractions were pooled, concentrated, and applied to a Superdex-75 SEC column (Figure 4.6). When the

resulting CfXEG fractions were analyzed by SDS-PAGE, a single band was detected with an apparent molecular weight of 24 kDa (Figure 4.8), which is similar to the theoretical molecular weight (23.7 kDa) of XEG from *Aspergillus aculeatus*. It should be noted that the mobility of XEG corresponds to a molecular weight of ~27 KDa in the same SDS-PAGE system (data not shown).

XEGIP interacts with CfXEG

The tomato extracellular protein XEGIP binds to *C. fulvum* extracellular protein CfXEG. This is first demonstrated by the retention time shift experiment on size exclusion chromatography as described (Chapter 2). When injected together with purified XEGIP, the CfXEG peak completely disappears while the peak corresponding to XEGIP elutes slightly earlier, suggesting that a complex is formed (Figure 4.7).

An immunoaffinity based technique (see Chapter 3) was used to identify proteins in the culture medium of *C. fulvum* that interact with XEGIP. A fraction enriched in CfXEG was prepared by anion exchange column at pH 7.6, as described above. The CfXEG-enriched fraction was divided into 3 aliquots. The first was applied directly to the immunoaffinity column prepared by attaching anti-XEGIP antibodies to the gel matrix. The column was washed with loading buffer and then eluted with a low pH buffer that disrupts binding of the anti-XEGIP antibodies to XEGIP. The low pH eluted fraction was assayed with SDS-PAGE, acting as a "blank" control. As expected, Coomassie blue staining of the gel did not reveal much protein in this fraction (Figure 4.9A, Lane 1),

although silver staining indicated some non-specific bindings to the anti-XEGIP polyclonal antibodies immobilized onto the matrix (Figure 4.9B, Lane 1). When the CfXEG enriched fraction was preincubated with XEGIP and the mixture was loaded onto the immunoaffinity column, Coomassie blue staining of SDS-PAGE of the low pH eluted fraction indicated only the presence of XEGIP (Figure 4.9B, Lane 2). However, silver staining indicated that the fraction contained XEGIP along with two additional proteins (compared with the "blank" control, Lane 1 in Figure 4.9B) with approximate molecular weights of 110 kDa and 24 kDa respectively (Figure 4.9, Lane 2). This result indicated that each of these two proteins forms a complex with XEGIP, and the complex binds to the anti-XEIGP column. The third aliquot was passed through a cation exchange column at pH 3.4, as described above, to obtain a fraction that is further enriched in CfXEG. SDS-PAGE (Figure 4.9, Lane 3) of the further enriched CfXEG indicated that it contained, *inter alia*, a 24 kDa protein that has the same electrophoretic mobility as one of the proteins identified as an XEGIP binding protein by affinity chromatography. The lack of ~110 kDa band in lane 3, suggests that the 24 kDa band is CfXEG. Indeed, the purified CfXEG has the same mobility as this 24 kDa band on SDS-PAGE (Figure 4.8). The identity of the ~110 kDa XEGIP interacting protein in *C. fulvum*. medium is unknown.

Experimental procedures

Fungal culture

Cladosporium fulvum AOTC 62079 (Race 2,4,5,9) (de Wit et al., 1984) was inoculated on PDAY plate from liquid nitrogen storage. The plates were kept under black light for 10-14 days. The liquid culture was started by putting three ~ 10 mm^2 pieces of colonized agar directly in the autoclaved medium containing Gamburg B-5 salts with minimum organics and glucose, cellulose powder (Whatman CC31), carboxymethylcellulose, and tamarind seed powder respectively, as carbon sources at a concentration of 1% (w/v). The pH of the medium was adjusted to 5.8 before autoclaving. For all polysaccharide-containing medium, 0.05% glucose was added to help initiate fungal growth. Fungi were grown on a shaker at 130 rpm under UV light at room temperature (24-26 °C) (de Wit et al., 1984). Ten days culture medium were harvested and filtrated by glass fiber filter. The clarified medium was used for enzyme assays.

Enzyme activity test

AZCL-xyloglucan and AZCL-HE-cellulose were used for XGase and HE-cellulase activity assay. About 1 mg insoluble substrates were suspended in 400 µl filtered *C. fulvum* medium. The reaction was incubated on a 37 °C shaker for a specified time period. The reaction mixture was then centrifuged on a bench top centrifuge at

maximum speed (> 10,000 rpm) for 5 min. Supernatant (200 µl) was transformed to an ELISA plate and the absorbance at 620 nm was recorded.

Purification of tomato XEGIP and its inhibition test

Tomato XEGIP was purified from culture medium of suspension-cultured tomato cells as described (Chapter 2). Two ml of filtered Cf medium was mixed with ~ 5 mg insoluble AZCL-XG or AZCL-HE-cellulose and vortexed. Eight 200 µl aliquots were transferred into non-stick Eppendorf tubes. Twenty µl of 0.57 µg/µl XEGIP was added to 4 of them and 20 µl water to the other 4. The tubes were vortexed and put on a 37 °C shaker for a specified time period. The reaction products were measured as described above.

Detection of xyloglucan oligosaccharides in culture medium

Two ml of filtered Cf culture medium was concentrated by Microcon MWCO 3K and the flow through solution was collected and applied to C-18 cartridge primed as described in cartridge manual. The column was washed with 5 ml water and then carbohydrates were eluted with 70% methanol. The elute was dried on vacuum and redissovled in 100 µl water. One µl of this solution was used for MALDI-MS as described (Chapter 2).

Immunoaffinity identification of interacting proteins of XEGIP

Purified tomato XEGIP was used to raise polyclonal antibodies according to standard procedures (Coligan, 1995) by the Monoclonal Facility at the University of Georgia. Immobilized anti-XEGIP polyclonal antibody column was made with Seize™ Primary Immnoprecipitation Kit (Pierce, Rockford, IL) according to manufacture's instruction. Briefly, mouse serum was diluted with PBS to ~1mg/ml (Bradford) and 500 µl was added to about 250 µl AminoLink Plus coupling gel in a microspin column. Five µl of 5 M sodium cyanoborohydride was added and the coupling reaction was incubated overnight on an end-over-end rocker. Unreacted active groups on the gel were blocked by quenching reagent provided in the kit.

Approximately 2~5 µg purified tomato XEGIP was mixed with 5 ml of Cf culture medium that had been passed through a Q-Sepharose column and then exchanged to binding buffer (Dulbecco's PBS). The mixture was incubated at room temperature for 5 min and then applied to the anti-XEGIP serum immunoaffinity gel slurry and incubated for 1 hr on a rocker at room temperature. The gel was then thoroughly washed with TBS buffer (0.025 M Tris, 0.15 M NaCl, pH 7.2) to remove unbound proteins. The protein-XEGIP complex or XEGIP bound to the antibody column were eluted by ~ 200 µl elution buffer (pH 2.8) provided in the kit. Elution was repeated 3 times and the fractions were collected into separate tubes. The low pH eluted material was immediately neutralized by 15 µl 1 M Tris, pH 9.5 buffer to prevent possible denaturation. The culture medium (no

incubation with XEGIP) was subject to the same process, and the eluted fractions were used as controls.

Purification of CfXEG

Ten days post-inoculation culture medium (pH ~ 7.6) of *C. fulvum* grown on tamarind seed powder was sequentially filtrated through glass fiber filter (Whatman, Clifton, NJ) and a 0.45 μm membrane (Nalgene, Rochester, NY). The filtrate was then sequentially applied to cation and anion exchange columns (HiTrap-SP and Q, Amersham Biosciences, Piscataway, NJ) that had been equilibrated in H_2O. The pH of the flow-through solution was adjusted to pH 3.4 using concentrated acetic acid. The solution was loaded onto a HiTrap-SP column equilibrated with 20 mM pH 3.4 NaOAc buffer. Bound proteins were eluted by a salt gradient (eluent A, 20 mM NaOAc pH 3.4; eluent B, 20 mM pH 5.2 NaOAc buffer with 0.3 M NaCl; 0-100% B in 60 min at 0.5 mL/min) (Figure 4.5). Fractions containing *endo*glucanase activities were pooled, concentrated, and applied to a Superdex-75 size exclusion column and eluted with 20 mM NaOAc containing 0.3 M NaCl, pH 5.2 at a flow rate of 0.5 ml/min (Figure 4.6). The *endo*glucanase activity was pooled and analyzed by SDS-PAGE (Figure 4.9).

Discussion

A xyloglucan specific *endo*glucanase (CfXEG) was detected and purified from culture medium of the tomato pathogen *Cladosporium fulvum* grown on xyloglucan-rich

tamarind seed powder as its sole carbon source. XEG, the first and only microbial xyloglucan-specific *endo*glucanase reported to date is produced by the saprophytic fungus *Aspergillus aculeatus*. XEG hydrolyzes xyloglucan but does not digest cellulose or other cell wall polysaccharides. It has been speculated (Chapter 2) that other fungi, including those pathogenic to plants, may secrete enzymes with XEG-activity as well. The identification of such a xyloglucan-specific *endo*glucanase from the tomato pathogen *C. fulvum* suggests that xyloglucan-specific *endo*glucanases may widely exist in fungi and may be involved in the fungal colonization of plants. The late discovery of this class of enzymes may have been the result of using substrates other than xyloglucan when assaying for cell wall hydrolyzing enzymes. The use of cellulose or its derivatives as substrates would not allow the detection of such xyloglucan-specific *endo*glucanases. For the same reason, many cellulases and other beta-1,4-*endo*glucanases have not been widely tested for their activity on xyloglucan. The presence of only HE-cellulase and CMCase activity in the culture medium of *C. fulvum* grown on CMC suggests the existence of *endo*glucanase capable of digesting cellulose but not xyloglucan. The implication of microbes' capability to synthesize such substrate specific *endo*glucanases is not clear.

XEGIP, an extracellular protein in suspension-cultured tomato cell medium, inhibits the XGase activity in the culture medium of *C. vulvum* grown on xyloglucan-rich tamarind seed powder. XEGIP has recently been found to inhibit XEG from the non-pathogenic fungus *Aspergillus aculeatus* by forming a 1:1 protein:protein complex with a $K_i \approx 0.5$ nM (Chapter 2). XEGIP like proteins are widely present in the plant kingdom. It

has been proposed that XEGIP like protein may be involved in plant disease resistance because of its observed *in vitro* function (inhibition of cell wall hydrolyzing enzyme), localization, and expression pattern under certain stimuli (Chapter 2). The inhibition of an extracelluar cell wall hydrolyzing enzyme (CfXEG) of a tomato pathogen by a tomato extracellular protein (XEGIP) further supports the hypothesis that XEGIP plays a role in plant disease defense. Although the inhibition has been observed only *in vitro* to date, both proteins are extracellular and the growth of *C. fulvum* is limited to the apoplast of plant cells, so it is likely that such inhibition occurs *in vivo* as well.

Tomato-*Cladosporium fulvum* has been used extensively as a model system to study plant-pathogen interactions. This study shows that this system is also very useful in studying the interaction of fungal cell wall hydrolyzing enzymes and their inhibitors. As shown here, the selective induction of extracellular CWDEs by supplying different carbohydrates as carbon sources and biochemical analysis of these CDWEs using well-defined substrates should facilitate the detection and characterization of molecular interactions involved in this plant pathogenesis system.

Acknowledgments

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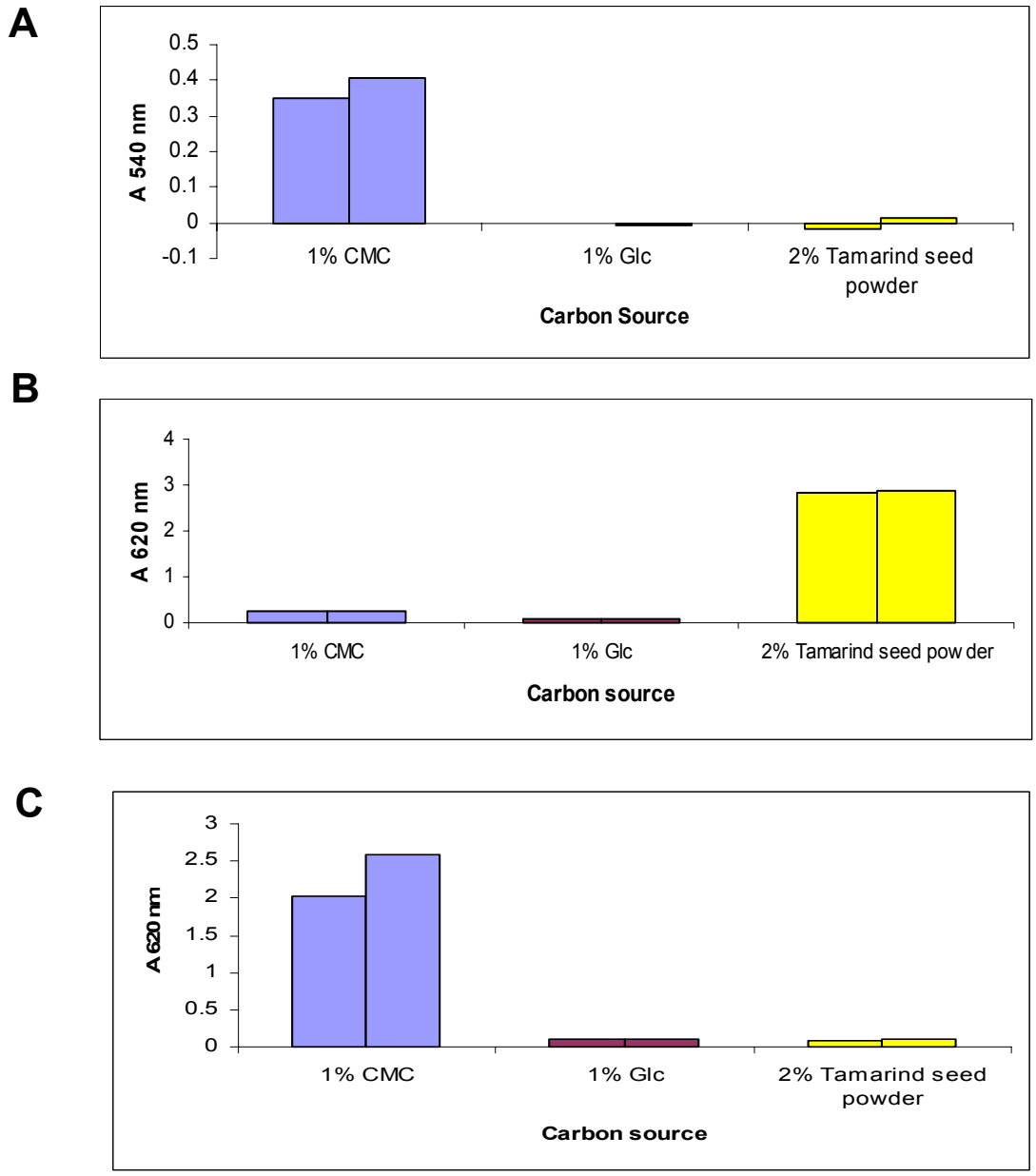

Figure 4.1. *Endo*glucanase activity assays (duplicates are shown for each assay) of Cf culture medium (B-5 salts) grown using different carbon sources. The endoglucanase substrates used were **A.** OBR-HE-cellulose **B**. AZCL-xyloglucan **C**. AZCL-HE-cellulose. **A** and **C** show HE-cellulase activity while **B** shows XGase activity.

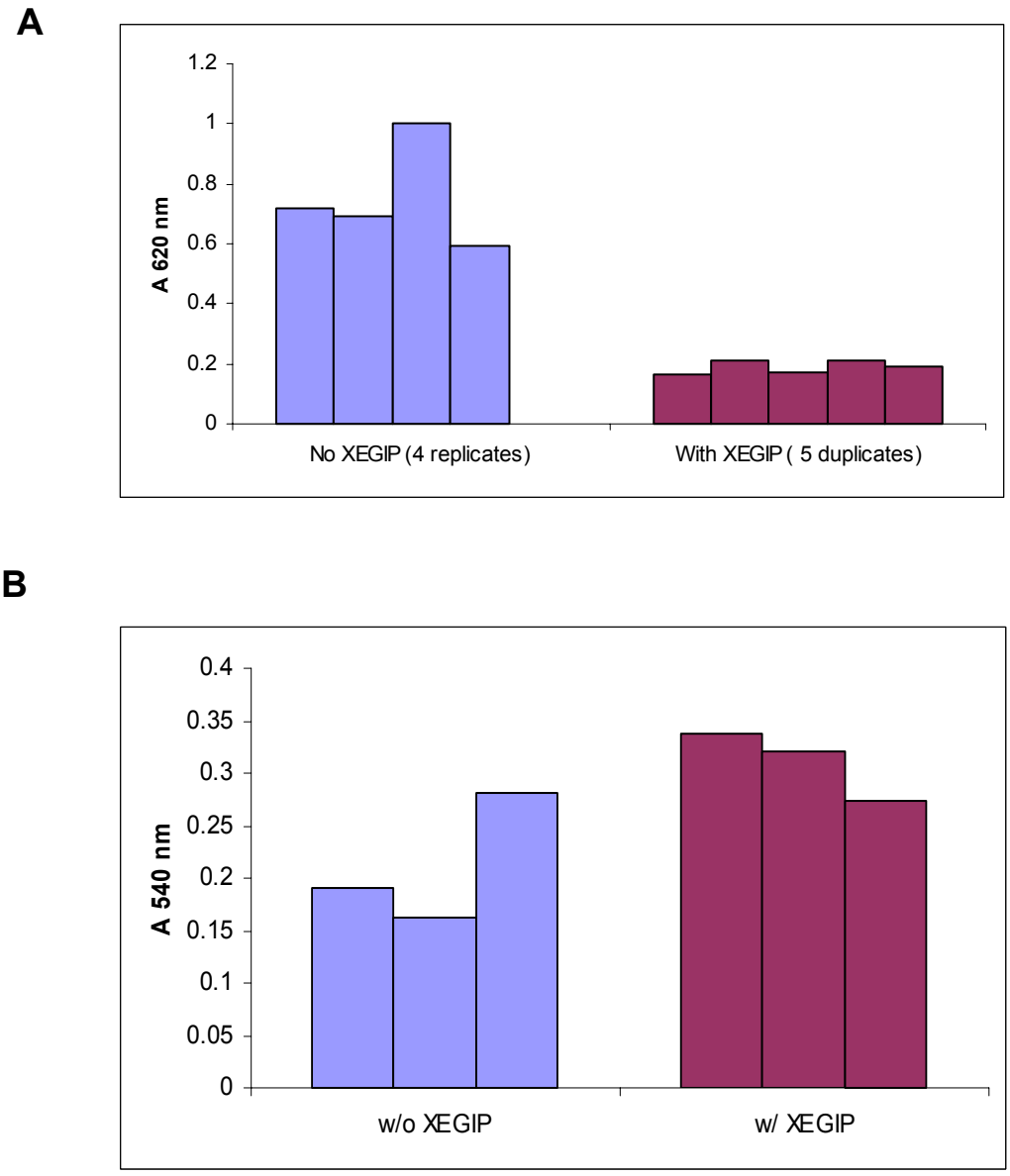

Figure 4.2. Effect of tomato protein XEGIP on **A.** XGase **B.** HE-cellulase activities in the culture medium of Cf grown on tamarind seed powder (**A**) and CMC (**B**) as the sole carbon source, respectively.

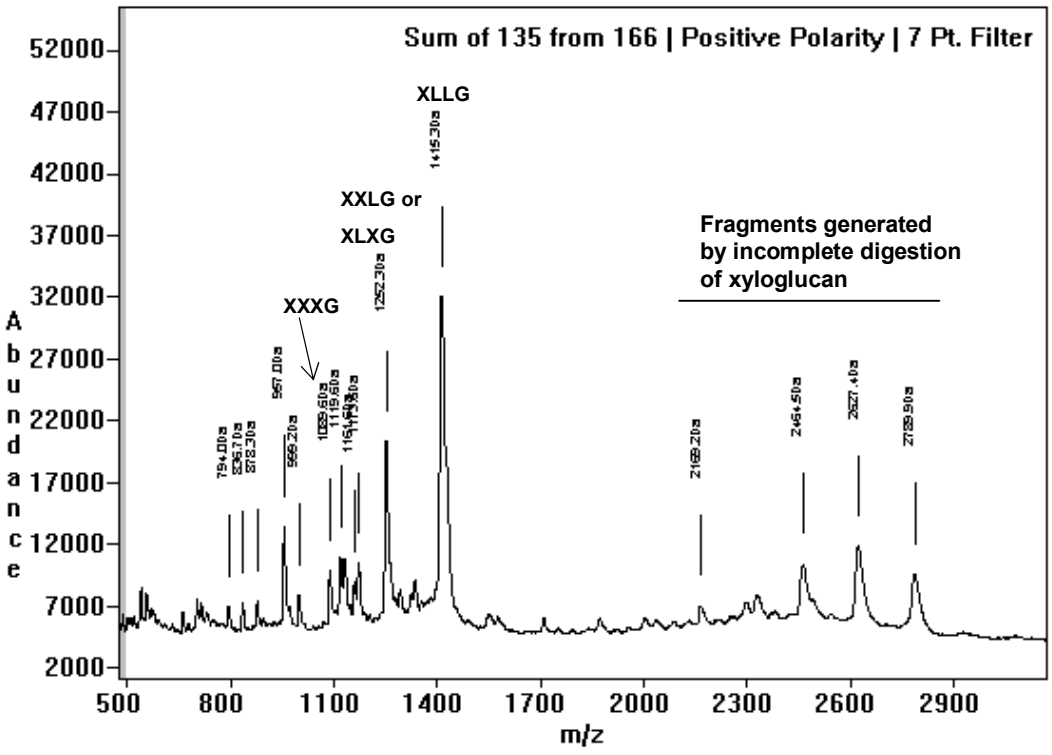

Figure 4.3. MALDI-MS of XGO isolated from *C. fulvum* culture medium grown on a mixture of purified xyloglucans extracted from tomato suspension-culture medium and tamarind seeds. Well-known oligosaccharides from tamarind xyloglucan are identified by the commonly used nomenclature (Fry et al., 1993).

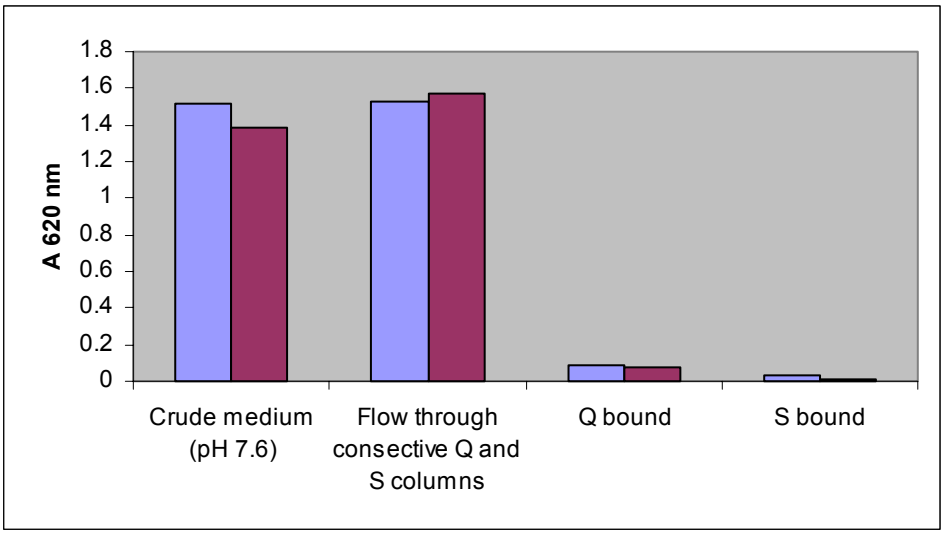

Figure 4.4. XGase activity assay (duplicates are shown for each assay) of *C. fulvum* culture medium (grown on xyloglucan rich tamarind seed powder) passed through ion exchange columns at pH 7.6 using AZCL-xyloglucan as substrates

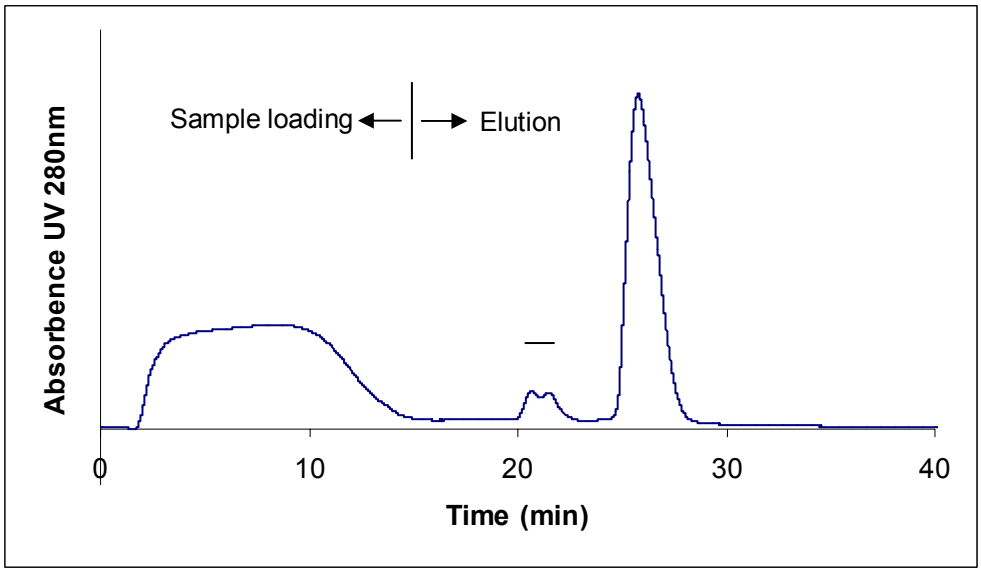

Figure 4.5. Elution of CfXEG from cation exchange column (HiTrap-S) at pH 3.4. *C. fulvum* medium (pH ~ 7.6) that had been passed through HiTrap-Q and HiTrap-S columns was titrated to pH 3.4 and loaded to a HiTrap-SP column in a starting buffer of 20 mM NaOAc, pH 3.4. The column was eluted at a flow rate of 0.5 ml/min with a gradient (0-100% over 60 min) of 20 mM NaOAc buffer (pH 5.2) containing 0.3 M NaCl. *Endo*glucanase activity of each fraction was assayed using AZCL-xyloglucan as a substrate. Active fractions are marked with a bar.

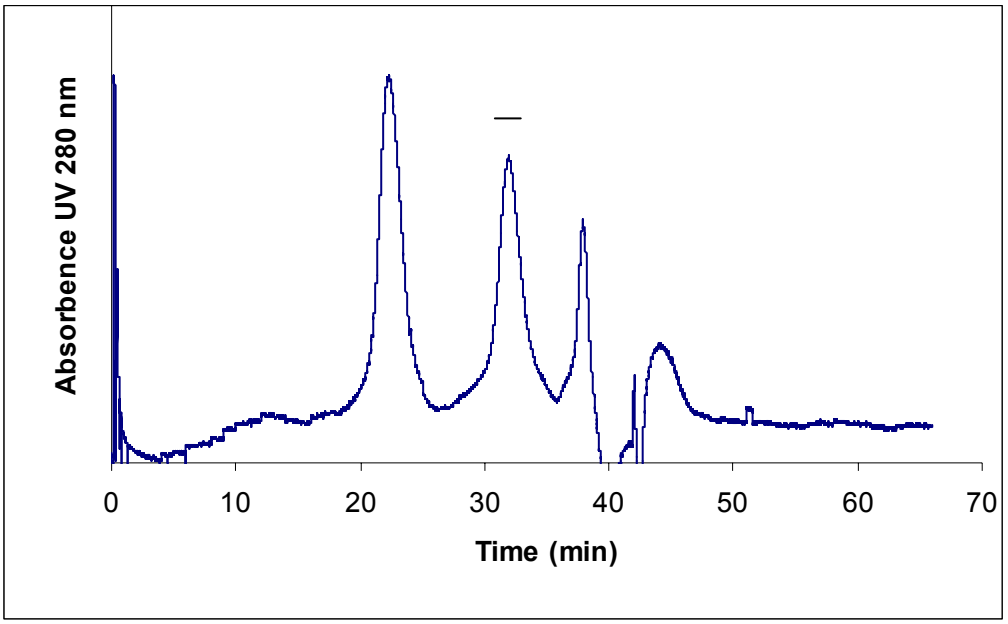

Figure 4.6. SEC (Superdex-75) profile of CfXEG enriched fractions from the HiTrap-SP column. Eluent is 20 mM NaOAc with 0.3 M NaCl, pH 5.2 at a flow rate of 0.5 ml/min. *Endo*glucanase activity of each fraction was assayed using AZCL-xyloglucan as a substrate. Active fractions are marked with a bar.

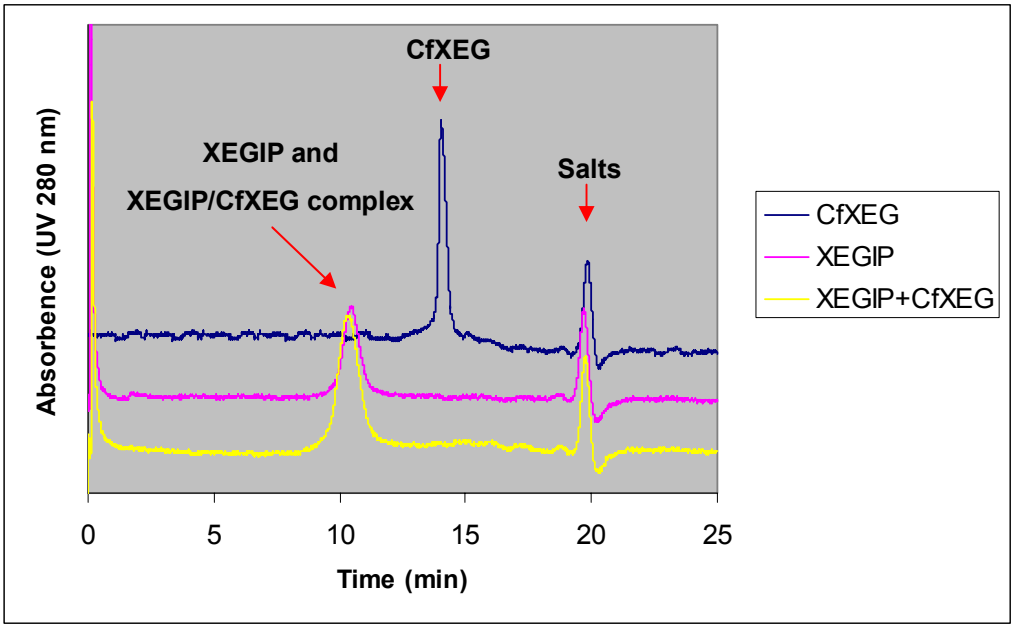

Figure 4.7. Interaction of CfXEG and XEGIP as determined by SEC on Superdex-75. Pure CfXEG, pure XEGIP and mixtures of the two were chromatographed (Eluant: 20 mM pH 5.2 NaOAc containing 0.3 M NaCl; flowrate: 1ml/min).When the CfXEG/XEGIP mixture, which contained the same amount of each protein when injected separately, was injected onto the column, the peak corresponding to pure CfXEG disappeared, while a peak appeared slightly earlier than that of pure XEGIP. This peak was larger than the XEGIP peak when pure XEGIP was chromatographed alone, indicating a CfXEG-XEGIP complex had formed and eluted earlier in SEC because of its increased molecular weight and size.

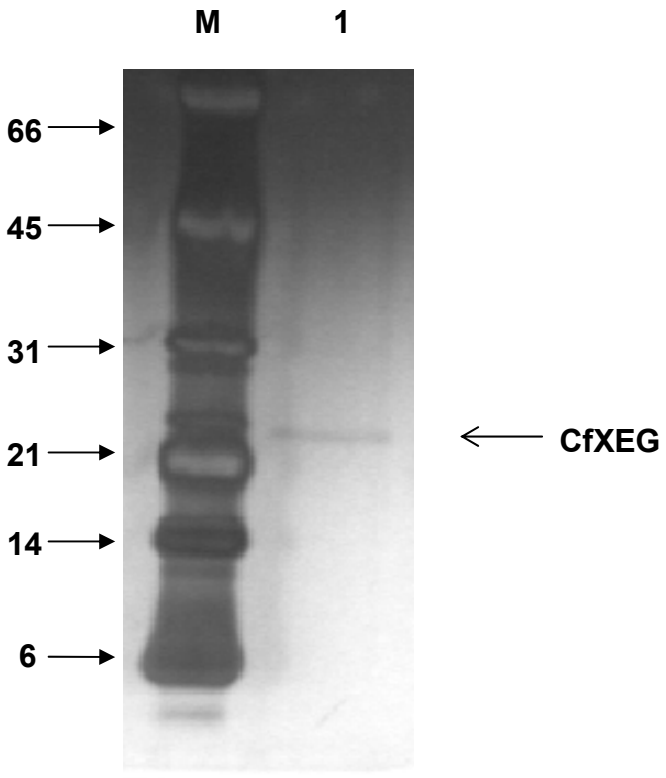

Figure 4.8. SDS-PAGE of purified xyloglucan-specific *endo*glucanase (CfXEG) from culture medium of *Cladosporium fulvum* grown on xyloglucan-rich tamarind seed powder. Lane 1 shows the final purified protein after ion exchange (Figure 4.5) and SEC (Figure 4.6) chromatography.

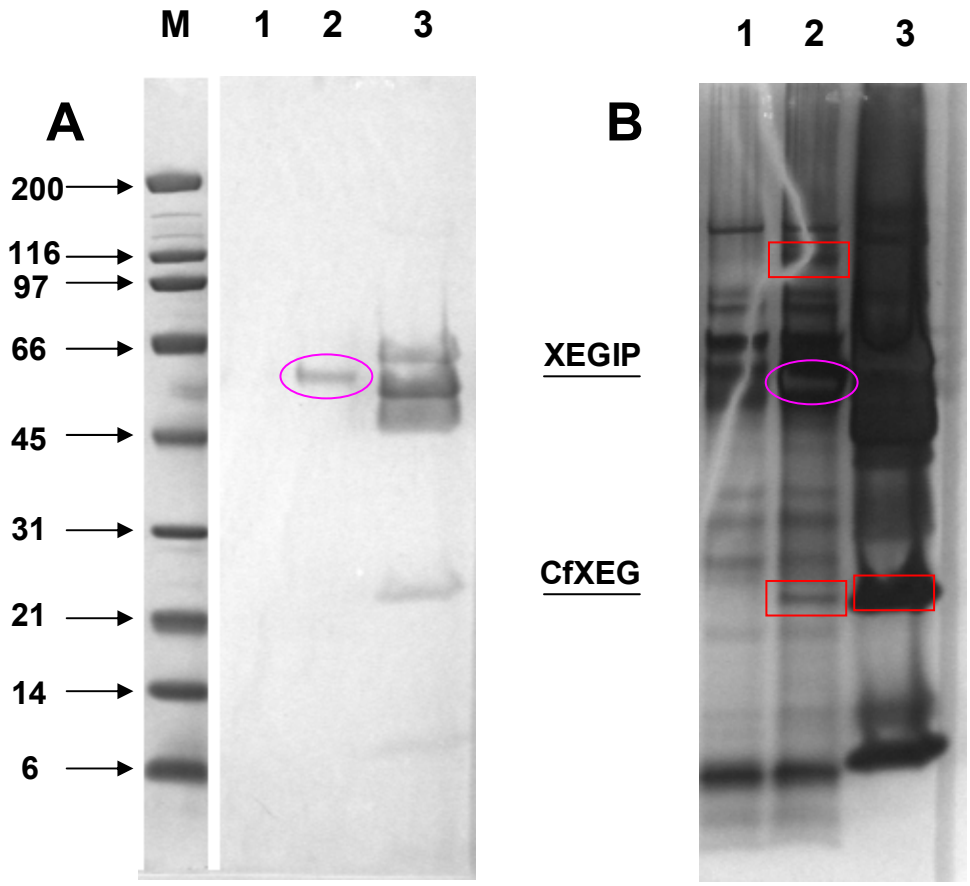

Figure 4.9. Coomassie blue (A) and silver (B) stained SDS-PAGE of CfXEG enriched fractions analyzed by immunoaffinity chromatography. *C. fulvum* culture medium was passed through an anion exchange column at pH 7.6 to prepare a CfXEG enriched sample. This material was applied to the anti-XEGIP affinity column after preincubation with or without XEGIP. The affinity column was washed and XEGIP complexes were then eluted with low pH buffer (Lane 1, without XEGIP, Lane 2 with XEGIP). Bands, corresponding to XEGIP interacting proteins, are marked with a red rectangle. XEGIP bands are marked with a pink oval. For comparison, a sample further enriched in CfXEG by cation exchange at pH 3.4 is shown in Lane 3. Among other unknown proteins (especially in the 50-70 kDa range), this fraction also contains a 24 kDa band but not the 110 kDa band contained in Lane 2, indicating it is the 24 kDa band that represents CfXEG.

CHAPTER 5

CONCLUSIONS

This dissertation describes the discovery and characterization of a family of glycosyl hydrolase inhibitors produced by diverse species of higher (vascular) plants. These proteins are called XEGIP-like proteins, relative to XEGIP, a xyloglucan-specific *endo*glucanase inhibitor protein from tomato (*Lycopersicon esculentum*). The discovery and characterization of this protein family provides further insight into the interaction of plants with potential microbial pathogens.

Chapter 2 describes the purification and molecular characterization of XEGIP. This protein, which inhibits the hydrolytic activity of a xyloglucan-specific fungal *endo*glucanase (XEG) from the fungus *Aspergillus aculeatus*, was isolated from the medium of suspension-cultured tomato cells. XEGIP is the first protein from plants or any other source that has been shown to inhibit an *endo*-β-1,4-glucanase. It shows no detectable proteolytic activity and inhibits XEG by forming a tightly bound 1:1 complex with a K_i of approximately 5×10^{-10}M. The XEG-XEGIP complex has no detectable *endo*glucanase activity, but active XEG and XEGIP can be regenerated upon dissociation of the immobilized complex with 2M imidazole buffer.

XEGIP represents a class of plant-derived proteins that protect plant cell walls from digestion by microbial enzymes. Microbes have evolved many different enzymes to efficiently hydrolyze the complex polysaccharides in plant cell walls and *endo*glucanases constitute a major class of these enzymes. For example, seven *endo*glucanases, including cellulase and XEG, have been identified in *Aspergillus aculeatus* (de Vries and Visser, 2001). Although *A. aculeatus* is considered to be a saprophyte, it secrets a spectrum of

plant cell wall hydrolases that are similar to those produced by pathogenic fungi and bacteria. In Chapter 4, experiments were performed to determine whether tomato XEGIP protein can also inhibit cell wall degrading enzymes from a known tomato pathogen, *Cladosporium fulvum*. A xyloglucan specific *endo*glucanase (CfXEG) was detected and purified from the culture medium of *C. fulvum* grown on xyloglucan-rich tamarind seed powder as its sole carbon source. The identification of such a xyloglucan-specific *endo*glucanase from *C.fulvum* suggests that xyloglucan-specific *endo*glucanases may exist widely in different fungi and may be involved in the fungal colonization of plants. Similar to its interaction with XEG from *A. aculeatus,* XEGIP inhibits CfXEG from *C. fulvum* by forming a complex. The inhibition of an extracellular cell wall hydrolyzing enzyme (CfXEG) of a tomato pathogen by a tomato extracellular protein (XEGIP) further supports the hypothesis that XEGIP plays a role in plant disease defense. Although the inhibition has thus far been observed only *in vitro*, both proteins are extracellular and the growth of *C. fulvum* is limited to the apoplast of plant cells, suggesting such inhibition can occur *in vivo* as well. It is possible that XEGIP-related proteins have evolved to counteract cellulolytic and hemicellulolytic enzymes produced by plant pathogens, analogous to the role proposed for PGIPs, which inhibit pathogenic polygalacturonases.

EDGP, the XEGIP homolog in carrot, is a 57 kDa protein that is expressed at high level in the dermal tissues of roots, petioles, leaves, as well as in developing seeds. It has been proposed that EDGP is involved in pathogen resistance due to its localization in dermal tissues and expression in response to wounding (Satoh et al., 1992). However, its function had not previously been established *in vivo* or *in vitro*. The sequence homology

between EDGP and XEGIP (62% identity) suggests that they have similar functions. Indeed, the results of preliminary experiments showed that the ethanol precipitate of carrot cell culture media contains a component that inhibits *A. aculeatus* XEG and that the inhibitory activity is heat sensitive. In Chapter 3, a protein from carrot culture medium that exhibits XEG-inhibition activity was purified to approximately 95% homogeneity and was identified as EDGP by its apparent molecular weight on SDS-PAGE and peptide fingerprinting. Similar to XEGIP, EDGP forms a complex with XEG.

Both XEGIP and EDGP are secreted into the medium by suspension-cultured cells that were derived from callus, which normally grows in response to wounding a plant tissue. As pointed out by Satoh et al. (1992), plant proteins that are typically expressed in response to wounding are also detected in suspension-cultured cells. These include invertase (Lauriere et al., 1988) hydroxyproline-rich glycoproteins (Lamport and Northcote, 1960; Pope and Lamport, 1974; Esaka et al., 1992; Brownleader and Dey, 1993; Hirsinger et al., 1997; Lamport, 2001), chitinase (Kunze et al., 1998; Wojtaszek et al., 1998; Arie et al., 2000), β-1,3-glucanase (Kunze et al., 1998), and peroxidase (Breda et al., 1993; Schnabelrauch et al., 1996). The high expression of XEGIP-related proteins in cultured cells is consistent with their putative role in plant stress responses.

A study (Ditt et al., 2001) showed that an *Ageratum conyzoide*s gene similar to EDGP and XEGIP is strongly up-regulated when the plant is infected by *Agrobacterium tumefaciens*. Furthermore, antifungal activity was reported for a 46 kDa basic protein from cotton seeds (Chung et al., 1997), and the N-terminal sequence of this cotton protein

is similar to XEGIP (50% identity). Taken together, these observations suggest that XEGIP-related proteins may be involved in a plant's defense against pathogenic attack.

Basic 7S globulin (Bg7S) from soybean seeds and conglutin γ (Cγ) from *Lupinus albus* seeds (Blagrove and Gillespie, 1975; Elleman, 1977; Blagrove et al., 1980) also have amino acid sequences that are 35% identical to those of XEGIP and EDGP (Chapter 2, Figure 2.6), suggesting a remote evolutionary relationship. Proteins that are closely related to Bg7S and Cγ are widely present in the seeds of leguminous plants (Kagawa et al., 1987), and have long been considered to be storage proteins (Blagrove et al., 1980; Kolivas and Gayler, 1993). However, recent research suggests that these proteins may have other physiological roles. When soybean or *Lupinus albus* seeds are immersed in 50-60 °C water, large amounts of Bg7S and Cγ, respectively, are released along with other proteins (Kagawa et al., 1987). The Cγ was found to be newly synthesized rather than constitutive (Duranti et al., 1994), consistent with its potential role in stress response. Bg7S binds insulin and insulin-like growth factors (Komatsu and Hirano, 1991), while Cγ was observed to have lectin-like activity (Duranti et al., 1995). Thus, it appears that two common characteristics of XEGIP-related proteins are their capacity to bind to specific proteins or peptides and their enhanced expression when the plant is exposed to a stressful stimulus such as heat-shock, wounding, or infection.

When the sequences of XEGIP-like proteins are aligned, it highlights that the cysteine residues are especially well conserved, suggesting that the three-dimensional structures of these proteins may have features in common with XEGIP. Interestingly, the

distribution pattern of seven cysteines near N-terminals of these XEGIP-like proteins is strikingly similar to a protein motif known as "cystine knot" existing in some growth factors (e.g. nerve growth factor (NGF), transforming growth factor β2 (TGFβ2), and platelet-derived growth factor (PDGF)) (McDonald and Hendrickson, 1993) and some toxic or inhibitory peptides (e.g. a neurotoxin ω-CgTx from *Conus geographus*, a cyclic polypeptide from plant *Oldenlandia affinis* DC, and a trypsin inhibitor from the seeds of pumpkin *Curcurbita maxima*) (Pallaghy et al., 1994). A cystine knot consists of a ring formed by the first two disulfide bonds (between the first and fourth cysteine and the second and fifth cysteine) and the intervening polypeptides backbone, through which the third disulfide bond (cysteine3 and 6) passes (Pallaghy et al., 1994). All known "cystine knot" containing polypeptides/proteins have been implicated in protein-protein interactions. It remains unknown whether such a motif exists in XEGIP-like proteins, as further experiments are required to map the disulfide bonds in XEGIP-like proteins. If such a cystine knot indeed exists in XEGIP-like proteins, it is tempting to hypothesize that the cystine knot may provide a molecular basis for the interaction of XEGIP-like proteins with their ligands.

Chapter 3 describes the heterologous expression of XEGIP in *E. coli*. XEGIP protein was successfully expressed only when fused to a thioredoxin leading sequence. Polyclonal antibodies raised against native XEGIP react to these recombinant proteins in Western blots. However, the protein was expressed as an insoluble, inactive form that could be recovered from inclusion bodies. Efforts to refold the recombinant protein failed.

Chapter 3 also describes that the development of an immunoaffinity chromatography based method to identify other XEGIP ligands in fungal protein preparations such as commercial mixtures of cell wall hydrolases. Polyclonal antibodies against XEGIP were covalently immobilized on gel matrix. Purified XEGIP was incubated with the protein(s) to be screened, allowing XEGIP-ligand complexes to form. The mixture was then applied to the immunoaffinity column which binds free XEGIP and any XEGIP-containing protein complexes. The affinity bound proteins were then eluted and assayed by SDS-PAGE. The protein mixture to be screened was also subject to the same procedure with no added XEGIP to act as a negative control. This method was successfully used to identify a XEGIP interacting protein in Sanzyme®, a mixture of cell wall hydrolyzing enzymes from the fungus *Aspergillus oryzae*. The XEGIP ligand was identified as a glycanase in the same family (12) as XEG. This method was also used to verify the interaction of CfXEG with XEGIP (Chapter 4).

Xylanase inhibitors have recently been isolated from wheat (*Triticum aestivum*) flour (Debyser et al., 1997; Rouau and Surget, 1998; Debyser et al., 1999), (McLauchlan et al., 1999; Gebruers et al., 2001). One of these, *Triticum aestivum* xylanase inhibitor, termed TAXI (Debyser et al., 1997; Debyser et al., 1999), was found to contain two different components, TAXI-I and TAXI-II (Gebruers et al., 2001). TAXI-I and II are distinct proteins with different pIs and different inhibition specificities for their xylanase ligands. TAXI-I inhibits xylanases from *A. niger* and *B. subtilis* (both Family 11 xylanases) whereas TAXI-II only inhibits the *B. subtilis* xylanase. Neither inhibits a Family 10 xylanase from *A. aculeatus*.

The N-terminal sequences of TAXI proteins showed no significant similarity to sequences available in databases at the time of their discovery (Gebruers et al., 2001). However, as a result of the recently completed analysis of the rice genome (Goff et al., 2002; Yu et al., 2002) and other ongoing sequencing projects, several sequences similar to TAXI proteins can now be found in the databases. Among these are five rice gene-products, which are surprisingly the same genes that I previously found to be similar to XEGIP (25-29% identity) (Chapter 2). The full length sequence of TAXI-I (accession number: CAD27730) was recently deposited into gene bank. Sequence alignment confirms similarities between TAXI with the rice genes referred in Chapter 2 (Rice 1-5). Among them, Rice-2 (accession number BAB89708) is 54% identical and 65% similar to TAXI, suggesting Rice-2 may be an ortholog of TAXI and may function as xylanase inhibitor in rice. Alignment of wheat xylanase inhibitor protein (TAXI) with tomato xyloglucan-specific *endo*glucanase inhibitor protein (XEGIP) indicates these two proteins are remotely related (Figure 5.1, 27% identity, 41% similarity). These unexpected results suggest that instead of acting as *endo*glucanase inhibitors (as they do in non-graminaceous plants), the XEGIP homologues may function as xylanase inhibitors in graminaceous plants. Xylanase inhibitor and xyloglucan-specific *endo*glucanase inhibitor may belong to a super family of proteins that inhibit cell wall hydrolyzing enzymes. In graminaceous plants, glucuronarabinoxylan is a major component of the plant cell wall (Type I walls, (Carpita and Gibeaut, 1993)). Like xyloglucan, unbranched xylans can hydrogen bond to cellulose and are proposed to form a load-bearing network in the cell wall. Hence, prevention of xyloglucan and xylan degradation by pathogenic

enzymes (xyloglucanase and xylanase) may increase the infected plant's chances for survival.

The close relationship between XEGIP-like proteins and TAXIs are also hinted by the similarities among their ligands (Family 11 and 12 glycosyl hydrolases). Although the sequence identity between Family 12 *endo*glucanases and Family 11 xylanases is less than 20%, hydrophobic cluster analysis indicates that they are similar to each other (Torronen et al., 1993). X-ray structures (Sulzenbacher et al., 1997; Crennell et al., 2002) have confirmed that they share a very similar overall fold structure. In fact, it has been proposed that these two families of proteins form a "clan" characterized by a conserved complement of catalytic residues and common catalytic mechanisms (Henrissat and Davies, 1997).

Hemicellulose structure, degradation, and protection by glycanase-inhibiting proteins are similar in graminaceous and non-graminaceous plant species (Table 5.1). The sequence similarity of TAXI and XEGIP-like proteins suggests that they may represent a new super family of plant proteins, hemicellulase inhibitor proteins (HIPs), which can inhibit fungal enzymes that degrade the major hemicellulosic component in the cell walls of diverse plant species. These inhibitors may be considered to be weapons in an ongoing arms race between plants and their pathogens: a family of structurally related cell wall hydrolyzing enzymes evolves in microbes while the corresponding inhibitors co-evolve in plants, allowing some plants to survive pathogenic attack.

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Table 5.1 Similarities between graminae and non-graminae plants

Plant species	**Major hemicellulose**	**Associated fungal hydrolases**	**Plant inhibiting proteins**
Graminae	Xylan	Family 11 xylanases	Xylanase inhibitor, e.g. *Triticum aestivum* (wheat) xylanase inhibitor (TAXI)
Non-graminae	Xyloglucan	Family 12 endoglucanases	Endoglucanase inhibitor, e.g. tomato xyloglucan-specific endoglucanase inhibiting protein (XEGIP)
Comparison of Features	Both comprised of β-1,4-linkages; both bind noncovalently to cellulose	Share low sequence identity, but have very similar 3D structures, catalytic mechanism and molecular weight	Share low sequence similarity, but have similar molecular weights

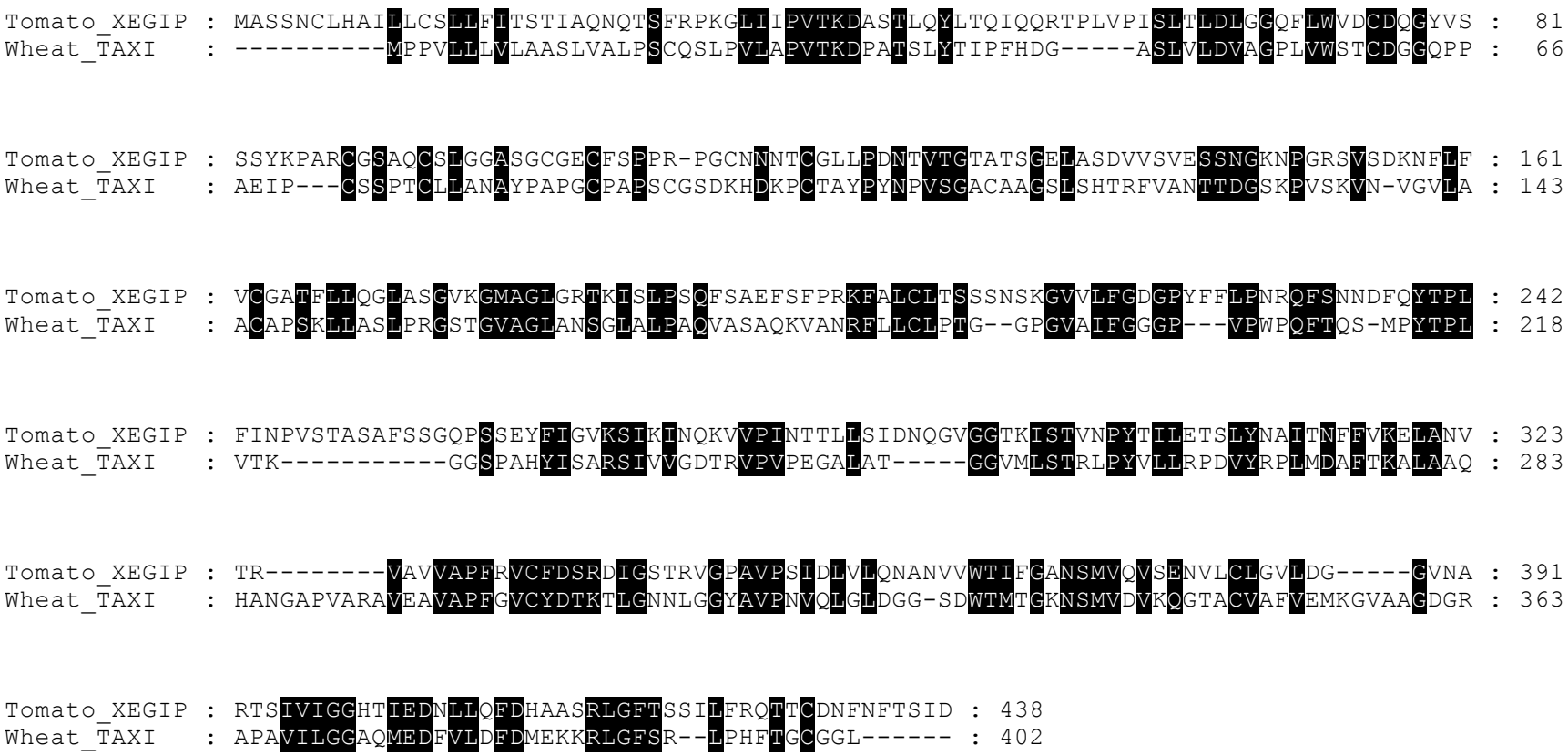

Figure 5.1. Alignment of xyloglucan-specific *endo*glucanase inhibitor (XEGIP) from tomato with *Triticum aestivum* xylanase inhibitor protein (TAXI).