**Safe sex in Douglas-fir**

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## Abstract

Approximately one week before fertilization in Douglas-fir, coincident with pollen tube initiation, an ovular secretion exudes from the nucellar apex and fills the micropylar chamber that houses engulfed pollen as well as any pathogens that may have also entered. Analysis has revealed that this liquid may not only play a role in pollen selection and development, but also serves as a delivery vehicle for pathogenesis-related (PR) proteins known to be antimicrobial. Although this ovular secretion does not extrude outside the ovule *in vivo*, the induction of a dissection droplet allows for its collection. Analysis of the secretions over a five year period showed that these liquids had similar protein concentrations and compositions; containing many different proteins with molecular weights ranging from 14 to 95 kDa.

Proteomic analysis has revealed that this secretion may not only provide beneficial nutrients to the pollen during pollen tube development, but may also initiate pollen tube formation. Using gel electrophoresis combined with N-terminal amino acid sequencing and quadrupole time-of-flight tandem mass spectrometry peptide sequencing, the most abundant proteins were identified as a 15 kDa phytocyanin, a 90 kDa xylosidase with an isoelectric point (pI) of 6.6, a 65 kDa xylosidase with a pI of 6.0, a 70 kDa invertase with a pI of 6.3, a 50 kDa invertase with a pI of 6.5, a 45 kDa galactosidase with a pI of 7.8, a 29 kDa galactosidase with a pI of 5.9, a 40 kDa aspartyl protease with a pI of 5.5, a 37

kDa peroxidase with a pI of 7.9, and a 33 kDa serine carboxypeptidase-like protein with a pI of 4.5. The presence of these proteins suggests that this secretion may play a significant role in pollen selection and development.

The Douglas-fir ovular secretion also contains many pathogenesis-related (PR) proteins to provide defence against pathogen attack. These include at least eight chitinases (PR-3, PR-4, PR-8, and PR-11), a thaumatin-like protein (PR-5), and a peroxidase (PR-9). It was first shown that the Douglas-fir ovular secretion had optimum chitinase activity at pH 6. The chitinases present in the secretion were identified and characterized using in-gel chitinase assays. These assays showed that there were at least 8 different chitinases present in the secretion with varying molecular weights and pIs. The majority of the chitinases identified using these assays were acidic. Two-dimensional gel electrophoresis (2D GE) was also used to separate PR proteins present in the Douglas-fir ovular secretion. Internal amino acid sequences were obtained by digesting these proteins with trypsin and then sequencing the generated peptides using quadrupole time-of-flight tandem mass spectrometry. Using this methodology, three PR proteins were successfully identified – a 37 kDa peroxidase with a pI of 7.9, a 28 kDa thaumatin-like protein with a pI of 4.3, and a basic 27 kDa chitinase (pI 7.8). To determine the origin of these PR proteins, a polyclonal serum was used. Western analysis showed that the Douglas-fir ovular secretion thaumatin-like protein originated from the nucellus, not from the megagametophyte or integument.

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## List of Abbreviations

| | |
|---|---|
| **ATP** : | adenosine 5'-triphosphate |
| **Avr** : | avirulence |
| **BSA** : | Bovine serum albumin |
| **DMAPP** : | dimethallyl pyrophosphate |
| **ESI** : | electrospray ionisation |
| **GM** : | genetically modified |
| **GTP** : | guanosine 5'-triphosphate |
| **HR** : | hypersensitive response |
| **IDP** : | isopentenyl diphosphate |
| **kDa** : | kiloDalton |
| **LRR** : | leucine-rich repeat |
| **MS** : | mass spectrometry |
| **NB** : | nucleotide binding |
| **PP** : | polyphenolic parenchyma |
| **POXs** : | extracellularly secreted plant peroxidases |
| **PR** : | pathogenesis-related |
| **PVDF** : | polyvinylidienedifluoride |
| ***R* genes** : | Resistance genes |
| ***R*GAs** : | *R* gene analogs |
| **ROIs** : | reactive oxygen intermediates |
| **RP-HPLC** : | reversed-phase high performance liquid chromatography |
| **SAR** : | systemic acquired resistance |
| **SEM** : | scanning electron micrograph |
| **TD** : | traumatic resin duct |
| **TFA** : | trifluoroacetic acid |
| **TL** : | thaumatin-like proteins |

| | |
|---|---|
| **1D SDS-PAGE** : | one-dimensional sodium dodecylsulphate polyacrylamide gel electrophoresis |
| **2D GE** : | two-dimensional gel electrophoresis |

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# Chapter 1

## General Introduction

Canada's forests not only dominate the landscape, but they have shaped our cultural, economic, and social life. From the conifers of the Pacific coast to the maples and oaks of the east, forests cover nearly half of our country. Canadians have had an intricate relationship with their forests since early times. Today, we not only enjoy our forests recreationally, but the commercial trading of their resources is critical to the welfare of our nation.

Conifers arose at least 200 million years ago and are the most dominant gymnosperms (i.e. "naked seed" plants) in the modern world. They are distributed worldwide with approximately 650 species identified to date arranged in 7 families (Pilger 1926). Conifers of the Pinaceae family dominate the boreal forest, a nearly continuous belt of coniferous trees across North America, Europe, and Asia. Our temperate forests in British Columbia are also dominated by pinaceous species including the conifer studied in this thesis, Douglas-fir (Figure 1) (*Pseudotsuga menziesii* (Mirbel) Franco).

Conifers have provided a wealth of beneficial products that have significantly enhanced the quality of life for human societies around the world. In addition to providing us with the more tangible products - timber, pulp, paper, and fuelwood - conifers also provide us with a wide range of beneficial non-wood products. Some of these are used to treat various medical conditions. The diterpene Paclitaxel (taxol), originally extracted from the bark of Pacific yew (*Taxus brevifolia* Nutt.), is used to treat various cancers (Wani *et al.* 1971). Diterpenes extracted from the cones of luchu pine

(*Pinus luchuensis* Mayr.) have exhibited antiviral properties (Minami *et al.* 2002). Homoharringtonine, first isolated from the plum yew (*Cephalotaxus harringtonia* Forbes), has been shown to have broad antitumour activity in rodents and antileukemic effects in humans (Ni *et al.* 2003). These medicinal compounds are very beneficial, but they pale in comparison to how much humans rely on conifer wood products.

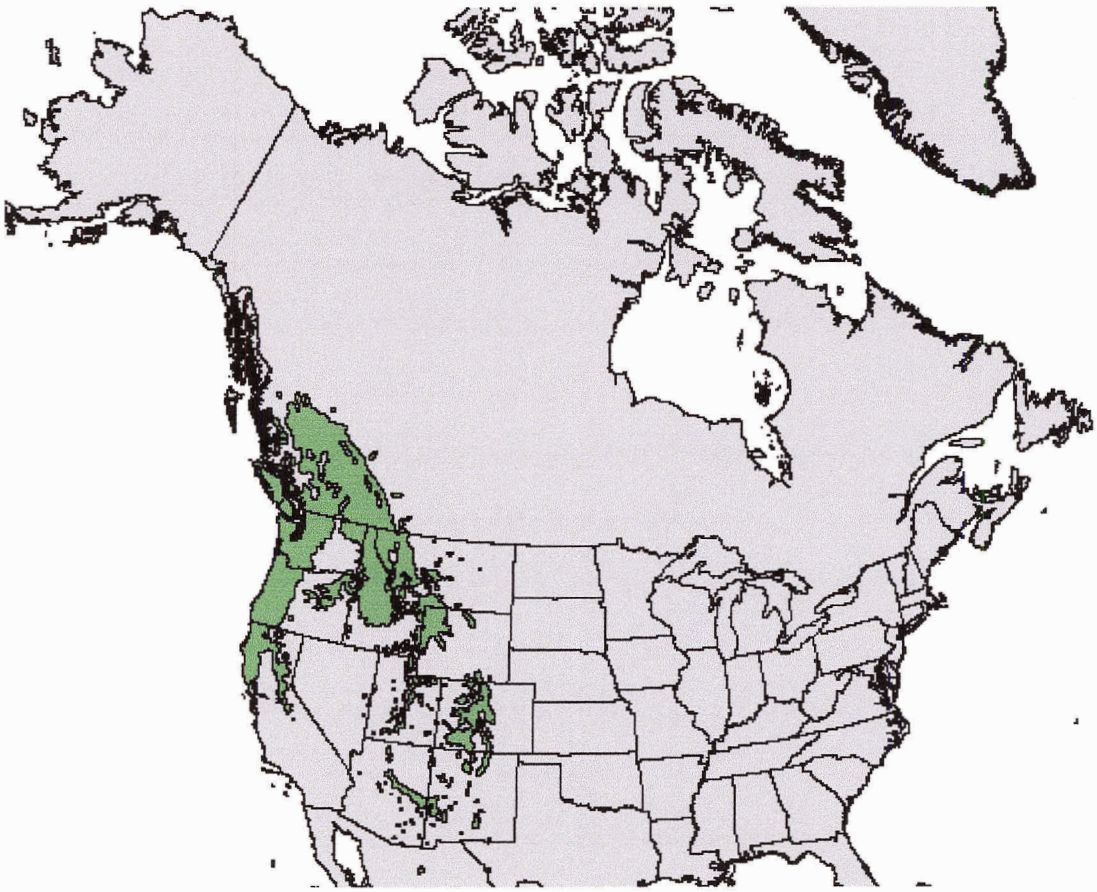


**Figure 1. Douglas-fir (*Pseudotsuga menziesii*) distribution in North America (Little 1971)**

Canada plays the dominant role in global forestry since 10 % of all forests worldwide are within our borders. Of the provinces, British Columbia is the major contributor. More than 30 % of the national revenue generated from exporting forest products comes from this province.

Forestry in British Columbia began in the mid-1800s, but remained rather modest until the arrival of railroads in the late 19$^{th}$ century. With the completion of this new transportation network, a critical link with an expanding North American economy was made. Eastern resource capitalists realized the potential for investment in west coast timber and a wave of business activity in the forestry sector of the Pacific Northwest was initiated. The onset of this new industrial order in British Columbia, combined with poor forestry practices, resulted in overproduction and waste. By 1930, the first predictions of a future timber famine were to be heard (Rajala 1998). Although our forestry practices have evolved significantly over the last 70 years, the creation of a sustainable forest is still a primary goal for both British Columbia and Canada.

Today, Canada is the largest exporter of forest products in the world. The value of these exports in 2002 was $42.9 billion dollars; $10.3 billion of this from conifer (i.e. softwood) exports. The province of British Columbia is the largest exporter of softwood lumber, averaging $7 billion dollars annually, or about 60 percent of the national average (NRC 2002-2003).

This softwood industry is critical to the British Columbian economy not only for the provincial revenue generated, but also for the jobs it has created. It is estimated that the forestry sector employs 55,000 British Columbians. Unfortunately, rapid harvesting of this slow-growing crop has reduced their numbers. We must protect our current

natural stand conifers from insects and disease and sustain their populations through systemic reforestation if we want to maintain this vital economy.

Conifer insects and diseases are a natural and important part of a healthy forest, but some are detrimental. In 1998, the total timber harvest in British Columbia was 67.6 million $m^3$. The amount of natural stand timber that was lost as a result of insect and pathogen infestation that same year was 12.8 million $m^3$, nearly 20 % of what was harvested (Forests 2000)! The discovery and application of new antimicrobial and antiherbivory molecules will help protect our forests thereby increasing their potential yield.

Reforestation is accomplished through both seedling planting programs and natural regeneration with seed. Around 250 million seedlings are planted annually in British Columbia. To maximize a seedling's growth potential they must have traits appropriate for the zone in which they will be planted. Genetic improvement and silvicultural management techniques allow for optimal production of these ideal seedlings in a nursery. In consequence, the Province of British Columbia has established a goal of producing 75 % of all seedlings from conifer orchard seed by 2007 (British Columbia Ministry of Forests 2000).

Since much time and effort goes into breeding, the conifer orchard tree seed is of great value. Unfortunately, seeds can carry pathogenic organisms such as bacteria and fungi. Most reside on the seed coat, but some can also reside within the seed. Having the ability to spread, these pathogens contaminate other seeds. Finding ways to protect these seeds either through treatment or encapsulation with antimicrobial molecules would greatly decrease seed losses.

In today's genetically modified (GM) sensitive world, using defensive molecules produced from the conifers themselves would be most appropriate in protecting our current conifer populations and their seed. Antimicrobial molecules produced from the same conifer organ that produces the seed would be ideal.

My thesis investigated the following three hypotheses:

**$H_o^1$ Proteins present in the Douglas-fir ovular secretion may play a role in pollen selection and development**

**$H_o^2$ The Douglas-fir ovular secretion contains antifungal proteins**

**$H_o^3$ Douglas-fir ovular secretion proteins originate in the nucellus**

# Chapter 2

## Conifer defense: a review

There are many conifer pests and pathogens. Conifer defense against pathogen and insect attack begins with physical barriers such as bark, the waxy cuticle of the needles, and resin production. They also constitutively produce antimicrobial and antiherbivory secondary metabolites to help impede pathogen ingress. If the invader happens to circumvent these preformed defense barriers, more conifer defense mechanisms are initiated to provide further resistance.

### 2.1 Preformed conifer defenses

Conifer bark, including the periderm and secondary phloem, provides an elaborate physical barrier to invading organisms. This barrier includes static and constitutive defenses such as cells containing calcium oxalate crystals (Srivastava 1963; Kartusch *et al.* 1991; Hudgins *et al.* 2003b), lignified stone cell masses (Wainhouse *et al.* 1990; Wainhouse *et al.* 1997), and the production of toxic secondary metabolites (Theis and Lerdau 2003).

Conifers continually produce a diverse array of secondary metabolites. These metabolites are those molecules produced by plants that are not directly essential for their survival (Bell 1981). Unlike primary metabolites that are present in all plant cells, secondary metabolite distribution varies throughout the plant. They are typically produced in specific organs, tissues, or cell types during distinct stages of development. There are three major classes of plant secondary metabolites: terpenes, alkaloids, and phenolics. Some members from each of these classes provide defense against invading

pathogens and herbivores. It has been theorized that the diversity of these defensive secondary metabolites is a direct result of their continual evolution and the stepwise evolutionary pressures put on them by phytopathogens and insect predators (Ehrlich and Raven 1964).

### 2.1.1 Terpenes

Terpenes are the largest class of secondary metabolites in plants with more than 30,000 identified (Buckingham 1998). The word terpene is derived from the German word for turpentine (Terpentin) from which these natural products were first isolated and characterized. They play many different roles in plants. Some are hormones, electron carriers, structural components of membranes, and photosynthetic pigments. Others provide defense against pathogens.

A first line of defense in conifers is a complex mixture of terpenes present in oleoresin, most commonly referred to as resin or pitch. Resin is roughly composed of equal amounts of turpentine and rosin. All terpenes can be classified according to the number of isoprene units ($C_5H_8$) that make up their chemical structures (Figure 2.1A). Turpentine consists of monoterpenes ($C_{10}$) and sesquiterpenes ($C_{15}$); whereas, rosin consists of diterpenoid ($C_{20}$) resin acids (Figure 2.1B) (Phillips and Croteau 1999).

There are various sites of resin production and accumulation in conifers. Cedars (*Thuja*) exhibit the simplest system with isolated resin cells scattered throughout their stems. In the wood and bark of true firs (*Abies*) and California redwoods (*Sequoia sempervirens* D. Don.), resin is accumulated in resin blisters - multicellular sack-like structures surrounded by a layer of epithelial cells (Bannon 1936). Members of the genera *Larix* and *Pseudotsuga* have an even higher organization of resin production and

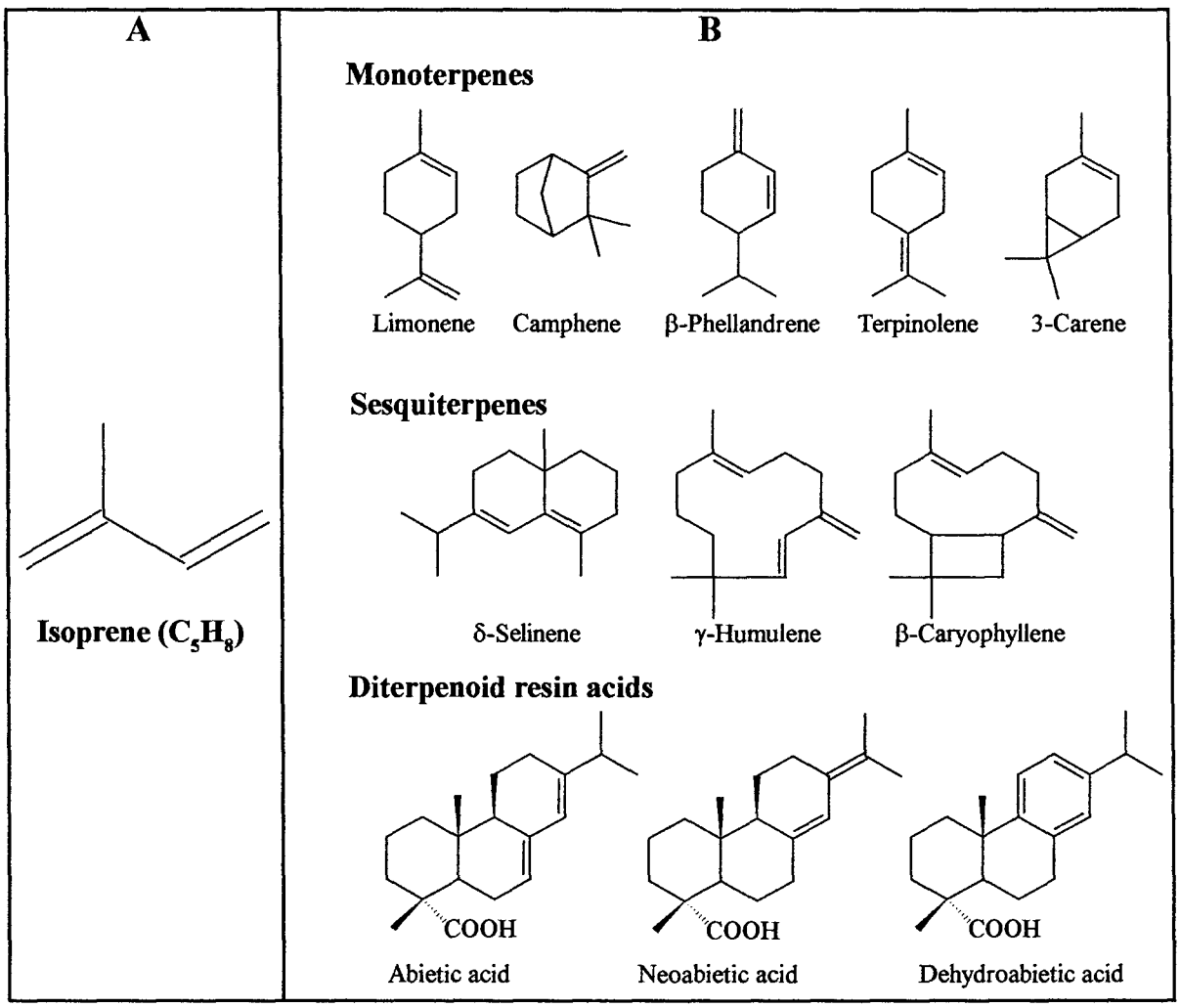



**Figure 2.1 Terpenes. A.** The various terpenes can be classified according to the number of isoprene units that make up their chemical structures. **B.** The common terpenes of conifer oleoresin (Phillips and Croteau 1999).

accumulation since their resin blisters are connected through a network of constricted resin passages and ducts (Trapp and Croteau 2001). More elaborate resin systems are observed for spruces (*Picea*) and pines (*Pinus*). Throughout the wood and bark of these conifers are highly branched networks of interconnected nonrestricted resin ducts (Werker and Fahn 1969) capable of transporting stored resin over several meters (Lewinsohn *et al.* 1991).

Resin accumulates within the lumen of these various structures and is mobilized upon wounding and infection. The turpentine fraction of resin carries out two functions. First, it acts as a solvent for transporting the diterpenoid resin acids of the rosin fraction to the site of injury or infection. Once exposed to the atmosphere, the volatile terpenes in the turpentine fraction evaporate and the oxidative polymerization of the remaining diterpenoid resin acids results in a hardened barrier that seals the wound and often traps the microbial and insect invaders within the matrix. Second, the turpentine fraction contains a range of terpenes (Figure 2.1B) directly toxic to insects and microbes (Phillips and Croteau 1999).

Terpenes are not only present in resin. They are also produced in conifer needles and wood. Terpenes present in Douglas-fir needles were shown to inhibit both deer and sheep rumen microbes (Oh *et al.* 1967) and provide a level of resistance to western spruce budworm (*Choristoneura occidentalis* Freeman), the most widely distributed and destructive defoliator of coniferous forests in Western North America (Chen *et al.* 2002). Pine (*Pinus*) needle terpenes have also been shown to exhibit antifungal activity (Krauze-Baranowska *et al.* 2002). Essential oils extracted from the wood of various Pacific Northwest conifers such as Alaska cedar (*Chamaecyparis nootkatensis* (D. Don) Spach),

western juniper (*Juniperus occidentalis* Hook.), and Douglas-fir contain terpenes that exhibit a range of antimicrobial activity against various anaerobic bacteria and yeast (Johnston *et al.* 2001).

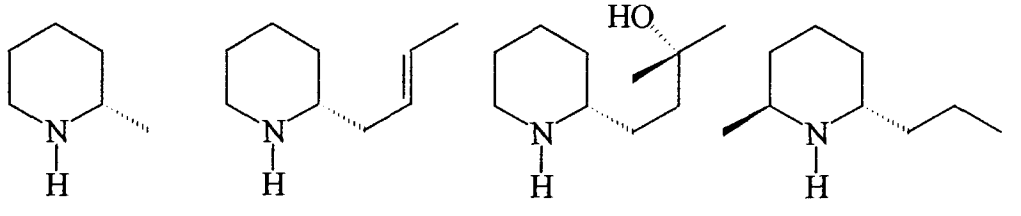
Terpene synthesis occurs in two subcellular locations. The production of triterpenes and sesquiterpenes via the classic acetate/mevalonic acid biochemical pathway occurs in the cytosol (Porter and Spurgeon 1981; Chappell 1995a; Chappell 1995b). The deoxyxylulose phosphate/methylerythritol (DOXP/MEP) biochemical pathway takes place in the plastids to produce the monoterpenes, diterpenes, and tetraterpenes (Lichtenthaler *et al.* 1997; Schwender *et al.* 1997; Eisenreich *et al.* 1998; Lichtenthaler 1999). These two pathways may also act synergistically since various intermediates can be shared (Jux *et al.* 2001).

Both pathways begin with two isoprene units, isopentenyl diphosphate (IDP) and dimethallyl pyrophosphate (DMAPP), being joined together in a head-to-tail fashion by prenyltransferases (Ruzicka 1953). The resulting molecules of varying lengths are then modified through dimerizations and cyclizations by terpene synthases to yield an assortment of different terpenes with an array of different functions (Davis and Croteau 2000). The majority of work on conifer terpene synthases has been carried out with grand fir (*Abies grandis* Lamb.) (Bohlmann *et al.* 1997; Bohlmann *et al.* 1998; Bohlmann and Croteau 1999; Bohlmann *et al.* 1999), but some have been isolated and characterized in other conifers including Sitka spruce (McKay *et al.* 2003), Norway spruce (*Picea abies* L.) (Faldt *et al.* 2003), loblolly pine (*Pinus taeda* L.) (Phillips *et al.* 1999; Phillips *et al.* 2003), and lodgepole pine (*Pinus contorta* Douglas ex Loudon) (Savage *et al.* 1994).

### 2.1.2 Alkaloids

Alkaloids are defined as non-peptidic and non-nucleosidic nitrogen containing compounds (Mann 1994a). Over 10,000 have been isolated and characterized including cocaine, caffeine, morphine, and nicotine. They are best known for their pharmacological effects on animals. In consequence, they are believed to be involved in plant defense against herbivores (Swain 1977).

Research on conifer alkaloids has been modest. The first Pinaceae alkaloids isolated were α-pipecoline and (-)-pinidine (Figure 2.2) from gray pine (*Pinus sabiniana* Douglas ex. D. Don) (Tallent *et al.* 1955; Tallent and Horning 1956). For the next 25 years there were no new Pinaceae alkaloids reported until the discovery of (-)-pinidinol (Figure 2.2) in Engelmann spruce (*Picea engelmannii* Parry ex. Engelm.) (Stermitz *et al.* 1990).




α-pipecoline (-)-pinidine (-)-pinidinol epidihydropinidine

**Figure 2.2 Conifer alkaloids.**

Even less research has been carried out to study the defensive capabilities of these molecules. In one of the earliest experiments, epidihydropinidine (Figure 2.2), an alkaloid extracted from Engelmann spruce, was shown to possess antifeedant activity against spruce budworm (*Choristoneura fumiferana* Clemens) (Schneider *et al.* 1991). Piperidine alkaloids of Sitka spruce have also been shown to provide a level of resistance to the white pine weevil (*Pissodes strobi* Peck), the most serious and economically

important native insect pest of spruce and pine trees in Canada (Gerson and Kelsey 2002).

### 2.1.3 Phenolics

Phenolics possess at least one aromatic ring attached to a hydroxyl group. Included in this class are molecules such as tannins, flavonoids, and lignins. They are all derived from the amino acids phenylalanine and tyrosine, both of which are produced via the shikimic acid pathway (Mann 1994b).

Conifer phenols provide defense in several forms. As previously mentioned, lignified stone cell masses in conifer bark tissue provides one level of insect defense because insect larvae that feed on high levels of lignin show a reduction in their survival and development (Wainhouse *et al.* 1990). Phenols present in the polyphenolic parenchyma (PP) cells of the secondary phloem in conifer bark provide another level of resistance. Active in the synthesis, storage, and modification of phenolics; PP cells at the site of infection immediately release their stored phenols (Franceschi *et al.* 1998; Franceschi *et al.* 2000; Hudgins *et al.* 2003a). Phenolics also accumulate at sites away from the infection, indicating the initiation of some induced responses (Nagy *et al.* 2000; Krekling *et al.* 2004). Some even act as signaling molecules. Salicylic acid and methyl salicylate have the ability to initiate further plant defense responses in both gymnosperms and the other group of seed plants, the angiosperms (Malamy *et al.* 1990; Metraux *et al.* 1990; Shulaev *et al.* 1997; Yu *et al.* 1997; Davis *et al.* 2002).

These preformed and constitutive defense mechanisms provide some resistance to pathogen or insect invasion, but conifers must also have other defenses in case the invaders breach these initial barriers.

## 2.2 Plant-pathogen interactions

When a pathogen attacks, an array of plant defenses are induced. Although interactions with pathogens are poorly understood in conifers, they have been extensively studied in angiosperms.

Initial activation of plant defenses depends on the ability of the host plant to first recognize elicitor molecules. Many elicitors have been characterized; they are classified as being either race-specific or non-race-specific. Typical race-specific elicitors are secreted peptides and proteins encoded for by pathogen avirulence (*Avr*) genes. They initiate plant defenses upon interaction, directly or indirectly, with host plant disease-resistance (*R*) gene products (de Wit 1997). This is the "gene-for-gene" concept for pathogenicity (Flor 1942; Oort 1944). Elicitors that initiate defensive responses in a different manner are referred to as non-race-specific elicitors (Hahn 1996).

### 2.2.1 Race-specific elicitors

Due to a continuous exchange of molecular information between plants and phytopathogens, an intricate relationship has coevolved between them in regards to plant disease and resistance. Independent research by Flor and Oort in the 1940s initiated the "gene-for-gene" concept for pathogenicity. Harold Flor was studying flax (*Phormium tenax* Forster & Forster f.) and flax rust fungus (*Melampsora lini* Ehrenb.) (Flor 1942; Flor 1971). Oort studied the *Ustilago tritici* (Pers.) – wheat interaction (Oort 1944). Since then, this concept has been fully established by the discovery of pathogen Avr ligands that, directly or indirectly, initiate plant defenses upon interaction with host plant *R* gene products.

In the past, this "gene-for-gene" interaction was interpreted as a receptor-ligand model in which the plant defense mechanisms were activated once the Avr ligand was directly bound to the corresponding plant R receptor. However, recent experiments have shown that even though the co-localization of the avirulence factor and the corresponding resistance protein is essential for successful initiation, direct interaction between these two proteins is not necessary. Other models exist that may account for this observed "gene-for-gene" relationship including a co-receptor model, a guard model, and a protease-dependent defense elicitation model. The co-receptor model suggests that defense elicitation occurs when the appropriate Avr ligand attaches to a high affinity binding site present on a co-receptor that is part of a multi-protein assembly complex along with the corresponding R protein (Bonas and Lahaye 2002). The guard model hypothesizes that the R protein associates and "guards" the actual plant protein to which the Avr factor is targeted. When the corresponding Avr molecule binds its target, the R protein initiates appropriate defense responses (Dangl and Jones 2001). Since several pathogen *Avr* genes also encode for proteases, a protease-dependent defense elicitation model has also been suggested (Bonas and Lahaye 2002).

### 2.2.2 Plant Resistance (*R*) genes

R receptors, or protein complexes with associated R proteins, must carry out at least two functions. First, they must have the ability to recognize their corresponding *Avr* gene product. Second, once this recognition takes place, signaling cascades need to be initiated to activate and coordinate the appropriate defense mechanisms required to impede pathogen ingress.

Although plant R proteins are effective against a variety of different pathogens, the ones characterized thus far all share structural similarities. Analysis shows that they have at least two different protein motifs. One of these motifs provides insight on how R proteins bind their respective Avr ligands. The other motif is believed to be involved in signal cascade initiation. Based on the different motif combinations that have been characterized, five different classes of *R* genes have been identified.

The largest class is the *NB-LRR* genes which encode cytoplasmic proteins with both a leucine-rich repeat (LRR) domain for molecular binding and a nucleotide binding (NB) site for signal cascade initiation. LRR domains have been implicated in molecular binding since they have been shown to mediate protein-protein interactions, protein-carbohydrate interactions, as well as peptide-ligand binding (Kajava 1998). NB sites are believed to play a role in signal initiation since they are critical for binding ATP or GTP (Saraste *et al.* 1990). Transference of the available phosphate from this ATP or GTP bound molecule to G proteins or kinases could trigger the appropriate signaling cascades needed to elicit an effective defense response (Hammond-Kosack and Jones 1997). Recent proteomic studies has shown that rapid protein phosphorylations take place when plants and pathogens interact (Peck *et al.* 2001; Xing *et al.* 2002).

Many members of this class have been isolated and characterized in angiosperms including tomato, rice, potato, wheat, pepper, corn, and *Arabidopsis* (Takken and Joosten 2000). Conifer *R* gene discovery began only recently. White pine blister rust is a devastating introduced disease of five-needled pines in North America. To better understand this disease, a polymerase chain reaction (PCR) strategy was employed to characterize *R* gene analogs (RGAs) from western white pines (*Pinus monticola* Dougl.

ex D. Don.) resistant to this fungus (*Cronartium ribicola* J. C. Fisch. ex Rab.). Using oligonucleotide primers constructed from conserved sequences in the NB sites of angiosperm *NB-LRR* genes, 67 *NB* sequences were cloned from these resistant western white pine trees. The results obtained in these preliminary experiments provide evidence for the presence of conifer RGAs and their potential role in conifer disease resistance (Liu and Ekramoddoullah 2003).

### 2.2.3 Non-race-specific elicitors

Non-race-specific elicitors are molecules that have the ability to activate defense mechanisms in a manner different from the aforementioned "gene-for-gene" models. The largest class of non-race-specific elicitors is comprised of biologically active oligosaccharides released from pathogen cell walls by hydrolases secreted from the host plant (Hahn 1996). Only a few of these biologically active oligosaccharides have had their structure and biological function characterized (Cote and Hahn 1994; Fritig *et al.* 1998).

Chitin (β-1,4-linked polymer of *N*-acetylglucosamine) is a polysaccharide that occurs in both fungal cell walls and in invertebrate exoskeletons. Chitin fragments, *N*-acetylchitooligosaccharides generated from enzymatic hydrolysis, have been shown to elicit many plant resistance responses. Defensive responses such as reactive oxygen intermediate (ROI) production (Kuchitsu *et al.* 1995) and the increased expression of defense related genes (Nishizawa *et al.* 1999; Takai *et al.* 2001) have been reported in suspension-cultured rice cells treated with chitin fragments. Phytoalexins were also produced (Yamada *et al.* 1993). Phytoalexins are defined as any low molecular weight antimicrobial molecules produced after microbial attack (Bailey and Mansfield 1982).

Along with chitin, β-glucans are also present in fungal cell walls. β-glucan fragments were first recognized to be actively involved in plant-pathogen interactions in the mid 1970s (Ayers *et al.* 1976). Since then, many β-glucans isolated from the cell walls of various fungi have been studied. Some of the most well characterized β-glucan fragments are those generated from the pathogenic fungus *Phytophthora sojae* (Kaufmann and Gerdemann), the causative agent of root and stem rot in soybean (*Glycine max* (L.) Merr.). These fragments have been shown to elicit phytoalexin biosynthesis in soybean cotyledon cells (Sharp *et al.* 1984). Although β-glucan elicitor activity has been primarily studied in leguminous plants, it was also shown that the treatment of tobacco (*Nicotiana tabacum* L.) with these β-glucans resulted in antiviral protection (Kopp *et al.* 1989).

Structural studies have been carried out on β-glucan elicitors in an attempt to tease out the intricacies of their interactions with plants. The minimal requirements needed to elicit phytoalexin biosynthesis in soybean was a succession of five β-1,6-linked glucosyl residues with two side branches of β-1,3-glucan (Cheong *et al.* 1991).

Research on the ability of fungal cell wall components to initiate responses in conifers is limited. Much of this research has been focused on elicitors released from the ectomycorrhizal fungus *Hebeloma crustuliniforme* and their effect on cultured Norway spruce cells. Although this research focuses on mutualistic associations with an ectomycorrhizal fungus, it may also provide insights on the communication between pathogenic fungi and their conifer hosts as well. Treatment of cultured Norway spruce cells resulted in signaling responses such as cellular $Ca^{2+}$ influx and protein phosphorylation changes (Salzer *et al.* 1996; Salzer *et al.* 1997; Hebe *et al.* 1999).

Defensive responses such as extracellular alkalinization along with the production of reactive oxygen intermediates (ROIs) also took place (Schwacke and Hager 1992; Salzer *et al.* 1996; Salzer *et al.* 1997). Antimicrobial protein production also increased (Sauter and Hager 1989; Salzer and Hager 1993; Salzer *et al.* 1996). Recently, similar defensive responses were reported for conifer cell cultures of Yunnan yew (*Taxus yunnanensis* Cheng & L. K. Fu) that were treated with chitosan fragments (Zhang *et al.* 2002).

**2.3 Induced defenses**

When a pathogen attacks, an array of plant defenses are induced upon recognition of elicitor molecules. These interactions trigger responses both locally at the area of infection and systemically in distant unaffected areas of the plant. Experiments have even indicated that infected plants may induce a systemic response in neighbouring plants via a volatile chemical messenger (Shulaev *et al.* 1997).

Defensive physiological responses are triggered as a result of pathogen infection. The hypersensitive response (HR) is initiated first. An early characteristic of HR is the rapid generation of superoxide and accumulation of peroxide (Doke 1983a; Doke 1983b). Treatment of cultured Norway spruce cells with elicitors released from the fungus *Hebeloma crustuliniforme* resulted in the production of these ROIs (Schwacke and Hager 1992; Salzer *et al.* 1996; Salzer *et al.* 1997). It is unclear if ROIs induce HR directly by killing the pathogen (Levine *et al.* 1994) or indirectly by eliciting other defensive responses (Jabs *et al.* 1997). Activation of HR ultimately results in localized tissue cell death and prevents further spread of the disease by restricting the pathogen to the small area immediately surrounding the infected cells (Lamb *et al.* 1989).

Once the HR is initiated, a longer lasting nonspecific resistance throughout the plant is also triggered. This response is known as systemic acquired resistance (SAR) (Ryals *et al.* 1996). Well characterized nonspecific defense mechanisms activated during SAR include cell wall lignification, callose deposition around dead cell foci, and the formation of antimicrobial phytoalexins. All of these responses attempt to inhibit further growth of the phytopathogen (Dietrich *et al.* 1994).

An additional nonspecific defense mechanism activated during gymnosperm SAR is the alteration of the phenolic composition in polyphenolic parenchyma (PP) cells. PP cells - a major proportion of living cells in conifer secondary phloem – are active in the synthesis, storage, and modification of defensive phenols (Franceschi *et al.* 1998; Franceschi *et al.* 2000; Hudgins *et al.* 2003a). Although they provide important initial resistance to pathogen and herbivore attack, these cells also expand in size and alter their phenolic content when mechanically wounded. Activated within a few days of wounding, this response more than likely contributes to increased resistance to fungal infections (Franceschi *et al.* 2000).

Traumatic resin duct (TD) formation in sapwood is another nonspecific SAR defense mechanism. Depending on the conifer species and the invasiveness of the pathogen, the initial conifer resin system may not be able to provide an adequate defense. It has been observed that upon wounding and inoculation with fungi, TDs begin to form throughout Norway spruce sapwood. The formation of these TDs is believed to provide further resistance by producing more resin than was initially available (Nagy *et al.* 2000; Krokene *et al.* 2003).

In both gymnosperms and angiosperms, SAR also results in the increased expression of many genes (Ward *et al.* 1991; Uknes *et al.* 1992; Davis *et al.* 2002). The largest class of *SAR* genes is the pathogenesis-related (PR) proteins. Functional analysis of many of these PR proteins has shown them to exhibit antimicrobial activity (Selitrennikoff 2001).

In summary, there are many conifer pests and pathogens. Conifers have several preformed and constitutive defenses to provide an ever present initial level of resistance. If these initial barriers are circumvented, elicitor recognition results in more defenses being initiated both locally at the site of infection and systemically in uninfected areas.

# Chapter 3

## Identification of the most abundant proteins present in the Douglas-fir ovular secretion: an insight into conifer pollen selection and development

### 3.1 Introduction

Coordinated development between male and female organs is essential for reproductive success in plants (Herrero 2003). This holds especially true in conifers for several reasons: their methods of pollen capture are not very discriminatory (Owens *et al.* 1998), they exhibit extended periods of time between pollination and fertilization compared with angiosperms, and these pollination-fertilization intervals show variation between species as well as within a species (Singh 1978).

All conifers are wind pollinated. Complex wind eddies generated by the unique geometries of the female cones direct airborne pollen toward them (Niklas 1981; Niklas 1982; Owens *et al.* 1998). Some of the pollen that settle on these cones make their way down the cone axis and land just outside the receptive ovules. The pollen must then be drawn into the micropyle from outside the ovule (Figure 3.1). The method in which this is accomplished is referred to as the pollination mechanism and differs according to species. It may involve the production of a pollination drop outside the ovule, engulfment of pollen by ovular extensions (Figure 3.2), or even extreme siphonogamy where the pollen germinates and forms an especially long pollen tube while remaining outside the ovule (Owens *et al.* 1998).

For successful fertilization, pollen germination must culminate with the formation of a pollen tube - an event known as siphonogamy. The pollen tube then needs to penetrate an egg cell of the megagametophyte and deliver its male gametes (Figure 3.1) (Singh 1978). Although this is developmentally less complex than that found in

angiosperms, the intricacy of the many interactions taking place between the pollen and ovule in conifer reproduction shouldn't be underemphasized. These pollen-ovule interactions evolved to not only coordinate pollen and megagametophyte development, but also to ensure that appropriate mating partners are matched since foreign pollen may also enter the ovule. How are these pollen-ovule interactions carried out?

Whether it is in the form of a pollination drop or an active secretion into the micropyle, most conifer ovules produce at least one liquid secretion between pollination and fertilization (Owens *et al.* 1998; Gelbart and von Aderkas 2002). In Douglas-fir, an ovular secretion fills the micropyle of the ovule that houses the engulfed pollen and initiates an essential transition in pollen development – siphonogamy (Owens and Morris 1990; von Aderkas and Leary 1999). The pollen tube then elongates through the nucellus and eventually penetrates an egg cell of the megagametophyte approximately one week later (Owens and Morris 1990).

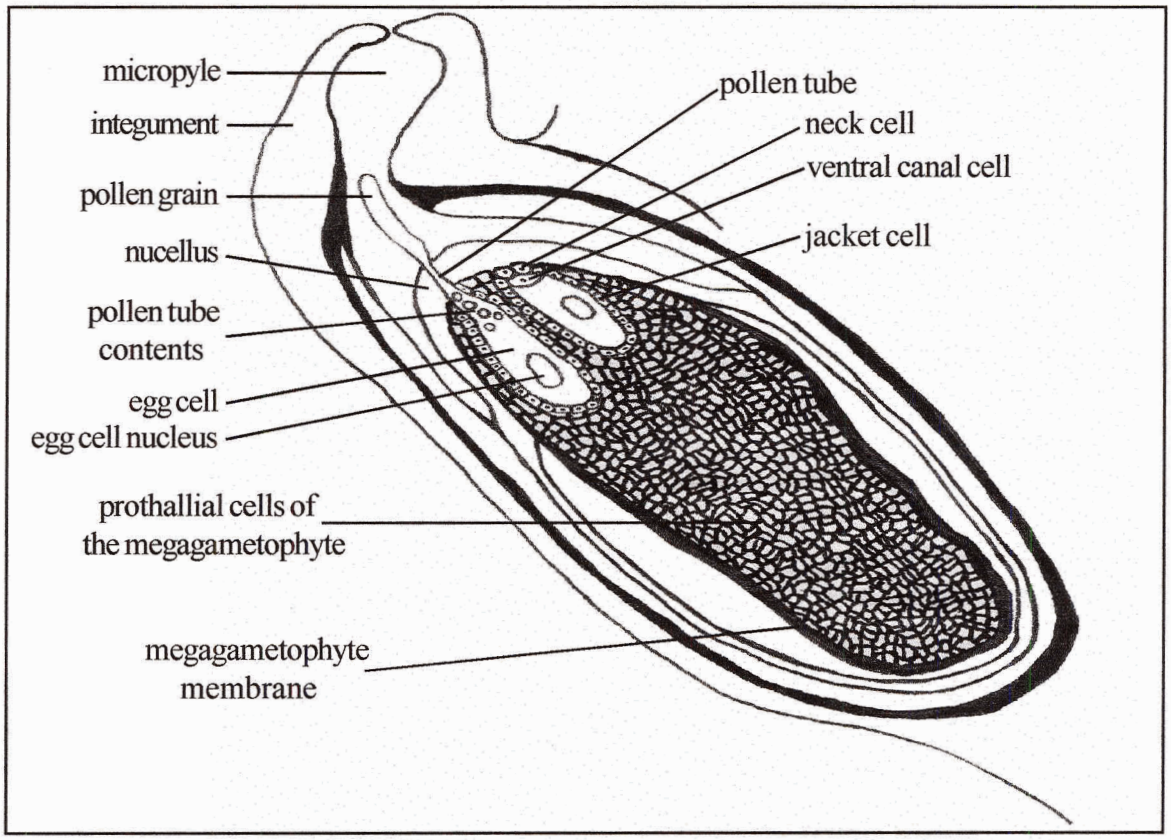



**Figure 3.1 A schematic of a mature ovule in Douglas-fir.** Inside the ovule, a pollen tube is penetrating an egg cell of the megagametophyte and delivering its contents. Adapted from Allen and Owens (1972).

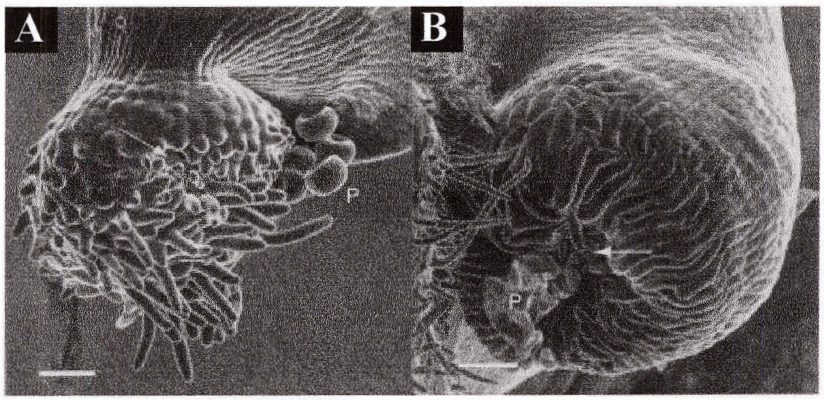



**Figure 3.2 The Douglas-fir engulfment pollination mechanism. A.** Scanning electron micrograph (SEM) showing a Douglas-fir ovule with pollen (P) adhering to the ovular extensions. Scale bar = 75 μm. **B.** SEM of a Douglas-fir stigmatic tip nearly finished pollen engulfment. Scale bar = 100 μm. (Owens *et al.* 1998)

**3.2 Results**

Although the ovular secretion of Douglas-fir does not extrude outside the ovule *in vivo*, the induction of a dissection droplet allows for its collection (Figure 3.3) (von Aderkas and Leary 1999). The Douglas-fir ovular secretion contains many different proteins with molecular weights ranging from 14 to 95 kDa (Figures 3.4 and 3.5).

Secretions collected over a five year period in 1999, 2000, 2001, 2002, and 2003 were determined to have protein concentrations of 1.61, 0.557, 0.867, 0.940, and 0.917 mg/mL, respectively (Figure 3.6 and Table 3.1). They also had similar protein compositions from season to season based on their one one-dimensional sodium dodecylsulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) protein profiles (Figure 3.7).

Prior to N-terminal amino acid sequencing, ovular secretion proteins were first separated by their relative hydrophobicity using reversed-phase high performance liquid chromatography (RP-HPLC) (Figure 3.8). The proteins eluted in each RP-HPLC fraction were then subjected to further separation based on their relative sizes using 1D SDS-PAGE (Figure 3.9) and subsequently transferred onto a polyvinylidienedifluoride (PVDF) membrane. The area of the PVDF membrane that contained a protein of interest was excised and submitted to the UVic Protein Chemistry Center for N-terminal amino acid sequencing.

A 15 kDa protein with an N-terminal amino acid sequence of TPYDVGGSSGXTIPXSNA was identified as a phytocyanin using Bork Group's MS blast search engine at EMBL (Protein A, Table 3.2). Other proteins that were N-terminally sequenced included an 8 kDa protein with an N-terminal sequence of

ATSXNQSPN (Protein B, Table 3.2) and a 19 kDa protein with an N-terminal sequence of SKQLXDHSVXRFXA (Protein C, Table 3.2). These proteins could not be identified using any protein search engines.

Two-dimensional gel electrophoresis (2D GE) was also used to separate proteins present in the Douglas-fir ovular secretion. These 2D gels indicate there are many proteins with varying sizes and isoelectric points (pIs) (Figure 3.10). Internal amino acid sequences were obtained by digesting the proteins with trypsin and then sequencing the generated peptides using quadrupole time-of-flight tandem mass spectrometry. These sequences were then submitted to Bork Group's MS blast search engine at EMBL for protein identification.

Six of the most abundant proteins present are glycosyl hydrolases including two xylosidases (Figures 3.11 & 3.13, Tables 3.3 & 3.5), two invertases (Figures 3.12 & 3.14, Tables 3.4 & 3.6), and two galactosidases (Figures 3.15 & 3.19, Tables 3.7 & 3.11). Two proteases - an aspartyl protease (Figure 3.16 and Table 3.8) and a serine carboxypeptidase-like protein (Figure 3.18 and Table 3.10) - have also been identified. A peroxidase is also present, but in less abundance (Figure 3.17 and Table 3.9). A summary for each protein identified including their approximate molecular weight, pI, and peptide amino acid sequences can be found in Tables 3.12 and 3.13.

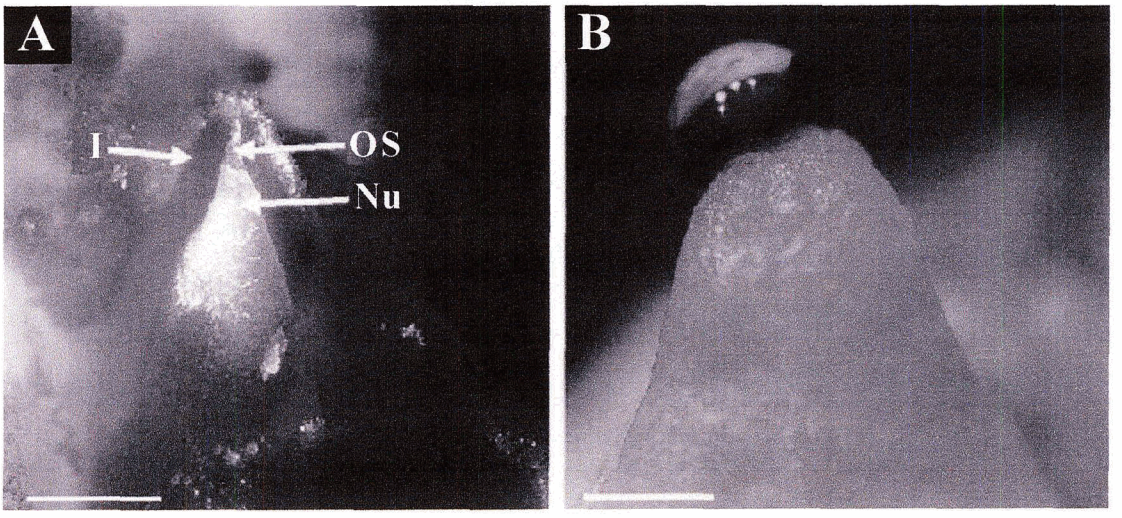



**Figure 3.3 Collecting the ovular secretion. A.** Photomicrograph of a Douglas-fir ovule frozen in liquid nitrogen *in situ* and dissected to reveal the frozen ovular secretion (OS), located between the integuments (I) and above the nucellus (N). *Bar* = 500 μm **B.** Photomicrograph of a Douglas-fir dissection drop on the ovular apex. *Bar* = 1.0 mm. The Douglas-fir ovular secretions studied were extracted from trees at the University of Victoria. The ovuliferous scales were dissected from the female cones collected in the field and placed in Fisherbrand® Petri dishes (Fisher Scientific, Canada) that had been kept humid with wetted Whatman® filter paper (Whatman International Ltd., Maidstone, England). Ten minutes later under a dissecting microscope, dissection droplets that formed were collected. Both photomicrographs are from the von Aderkas lab (von Aderkas and Leary 1999).

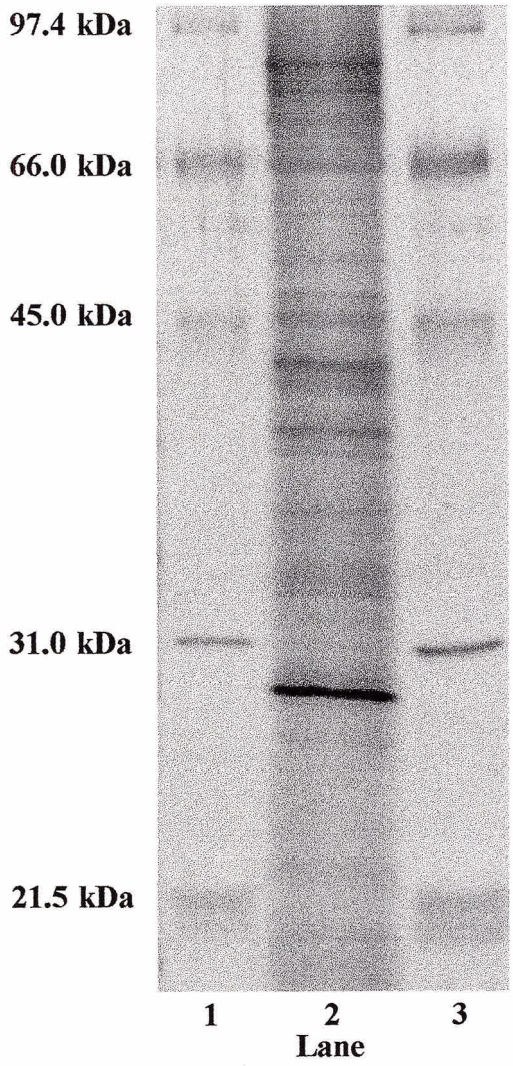



**Figure 3.4 Separation of Douglas-fir ovular secretion proteins using 1D SDS-PAGE.** Prior to loading, 3.0 μL of Douglas-fir ovular secretion was diluted to 10.0 μL with 2X glycine gel sample buffer and immersed in boiling water for 3 min. Electrophoresis was then carried out in a Bio-Rad Mini-Protean 3 Electrophoresis system (Bio-Rad Laboratories, Hercules, CA) through a 4 % acrylamide stacking gel and a 12 % acrylamide resolving gel until the tracking dye reached the bottom of the gel. The stacking and resolving gels were run at 10 and 20 mA, respectively. The buffers used were standard Tris-glycine buffers (Laemmli 1970). To visualize the proteins in the gel, they were silver-stained.

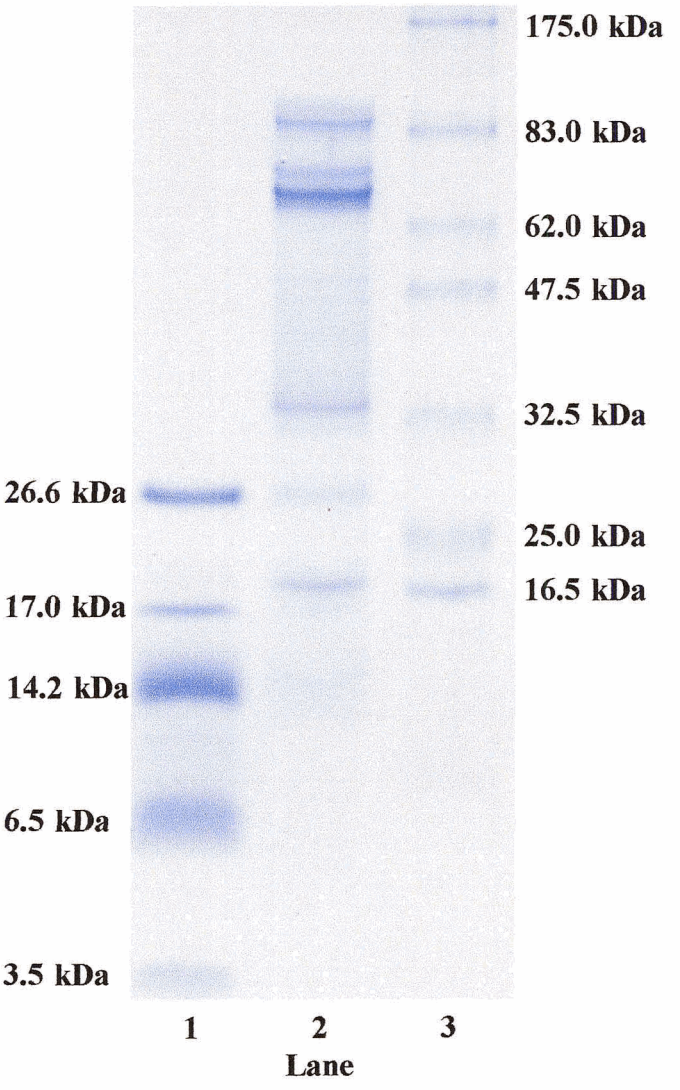



**Figure 3.5 Separation of Douglas-fir ovular secretion proteins using Tris-tricine 1D SDS-PAGE.** Prior to loading, 3.0 µL of Douglas-fir ovular secretion was diluted to 15.0 µL with 1X tricine gel sample buffer and immersed in boiling water for 3 min. Prepared samples were then electrophoresed in a Bio-Rad Mini-Protean 3 Electrophoresis system through a 4 % acrylamide stacking gel and 15 % acrylamide resolving gel at 100 V until the tracking dye reached the bottom of the gel. For protein visualization, the gel was stained with GelCode® Blue stain reagent.

**Lane 1:** Ultra-low range molecular weight markers (Sigma, St. Louis, MO)

**Lane 2:** 3.0 µL of Douglas-fir ovular secretion

**Lane 3:** Broad range molecular weight markers (New England BioLabs Inc., Beverly, MA)

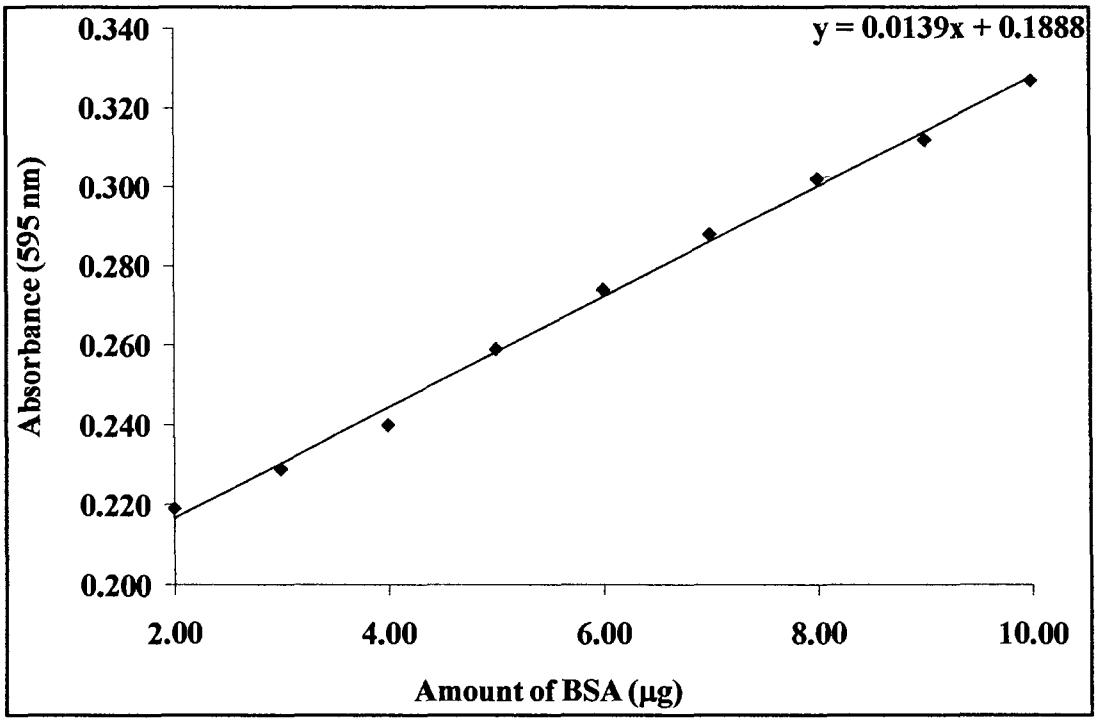



**Figure 3.6 Bovine serum albumin (BSA) standard curve.** The absorbances (595 nm) obtained for the various BSA standard solutions with concentrations ranging from 2.00 to 10.00 µg/mL.

| Date that Douglas-fir ovular secretions were collected | Average Absorbance (595 nm) | Amount of protein (μg) | Concentration (mg/mL) |
|---|---|---|---|
| June 7th, 1999 | 0.256 | 4.83 | 1.61 |
| June 9th, 2000 | 0.212 | 1.67 | 0.557 |
| June 12th, 2001 | 0.225 | 2.60 | 0.867 |
| June 16th, 2002 | 0.228 | 2.82 | 0.940 |
| June 7th, 2003 | 0.227 | 2.75 | 0.917 |

**Table 3.1 Douglas-fir ovular secretion protein concentration.** The protein concentrations were determined using data obtained from the Bradford Assay. The Bradford reagent (Sigma, St. Louis, MO) was gently mixed and brought to room temperature prior to the assay. A series of bovine serum albumin (BSA) standards were prepared in $dH_2O$ with concentrations ranging from 2.0 to 10.0 μg/mL. Three μL of Douglas-fir ovular secretion collected from each season were diluted to 1.0 mL with $dH_2O$. The assay was performed by first adding 1.0 mL of each standard and ovular secretion test sample to 1.0 mL of Bradford reagent in separate test tubes. They were then vortexed for approximately 10 s and incubated at room temperature for 30 min prior to measuring the absorbances at a wavelength of 595 nm. Each standard and test sample was performed in triplicate. A BSA standard curve was constructed by plotting the average absorbance values versus amount of BSA protein. The protein concentrations of the Douglas-fir ovular secretions were determined through extrapolation with this BSA standard curve (Figure 3.6).

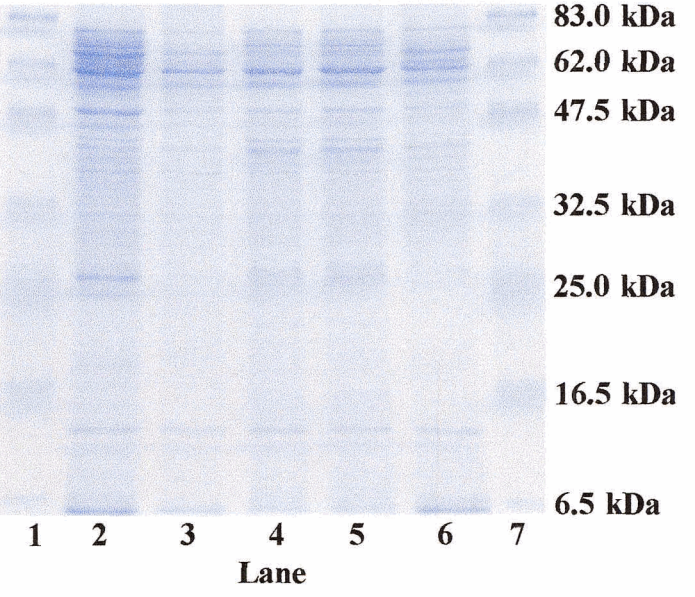



**Figure 3.7 Tris-glycine SDS-PAGE of Douglas-fir ovular secretions collected each season.** Prior to loading, 3.0 μL of Douglas-fir ovular secretions collected from each season were diluted to 10.0 μL with 2X glycine gel sample buffer and immersed in boiling water for 3 min. Electrophoresis was carried out in a Bio-Rad Mini-Protean 3 Electrophoresis system (Bio-Rad Laboratories, Hercules, CA) through a 4 % acrylamide stacking gel and a 12 % acrylamide resolving gel until the tracking dye reached the bottom of the gel. The stacking and resolving gels were run at 10 and 20 mA, respectively. The buffers used were standard Tris-glycine buffers (Laemmli 1970). For protein visualization, the gel was stained with GelCode® Blue stain reagent.
**Lanes 1 & 7 :** Broad range molecular weight markers
**Lane 2:** 3.0 μL of Douglas-fir ovular secretion collected on June 7, 1999
**Lane 3:** 3.0 μL of Douglas-fir ovular secretion collected on June 9, 2000
**Lane 4:** 3.0 μL of Douglas-fir ovular secretion collected on June12, 2001
**Lane 5:** 3.0 μL of Douglas-fir ovular secretion collected on June 16, 2002
**Lane 6:** 3.0 μL of Douglas-fir ovular secretion collected on June 7, 2003

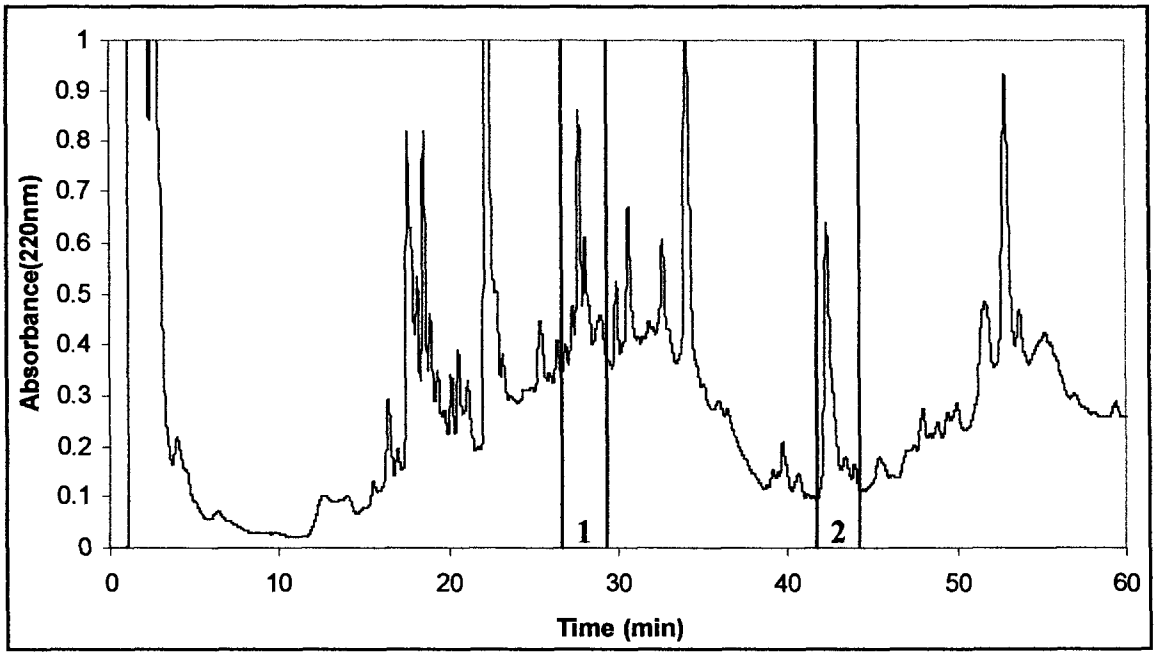



**Figure 3.8 Douglas-fir ovular secretion (50 µL) RP-HPLC profile.** Buffer A was 0.1 % TFA in water and Buffer B was 0.075 % TFA in acetonitrile. A gradient from 0-70 % Buffer B was carried out in 70 minutes. Detection was monitored at 220 nm. Proteins present in the RP-HPLC fractions indicated, 1 and 2, were further subjected to separation using 1D SDS-PAGE (Figure 3.9).

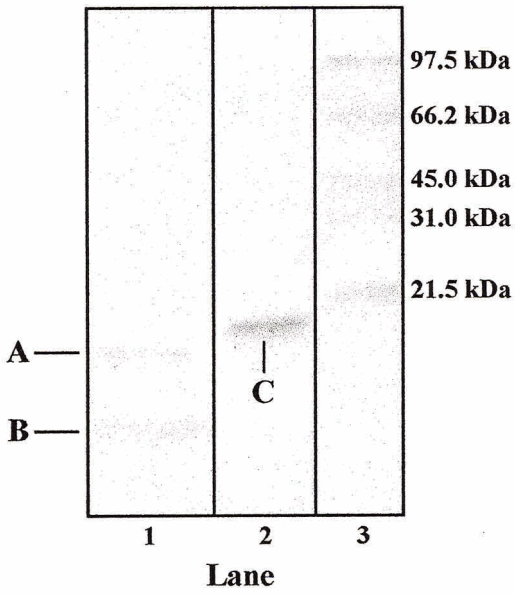



**Figure 3.9 SDS-PAGE of RP-HPLC fractions 1 and 2**. Prior to loading, the RP-HPLC fractions were solubilized in 10 µL of 1X glycine gel sample buffer and immersed in boiling water for 3 min. Fractions were then electrophoresed in a Bio-Rad Mini-Protean 3 Electrophoresis system (Bio-Rad Laboratories, Hercules, CA) through a 4 % acrylamide stacking gel and a 12 % acrylamide resolving gel until the tracking dye reached the bottom of the gel. The stacking and resolving gels were run at 10 and 20 mA, respectively. The buffers used were standard Tris-glycine buffers (Laemmli 1970). For protein visualization, the gels were stained with GelCode® Blue stain reagent (Pierce, Rockford, Il). See Figure 3.8 to correlate which RP-HPLC peaks belong to each fraction electrophoresed.
**Lane 1:** RP-HPLC Fraction 1 (Figure 3.8)
**Lane 2:** RP-HPLC Fraction 2 (Figure 3.8)
**Lane 3:** Molecular weight markers

| Douglas-fir ovular secretion protein (Figure 3.9) | Molecular Weight (kDa) | N-terminal amino acid sequence | Protein Identification by MSBlast |
|---|---|---|---|
| **Phytocyanin (A)** | 15 | TPYDVGGSSGXTIPXSNA | *Pinus taeda* (loblolly pine) phytocyanin homolog (AF101788) |
| **B** | 8 | ATSXNQSPN | No positive identification |
| **C** | 19 | SKQLXDHSVXRFXA | No positive identification |

**Table 3.2 N-terminal amino acid sequences obtained for various Douglas-fir ovular secretion proteins.** Proteins were separated using RP-HPLC (Figure 3.8) and 1D SDS-PAGE (Figure 3.9). The protein molecular weights were approximated based on their position relative to protein standards. Sequence searching was performed using Bork Group's MS blast search at EMBL.

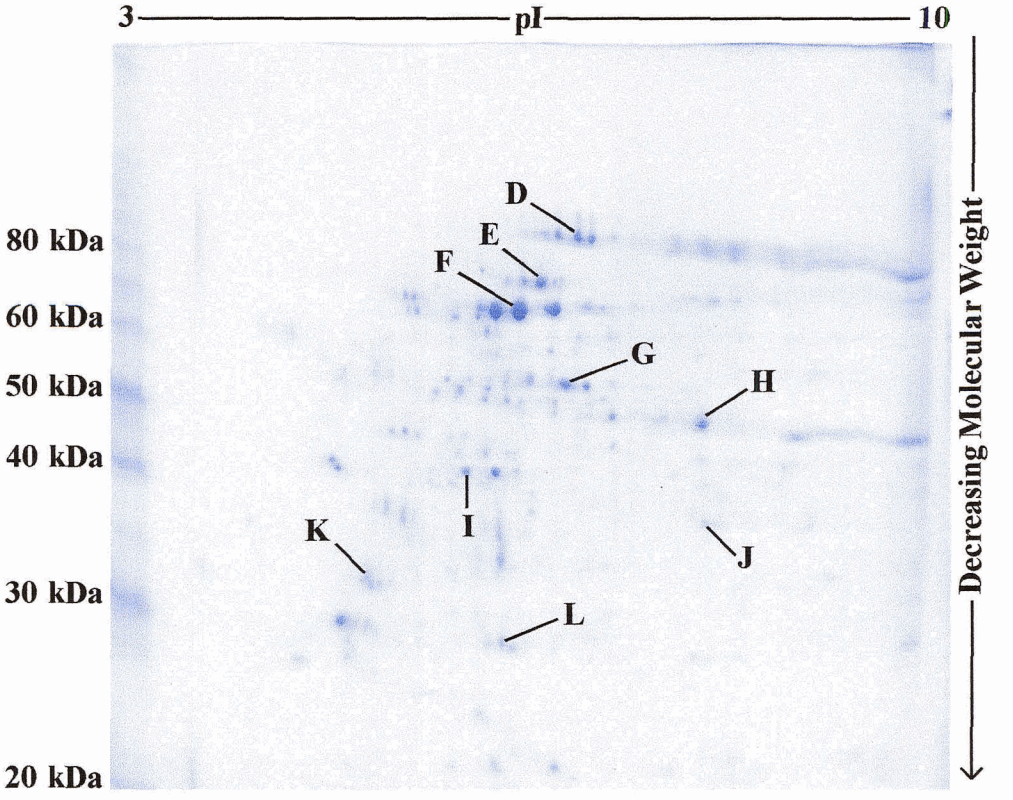



**Figure 3.10 2D gel of Douglas-fir ovular secretion proteins.** The Douglas-fir ovular secretion pellet was solubilized in 30 µL of SDS-MIX. Prior to electrophoresing the proteins, the isoelectric focusing (IEF) tube gels were first pre-electrophoresed for 1 h at 200 V. Once the protein samples were loaded, they were electrophoresed within the IEF tube gels for 17.5 h at 800 V. Before the proteins were further separated in the second dimension, the IEF tube gels were incubated in equilibration buffer for approximately 15 min. The proteins were then separated in the second dimension (10 % acrylamide) by electrophoresing them at 1 A until the blue tracking dye reached the end of the gels. To visualize the proteins present in the 2D gels, they were stained using a Colloidal Coomassie protocol (Neuhoff *et al.* 1988).

| | **Protein Identification** | **MS Data** |
|---|---|---|
| **D** | **Xylosidase I** | (Figure 3.11, Table 3.3) |
| **E** | **Invertase I** | (Figure 3.12, Table 3.4) |
| **F** | **Xylosidase II** | (Figure 3.13, Table 3.5) |
| **G** | **Invertase II** | (Figure 3.14, Table 3.6) |
| **H** | **Galactosidase I** | (Figure 3.15, Table 3.7) |
| **I** | **Aspartyl protease** | (Figure 3.16, Table 3.8) |
| **J** | **Peroxidase** | (Figure 3.17, Table 3.9) |
| **K** | **Serine carboxypeptidase-like protein** | (Figure 3.18, Table 3.10) |
| **L** | **Galactosidase II** | (Figure 3.19, Table 3.11) |

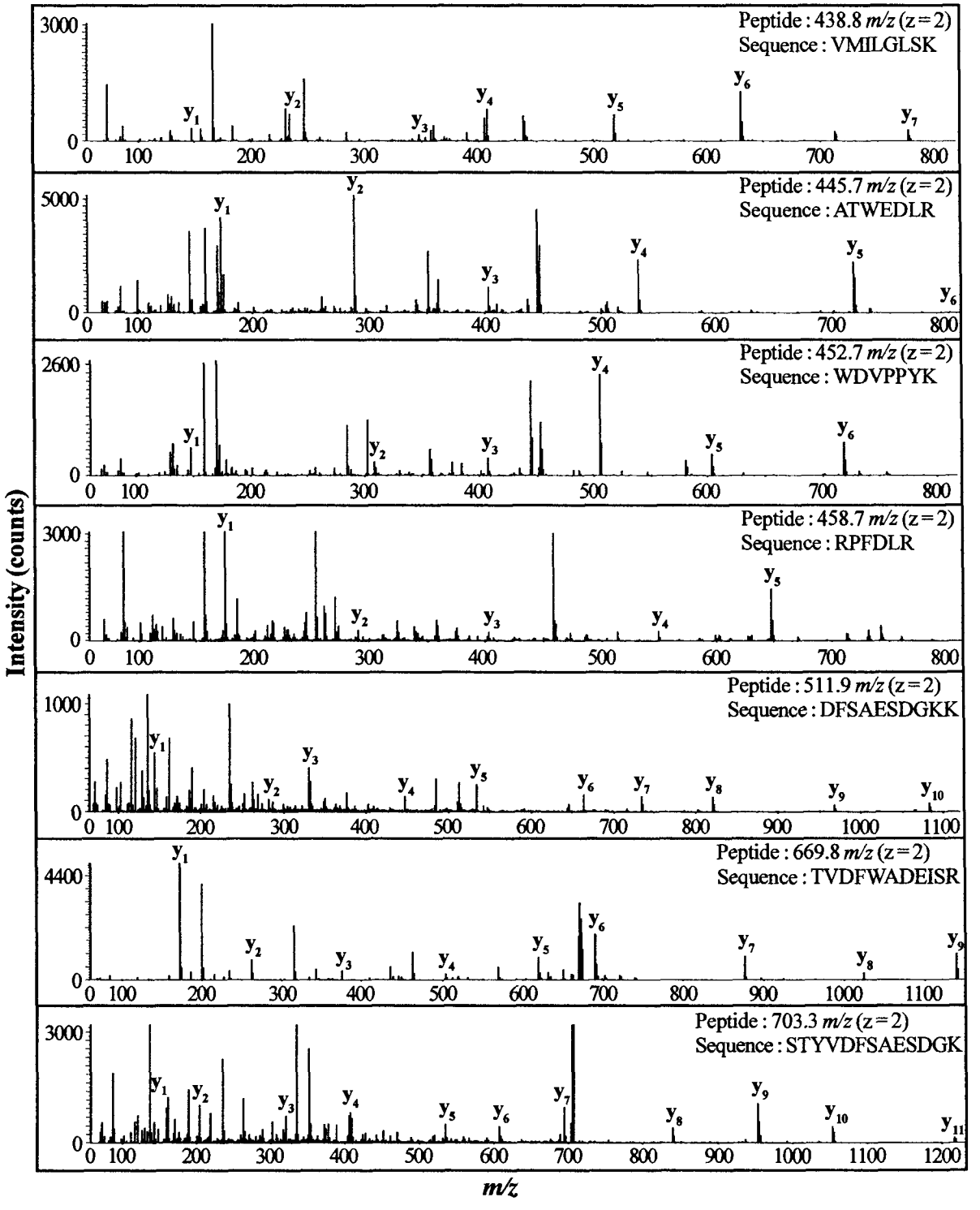



**Figure 3.11 Xylosidase I (Protein D) tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of Douglas-fir ovular secretion protein D (Figure 3.10). Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **438.8** | **445.7** | **452.7** | **458.7** | **511.9** | **669.8** | **703.3** |
| | **K** | **R** | **K** | **R** | **K** | **R** | **K** |
| **1** | 147.11 | 175.12 | 147.11 | 175.12 | 147.11 | 175.12 | 147.11 |
| | **S** | **L** | **Y** | **L** | **K** | **S** | **G** |
| **2** | 234.14 | 288.20 | 310.18 | 288.20 | 275.20 | 262.15 | 204.13 |
| | **L** | **D** | **P** | **D** | **G** | **I** | **D** |
| **3** | 347.23 | 403.23 | 407.23 | 403.23 | 332.23 | 375.24 | 319.16 |
| | **G** | **E** | **P** | **F** | **D** | **E** | **S** |
| **4** | 404.25 | 532.27 | 504.28 | 550.30 | 447.24 | 504.28 | 406.19 |
| | **L** | **W** | **V** | **P** | **S** | **D** | **E** |
| **5** | 517.33 | 718.35 | 603.35 | 647.35 | 534.28 | 619.30 | 535.24 |
| | **I** | **T** | **D** | | **E** | **A** | **A** |
| **6** | 630.42 | 819.40 | 718.38 | - | 663.32 | 690.34 | 606.27 |
| | **M** | | | | **A** | **W** | **S** |
| **7** | 777.49 | - | - | - | 734.37 | 876.42 | 693.31 |
| | | | | | **S** | **F** | **F** |
| **8** | - | - | - | - | 821.37 | 1023.49 | 840.37 |
| | | | | | **F** | **D** | **D** |
| **9** | - | - | - | - | 968.45 | 1138.52 | 955.40 |
| | | | | | **D** | | **V** |
| **10** | - | - | - | - | 1083.47 | - | 1054.47 |
| | | | | | | | **Y** |
| **11** | - | - | - | - | - | - | 1217.53 |

**Table 3.3 Xylosidase I (Protein D) peptide amino acid sequences.** Y ion masses obtained from tandem MS fragmentation of peptides generated from the digestion of protein D (Figure 3.11) from Douglas-fir ovular secretions and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

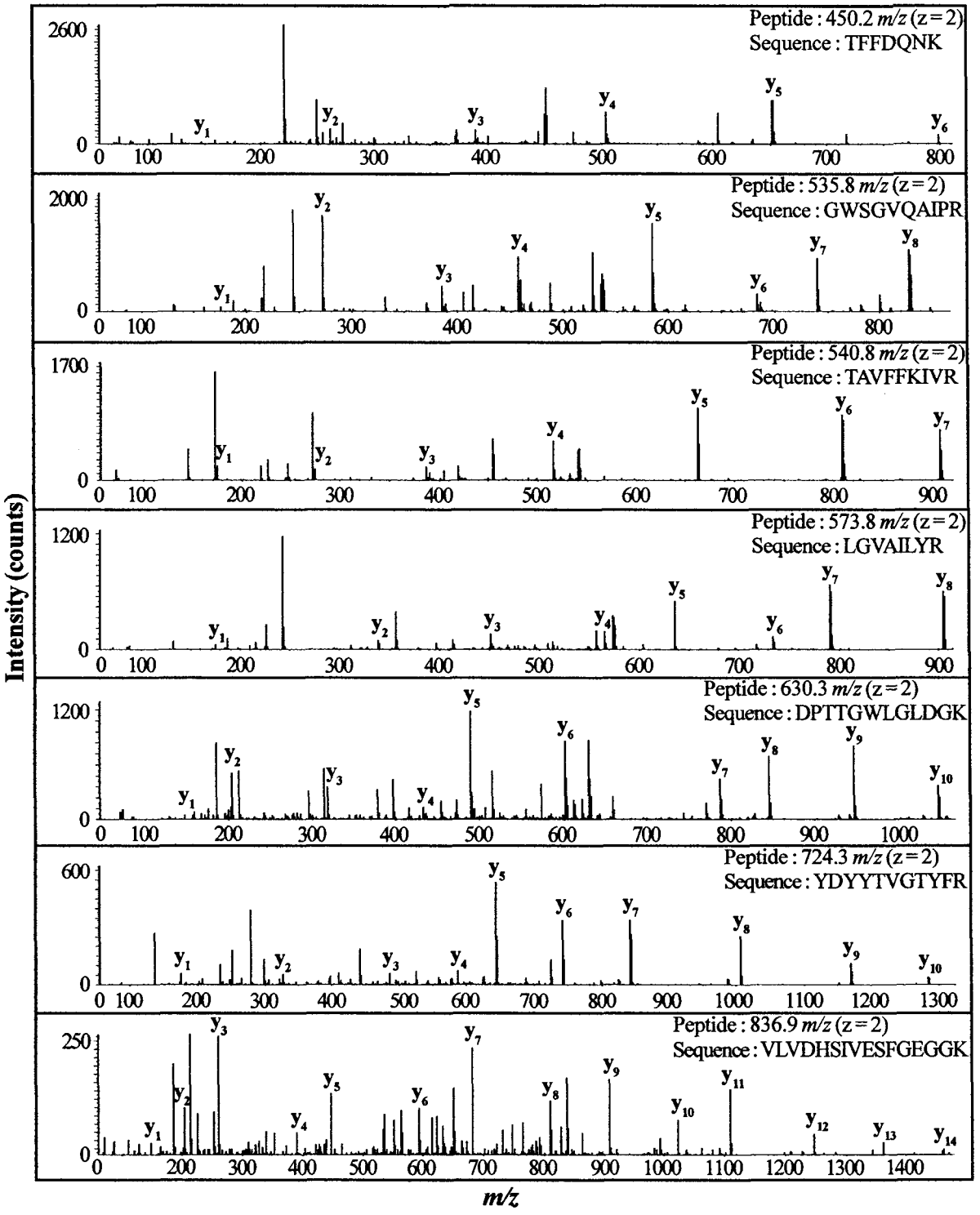



**Figure 3.12 Invertase I (Protein E) tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of Douglas-fir ovular secretion protein E (Figure 3.10). Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **450.2** | **535.8** | **540.8** | **573.8** | **630.3** | **724.3** | **836.9** |
| | **K** | **R** | **R** | **R** | **K** | **R** | **K** |
| **1** | 147.11 | 175.12 | 175.12 | 175.12 | 147.11 | 175.12 | 147.11 |
| | **N** | **P** | **V** | **Y** | **G** | **F** | **G** |
| **2** | 261.16 | 272.17 | 274.19 | 338.18 | 204.13 | 322.19 | 204.13 |
| | **Q** | **I** | **I** | **L** | **D** | **Y** | **G** |
| **3** | 389.21 | 385.26 | 387.27 | 451.27 | 319.16 | 485.25 | 261.16 |
| | **D** | **A** | **K** | **I** | **L** | **T** | **E** |
| **4** | 504.24 | 456.29 | 515.37 | 564.35 | 432.25 | 586.30 | 390.20 |
| | **F** | **Q** | **F** | **A** | **G** | **G** | **G** |
| **5** | 651.31 | 584.35 | 662.43 | 635.39 | 489.27 | 643.32 | 447.22 |
| | **F** | **V** | **F** | **V** | **L** | **V** | **F** |
| **6** | 798.38 | 683.42 | 809.50 | 734.46 | 602.35 | 742.39 | 594.29 |
| | | **G** | **V** | **G** | **W** | **T** | **S** |
| **7** | - | 740.44 | 908.57 | 791.48 | 788.43 | 843.44 | 681.32 |
| | | **S** | | **L** | **G** | **Y** | **E** |
| **8** | - | 827.47 | - | 904.56 | 845.45 | 1006.50 | 810.36 |
| | | | | | **T** | **Y** | **V** |
| **9** | - | - | - | - | 946.50 | 1169.56 | 909.43 |
| | | | | | **T** | **D** | **I** |
| **10** | - | - | - | - | 1047.55 | 1284.59 | 1022.52 |
| | | | | | | | **S** |
| **11** | - | - | - | - | - | - | 1109.55 |
| | | | | | | | **H** |
| **12** | - | - | - | - | - | - | 1246.61 |
| | | | | | | | **D** |
| **13** | - | - | - | - | - | - | 1361.63 |
| | | | | | | | **V** |
| **14** | - | - | - | - | - | - | 1460.70 |

**Table 3.4 Invertase I (Protein E) peptide amino acid sequences.** Y ion masses obtained from tandem MS fragmentation of peptides generated from the digestion of protein E (Figure 3.12) from Douglas-fir ovular secretions and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

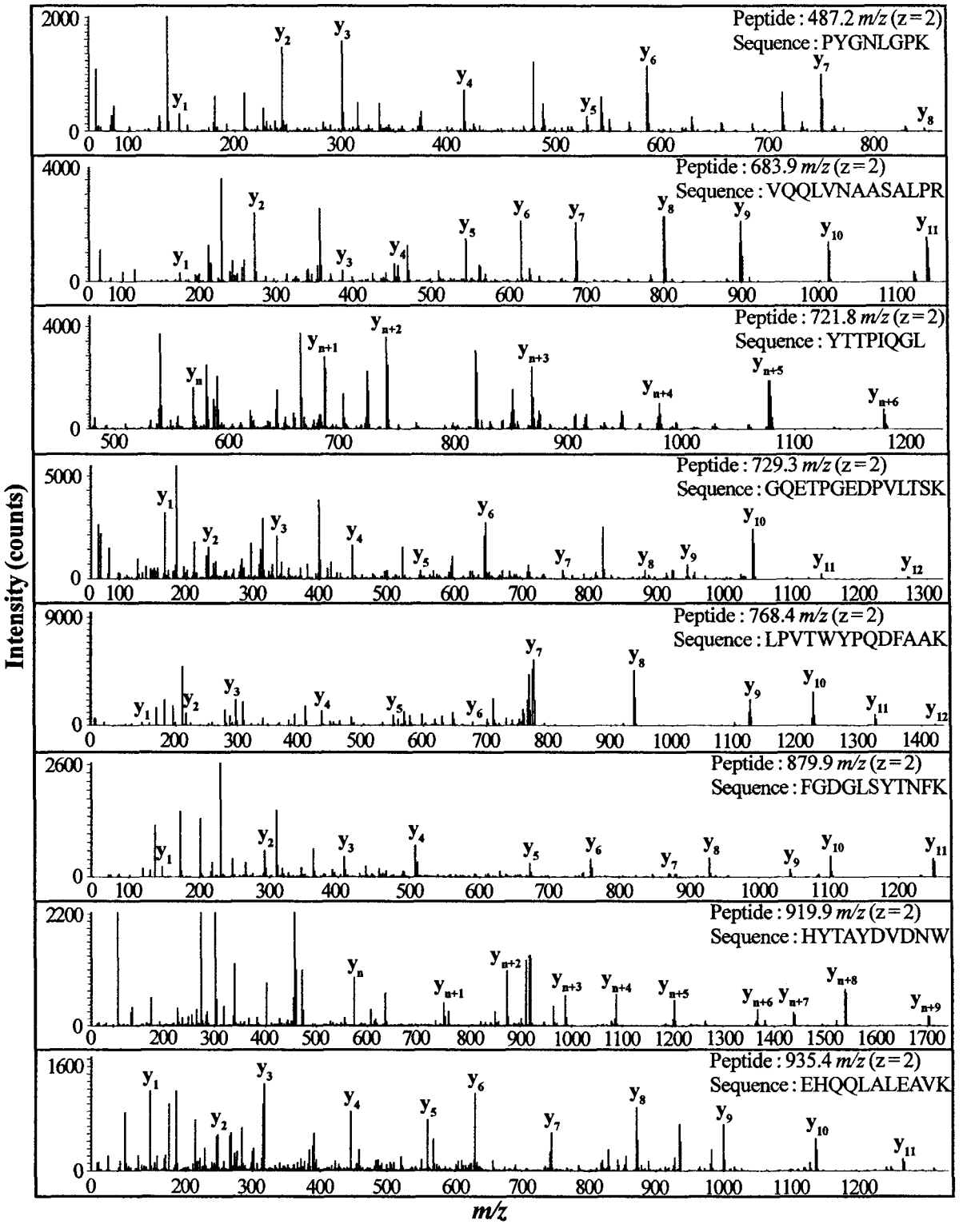



**Figure 3.13 Xylosidase II (Protein F) tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of Douglas-fir ovular secretion protein F (Figure 3.10). Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **487.2** | **683.9** | **721.8** | **729.3** | **768.4** | **879.9** | **919.9** | **935.4** |
| | K | R | | K | K | K | | K |
| 1 | 147.11 | 175.12 | 569.30 | 147.11 | 147.11 | 147.11 | 574.29 | 147.11 |
| | P | P | L | S | A | F | W | V |
| 2 | 244.17 | 272.17 | 682.38 | 234.14 | 218.15 | 294.18 | 760.36 | 246.18 |
| | G | L | G | T | A | N | N | A |
| 3 | 301.19 | 385.26 | 739.40 | 335.19 | 289.19 | 408.22 | 874.41 | 317.22 |
| | L | A | Q | L | F | T | D | E |
| 4 | 414.31 | 456.29 | 867.46 | 448.28 | 436.26 | 509.27 | 989.44 | 446.26 |
| | N | S | I | V | D | Y | V | L |
| 5 | 528.31 | 543.32 | 980.54 | 547.35 | 551.28 | 672.34 | 1088.50 | 559.35 |
| | G | A | P | P | Q | S | D | A |
| 6 | 585.34 | 614.36 | 1077.60 | 644.40 | 679.34 | 759.37 | 1203.53 | 630.38 |
| | Y | A | T | D | P | L | Y | L |
| 7 | 748.40 | 685.40 | 1178.65 | 759.42 | 776.39 | 872.45 | 1366.59 | 743.47 |
| | P | N | | E | Y | G | A | Q |
| 8 | 845.45 | 799.44 | - | 888.47 | 939.46 | 929.47 | 1437.63 | 871.20 |
| | | V | | G | W | D | T | Q |
| 9 | - | 898.51 | - | 945.49 | 1125.54 | 1044.50 | 1538.68 | 999.58 |
| | | L | | P | T | G | Y | H |
| 10 | - | 1011.59 | - | 1042.54 | 1226.58 | 1101.52 | 1701.74 | 1136.64 |
| | | Q | | T | V | F | | E |
| 11 | - | 1139.65 | - | 1143.59 | 1325.65 | 1248.59 | - | 1265.68 |
| | | | | E | P | | | |
| 12 | - | - | - | 1272.63 | 1422.71 | - | - | - |

**Table 3.5 Xylosidase II (Protein F) peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of protein F (Figure 3.13) from Douglas-fir ovular secretions and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

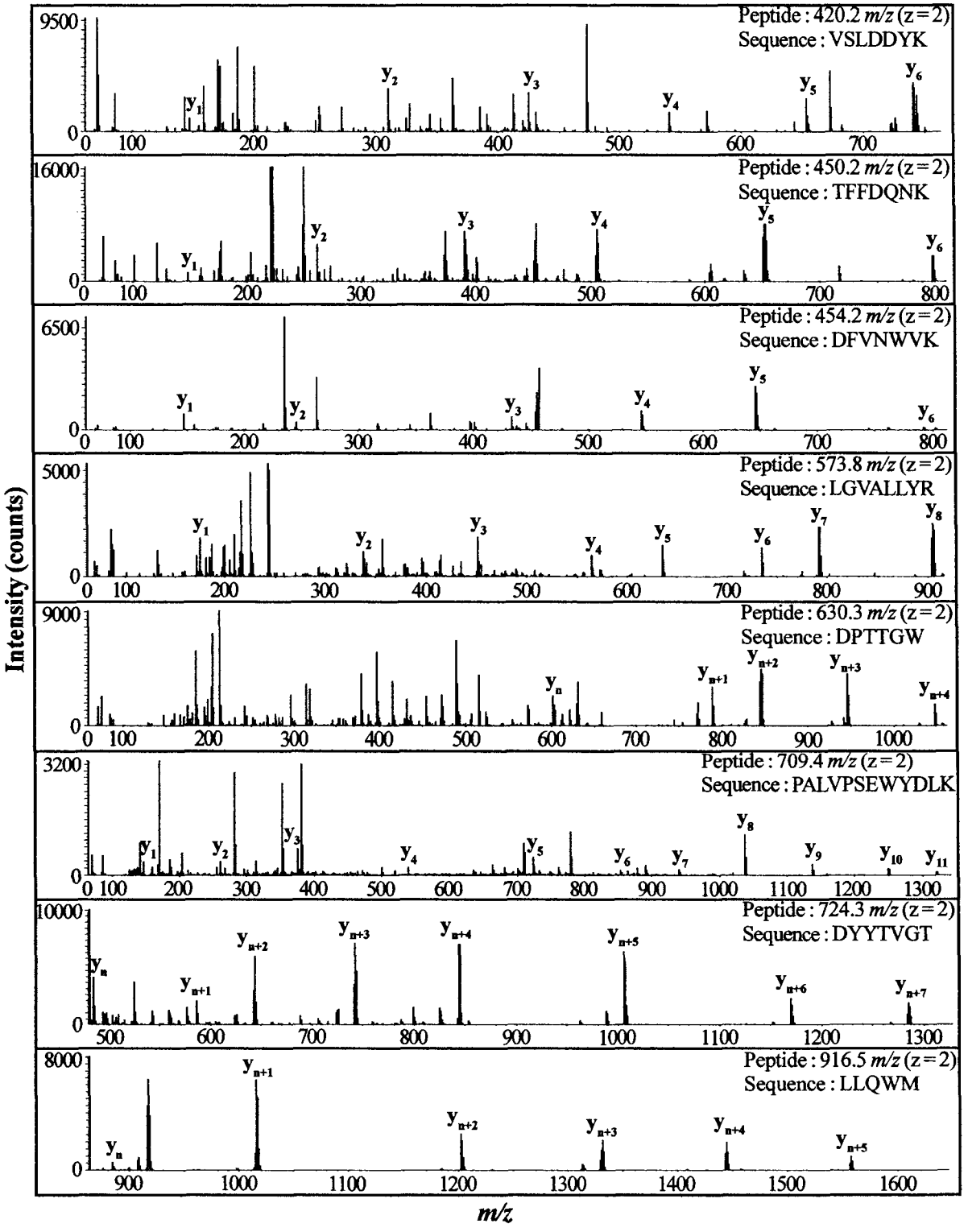



**Figure 3.14 Invertase II (Protein G) tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of Douglas-fir ovular secretion protein G (Figure 13.10). Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **420.2** | **450.2** | **454.2** | **573.8** | **630.3** | **709.4** | **724.3** | **916.5** |
| | **K** | **K** | **K** | **R** | | **K** | | |
| **1** | 147.11 | 147.11 | 147.11 | 175.12 | 602.41 | 147.11 | 485.29 | 884.51 |
| | **Y** | **N** | **V** | **Y** | **W** | **L** | **T** | **M** |
| **2** | 310.18 | 261.16 | 246.18 | 338.18 | 788.49 | 260.20 | 586.33 | 1015.60 |
| | **D** | **Q** | **W** | **L** | **G** | **D** | **G** | **W** |
| **3** | 425.20 | 389.21 | 432.26 | 451.27 | 845.51 | 375.22 | 643.36 | 1201.68 |
| | **D** | **D** | **N** | **L** | **T** | **Y** | **V** | **Q** |
| **4** | 540.23 | 504.24 | 546.30 | 564.35 | 946.56 | 538.29 | 742.42 | 1329.74 |
| | **L** | **F** | **V** | **A** | **T** | **W** | **T** | **L** |
| **5** | 653.31 | 651.31 | 645.37 | 635.39 | 1047.61 | 724.37 | 843.47 | 1442.82 |
| | **S** | **F** | **F** | **V** | | **E** | **Y** | **L** |
| **6** | 740.34 | 798.38 | 792.44 | 734.46 | - | 853.41 | 1006.54 | 1555.90 |
| | | | | **G** | | **S** | **Y** | |
| **7** | - | - | - | 791.48 | - | 940.44 | 1169.60 | - |
| | | | | **L** | | **P** | **D** | |
| **8** | - | - | - | 904.56 | - | 1037.49 | 1284.63 | - |
| | | | | | | **V** | | |
| **9** | - | - | - | - | - | 1136.56 | - | - |
| | | | | | | **L** | | |
| **10** | - | - | - | - | - | 1249.65 | - | - |
| | | | | | | **A** | | |
| **11** | - | - | - | - | - | 1320.68 | - | - |

**Table 3.6 Invertase II (Protein G) peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of protein G (Figure 3.14) from Douglas-fir ovular secretions and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

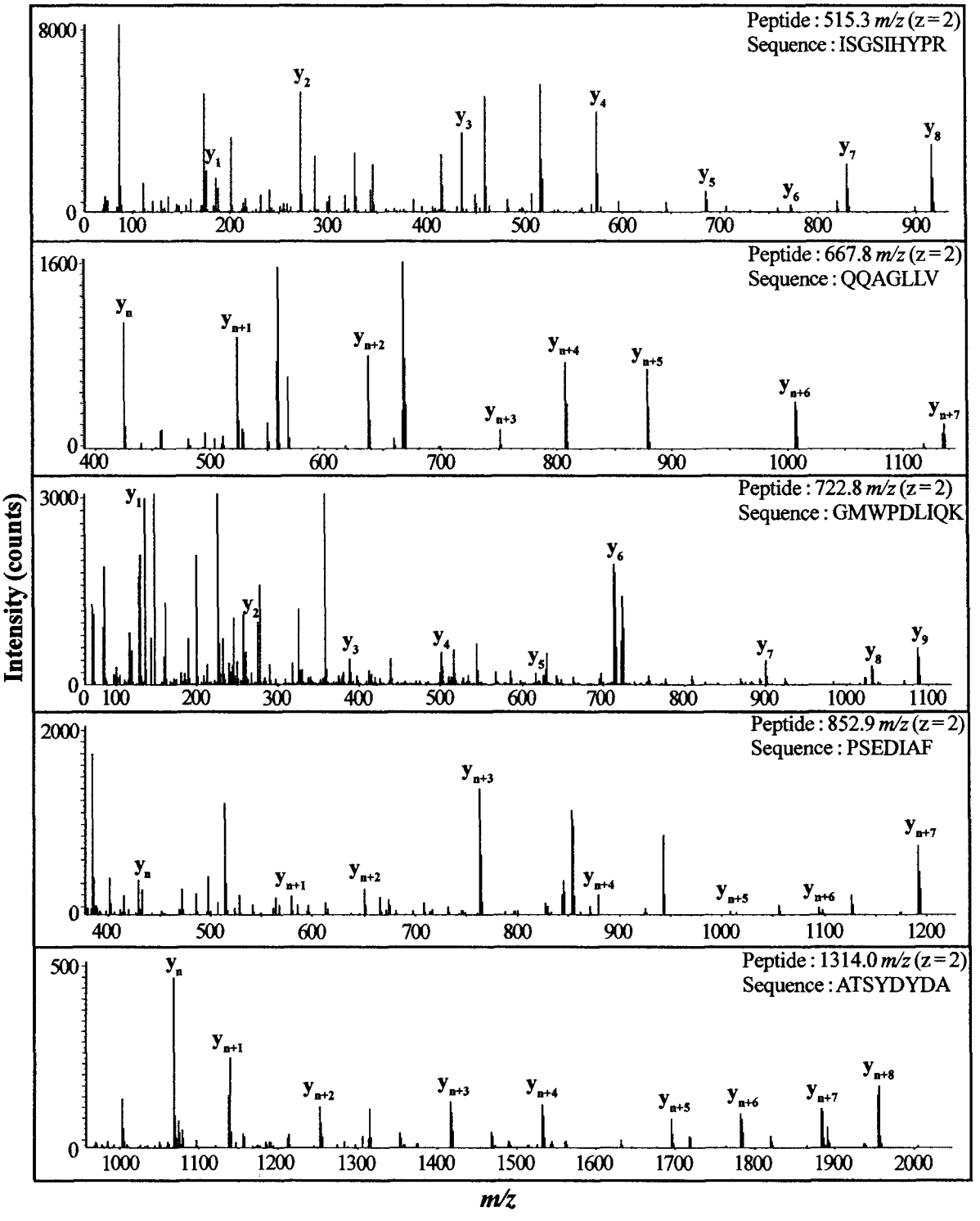



**Figure 3.15 Galactosidase I (Protein H) tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of Douglas-fir ovular secretion protein H (Figure 3.10).Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y | Peptide *m/z* (z = 2) | | | | |
|---|---|---|---|---|---|
| ions | 515.3 | 667.8 | 722.8 | 852.9 | 1314.0 |
| | R | | K | | |
| 1 | 175.12 | 425.24 | 147.11 | 432.24 | 1067.45 |
| | P | V | Q | F | A |
| 2 | 272.17 | 524.31 | 275.17 | 579.31 | 1138.49 |
| | Y | L | I | A | D |
| 3 | 435.24 | 637.40 | 388.25 | 650.34 | 1253.51 |
| | H | L | L | I | Y |
| 4 | 572.29 | 750.48 | 501.34 | 763.43 | 1416.58 |
| | I | G | D | D | D |
| 5 | 685.38 | 807.50 | 616.36 | 878.46 | 1531.60 |
| | S | A | P | E | Y |
| 6 | 772.41 | 878.54 | 713.42 | 1007.50 | 1694.67 |
| | G | Q | W | S | S |
| 7 | 829.43 | 1006.60 | 899.49 | 1094.53 | 1781.70 |
| | S | Q | M | P | T |
| 8 | 916.46 | 1134.66 | 1030.54 | 1191.58 | 1882.75 |
| | | | G | | A |
| 9 | - | - | 1087.56 | - | 1953.78 |

**Table 3.7 Galactosidase I (Protein H) peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of protein H (Figure 3.15) from Douglas-fir ovular secretions and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

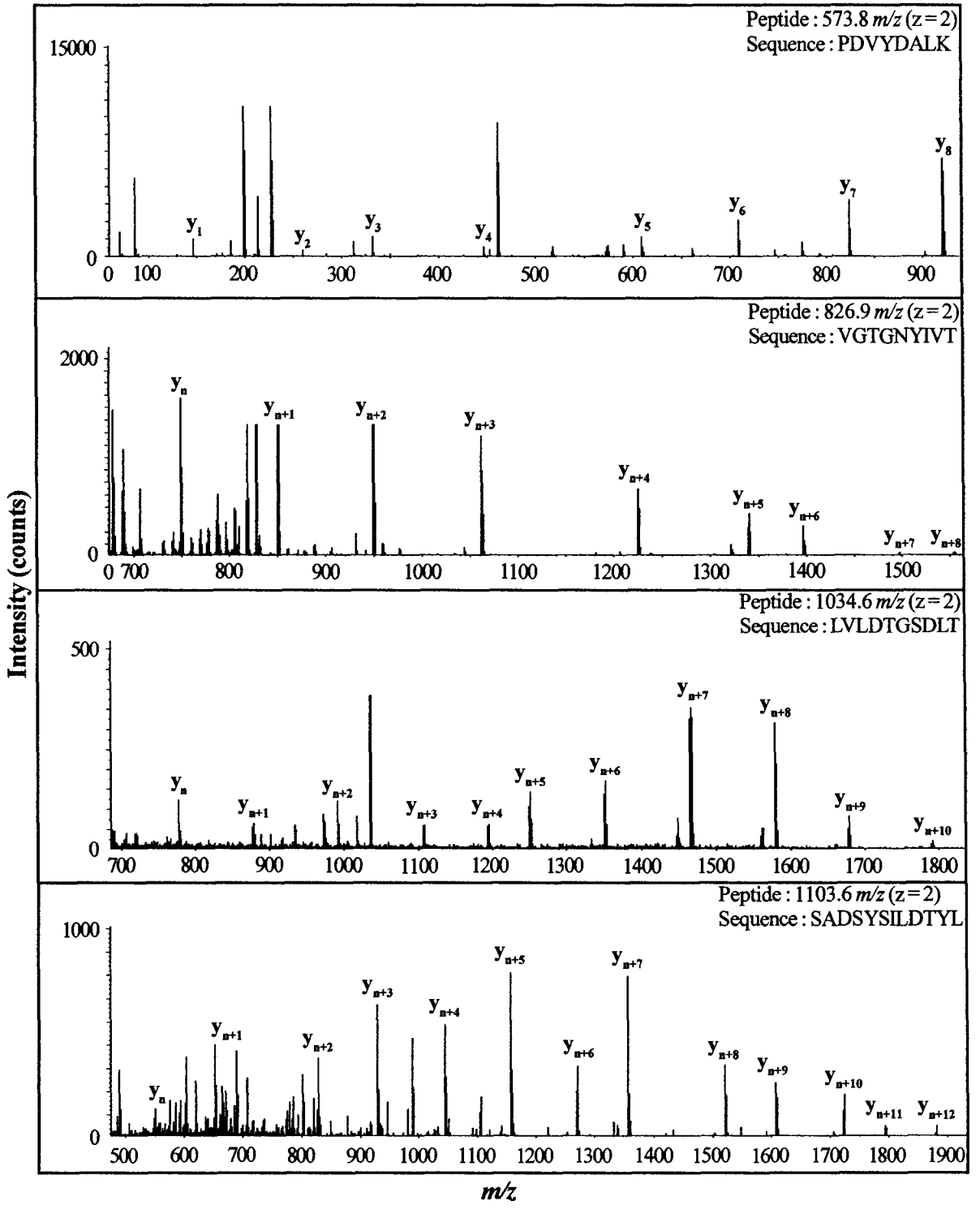



**Figure 3.16 Aspartyl protease (Protein I) tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of Douglas-fir ovular secretion protein I (Figure 3.10). Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | | |
|---|---|---|---|---|
| | 573.8 | 826.9 | 1034.6 | 1103.6 |
| | K | | | |
| 1 | 147.11 | 748.62 | 776.62 | 551.50 |
| | L | T | T | L |
| 2 | 260.20 | 849.67 | 877.67 | 664.59 |
| | A | V | L | Y |
| 3 | 331.23 | 948.74 | 990.75 | 827.65 |
| | D | I | D | T |
| 4 | 446.26 | 1061.82 | 1105.78 | 928.70 |
| | Y | Y | S | D |
| 5 | 609.32 | 1224.88 | 1192.81 | 1043.73 |
| | V | N | G | L |
| 6 | 708.39 | 1338.93 | 1249.83 | 1156.81 |
| | D | G | T | I |
| 7 | 823.42 | 1395.95 | 1350.88 | 1269.89 |
| | P | T | D | S |
| 8 | 920.47 | 1496.99 | 1465.91 | 1356.93 |
| | | G | L | Y |
| 9 | - | 1554.02 | 1578.99 | 1519.99 |
| | | | V | S |
| 10 | - | - | 1678.06 | 1607.02 |
| | | | L | D |
| 11 | | - | 1791.14 | 1722.05 |
| | | | | A |
| 12 | - | - | - | 1793.09 |
| | | | | S |
| 13 | - | - | - | 1880.12 |

**Table 3.8 Aspartyl protease (Protein I) peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of protein I (Figure 3.16) from Douglas-fir ovular secretions and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

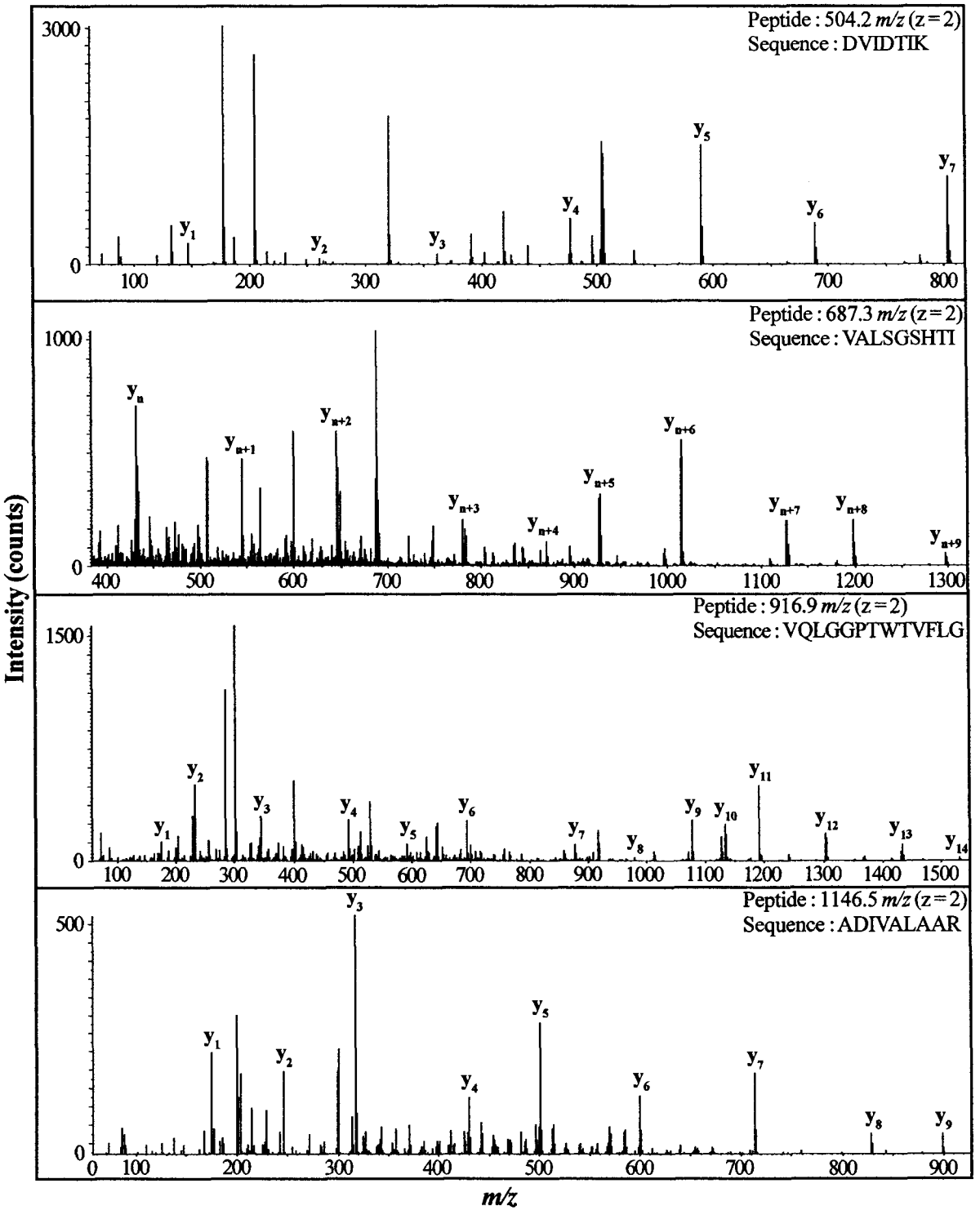



**Figure 3.17 Peroxidase (Protein J) tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of Douglas-fir ovular secretion protein J (Figure 3.10).Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | | |
|---|---|---|---|---|
| | 504.2 | 687.3 | 916.9 | 1146.5 |
| | K | | R | R |
| 1 | 147.11 | 431.18 | 175.12 | 175.12 |
| | I | I | G | A |
| 2 | 260.20 | 544.27 | 232.02 | 246.16 |
| | T | T | L | A |
| 3 | 361.24 | 645.31 | 345.11 | 317.19 |
| | D | H | F | L |
| 4 | 476.27 | 782.37 | 492.18 | 430.28 |
| | I | S | V | A |
| 5 | 589.36 | 869.40 | 591.25 | 501.31 |
| | V | G | T | V |
| 6 | 688.42 | 926.43 | 692.30 | 600.38 |
| | D | S | W | I |
| 7 | 803.45 | 1013.46 | 878.38 | 713.47 |
| | | L | T | D |
| 8 | - | 1126.54 | 979.42 | 828.49 |
| | | A | P | A |
| 9 | - | 1197.58 | 1076.48 | 899.53 |
| | | V | G | |
| 10 | - | 1296.65 | 1133.50 | - |
| | | | G | |
| 11 | - | - | 1190.52 | - |
| | | | L | |
| 12 | - | - | 1303.60 | - |
| | | | Q | |
| 13 | - | - | 1431.66 | - |
| | | | V | |
| 14 | - | - | 1530.73 | - |

**Table 3.9 Peroxidase (Protein J) peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of protein J (Figure 3.17) from Douglas-fir ovular secretions and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

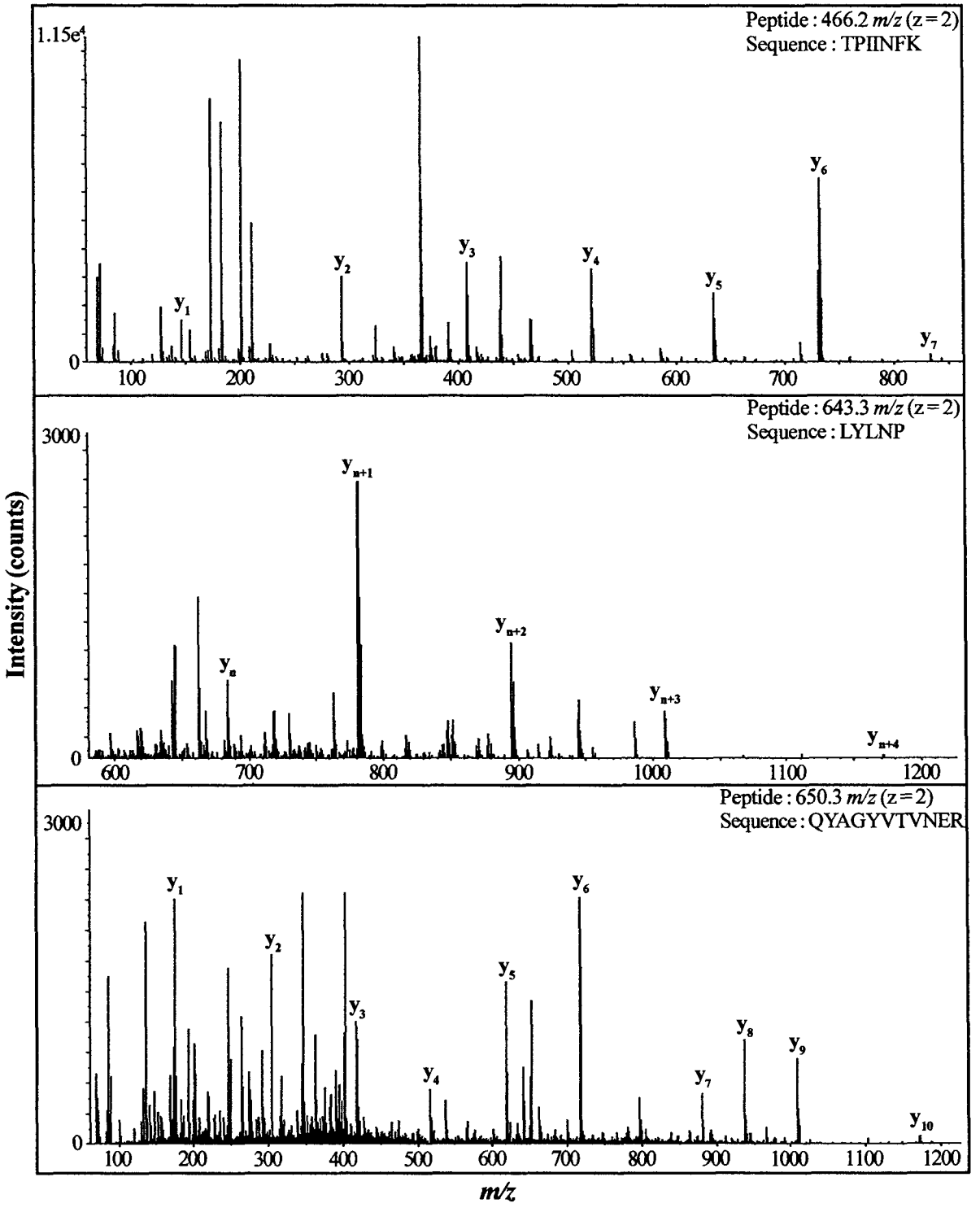



**Figure 3.18 Serine carboxypeptidase-like protein (Protein K) tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of Douglas-fir ovular secretion protein K (Figure 3.10).Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | |
|---|---|---|---|
| | 466.2 | 643.3 | 650.3 |
| | K | | R |
| 1 | 147.11 | 684.37 | 175.12 |
| | F | P | E |
| 2 | 294.18 | 781.43 | 304.16 |
| | N | N | N |
| 3 | 408.22 | 895.47 | 418.20 |
| | I | L | V |
| 4 | 521.31 | 1008.55 | 517.27 |
| | I | Y | T |
| 5 | 634.39 | 1171.62 | 618.32 |
| | P | L | V |
| 6 | 731.45 | 1284.70 | 717.39 |
| | T | | Y |
| 7 | 832.49 | - | 880.45 |
| | | | G |
| 8 | - | - | 937.47 |
| | | | A |
| 9 | - | - | 1008.51 |
| | | | Y |
| 10 | - | - | 1171.57 |
| | | | Q |
| 11 | - | - | 1299.59 |

**Table 3.10 Serine carboxypeptidase-like protein (Protein K) peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of protein K (Figure 3.18) from Douglas-fir ovular secretions and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

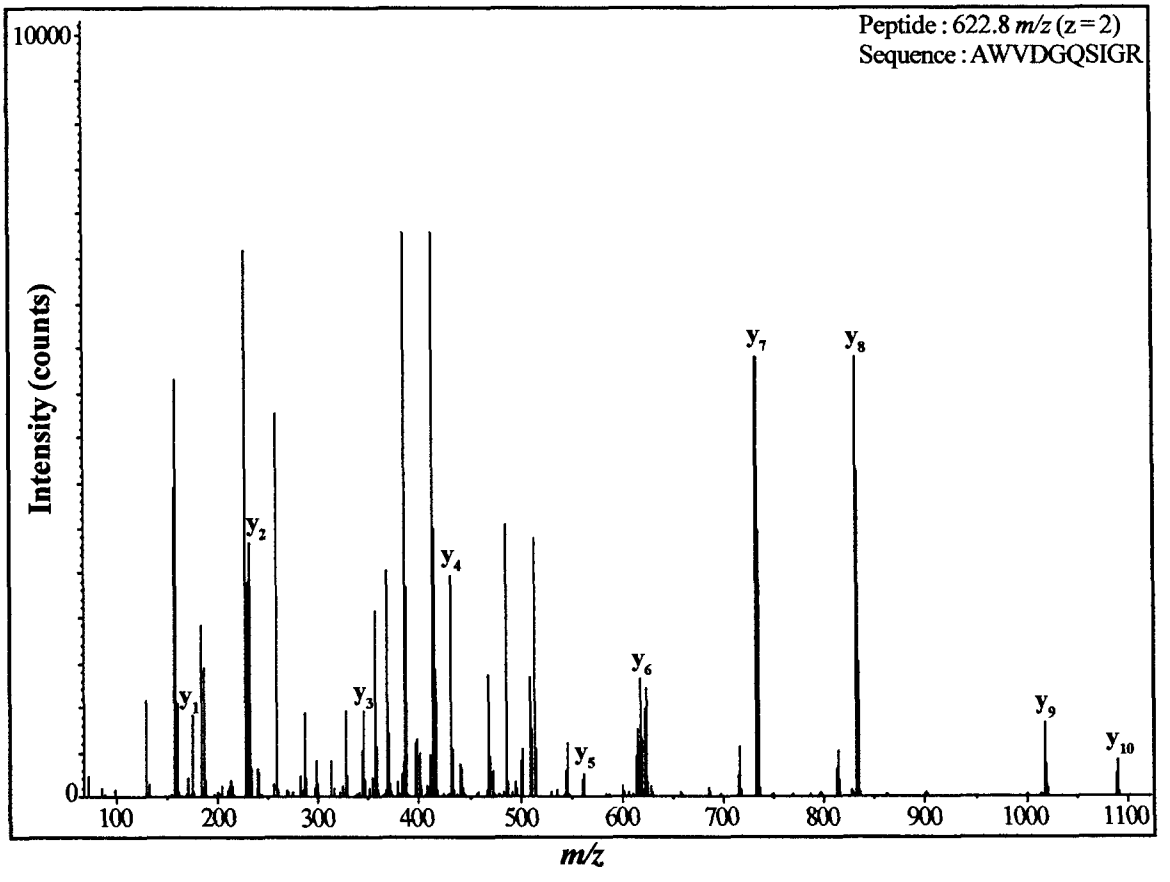



**Figure 3.19 Galactosidase II (Protein L) tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of Douglas-fir ovular secretion protein L (Figure 3.10). Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide 622.8 *m/z* (z = 2) |
|---|---|
| | **R** |
| **1** | 175.12 |
| | **G** |
| **2** | 232.14 |
| | **I** |
| **3** | 345.22 |
| | **S** |
| **4** | 432.26 |
| | **Q** |
| **5** | 560.32 |
| | **G** |
| **6** | 617.32 |
| | **D** |
| **7** | 732.36 |
| | **V** |
| **8** | 831.43 |
| | **W** |
| **9** | 1017.51 |
| | **A** |
| **10** | 1088.55 |

**Table 3.11 Galactosidase II (Protein L) peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of a peptide generated from the digestion of protein L (Figure 3.19) from Douglas-fir ovular secretions and the deduced amino acid sequence of this peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

| Douglas-fir ovular secretion protein (Figure 3.10) | Molecular weight (kDa) | pI | Peptide amino acid sequences obtained | Protein identification by MSBlast |
|---|---|---|---|---|
| **xylosidase I (D)** (Figure 3.11 & Table 3.3) | 90 | 6.6 | VMILGLSK<br>ATWEDLR<br>WDVPPYK<br>RPFDLR<br>DFSAESDGKK<br>TVDFWADEISR<br>STYVDFSAESDGK | *Pinus pinaster* (Maritime Pine) putative alpha-xylosidase (AF448201) |
| **invertase I (E)** (Figure 3.12 & Table 3.4) | 70 | 6.3 | TFFDQNK<br>GWGVQAIPR<br>TAVFFKIVR<br>LGVAILYR<br>DPTTGWLGLDGK<br>YDYYTVGTYFR<br>VLVDHSIVESFGEGGK | *Fragaria ananassa* (Hybrid Strawberry) cell wall invertase (AF000521) |
| **xylosidase II (F)** (Figure 3.13 & Table 3.5) | 65 | 6.0 | PYGNLGPK<br>VQQLVNAASALPR<br>YTTPIQGL<br>GQETPGEDPVLTSK<br>LPVTWYPQDFAAK<br>FGDGLSYTNFK<br>HYTAYDVDNW<br>EHQQLALEAVK | *Arabidopsis thaliana* (Arabidopsis) beta-1,4-xylosidase (AP000417) |
| **invertase II (G)** (Figure 3.14 & Table 3.6) | 50 | 6.5 | VSLDDYK<br>TFFDQNK<br>DFVNWVK<br>LGVALLYR<br>DPTTGWLGLDGK<br>PALVPSEWYDLK<br>DYYTVGT<br>LLQWM | *Carica papaya* (Papaya) cell wall invertase (AF420223) |

**Table 3.12 Summary of Douglas-fir ovular secretion proteins D, E, F, and G.** Peptide amino acid sequences obtained for Douglas-fir ovular secretion proteins separated using 2D gel electrophoresis (Figure 3.10). Proteins were reduced, alkylated, and digested with trypsin. Peptides generated were sequenced using tandem MS sequencing (Figures 3.11-3.14 and Tables 3.3-3.6). Sequence searching was performed using Bork Group's MS Blast search at EMBL on March 6$^{th}$, 2004. Protein molecular weights and their respective pI values were approximated by their position on the 2D gel.

| Douglas-fir ovular secretion protein (Figure 3.10) | Molecular weight (kDa) | pI | Peptide amino acid sequences obtained | Protein identification by MSBlast |
|---|---|---|---|---|
| **galactosidase I (H)** (Figure 3.15 & Table 3.7) | 45 | 7.8 | ISGSIHYPR<br>QQAGLLV<br>GMWPDLIQK<br>PSEDIAF<br>ATSYDYDA | *Lupinus angustifolius* (Blue Lupin) exo-galactanase (AJ011047) |
| **aspartyl protease (I)** (Figure 3.16 & Table 3.8) | 40 | 5.5 | PDVYDALK<br>VGTGNYIVT<br>LVLDTGSDLT<br>SADSYSILDTYL | *Arabidopsis thaliana* putative aspartyl protease (AY088536) |
| **peroxidase (J)** (Figure 3.17 & Table 3.9) | 37 | 7.9 | DVIDTIK<br>VALSGSHTI<br>VQLGGPTWTVFLG<br>ADIVALAAR | *Triticum aestivum* (Wheat) Pox1 protein (X85227) |
| **serine carboxypeptidase-like protein (K)** (Figure 3.18 & Table 3.10) | 33 | 4.5 | TPIINFK<br>LYLNP<br>QYAGYVTVNER | *Arabidopsis thaliana* serine carboxypeptidase-like protein (AL163972) |
| **galactosidase II (L)** (Figure 3.19 & Table 3.11) | 29 | 5.9 | AWVDGQSIGR | *Arabidopsis thaliana* (Arabidopsis) putative beta-galactosidase precursor (AJ270304) |

**Table 3.13 Summary of Douglas-fir ovular secretion proteins H, I, J, K, and L.** Peptide amino acid sequences obtained for Douglas-fir ovular secretion proteins separated using 2D gel electrophoresis (Figure 3.10). Proteins were reduced, alkylated, and digested with trypsin. Peptides generated were sequenced using tandem MS sequencing (Figures 3.15-3.19 and Tables 3.7-3.11). Sequence searching was performed using Bork Group's MS Blast search at EMBL on March 6th, 2004. Protein molecular weights and their respective pI values were approximated by their position on the 2D gel.

## 3.3 Discussion

For successful fertilization in conifers, a receptive egg cell must receive healthy male gametes from the pollen. In Douglas-fir, the egg cell only remains viable for little more than a week (Fernando *et al.* 1997); therefore, coordinating egg viability with pollen tube formation is absolutely essential. Initiation of pollen tube formation occurs approximately one week before fertilization. Coincident with this initiation is the secretion of a liquid into the micropyle of the ovule. This ovular secretion not only contains beneficial nutrients for the developing pollen during pollen tube elongation, but also delivers molecules that may coordinate pollen and megagametophyte development.

### 3.3.1 Pollen tube initiation

Delivering beneficial nutrients to the pollen during pollen tube elongation is an important function of the ovular secretion, but difficulties encountered when attempting to germinate Douglas-fir pollen *in vitro* suggest that this secretion is more than just a nutrient medium (Fernando *et al.* 1997; Dumont-BeBoux *et al.* 1999). Preliminary experiments have shown that there are molecules in the Douglas-fir ovular secretion derived from sources external to the pollen that initiate siphonogamy (Takaso *et al.* 1996; Fernando *et al.* 1997). These ovular initiating factors may be present to coordinate pollen tube formation with egg viability.

Megagametophyte development results in the formation of mature and viable egg cells approximately 9 weeks after pollination in Douglas-fir (Owens and Morris 1990). Once the egg cells mature, they only remain receptive for little more than a week, at which time any unfertilized egg cells degenerate (Owens *et al.* 1991; Fernando *et al.*

1997). A viable egg cell must receive healthy gametes from the pollen tube during this one week window for successful fertilization.

Within a day of being brought inside the Douglas-fir ovule, the pollen hydrates and swells causing the outer exine to burst and shed (Owens and Morris 1990). It then elongates within the micropyle for the next 6-9 weeks (Owens and Morris 1990; Owens *et al.* 1998). The ovular secretion exudes from the nucellar tip and fills the micropylar chamber of the ovule that houses the elongated pollen (Owens and Morris 1990; von Aderkas and Leary 1999). This secretion contains factors that initiate pollen tube formation (Owens and Morris 1990; Takaso *et al.* 1996; von Aderkas and Leary 1999). What are these ovular secretion initiation factors?

Pollen tubes are initiated when pollen cell walls are loosened (Derksen 1996). This loosening enables the cell to yield to internal turgor-driven expansive forces (Cosgrove 2001). When this ovular liquid exudes and makes contact with the elongated pollen, the outermost region of the pollen interacting with this secretion is the intine (Owens and Morris 1990). Intine cell walls of conifer pollen typically consist of a cellulose framework interpenetrated by a cross-linked matrix of noncellulosic molecules such as hemicelluloses, unesterified pectins, and arabinogalactan proteins (Said 1989; Mogami *et al.* 1999; Yatomi *et al.* 2002). The most abundant conifer hemicelluloses are polysaccharides composed of the monosaccharides glucose, fucose, xylose, and galactose (Acebes *et al.* 1993a; Acebes *et al.* 1993b; Barrachina and Lorences 1998; Crombie *et al.* 1998; Kakegawa *et al.* 1998; Sampedro *et al.* 2001). These polysaccharides are referred to as xyloglucans and because they tether the cellulose microfibrils directly, they are considered to be load-bearing components (Cosgrove 2001; Sampedro *et al.* 2001).

Hydrolytic degradation of the xyloglucan linkages causes the cell walls to loosen, resulting with pollen tube initiation (Derksen 1996; Cosgrove 1999; Cosgrove 2000; Cosgrove 2001).

Four of the most abundant proteins present in the Douglas-fir ovular secretion are xylosidases and galactosidases may help to initiate pollen tube formation (Tables 3.10 and 3.11). Previous experiments have shown that xylosidases and galactosidases can hydrolyze xyloglucans (Edwards *et al.* 1988; Sampedro *et al.* 2001). These enzymes may be delivered to the elongated pollen from the ovule to coordinate pollen tube initiation with egg viability. Using electron microscopy, it was shown that when Douglas-fir pollen encounters the Douglas-fir ovular secretion *in situ*, intine dissolution followed by pollen tube initiation occurs (Takaso and Owens 1994; Takaso *et al.* 1996).

The presence of a 15 kDa phytocyanin protein also suggests that this secretion may influence pollen tube initiation and development. It has been theorized that phytocyanins, copper-binding proteins in nonphotosynthetic plant tissues, may be involved in the control of cell wall reorganization (Yoshizaki *et al.* 2000).

### 3.3.2 A source of nutrition for developing pollen tubes

Once pollen tube formation has been initiated, it elongates towards the megagametophyte. Having beneficial nutrients available for absorption during this stage of development would be advantageous to the pollen. Research on conifer ovular secretions has been restricted due to their small volumes, but initial findings implicate them as sources of nutrition for pollen during pollen tube development. Early research identified calcium as a common constituent (Fujii 1903; Tison 1911). Calcium has long

been known to be necessary for pollen development (Brewbaker and Kwack 1963; Picton and Steer 1983; Steer and Steer 1989). It has also been theorized that calcium directs pollen tube growth (Mascarenhas and Machlis 1962), but more recent research indicates that this may not be the case (Higashiyama *et al.* 2003).

An assortment of amino acids are also present in these liquids (Fujii 1903; Tison 1911; Ziegler 1959; Seridi-Benkaddour and Chesnoy 1988; Chesnoy 1993). The addition of these ovular amino acids to the growth medium used for germinating juniper (*Juniperus communis* L.) pollen resulted in improved pollen tube growth (Duhoux and Pham Thi 1980). The two proteases identified in the Douglas-fir ovular secretion – an aspartyl protease and a serine carboxypeptidase-like protein - would result with amino acids being present in this nutritive liquid since proteases are usually involved with protein turnover associated with storage material mobilization (Granell *et al.* 1998; Cercós *et al.* 2003). In angiosperms, serine carboxypeptidase-like proteins have also been implicated with transferring solutes from the nucellar cells to developing endosperm (Cercós *et al.* 2003). In conifers, this enzyme may be transferring solutes to the developing pollen during tube elongation.

Monosaccharides are the monomer building blocks from which disaccharides, polysaccharides, and other essential carbohydrates are constructed in living plant cells. During pollen tube elongation, conifer pollen absorb monosaccharides from their environment (Nygaard 1977). Consequently, it would be beneficial for pollen to have these monosaccharides as a source of nutrition during this developmental period.

Sucrose, a disaccharide, is the major transport sugar in plants. It is noticeable by its absence in ovular secretions. In those that do have it, it is in very low amounts

(Chesnoy 1993). However, its constituent glucose and fructose monosaccharides are common (McWilliam 1958; Seridi-Benkaddour and Chesnoy 1988; Chesnoy 1993). Two of the most abundant proteins present in the Douglas-fir ovular secretion are invertases (Tables 3.10 and 3.11) which hydrolyze sucrose into its monomer constituents (Avigad 1982; ap Rees 1984). The presence of these enzymes would result in a nutrition enrichment of the liquid that feeds the pollen during pollen tube development.

Another source of monosaccharides is from polysaccharides. As previously mentioned, xyloglucans are the major hemicellulosic polysaccharides present in conifer cell walls (Acebes *et al.* 1993a; Acebes *et al.* 1993b; Barrachina and Lorences 1998; Kakegawa *et al.* 1998). They also serve as storage polysaccharides (Reid 1985). Xyloglucan hydrolysis would release these monosaccharides into the secretion. They would then be available for absorption by developing pollen.

### 3.3.3 Pollen selection

When conifer cones begin to collect pollen, any airborne pollen in the vicinity may settle on them. This is necessary to capture their compatible pollen, but allows foreign and incompatible pollen to also enter the ovule. Pollen selection barriers are present that minimize this pollen pollution. The first level of pollen selection is that of the pollination mechanism that the conifer employs to bring pollen inside the ovule. There are five pollination mechanisms. Each one has the ability to limit the type of pollen that can be drawn into the micropyle.

The simplest conifer pollination mechanism involves the active production of a pollination drop outside of erect or horizontal ovules. Only pollen that will sink upon

contact with the pollination drop will enter the ovule (Figure 3.20a). Pollen lacking air sacs (i.e. non-saccate pollen) will sink, as will porous saccate pollen. Any non-porous saccate pollen that contacts this pollination drop will simply float and not sink into the micropyle. The drop then undergoes rapid evaporation and recedes into the interior of the ovule (Owens *et al.* 1998). Five of the seven conifer families - Cupressaceae, Cephalotaxaceae, Taxaceae, Taxodiaceae, and some Podocarpaceae - have members that employ this pollination mechanism. This implies that pollen from all these species could enter each other's ovules freely.

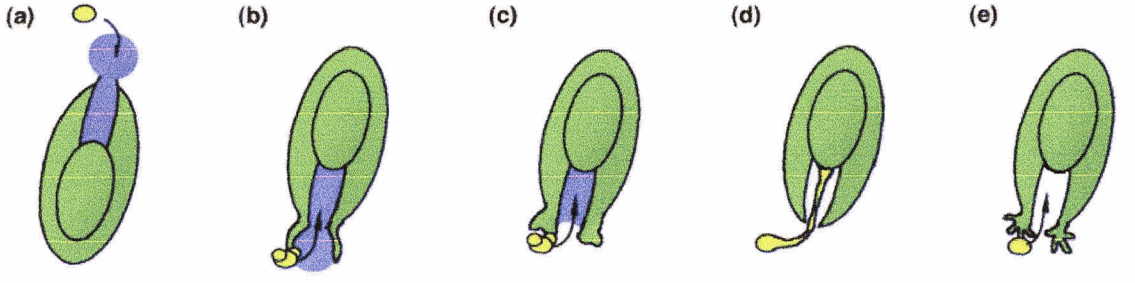



**Figure 3.20 Conifer pollination mechanisms. (a).** Erect or horizontal ovules that produce pollination drops for pollen capture (Cupressaceae, Cephalotaxaceae, Taxodiaceae, Taxaceae, and some members of Podocarpaceae). **(b).** Inverted ovules that produce pollination drops to capture saccate pollen (Some Pinaceae and Podocarpaceae). **(c).** Inverted ovules that capture saccate pollen without producing pollination drops (Some Pinaceae). **(d).** Extreme siphonogamy where pollen grains germinate outside of the ovule and their pollen tubes grow into the ovule (Some Pinaceae and Araucariaceae). **(e).** Inverted ovules that capture pollen via an engulfment mechanism (Some Pinaceae including Douglas-fir and *Larix*) (Owens *et al.* 1998).

The second type of pollination mechanism, exhibited by some members of the Pinaceae and Podocarpaceae, has inverted ovules that produce pollination drops to capture saccate pollen (Figure 3.20b). Saccate pollen have at least one air sac attached to their central body. When these pollen come into contact with inverted pollination drops,

their buoyancy allows them to float upward into the ovule through the micropyle and toward the nucellar surface (Owens *et al.* 1998). Non-saccate pollen that come into contact with these pollination drops do not float upward into the ovule.

Some Pinaceae have inverted ovules that capture saccate pollen without producing pollination drops (Figure 3.20c). The mode-of-action of this third type of pollination mechanism is the same as the second type except that rainwater carries out the role of the pollination drop (Takaso and Owens 1995; Runions and Owens 1996). The nucellus is still believed to secrete remnants of a pollination drop (Owens *et al.* 1998). Perhaps the secreted ovular molecules are still needed for successful fertilization.

All of the Araucariaceae along with most *Tsuga* species within the Pinaceae employ a fourth type of pollination mechanism (Figure 3.20d). Pollen from these species exhibit extreme siphonogamy. The pollen germinates outside of the ovule and produces an especially long pollen tube that grows through the micropyle and eventually penetrates an egg cell of the megagametophyte (Haines *et al.* 1984; Owens *et al.* 1998).

Douglas-fir, as well as *Larix*, exhibits a fifth and unique pollination mechanism - engulfment (Figure 3.20e). Airborne pollen that settle on the female cones make their way down the adaxial surface of the bracts and adhere to integumentary papillae located on the tips of the receptive ovules. Pollen is collected by these papillae for several days prior to being engulfed into the micropyle. This engulfment results from outer papillae surface cells elongating while cells around the micropylar opening collapse. This event always takes place at the same time and in the same manner whether or not pollen has adhered to the papillae (Owens *et al.* 1981).

Although an initial level of pollen selection is provided by these pollination mechanisms, they are not completely discriminatory. Foreign and incompatible pollen also enter the ovule. It has been widely accepted that there are no prezygotic breeding barriers in conifers (Willson and Burley 1983), but more recent evidence suggests that some selection barriers may be taking place within the ovule prior to fertilization. Research in our lab supports this hypothesis. *In vitro* studies demonstrated that larch (*Larix occidentalis* Nutt.), Sitka spruce, and white pine (*Pinus monticola* Douglas ex D. Don) pollen tubes could penetrate all three of the other species' megagametophytes. These intergeneric crosses show that there are no barriers to gamete delivery *in vitro*; however, *in vivo* events within the ovule must exhibit some form of pollen selection because these crosses do not occur naturally (Dumont-BeBoux *et al.* 1998).

One potential selection barrier within the ovule is the osmolarity of the ovular secretion. It has long been theorized that osmolarity may be one of the factors responsible for pollen selection (Lewis 1943). Depending on the conifer species, these ovular secretions may have different osmolarities due to their unique compositions. This is an interesting hypothesis and one that should be investigated further.

Another level of selection may be provided by the unique monosaccharide composition of the secretion. Conifer pollen may absorb monosaccharides preferentially during pollen tube development (Nygaard 1977). This suggests that conifer pollen tubes may benefit developmentally from the unique monosaccharide composition of their respective ovular feeding liquids. Upon exposure to the ovular secretion, compatible pollen tube development would thrive; whereas, foreign and incompatible pollen tube development would be impeded.

The unique monosaccharide compositions and varying osmolarities of the feeding liquids may provide some of this selection, but these may only provide an advantageous situation for compatible pollen. Other factors may be present that are directly detrimental to foreign and incompatible pollen. Pollen grains in self-pollinated Douglas-fir ovules showed increased incidents of intine dissolution and plasmolysis compared with those in cross-pollinated ovules (Takaso and Owens 1994). Using ovular dissections and statistical analysis, Marlies Rise showed that Douglas-fir ovules reduce or inhibit heterospecific pollen grain germination (Rise 2001). In addition, treating angiosperm pollen with English yew (*Taxus baccata* L.) pollination drops inhibits their germination (Zenkteler and Relska-Roszak 2003).

If the cell walls of germinating pollen are significantly compromised, they may undergo plasmolysis because of their internal turgor pressure. A peroxidase present in Douglas-fir ovular secretions may carry out this function (Table 3.11). Although the production of reactive oxygen intermediates by extracellularly secreted plant peroxidases is well characterized in plant defense (Schwacke and Hager 1992; Salzer *et al.* 1996; Salzer *et al.* 1997), research has also shown that hydroxyl radicals have the ability to cleave plant cell wall polysaccharides (Schopfer 2001; Kawano 2003). This non-enzymatic cleavage of wall polymers by hydroxyl radicals is non-specific and acts on pollen cell walls no matter their polysaccharide composition – an important characteristic if it is to be effective against multiple targets.

If peroxidases are detrimental to developing pollen, their activity must be removed or regulated when compatible pollen are present. This has been suggested previously in angiosperm compatibility. Peroxidases were present in non-pollinated and

self-pollinated styles of Petunia (*Petunia hybrida* Hort. Vilm.), but they were absent in cross-pollinated styles. These authors suggested that stylar peroxidase activity may be associated with the rejection of incompatible pollen tubes and that the removal of peroxidase activity would be necessary for a compatible pollination process (Carraro *et al.* 1986).

In conclusion, proteomic analysis on the most abundant proteins present in the Douglas-fir ovular secretion provides evidence that this liquid may not only provide beneficial nutrients to the developing pollen during pollen tube elongation through the nucellus, but may also play a significant role in pollen selection and development.

# Chapter 4

## The Douglas-fir ovular secretion contains antifungal proteins

### 4.1 Introduction

There are many conifer diseases caused by many different pathogens and pests including bacteria, fungi, nematodes, and insects. Conifer defense against pathogen and insect attack begins with physical barriers such as bark, the waxy cuticle of the needles, and resin production. Constitutive production of antimicrobial and antiherbivory molecules also helps to impede pathogen ingress. If the invader happens to circumvent these preformed defense barriers, more conifer defenses are initiated to provide further resistance. Pathogenesis-related (PR) protein synthesis is one of these defensive responses. Functional analysis of many of these PR proteins has shown them to exhibit antimicrobial activity (Selitrennikoff 2001).

During pollination, conifers are extremely susceptible to pathogen attack. Since all conifers are wind pollinated, they must employ pollination mechanisms to capture the airborne pollen. Unfortunately, these methods of pollen capture not only bring pollen inside the ovule, but also bring potentially devastating pathogens into this site of fertilization. What molecules do conifer ovules produce to protect themselves from these disease causing organisms? None have been characterized, but potential delivery systems have been reported. Whether it is a pollination drop or an active secretion into the micropyle, most conifer ovules produce at least one secretion between pollination and fertilization (Owens *et al.* 1998; Gelbart and von Aderkas 2002).

The ovular secretion in Douglas-fir exudes from the nucellar surface approximately one week before fertilization (Owens and Morris 1990). It fills the

micropyle of the ovule where any pathogens that may have accompanied the pollen into the ovule would be located (Owens and Morris 1990; von Aderkas and Leary 1999).

**4.2 Results**

Using a 0.20 mM para-nitrophenyl-N-acetyl-β-glucosaminide solution, it was shown that the Douglas-fir ovular secretion had chitinase activity (Figure 4.1). Using this same assay at varying pHs, it was determined that the optimum chitinase activity for the secretion was approximately 6.0 (Figure 4.2). After these preliminary experiments, the chitinases present in the secretion needed to be identified and characterized. This was performed using in-gel chitinase assays. These assays showed that there were at least eight different chitinases of varying molecular weights (Figure 4.3 & Table 4.1) and isoelectric points (pI) (Figure 4.4 & Table 4.1). The majority of the chitinases identified using this assay were acidic (Figure 4.4 & Table 4.1).

Two-dimensional gel electrophoresis (2D GE) was also used to separate proteins present in the Douglas-fir ovular secretion. These 2D gels indicate there are many proteins with varying sizes and isoelectric points (pIs) (Figure 4.5). Internal amino acid sequences were obtained by digesting the proteins with trypsin and then sequencing the generated peptides using quadrupole time-of-flight tandem mass spectrometry. These sequences were then submitted to Bork Group's MS blast search engine at EMBL for protein identification. Using this methodology, three PR proteins were unequivocally identified – a 37 kDa peroxidase with a pI of 7.9 (Figure 4.6 & Table 4.2), a 28 kDa thaumatin-like protein with a pI of 4.3 (Figure 4.7 & Table 4.3), and a 27 kDa chitinase with a pI of 7.8 (Figure 4.8 & Table 4.4). A summary for each of these PR proteins identified including their approximate molecular weight, pI, and peptide amino acid sequences can be found in Table 4.5.

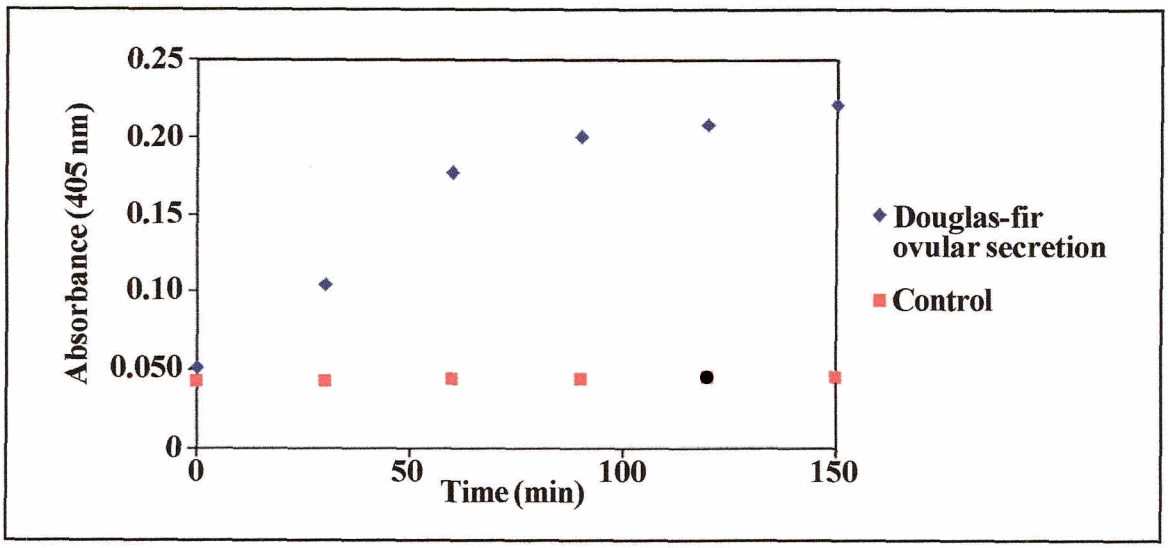



**Figure 4.1 Chitinase activity of the Douglas-fir ovular secretion.** Into 100.0 μL of a 0.20 mM para-nitrophenyl-N-acetyl-β-D-glucosaminide [*p*-NP-(GlcNAc), Sigma, St. Louis, MO] solution in $dH_2O$, 1.0 μL of Douglas-fir ovular secretion was added. Samples were vortexed and incubated at 30 °C for 2.5 h. Absorbances were measured at a wavelength of 405 nm every 30 min. The control was 101.0 μL of the 0.20 mM *p*-NP-(GlcNAc) solution.

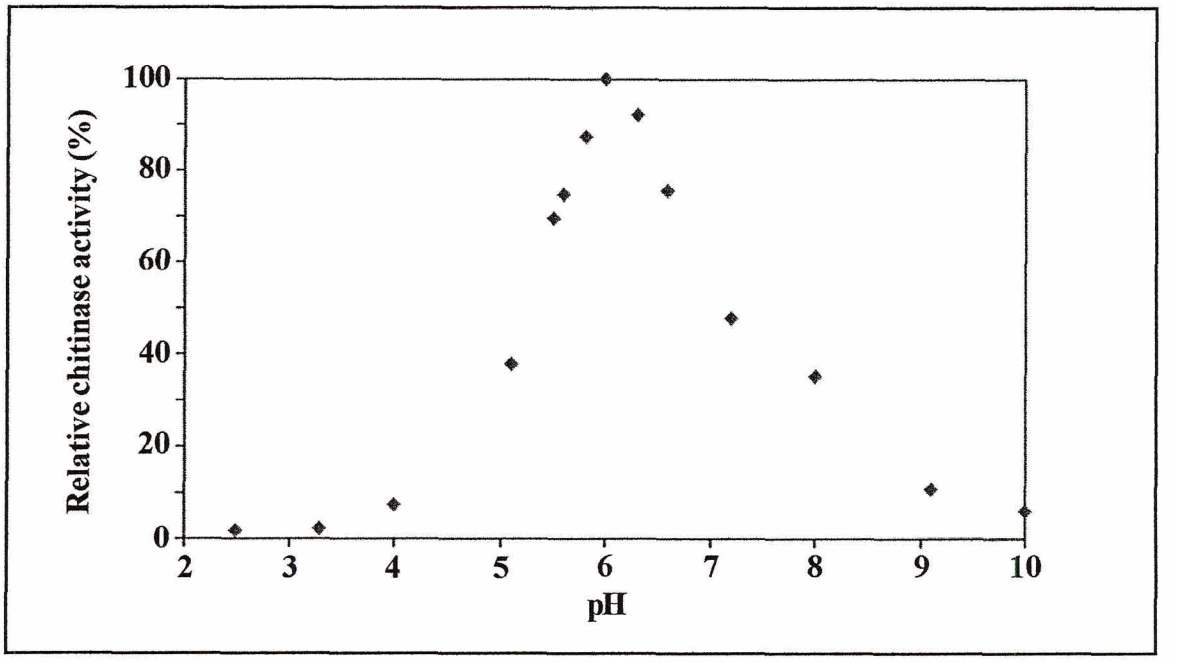



**Figure 4.2 pH dependence of the Douglas-fir ovular secretion chitinase activity.** Into 100.0 μL of 0.20 mM *p*-NP-(GlcNAc) solutions of varying pH, 1.0 μL of Douglas-fir ovular secretion was added. The following solutions were used: 50 mM glycine pH 2.5, 50 mM glycine pH 3.3, 50 mM sodium acetate pH 4.0, 50 mM sodium acetate pH 5.1, 50 mM sodium acetate pH 5.5, 50 mM sodium phosphate pH 5.6, 50 mM sodium phosphate pH 5.8, 50 mM sodium phosphate 6.0, 50 mM sodium phosphate pH 6.3, 50 mM sodium phosphate pH 6.6, 50 mM sodium phosphate pH 7.2, 50 mM sodium phosphate pH 8.0, 50 mM glycine pH 9.1, and 50 mM glycine pH 10.0. Samples were vortexed and incubated at 30 °C for 2.0 h. Absorbances were measured at a wavelength of 405 nm.

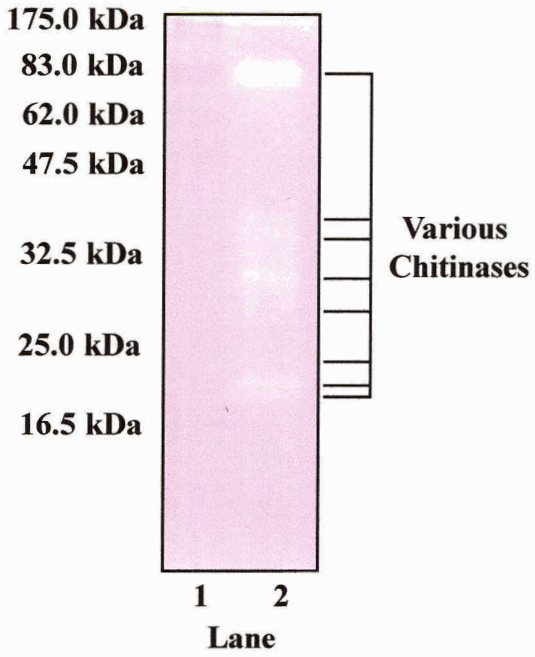



**Figure 4.3 In-gel chitinase assay on the Douglas-fir ovular secretion proteins separated using 1D SDS-PAGE.** Prior to loading, 3.0 µL of Douglas-fir ovular secretion was diluted to 10.0 µL with 2X glycine gel sample buffer (without the reducing agent 2-mercaptoethanol) and immersed in boiling water for 3 min. Electrophoresis was carried out in a Bio-Rad Mini-Protean 3 Electrophoresis system (Bio-Rad Laboratories, Hercules, CA) through a 4 % acrylamide stacking gel and a 12 % acrylamide resolving gel until the tracking dye reached the bottom of the gel. The concentration of the CM-chitin-RBV reagent (Loewe Biochemica GmbH, Sauerlach, Germany) in the resolving gel was 0.68 g/mL. The stacking and resolving gels were run at 10 and 20 mA, respectively. The buffers used were standard Tris-glycine buffers (Laemmli 1970). After electrophoresis, the gel was incubated in a 50.0 mM sodium phosphate, 0.1 % Tween-20 solution with a pH of 6 until clearance bands could be observed. For protein visualization, the gel was stained with GelCode® Blue stain reagent.
**Lane 1:** Broad range molecular weight markers
**Lane 2:** 5.0 µL of Douglas-fir ovular secretion

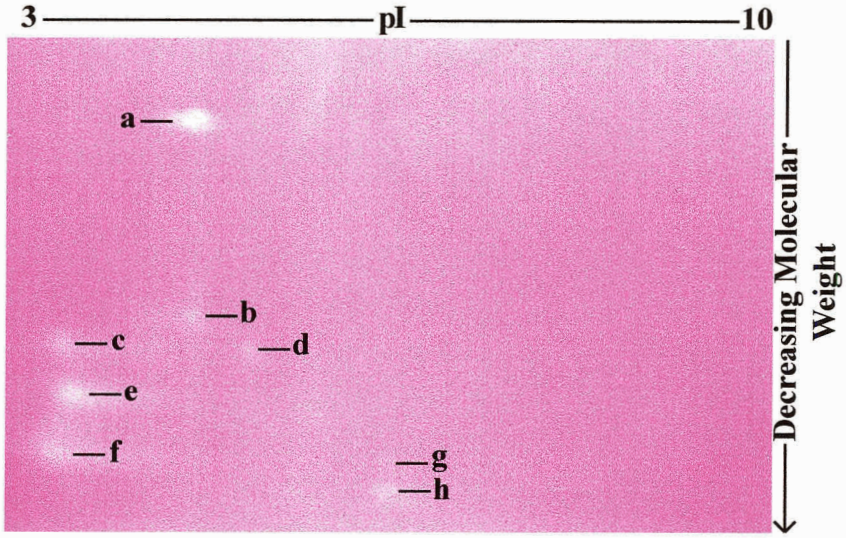



**Figure 4.4 In-gel chitinase assay on the Douglas-fir ovular secretion proteins separated using 2D GE.** Ten microliters of Douglas-fir ovular secretion was solubilized in 10 µL of isoelectric focusing (IEF) non-reducing sample buffer (8.0 M urea, 2.0 % v/v NP-40, 2.0 % 3-10 ampholytes). Prior to electrophoresing the proteins, the IEF tube gels were first pre-electrophoresed for 10 min at 200 V, 15 min at 300 V, and 15 min at 400 V. Before the proteins were further separated in the second dimension, the IEF tube gels were incubated in equilibration buffer (with no reducing agent) for approximately 10 min. The IEF tube gels were then placed on top of the 10 % second dimension acrylamide gels and electrophoresed at 20 mA until the blue tracking dye reached the end of the gels. The concentration of the CM-chitin-RBV reagent (Loewe Biochemica GmbH, Sauerlach, Germany) in the resolving gel was 0.68 g/mL. After electrophoresis, the gel was incubated in a 50.0 mM sodium phosphate, 0.1 % Tween-20 solution with a pH of 6 until clearance zones could be observed.

| Douglas-fir ovular secretion chitinase (Figures 4.3 & 4.4) | Molecular weight (kDa) (Figure 4.3)* | pI (Figure 4.4) |
|---|---|---|
| **a** | 83 | 4.8 |
| **b** | 38 | 4.8 |
| **c** | 36 | 3.5 |
| **d** | 31 | 5.2 |
| **e** | 28 | 3.6 |
| **f** | 23 | 3.5 |
| **g** | 21 | 6.5 |
| **h** | 20 | 6.5 |

**Table 4.1 Summary of Douglas-fir ovular secretion chitinases a - h.** Their molecular weights and respective pI values were approximated by their position on the 1D SDS-PAGE gel (Figure 4.3) and 2D gel (Figure 4.4), respectively. * Without reducing agent added to the 1D SDS-PAGE, the molecular weights may contain significant error.

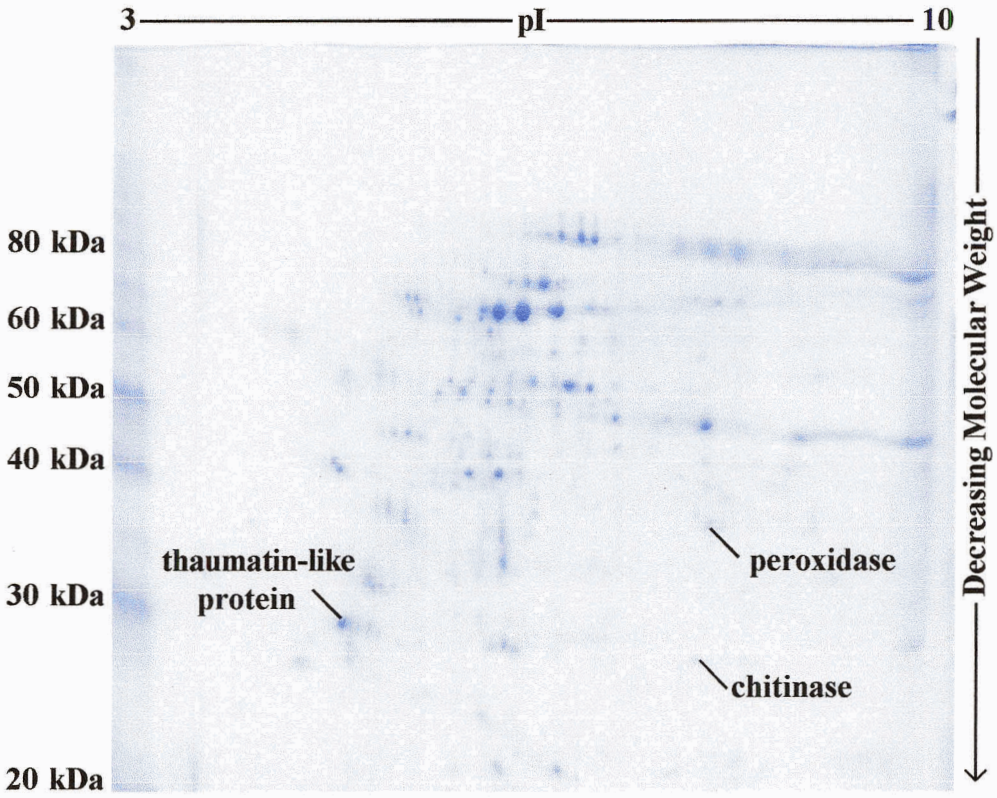



**Figure 4.5 2D gel of Douglas-fir ovular secretion proteins.** The Douglas-fir ovular secretion pellet was solubilized in 30 µL of SDS-MIX. Prior to electrophoresing the proteins, the isoelectric focusing (IEF) tube gels were first pre-electrophoresed for 1 h at 200 V. Once the protein samples were loaded, they were electrophoresed within the IEF tube gels for 17.5 h at 800 V. Before the proteins were further separated in the second dimension, the IEF tube gels were incubated in equilibration buffer for approximately 15 min. The proteins were then separated in the second dimension (10 % acrylamide) by electrophoresing them at 1 A until the blue tracking dye reached the end of the gels. To visualize the proteins present in the 2D gels, they were stained using a Colloidal Coomassie protocol (Neuhoff *et al.* 1988).

| Protein Identification | MS Data |
|---|---|
| **peroxidase** | (Figure 4.6, Table 4.2) |
| **thaumatin-like protein** | (Figure 4.7, Table 4.3) |
| **chitinase** | (Figure 4.8, Table 4.4) |

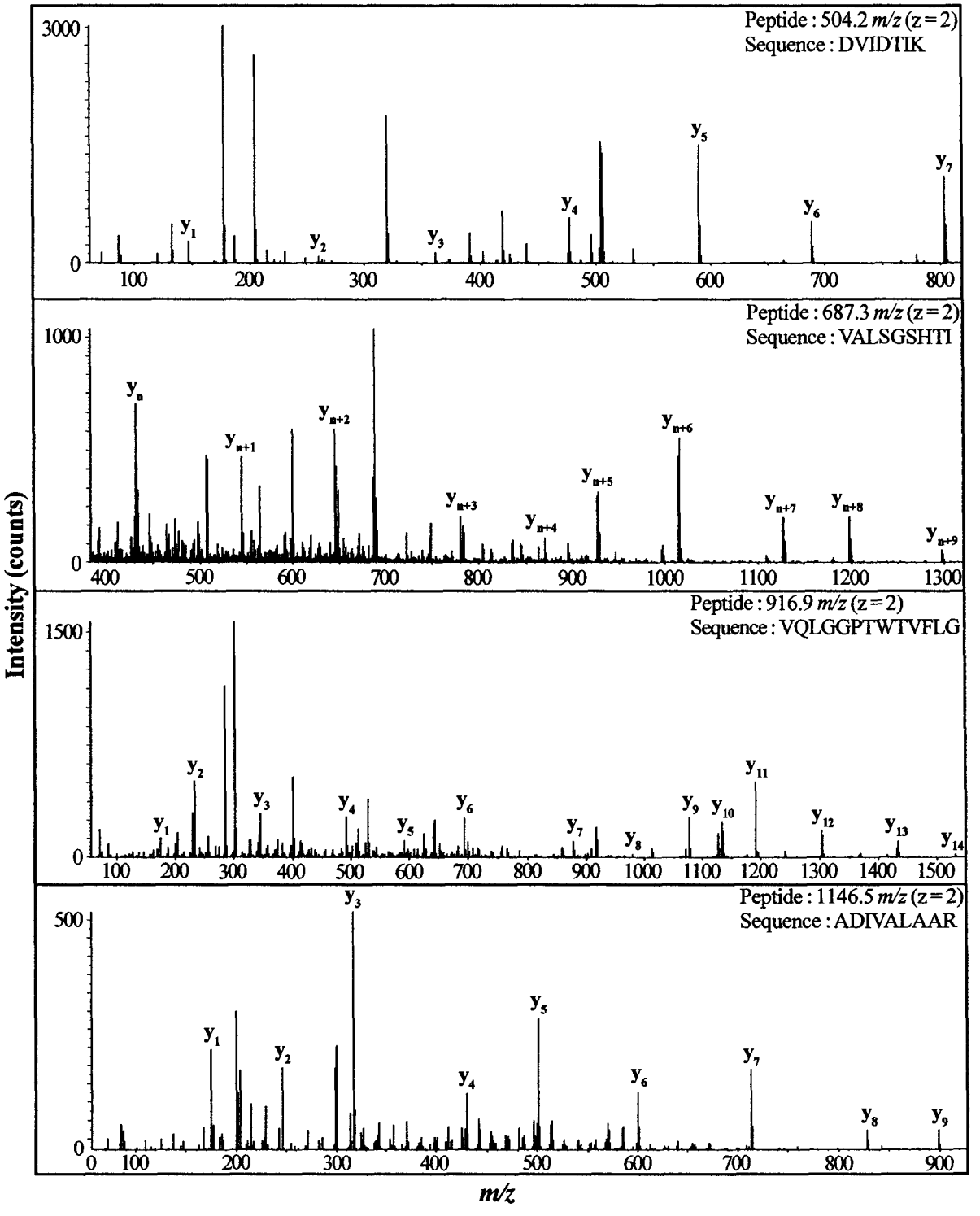



**Figure 4.6 Peroxidase tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of the Douglas-fir ovular secretion peroxidase (Figure 4.5). Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | | |
|---|---|---|---|---|
| | **504.2** | **687.3** | **916.9** | **1146.5** |
| | **K** | | **R** | **R** |
| **1** | 147.11 | 431.18 | 175.12 | 175.12 |
| | **I** | **I** | **G** | **A** |
| **2** | 260.20 | 544.27 | 232.02 | 246.16 |
| | **T** | **T** | **L** | **A** |
| **3** | 361.24 | 645.31 | 345.11 | 317.19 |
| | **D** | **H** | **F** | **L** |
| **4** | 476.27 | 782.37 | 492.18 | 430.28 |
| | **I** | **S** | **V** | **A** |
| **5** | 589.36 | 869.40 | 591.25 | 501.31 |
| | **V** | **G** | **T** | **V** |
| **6** | 688.42 | 926.43 | 692.30 | 600.38 |
| | **D** | **S** | **W** | **I** |
| **7** | 803.45 | 1013.46 | 878.38 | 713.47 |
| | | **L** | **T** | **D** |
| **8** | - | 1126.54 | 979.42 | 828.49 |
| | | **A** | **P** | **A** |
| **9** | - | 1197.58 | 1076.48 | 899.53 |
| | | **V** | **G** | |
| **10** | - | 1296.65 | 1133.50 | - |
| | | | **G** | |
| **11** | - | - | 1190.52 | - |
| | | | **L** | |
| **12** | - | - | 1303.60 | - |
| | | | **Q** | |
| **13** | - | - | 1431.66 | - |
| | | | **V** | |
| **14** | - | - | 1530.73 | - |

**Table 4.2 Peroxidase peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of the Douglas-fir ovular secretion peroxidase (Figure 4.6) and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

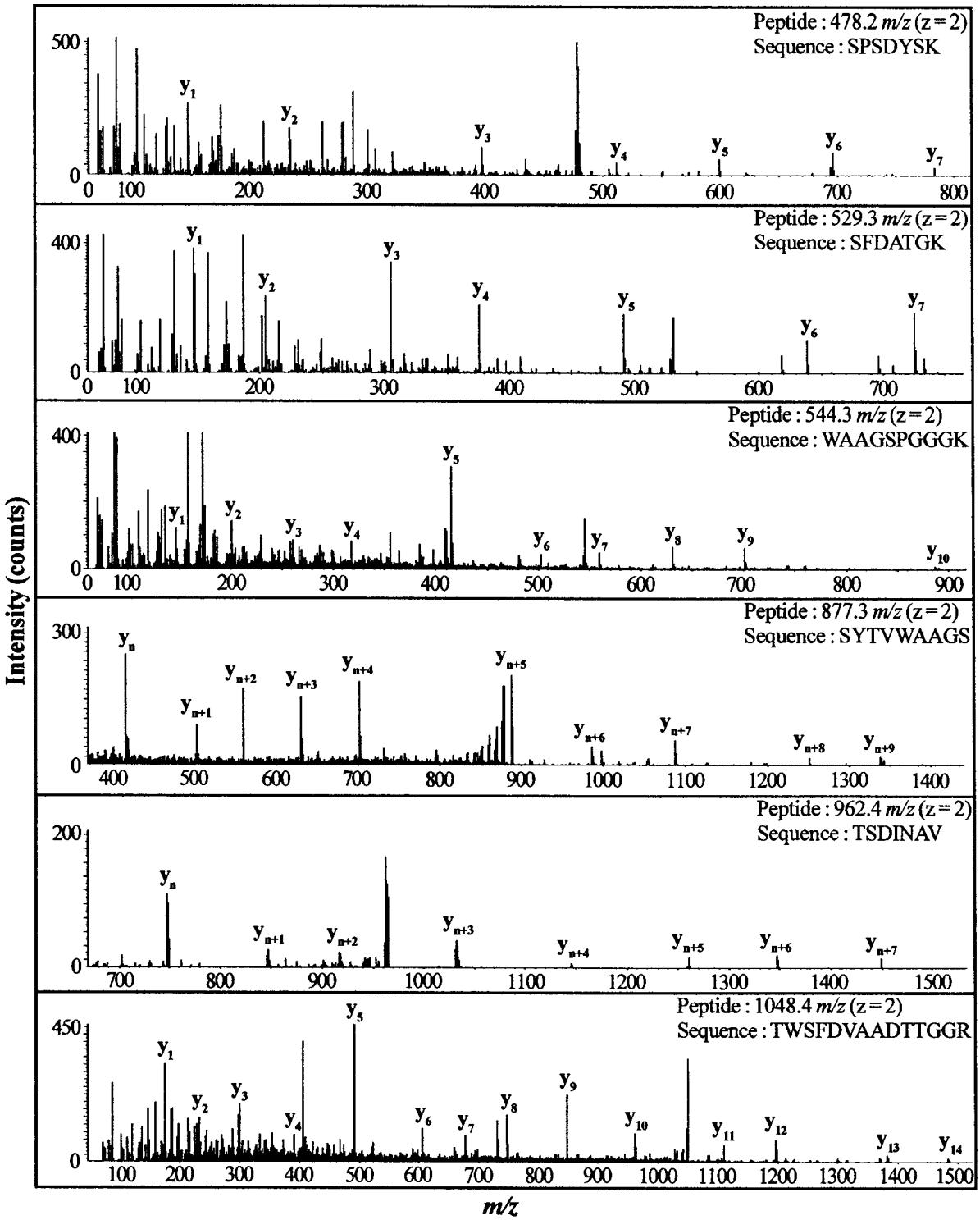



**Figure 4.7 Thaumatin-like protein tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of the Douglas-fir ovular secretion thaumatin-like protein (Figure 4.5). Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | | | | |
|---|---|---|---|---|---|---|
| | 478.2 | 529.3 | 544.3 | 877.3 | 962.4 | 1048.4 |
| | K | K | K | | | R |
| 1 | 147.11 | 147.11 | 147.11 | 415.22 | 747.38 | 175.12 |
| | S | G | G | S | V | G |
| 2 | 234.14 | 204.13 | 204.13 | 502.25 | 846.45 | 232.14 |
| | Y | T | G | G | A | G |
| 3 | 397.21 | 305.18 | 261.16 | 559.27 | 917.49 | 289.17 |
| | D | A | G | A | N | T |
| 4 | 512.24 | 376.22 | 318.18 | 630.31 | 1031.53 | 390.21 |
| | S | D | P | A | I | T |
| 5 | 599.70 | 491.25 | 415.23 | 701.34 | 1144.62 | 491.26 |
| | P | F | S | W | D | D |
| 6 | 696.32 | 638.31 | 502.26 | 887.42 | 1259.64 | 606.28 |
| | S | S | G | V | S | A |
| 7 | 783.35 | 725.35 | 559.28 | 986.49 | 1346.68 | 677.32 |
| | | | A | T | T | A |
| 8 | - | - | 630.32 | 1087.54 | 1447.72 | 748.36 |
| | | | A | Y | | V |
| 9 | - | - | 701.36 | 1250.60 | - | 847.43 |
| | | | W | S | | D |
| 10 | - | - | 887.44 | 1337.63 | - | 962.45 |
| | | | | | | F |
| 11 | - | - | - | - | - | 1109.52 |
| | | | | | | S |
| 12 | - | - | - | - | - | 1196.55 |
| | | | | | | W |
| 13 | - | - | - | - | - | 1382.63 |
| | | | | | | T |
| 14 | - | - | - | - | - | 1483.68 |

**Table 4.3 Thaumatin-like protein peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of the thaumatin-like protein from the Douglas-fir ovular secretion (Figure 4.7) and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

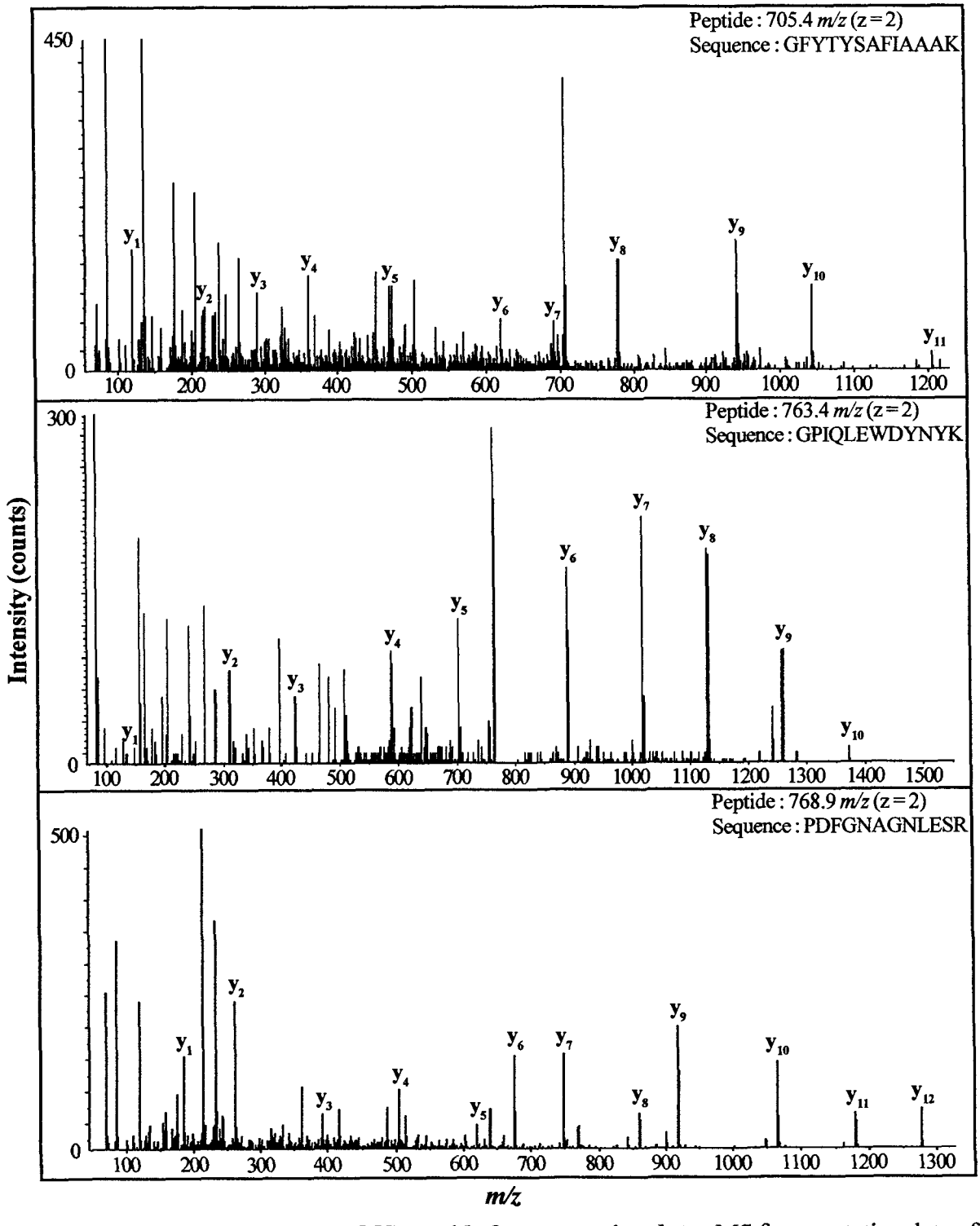



**Figure 4.8 Chitinase tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of the Douglas-fir ovular secretion chitinase (Figure 4.5).Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | |
|---|---|---|---|
| | 705.4 | 763.4 | 768.9 |
| | K | K | R |
| 1 | 147.11 | 147.11 | 175.12 |
| | A | Y | S |
| 2 | 218.15 | 310.17 | 262.15 |
| | A | N | E |
| 3 | 289.15 | 424.22 | 391.19 |
| | A | Y | L |
| 4 | 360.22 | 587.28 | 504.28 |
| | I | D | N |
| 5 | 473.31 | 702.31 | 618.32 |
| | F | W | G |
| 6 | 620.38 | 888.39 | 675.34 |
| | A | E | A |
| 7 | 691.41 | 1017.43 | 746.38 |
| | S | L | N |
| 8 | 778.45 | 1130.52 | 860.42 |
| | Y | Q | G |
| 9 | 941.51 | 1258.57 | 917.44 |
| | T | I | F |
| 10 | 1042.56 | 1371.66 | 1064.51 |
| | Y | P | D |
| 11 | 1205.62 | 1468.71 | 1179.54 |
| | F | G | P |
| 12 | 1352.69 | 1525.73 | 1276.59 |
| | G | | |
| 13 | 1409.71 | - | - |

**Table 4.4 Chitinase peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of a chitinase from the Douglas-fir ovular secretion (Figure 4.8) and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

| Douglas-fir ovular secretion PR protein (Figure 4.5) | Molecular weight (kDa) | pI | Peptide amino acid sequences obtained | Protein identification by MSBlast |
|---|---|---|---|---|
| **peroxidase** (Figure 4.6 & Table 4.2) | 37 | 7.9 | DVIDTIK<br>VALSGSHTI<br>LVLDTGSDLT<br>SADSYSILDTYL | *Triticum aestivum* (Wheat) Pox1 protein (X85227) |
| **thaumatin-like protein** (Figure 4.7 & Table 4.3) | 28 | 4.3 | SPSDYSK<br>SFDATGK<br>WAGSPGGGK<br>SYTVWAAGS<br>TSDINAV<br>TWSFDVAADTTGGR | *Pseudotsuga menziesii* (Douglas-fir) thaumatin-like protein (AJ131731) |
| **chitinase** (Figure 4.8 & Table 4.4) | 27 | 7.8 | GFYTYSAFIAAAK<br>GPIQLEWDYNYK<br>PDFGNAGNLESR | *Picea abies* (Norway Spruce) putative class II chitinase (AY450923) |

**Table 4.5 Summary of Douglas-fir ovular secretion PR proteins.** Peptide amino acid sequences obtained for Douglas-fir ovular secretion PR proteins separated using 2D gel electrophoresis (Figure 4.5). Proteins were reduced, alkylated, and digested with trypsin. Peptides generated were sequenced using tandem MS sequencing (Figures 4.6 - 4.8 and Tables 4.2 - 4.4). Sequence searching was performed using Bork Group's MS Blast search at EMBL on April 23rd, 2004. Protein molecular weights and their respective pI values were approximated by their position on the 2D gel.

## 4.3 Discussion

Pathogenesis-related (PR) protein synthesis is one of the responses plants employ to better defend themselves against invading pathogens. PR proteins were originally identified as novel proteins that accumulated after infection of tobacco leaves with tobacco mosaic virus (Van Loon 1985). Currently, PR proteins have been identified and characterized in more than 40 species belonging to at least 11 groups. Functional analysis of many of these PR proteins has shown them to exhibit antimicrobial activity (Selitrennikoff 2001).

### 4.3.1 Chitinases

Chitinases hydrolyze chitin, polymers of β-1,4-linked *N*-acetylglucosamines present in fungal cell walls and invertebrate exoskeletons, but not in plants (Balasubramanian and Manocha 1992). These enzymes have been isolated and characterized from many different plant sources including conifers (Selitrennikoff 2001; Davis *et al.* 2002; Pirttila *et al.* 2002).

It is this ability to cleave chitin that gives these enzymes their antifungal ability. Fungal cell walls are typically composed of β-glucans, chitin, lipids, and peptides embedded in a protein matrix (Figure 4.9). This rigid structure not only protects them against hostile environments, but also provides a clear and discernable difference between themselves and their plant hosts; thereby, yielding an obvious target for plant antifungal compounds. Compromising the integrity of these cell walls can result in death by lysis since fungi have high internal turgor pressure (Selitrennikoff 2001).

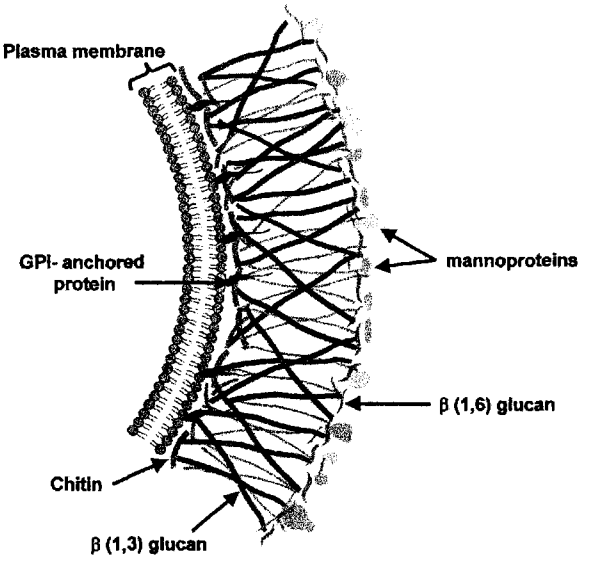



**Figure 4.9** Fungal cell wall schematic (Selitrennikoff 2001)

Chitinase production in plants is considered to be both an active and passive defense mechanism against pathogens for several reasons: their substrates are not found in plants, the majority are induced by pathogen infection, and many show antifungal properties *in vitro* and *in planta* (Mauch *et al.* 1988; Benhamou *et al.* 1990; Hamel and Bellemare 1995; Krishnaveni *et al.* 1999; Regalado *et al.* 2000; Kasprzewska 2003). It is believed they carry out a two-pronged attack against fungi. Upon initial invasion, fungi are attacked by constitutively secreted apoplastic chitinases. The chitin fragments that are released elicit further defensive responses including an increase in apoplastic and vacuolar chitinase production. Once these primed cells are compromised, the more potent vacuolar chitinases are released (Kasprzewska 2003). There is a profound difference in substrate specificity exhibited by the vacuolar chitinases compared to secreted apoplastic chitinases. Whereas the acidic apoplastic chitinases hydrolyze

soluble chitin better and exhibit little antifungal activity, the basic vacuolar ones are more active against crystalline chitin and are quite antifungal (Collinge *et al.* 1993; Neuhaus 1999; Kasprzewska 2003). Using in-gel chitinase assays, it was discovered that the Douglas-fir ovular secretion contains at least 8 chitinases – all with pI values less than 7.

Due to differences in their localization, molecular structure, and substrate specificity, chitinases have been divided into four PR groups: PR-3, PR-4, PR-8, and PR-11 (Neuhaus *et al.* 1996). Although many chitinases are present in the Douglas-fir ovular secretion, the only one unequivocally identified to date using quadrupole time-of-flight tandem mass spectrometry peptide sequencing is a class II chitinase of the PR-3 group. Other members of this group are plant-specific endochitinases, with the exception of one from the bacterium *Streptomyces griseus* (Ohno *et al.* 1996). Endochitinases degrade chitin polymers from within the chitin chains. In contrast, exochitinases can only hydrolyze chitin residues at their non-reducing ends (Graham and Stricklen 1994).

Chitinases in the PR-3 group share a common catalytic domain. Its tertiary structure has been determined in barley (*Hordeium vulgare* L.) (Hart *et al.* 1993; Hart *et al.* 1995). The crystal structure is mostly α-helical, forming globular domain with a groove containing the two catalytic glutamate residues.

### 4.3.2 Thaumatin-like proteins (PR-5)

Members of the fifth group of PR proteins (PR-5) are referred to as thaumatin-like (TL) proteins due to their amino acid sequence similarity with thaumatin - a sweet-tasting protein originally isolated from the arils of South African ketemfe bushes (*Thaumatococcus daniellii* (Bennett) Benth.) (Van der Wel and Loeve 1972). TL

proteins have since been isolated from a variety of plant sources including cherries (Fils-Lycaeon *et al.* 1996), *Brassica* flower buds (Cheong *et al.* 1997), and various seeds (Roberts and Selitrennikoff 1990; Vigers *et al.* 1991; Garcia-Casado *et al.* 2000). In conifers, TL proteins have been identified in the pollen of Ashe juniper (*Juniperus ashei* Buchh.) and radiata pine (*Pinus radiata* D. Don) (Walden *et al.* 1999; Soman *et al.* 2000). It has been theorized that PR proteins such as the TL proteins are abundant in fruits, seeds, and other storage organs because these storage tissues are good substrates for many heterotrophic organisms including pathogenic fungi (Garcia-Casado *et al.* 2000).

TL proteins are highly soluble. Some accumulate intracellularly within the vacuole under appropriate conditions; whereas, others are secreted extracellularly (Velazhahan *et al.* 1999). They can be acidic, neutral, or basic. Extracellularly secreted TL proteins tend to be acidic (Stintzi *et al.* 1993). Mature TL proteins fall into two mass ranges. One range is from approximately 22 – 26 kDa while the second has proteins with an approximate size of 16 kDa. The smaller TL proteins lack an internal 58 amino acid segment (Velazhahan *et al.* 1999). The TL protein identified in the Douglas-fir ovular secretion had an approximate molecular weight of 28 kDa and an isoelectric point (pI) of 4.3.

Structural analysis of the larger TL proteins has revealed that they have internal cores with 16 cysteine amino acids that form 8 disulfide bonds (Roberts and Selitrennikoff 1990), the locations of which are highly conserved (Velazhahan *et al.* 1999). This reinforced architecture makes them quite resistant to proteolysis and pH- or heat-induced denaturation.

Purified TL proteins from several different plant sources possess *in vitro* antifungal activity by inhibiting fungal hyphal growth and spore germination (Vigers *et al.* 1992; Stintzi *et al.* 1993). Direct evidence for their *in planta* antifungal ability was demonstrated using transgenic potato plants that constitutively overexpressed osmotin - a tobacco TL protein originally characterized as being upregulated upon osmotic challenge (Singh *et al.* 1987). These genetically modified (GM) plants exhibited reduced fungal disease symptoms (Liu *et al.* 1994).

Although many of these experiments have conclusively shown that TL proteins exhibit antifungal activity, their exact modes of action remain a mystery. Osmotin has the ability to disrupt the regulation of fungal cell wall assembly (Yun *et al.* 1997; Yun *et al.* 1998). Permatins, as their name implies, are constitutively produced antifungal seed TL proteins that permeabilize fungal cell plasma membranes (Roberts and Selitrennikoff 1990; Vigers *et al.* 1991; Vigers *et al.* 1992). Others have been shown to both bind and hydrolyze β-1,3-glucans *in vitro* (Grenier and Asselin 1993; Grenier *et al.* 1993; Trudel *et al.* 1998). Hydrolyzing these polysaccharides weakens the fungal cell walls. Since fungi have significant internal turgor pressure, their cells undergo death by lysis. Regardless of how TL proteins function, they are nevertheless fungicidal.

As well as having antifungal properties, TL proteins may also provide defense against insect herbivory. A maize (*Zea mays* L.) protein with high amino acid sequence similarity to thaumatin is known to inhibit α-amylase activity (Richardson *et al.* 1987). Inhibition of this insect protein impedes their digestion of plant carbohydrates (Franco *et al.* 2002). This is the only TL protein tested to date that exhibits antiherbivory properties (Hejgaard *et al.* 1991; Vigers *et al.* 1991).

Conifer cones would benefit from having antiherbivory molecules in their ovular secretions since they are subject to many different insect infestations. Conophytes can wipe out entire cone crops. In British Columbia, there are some particularly devastating conophytes. The infestation of a single cone with one Douglas-fir cone moth larva (*Barbara colfaxiana*) can result in a 65 % reduction in that cone's seed yield. The Ponderosa pine cone moth (*Cydia piperana*) can destroy 50 % of total seeds per cone. In some areas, the red cedar cone midge (*Mayetiola thujae*) regularly consumes all red cedar seeds (de Groot *et al.* 1994; Turgeon *et al.* 1994). By studying the ovular secretions of insect resistant cones, we may be able to identify new antiherbivorous molecules.

PR-5 proteins have also been implicated as anti-freeze proteins. Apoplastic extracts from cold-acclimated winter rye (*Secale cereale* L.) exhibited anti-freeze activity; whereas, apoplastic extracts from the control plants did not. N-terminal sequencing of some of the cold-acclimated apoplastic proteins showed that they had amino acid sequence similarities with TL proteins from barley and rice (Hon *et al.* 1995). In conifers, pollen tube initiation and subsequent elongation through the nucellus occurs in the spring during periods of enormous temperature fluctuations. Protecting these events may involve PR protein production.

### 4.3.3 Peroxidases (PR-9)

Peroxidases (PR-9) participate in various defensive physiological processes including wound healing and defense mechanisms against pathogen infection (Hiraga *et al.* 2001). Extracellularly secreted plant peroxidases (POXs) present in the apoplastic spaces are believed to generate aromatic oxyl radicals from various aromatic compounds

(Takahama and Yoshitama 1998). The production of these radicals often results in reactive oxygen intermediate (ROI) production (Kagan *et al.* 1990) known to be an early characteristic of the hypersensitive response (HR) during plant defense (Doke 1983a; Doke 1983b). ROI generation by POXs has been directly observed when pea plants (*Pisum sativum* L.) were treated with elicitors from the fungus *Mycosphaerella pinodes* ((Berk. & A. Bloxam) Vestergr.) (Kiba *et al.* 1996).

Peroxidases have been separated into two large groups: the animal peroxidase super-family and the plant peroxidase super-family. The plant peroxidases are further separated into three classes. The majority of the third class members are extracellular plant peroxidases secreted via the endoplasmic reticulum. Structural studies have shown that these particular enzymes typically contain structural calcium ions, carbohydrate side chains, and several disulfide bridges (Neuhaus 1999). The basic 37 kDa peroxidase identified in the Douglas-fir ovular secretion belongs to this class.

In summary, the rich variety of defense compounds lends support to the concept that their production has evolved concurrently with reproduction in order to protect the ovules from collateral damage during reproduction. More simply stated, the presence of these defensive compounds makes for safe sex in Douglas-fir.

# Chapter 5

## The 28 kDa thaumatin-like protein originates in the nucellus

### 5.1 Introduction

The Douglas-fir ovular secretion provides defense against pathogens during pollination. Pathogenesis related (PR) proteins including various chitinases, a peroxidase, and a thaumatin-like protein have been previously identified and characterized in this thesis. In these experiments, a polyclonal serum generated against a Douglas-fir thaumatin-like protein was used to show that the origin of these proteins may be from the nucellus.

Antibodies are powerful research tools because of their exquisite specificities. These proteins bind specific sites, referred to as epitopes, on their respective antigens. Antigens are typically proteins or other large molecules foreign to the host organism. The specificity and strength of the antibody-antigen interaction allows for the targeted identification of a specific protein within a complex mixture of proteins (Goldman 2000).

An antibody consists of two identical heavy chains and two identical light chains joined together by disulfide bonds. The C-terminal domains of these chains are referred to as constant regions because their amino acid sequences remain essentially the same for all of the antibodies produced by any given host. The uniqueness of each antibody is due to the N-terminal variable regions which contain amino acid sequences unique to each specific antibody. It is the variable regions that confer antigen (epitope) binding specificity (Marchalonis *et al.* 2002).

One antigen detection process commonly used is Western blotting, more formally referred to as immunoblotting. The proteins in a complex mixture are first separated

using gel electrophoresis. Subsequently, they are transferred onto a membrane, typically nitrocellulose or other analogous materials. This membrane is then incubated in a primary antibody solution containing antibodies specific to the target protein. The membrane is then exposed to a second antibody solution containing antibodies specific to the C-terminal constant regions of the primary antibodies. They are also linked to a detector enzyme. Upon incubation with the appropriate substrate, the target protein can be visualized on the membrane by conversion of the substrate to a coloured, fluorescent, or luminescent product. Western blotting is a powerful methodology used in many different research areas (Kurien and Scofield 2003).

## 5.2 Results

Western analysis showed that the polyclonal serum generated against a Douglas-fir thaumatin-like protein successfully detected the previously characterized 28 kDa thaumatin-like protein with an approximate isoelectric point (pI) of 4.3 present in the Douglas-fir ovular secretion (Figures 5.1-5.3). Internal amino acid sequences of this protein were obtained by digesting the protein with trypsin and then sequencing the generated peptides using quadrupole time-of-flight tandem mass spectrometry. These sequences were then submitted to Bork Group's MS blast search engine at EMBL for protein identification. The mass spectrometry data conclusively proved that the protein detected was indeed the same thaumatin-like protein characterized earlier in this thesis (Figure 5.4 & Tables 5.1-5.2).

To learn the origin of this protein prior to its presence in the Douglas-fir ovular secretion, proteins were first extracted from the different ovular tissues at the time of secretion production. The tissues analyzed were the nucellus, the megagametophyte, and the integument. Protein detection using 1D SDS-PAGE indicated that the protein extractions were successful (Figure 5.5). Western analysis with these extracted proteins indicated that the thaumatin-like protein originated in the nucellus (Figure 5.6).

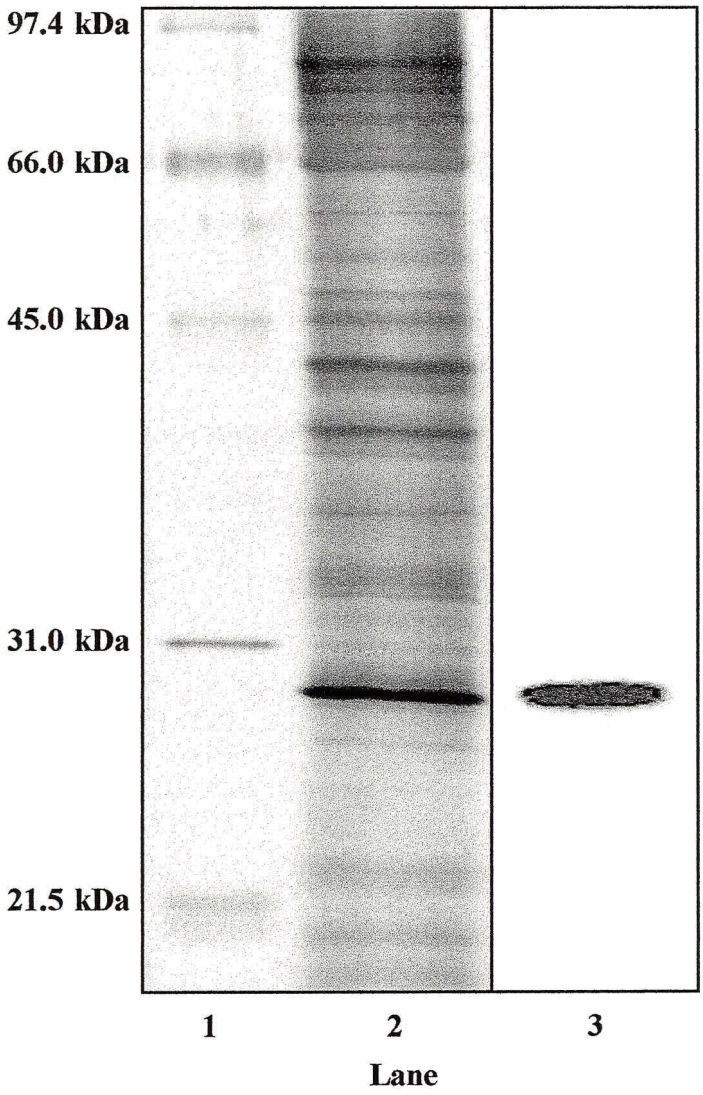



**Figure 5.1 Detection of a thaumatin-like protein in the Douglas-fir ovular secretion.** Prior to loading, 3.0 μL of Douglas-fir ovular secretion was diluted to 10.0 μL with 2X glycine gel sample buffer and immersed in boiling water for 3 min. Electrophoresis was then carried out in a Bio-Rad Mini-Protean 3 Electrophoresis system (Bio-Rad Laboratories, Hercules, CA) through a 4 % acrylamide stacking gel and a 12 % acrylamide resolving gel until the tracking dye reached the bottom of the gel. The stacking and resolving gels were run at 10 and 20 mA, respectively. The buffers used were standard Tris-glycine buffers (Laemmli 1970). To visualize the proteins in the gel, they were silver-stained. To probe for the presence of any thaumatin-like proteins present in Douglas-fir ovular secretions, Western blotting was performed using an antiserum generated against a Douglas-fir thaumatin-like protein.
**Lane 1:** 10.0 μL of molecular weight markers (silver-stained)
**Lane 2:** 3.0 μL of Douglas-fir ovular secretion (silver-stained)
**Lane 3:** 3.0 μL of Douglas-fir ovular secretion (western blot)

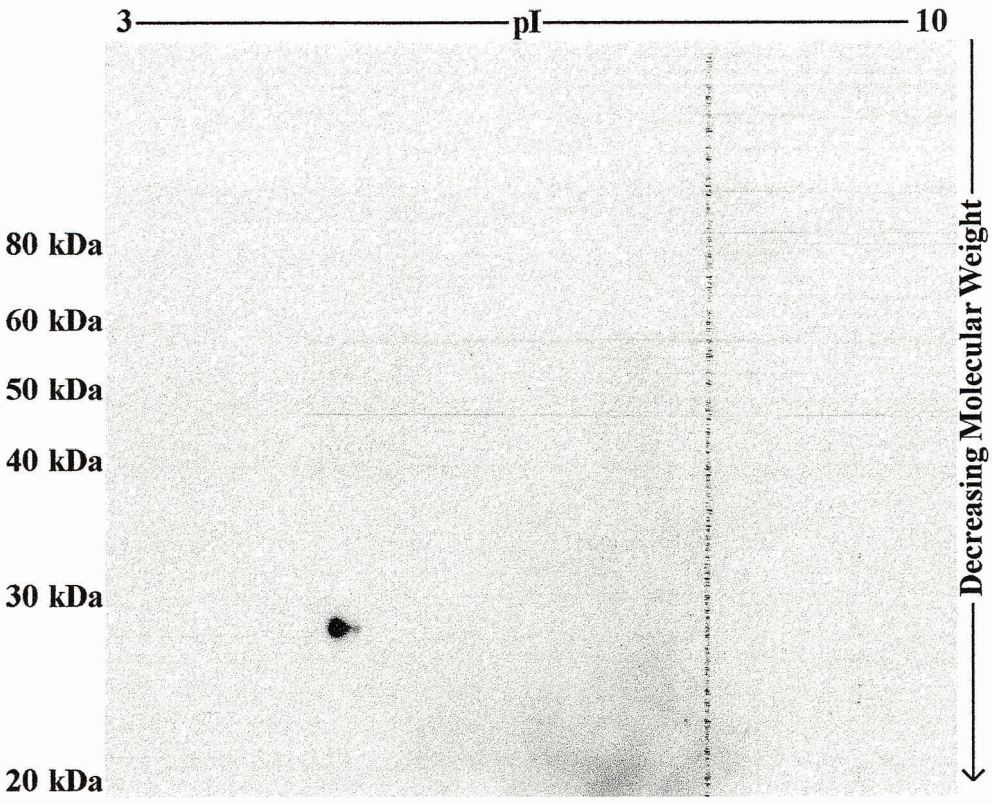



**Figure 5.2 Detection of the Douglas-fir ovular secretion thaumatin-like protein on a 2D gel.** Douglas-fir ovular secretion (50 μL) was vacuum-desiccated to dryness using a Jouan RC 10.22 Centrifugal Evaporator and then solubilized in 30 μL of SDS-MIX [a 1 % (w/v) 2-(cyclohexylamino)ethanesulfonic acid (CHES), 2 % (w/v) SDS, 1 % (w/v) dithiothreitol (DTT), and 10 % (v/v) glycerol solution in $dH_2O$]. Prior to electrophoresing the proteins, the isoelectric focusing (IEF) tube gels were first pre-electrophoresed for 1 h at 200 V. Once the protein samples were loaded, they were electrophoresed within the IEF tube gels for 17.5 h at 800 V. Before the proteins were further separated in the second dimension, the IEF tube gels were incubated in equilibration buffer for approximately 15 min. The IEF tube gels were then placed on top of the second dimension gels and overlaid with an agarose solution that polymerized almost immediately. This was done to keep the IEF tube gels on top of the second dimension gels during electrophoresis. The proteins were separated in the second dimension (10 % acrylamide) by electrophoresing them at 1 A until the blue tracking dye reached the end of the gels. Proteins were then transferred from the large 2D gel onto a PVDF membrane overnight at 20 mA and 4 °C using a large Bio-Rad Trans-Blot® Cell. To probe for the presence of any thaumatin-like proteins present in Douglas-fir ovular secretions, Western blotting was performed using an antiserum generated against a Douglas-fir thaumatin-like protein.

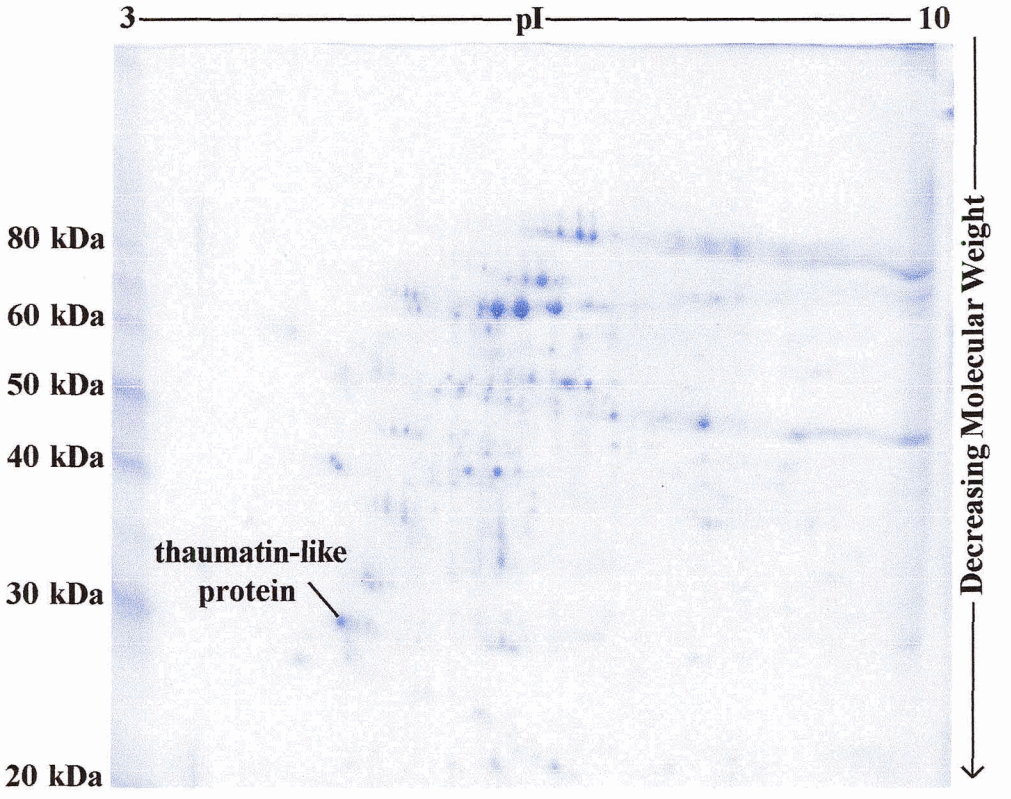



**Figure 5.3 Purification of the Douglas-fir ovular secretion thaumatin-like protein using 2D GE.** Douglas-fir ovular secretion (50 µL) was vacuum-desiccated to dryness using a Jouan RC 10.22 Centrifugal Evaporator and then solubilized in 30 µL of SDS-MIX [a 1 % (w/v) 2-(cyclohexylamino)ethanesulfonic acid (CHES), 2 % (w/v) SDS, 1 % (w/v) dithiothreitol (DTT), and 10 % (v/v) glycerol solution in $dH_2O$]. Prior to electrophoresing the proteins, the isoelectric focusing (IEF) tube gels were first pre-electrophoresed for 1 h at 200 V. Once the protein samples were loaded, they were electrophoresed within the IEF tube gels for 17.5 h at 800 V. Before the proteins were further separated in the second dimension, the IEF tube gels were incubated in equilibration buffer for approximately 15 min. The IEF tube gels were then placed on top of the second dimension gels and overlaid with an agarose solution that polymerized almost immediately. This was done to keep the IEF tube gels on top of the second dimension gels during electrophoresis. The proteins were separated in the second dimension (10 % acrylamide) by electrophoresing them at 1 A until the blue tracking dye reached the end of the gels. To visualize the proteins present in the 2D gels, they were stained using a Colloidal Coomassie protocol (Neuhoff *et al.* 1988).

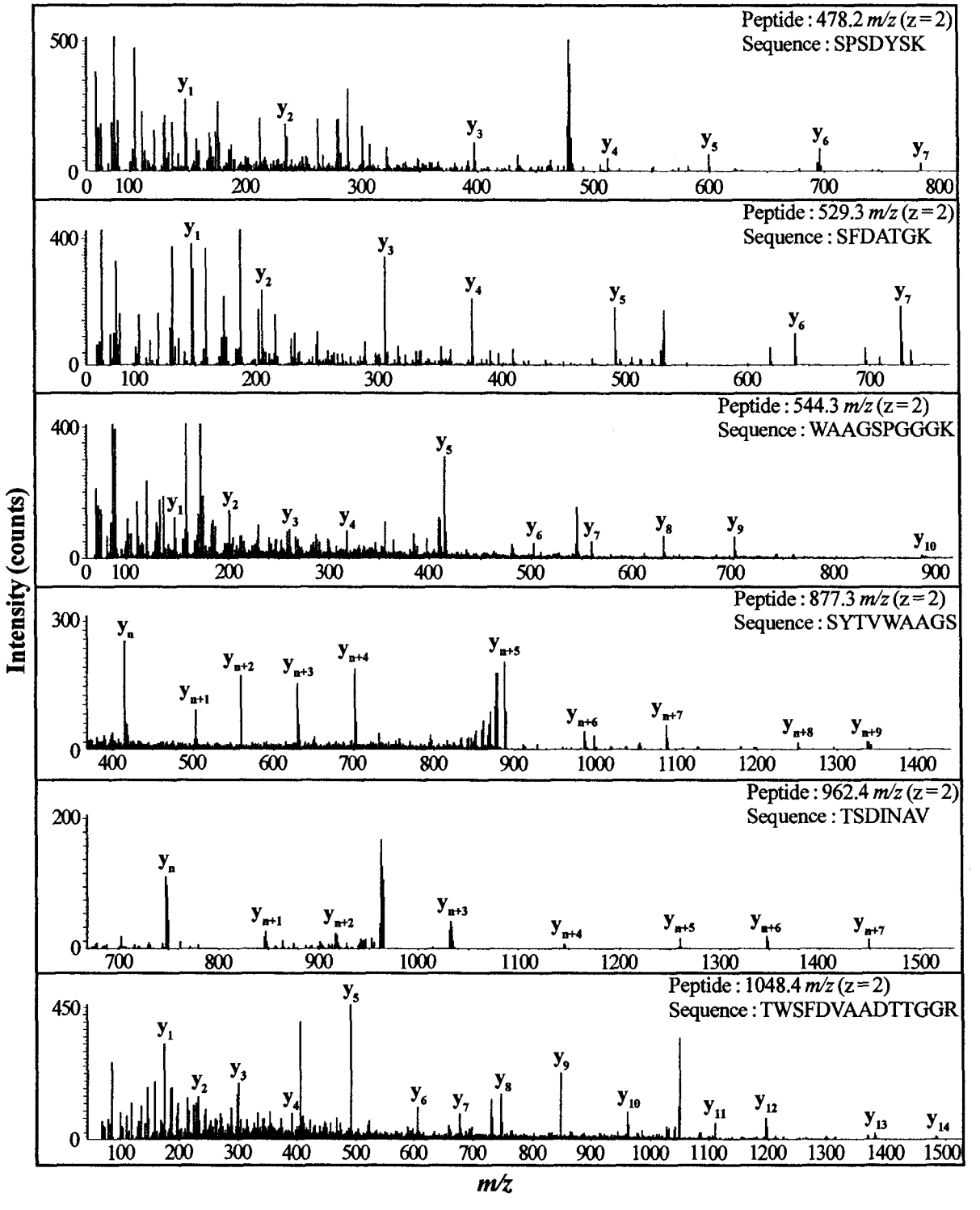



**Figure 5.4 Douglas-fir ovular secretion thaumatin-like protein tandem MS peptide fragmentation data.** MS fragmentation data of peptides generated from the digestion of the Douglas-fir ovular secretion thaumatin-like protein (Figure 5.3). Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

| y ions | Peptide *m/z* (z = 2) | | | | | |
|---|---|---|---|---|---|---|
| | 478.2 | 529.3 | 544.3 | 877.3 | 962.4 | 1048.4 |
| | K | K | K | | | R |
| 1 | 147.11 | 147.11 | 147.11 | 415.22 | 747.38 | 175.12 |
| | S | G | G | S | V | G |
| 2 | 234.14 | 204.13 | 204.13 | 502.25 | 846.45 | 232.14 |
| | Y | T | G | G | A | G |
| 3 | 397.21 | 305.18 | 261.16 | 559.27 | 917.49 | 289.17 |
| | D | A | G | A | N | T |
| 4 | 512.24 | 376.22 | 318.18 | 630.31 | 1031.53 | 390.21 |
| | S | D | P | A | I | T |
| 5 | 599.70 | 491.25 | 415.23 | 701.34 | 1144.62 | 491.26 |
| | P | F | S | W | D | D |
| 6 | 696.32 | 638.31 | 502.26 | 887.42 | 1259.64 | 606.28 |
| | S | S | G | V | S | A |
| 7 | 783.35 | 725.35 | 559.28 | 986.49 | 1346.68 | 677.32 |
| | | | A | T | T | A |
| 8 | - | - | 630.32 | 1087.54 | 1447.72 | 748.36 |
| | | | A | Y | | V |
| 9 | - | - | 701.36 | 1250.60 | - | 847.43 |
| | | | W | S | | D |
| 10 | - | - | 887.44 | 1337.63 | - | 962.45 |
| | | | | | | F |
| 11 | - | - | - | - | - | 1109.52 |
| | | | | | | S |
| 12 | - | - | - | - | - | 1196.55 |
| | | | | | | W |
| 13 | - | - | - | - | - | 1382.63 |
| | | | | | | T |
| 14 | - | - | - | - | - | 1483.68 |

**Table 5.1 The Douglas-fir ovular secretion thaumatin-like protein peptide amino acid sequences.** Y ion masses obtained from the MS fragmentation of peptides generated from the digestion of the thaumatin-like protein from Douglas-fir ovular secretions (Figure 5.4) and the deduced amino acid sequence of each peptide based on these masses. The monoisotopic mass difference between individual Y ions is the monoisotopic residue mass of an amino acid.

| Molecular weight (kDa) | pI | Peptide amino acid sequences obtained | Protein identification by MSBlast |
|---|---|---|---|
| 28 | 4.3 | SPSDYSK<br>SFDATGK<br>TVWAAGSPGGGK<br>SYTVWAAGS<br>TSDINAV<br>TWSFDVAADTTGGR | *Pseudotsuga menziesii* (Douglas-fir) thaumatin-like protein (AJ131731) |

**Table 5.2 Identification of the Douglas-fir ovular secretion thaumatin-like protein.** Peptide amino acid sequences obtained for the Douglas-fir ovular secretion thaumatin-like protein separated using 2D gel electrophoresis (Figure 5.3). The protein was reduced, alkylated, and digested with trypsin. Peptides generated were sequenced using tandem MS sequencing (Figure 5.4 and Table 5.1). Sequence searching was performed using Bork Group's MS Blast search at EMBL on April 10, 2004. The molecular weight and pI values were approximated by their position on the 2D gel.

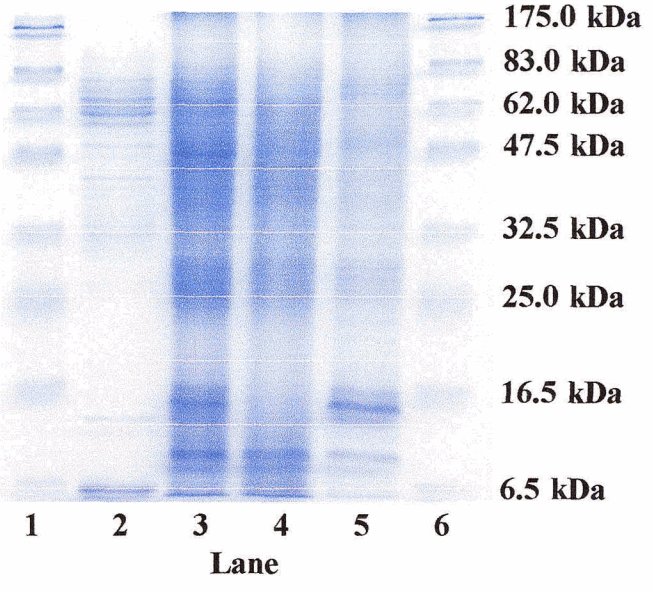



**Figure 5.5 1D SDS-PAGE of the Douglas-fir ovular secretion proteins extracted from the nucellus, megagametophyte, and integument.** Prior to loading, 3.0 µL of Douglas-fir ovular secretion was diluted to 10.0 µL with 2X glycine gel sample buffer. Each acetone pellet produced from the tissue extraction protocol was diluted with the appropriate amount of 2X glycine gel sample buffer. All the samples were immersed in boiling water for 3 min. Prepared samples were then electrophoresed in a Bio-Rad Mini-Protean 3 Electrophoresis system (Bio-Rad Laboratories, Hercules, CA) through a 4% acrylamide stacking gel and a 12% acrylamide resolving gel until the tracking dye reached the bottom of the gel. The stacking and resolving gels were run at 10 and 20 mA, respectively. For protein visualization, the gel was stained with GelCode® Blue stain reagent.

**Lane 1:** Broad range molecular weight markers
**Lane 2:** 3.0 µL of Douglas-fir ovular secretion
**Lane 3:** Proteins extracted from the nucellus
**Lane 4:** Proteins extracted from the megagametophyte
**Lane 5:** Proteins extracted from the integument
**Lane 6:** Broad range molecular weight markers

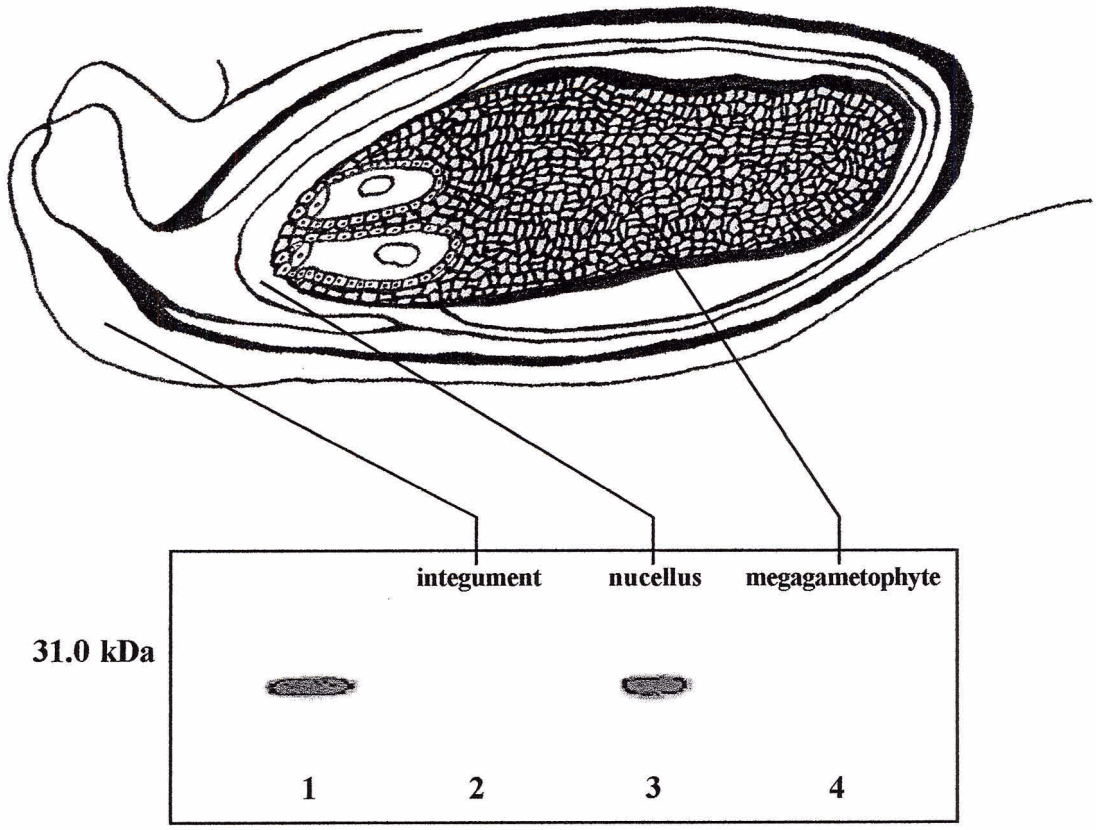



**Figure 5.6 The thaumatin-like protein in the Douglas-fir ovular secretion originates in the nucellus.** Prior to loading, 3.0 μL of Douglas-fir ovular secretion was diluted to 10.0 μL with 2X glycine gel sample buffer. Each acetone pellet produced from the tissue extraction protocol was diluted with the appropriate amount of 2X glycine gel sample buffer. All the samples were immersed in boiling water for 3 min. Prepared samples were then electrophoresed in a Bio-Rad Mini-Protean 3 Electrophoresis system (Bio-Rad Laboratories, Hercules, CA) through a 4% acrylamide stacking gel and a 12% acrylamide resolving gel until the tracking dye reached the bottom of the gel. The stacking and resolving gels were run at 10 and 20 mA, respectively. To probe for the presence of thaumatin-like proteins, Western blotting was performed using an antiserum generated against a Douglas-fir thaumatin-like protein.
**Lane 1:** 3.0 μL of Douglas-fir ovular secretion
**Lane 2:** Proteins extracted from the integument
**Lane 3:** Proteins extracted from the nucellus
**Lane 4:** Proteins extracted from the megagametophyte

**5.3 Discussion**

The Douglas-fir ovular secretion exudes from the nucellar surface approximately one week before fertilization and fills the micropyle of the ovule that houses the engulfed pollen (Owens and Morris 1990; von Aderkas and Leary 1999) as well as any pathogens that may have also been brought inside. Previous proteomic analysis detailed in this thesis has revealed that there are many different proteins present in this secretion including ones that may participate in pollen selection and development as well as several PR proteins. The origin of these proteins is unknown. It has been suggested that the secretion originates from either the nucellus (Owens and Morris 1990; Takaso and Owens 1995; O'Leary *et al.* 2004) or the megagametophyte (Takaso and Owens 1996).

Once the pollen has been engulfed, it elongates within the micropyle towards the nucellar surface for 6 to 9 weeks. During this time, cells at the base of the nucellus frequently divide causing the nucellus to grow towards the elongating pollen. Prior to the pollen and nucellus coming into close proximity with each other, the cells at the tip of the nucellus contain abundant starch and many organelles with only a few small vacuoles. However, once they come into close proximity, the starch disappears and the cells become vacuolate. The newly formed vacuoles fuse and their contents are secreted. Since these events coincide with secretion production, the hypothesis was made that the secretion originates from the nucellus (Owens and Morris 1990). More recently, it was shown that arabinogalactan proteins present in yew (*Taxus* x *media* Rehder) pollination drops originated from the nucellus (O'Leary *et al.* 2004).

Other research has suggested that the secretion originates from the prothallial cells of the megagametophyte (Takaso and Owens 1996; Takaso *et al.* 1996). As it is

produced, it is pushed into the micropyle through the nucellar cells since the nucellus has a thick hydrophobic cuticle between itself and the integument (Takaso and Owens 1996). Other experimental evidence also suggests a megagametophyte origin. Megagametophyte homogenates at the time of secretion production elicit similar responses on pollen development as the ovular secretion does (Takaso *et al.* 1996).

My research, in agreement with that of O'Leary *et al.* (2004), shows that the protein complement of the secretion probably originates from the nucellus. Western analysis on dissected ovular tissues conclusively showed that the 28 kDa thaumatin-like protein present in the Douglas-fir ovular secretion originated from the nucellus and not the megagametophyte or integument. My previous identification of a serine carboxypeptidase-like protein in this liquid that may be involved with transferring solutes from the nucellus to the developing pollen during pollen tube development also suggests a nucellar origin for the secretion proteins.

Although the immunolocalization studies carried out in our lab implicate the nucellus as the tissue that produces the ovular secretion proteins, I believe that the nucellus cannot be the only contributing tissue to the secretion. Research described previously in this thesis provides evidence that the secretion not only contains beneficial nutrients for the developing pollen during pollen tube elongation, but also delivers molecules that may coordinate pollen tube formation with egg viability. Since the egg cells are housed within the megagametophyte, this tissue probably provides some developmental cues. Perhaps this gametophytic tissue provides the liquid component containing the beneficial low molecular weight nutrients to the pollen, thereby initiating further pollen development. The fact that megagametophyte homogenates elicit similar

responses as the secretion does on pollen development supports this hypothesis (Takaso *et al.* 1996). However, most of the pollen that was treated with these homogenates had their pollen tubes burst, not something that occurs *in vivo*. Nucellar proteins incorporated into the secretion as it moves into the micropyle may also be needed for proper pollen development. Perhaps an interactive communication system between the ovule and pollen needs to take place for successful pollen development and fertilization.

# Chapter 6

## Closing thoughts and remarks

The biochemical interactions taking place within a conifer ovule may be much more sophisticated and complex than previously believed (Willson and Burley 1983). These interactive communications are not only involved with pollen selection and development, but also provide defense against any pathogens that gain entry into this site.

### 6.1 Plant defense

When a pathogen attacks, an array of plant defenses are induced. Initial activation of plant defenses depends on the ability of the host plant to first recognize elicitor molecules. Many elicitors have been characterized; they are classified as either being race-specific or non-race-specific. Race-specific elicitors are secreted peptides and proteins encoded for by pathogen avirulence (*Avr*) genes. They initiate plant defenses upon interaction with host plant disease-resistance (*R*) gene products (de Wit 1997). This is the "gene-for-gene" concept for pathogenicity (Flor 1942; Oort 1944). Molecules that have the ability to elicit defensive responses in a manner different from the aforementioned "gene-for-gene" interaction are referred to as non-race-specific elicitors (Hahn 1996).

Elicitor recognition triggers a variety of defensive physiological responses. The first is the hypersensitive response (HR). An early characteristic of HR is the rapid generation of superoxide and accumulation of peroxide (Doke 1983a; Doke 1983b). These reactive oxygen intermediates (ROIs) can either kill the pathogens directly (Levine *et al.* 1994) or initiate further pathogen resistance responses (Jabs *et al.* 1997). HR

induced cell death prevents further spread of the disease by restricting the pathogen to the immediate vicinity of the infected cells (Lamb *et al.* 1989).

Concurrent with HR initiation, a longer lasting nonspecific response is activated either as a part of HR or as a symptom of the disease. This response is known as systemic acquired resistance (SAR) (Ryals *et al.* 1996). Well characterized nonspecific defense mechanisms that are activated include cell wall lignification, callose deposition around dead cell foci, antimicrobial phytoalexin production, and the increased expression of pathogenesis-related (*PR*) genes. Functional analysis of many of these PR proteins including chitinases (PR-3, PR-4, PR-8, and PR-11), thaumatin-like (TL) proteins (PR-5), and extracellular peroxidases (PR-9), has shown them to exhibit antimicrobial activity (Selitrennikoff 2001).

The identification of PR proteins in the Douglas-fir ovular secretion described in this thesis suggests that an interactive pathogen defense system - elicitor recognition followed by defensive response induction – may be active within conifer ovules. I hypothesize that this same system, or one analogous to it, is involved with pollen selection and development.

### 6.2 Pollen selection and development

Coordinated development between the pollen and ovule is essential for reproductive success in conifers. Pollen germination must culminate with the formation of a pollen tube. The pollen tube must then elongate towards the megagametophyte and eventually penetrate an egg cell where it delivers its male gametes. Although this is developmentally less complex than angiosperms, the intricacy of the many interactions

taking place between the pollen and ovule in conifer reproduction cannot be underemphasized. These pollen-ovule interactions evolved to not only coordinate pollen and megagametophyte development, but also to ensure that appropriate mating partners are matched since the rather indiscriminate pollination mechanisms conifers employ for pollen capture also allow foreign pollen to enter the ovules.

It has been widely accepted that there are no prezygotic breeding barriers in conifers (Willson and Burley 1983); however, recent research suggests otherwise. *In vitro* studies showed that larch, Sitka spruce, and white pine pollen tubes could penetrate all three of the other species' megagametophytes. These intergeneric crosses show that there are no barriers to gamete delivery *in vitro.* However, *in vivo* events within the ovule must exhibit some form of pollen selection because these crosses do not occur naturally (Dumont-BeBoux *et al.* 1998). Using ovular dissections and statistical analysis, it was also shown that Douglas-fir ovules reduce heterospecific pollen grain germination (Rise 2001). In addition, treating angiosperm pollen with English yew pollination drops inhibits their germination (Zenkteler and Relska-Roszak 2003).

How are these prezygotic breeding barriers initiated? I hypothesize that there may be two pollen selection systems present in conifer ovules. The first is a "gene-for-gene" concept for pollen selection (Figure 6.1). The second involves the initiation of these pollen selection barriers in a manner different from this "gene-for-gene" concept (Figure 6.2).

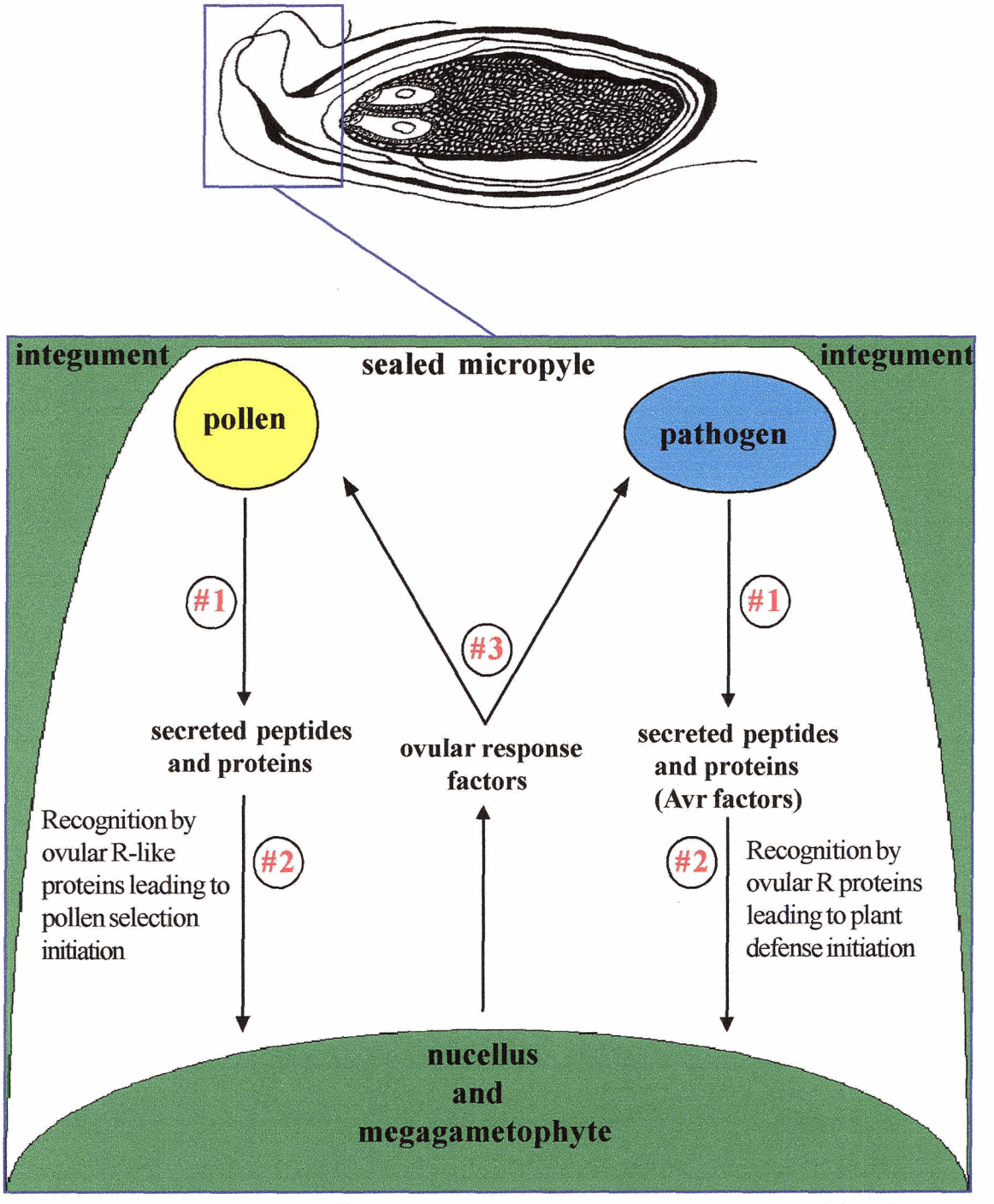



**Figure 6.1 The "gene-for-gene" concept for pollen selection and pathogenicity. #1).** Pollen (foreign and incompatible) and pathogens secrete peptides and proteins into the micropyle of the ovule. **#2).** The secreted pollen factors are recognized by R-like proteins leading to pollen selection initiation and the secreted pathogen factors (Avr factors) are recognized by their corresponding R proteins leading to plant defense initiation. **#3).** The ovular response factors generated as a result of these initiations are secreted into the micropyle and interact with their targets accordingly.

### 6.2.1 The "gene-for-gene" concept for pollen selection

Pollen are known to secrete many factors into their surrounding environment during germination including peptides and proteins (Stanley and Linskens 1965). Could these peptides and proteins from foreign or incompatible pollen initiate pollen selection responses in a similar fashion as pathogen Avr factors initiate defensive responses (Figure 6.1)? This is a very interesting hypothesis and one that is worth investigating further. To test this, the following experiments could be conducted. The first step would be to germinate foreign pollen and purify their secreted peptides and proteins. Conifer ovules would then be subjected to these peptides and proteins and their responses qualified and quantified. Serial purifications followed by identical conifer response experiments would eventually allow the specific "avirulence"-like pollen selection factors to be identified. Once these factors have been identified and characterized, one could use various interaction experiments to determine their corresponding ovular R-like proteins.

### 6.2.2 Pollen selection through elicitor recognition without "gene-for-gene interactions

Molecules that have the ability to activate plant defense mechanisms in a manner different from the "gene-for-gene" model are referred to as non-race-specific elicitors. The largest class of non-race-specific elicitors is the biologically active oligosaccharides (oligosaccharins) that are released from pathogen and host plant cell walls by hydrolases secreted from both organisms (Hahn 1996).

Only a few of these biologically active oligosaccharins have been fully characterized in terms of their structure and biological function. These oligosaccharins include β-glucans, chitin-derived oligomers, oligogalacturonides, and xyloglucans. The

β-glucans and chitin-derived oligomers released from pathogen cell walls upon hydrolysis by host plant glucanases and chitinases, respectively, are referred to as exogenous non-race-specific elicitors. Endogenous non-race-specific elicitors, such as the oligogalacturonides and xyloglucans, are derived from host plant cell surfaces upon pathogen invasion (Fritig *et al.* 1998).

The best characterized plant cell wall-derived elicitors are oligogalacturonides (OGs), homopolymers of α-1,4-linked D-galacturonic acid. Derived from homogalacturonan - the parent polysaccharide present in the pectic matrix - OGs have been known to elicit plant defensive responses such as lignification of cucumber cotyledons (Robertsen 1986), phytoalexin accumulation in soybean cotyledons (Nothnagel et al. 1983), and ROI production in cultured cells of soybean, cotton, and tobacco (Low and Heinstein 1986; Legendre et al. 1993). Plant receptors that bind OGs are currently the focus of several research groups, but to date, only one has been identified. Remorin, a 34 kDa plasma membrane protein from potatoes, binds OGs and is subsequently phosphorylated (Reymond et al. 1995; Reymond et al. 1996).

Another structural polysaccharide present in plant primary cell walls that elicits various plant defenses when hydrolyzed is xyloglucan. Compared to OGs, xyloglucan fragments have received little attention. One of the few experiments performed showed that high molecular weight xyloglucan fragments induced potato resistance to *Phytophthora infestans* (Perekhod et al. 1998), the causative agent of light blight disease.

Is there a pollen selection system within a conifer ovule that is initiated upon recognition of exogenous oligogalacturonides and xyloglucans derived from foreign or incompatible pollen cell surfaces (Figure 6.2)?

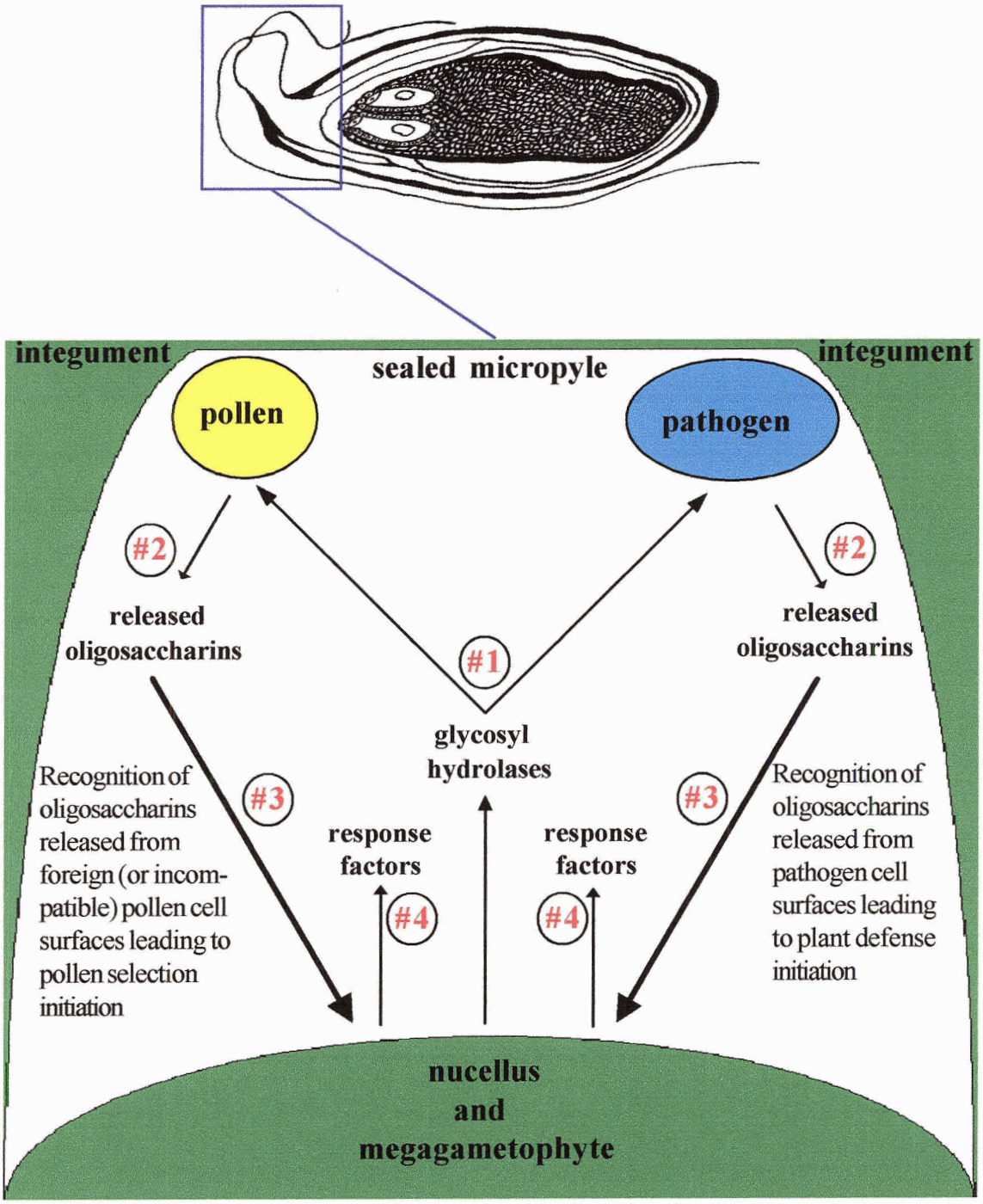



**Figure 6.2 Recognition of released oligosaccharins from foreign (or incompatible) pollen and pathogens leading to pollen selection initiation and plant defense initiation respectively. #1).** Glycosyl hydrolases are secreted by the ovule into the micropyle **#2).** The oligosaccharins are released from pollen (foreign and incompatible) and pathogen cell surfaces. **#3).** These elicitors are recognized by ovular receptor proteins **#4).** Appropriate ovular response factors generated as a result of these interactions are secreted into the micropyle and interact with their targets accordingly.

This thesis has shown that conifer ovular secretions contain glycosyl hydrolases that have the ability to cleave pollen cell wall polysaccharides. Are the released saccharides unique for each type of pollen? If so, do foreign and incompatible pollen cell wall polysaccharides initiate pollen selection responses in a similar manner as non-race-specific elicitors initiate plant defense (Figure 6.2)? One could run similar experiments as the ones previously discussed to tease out the "gene-for-gene" concept of pollen selection to study this hypothesis.

The work in this thesis suggests that conifer ovular secretions not only play significant roles in pollen selection and development, but also provide pathogen defense during conifer reproduction. Luckily for me, this is not where the story ends. This is where it begins.

# Chapter 7

# Methods and Materials

## 7.1 Chapter 3: The major proteins present in the Douglas-fir ovular secretion and their potential role in pollen selection and development

### 7.1.1 Collection of the Douglas-fir ovular secretion

The Douglas-fir ovular secretions studied were extracted from trees at the University of Victoria. The ovuliferous scales were dissected from the female cones collected in the field and placed in Fisherbrand® Petri dishes (Fisher Scientific, Canada) that had been kept humid with wetted Whatman® filter paper (Whatman International Ltd., Maidstone, England). Ten minutes later under a dissecting microscope, the dissection droplets that formed were collected bringing the ovular secretions with them. Collection was done with a 10 µL micropipette tip. Daily collections were pooled into 1.5 mL NUNC (Nalge NUNC International, Rochester, NY) storage tubes and frozen in liquid nitrogen. The frozen samples were stored at –20 °C until analysis. The Douglas-fir ovular secretions used in these experiments were collected during the following periods: June 3 - 22, 1999; June 5 - 18, 2000; June 8 - 28, 2001; June 12 - 26, 2002; and June 3 - 20, 2003. Prior to analysis, the ovular secretions were centrifuged at 14,000 rpm for 5 min to remove any micro-aggregates.

### 7.1.2 One-dimensional sodium dodecylsulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) using Tris-glycine

Prior to loading, 3.0 µL of Douglas-fir ovular secretion was diluted to 10.0 µL with 2X glycine gel sample buffer and immersed in boiling water for 3 min. Electrophoresis was then carried out in a Bio-Rad Mini-Protean 3 Electrophoresis system (Bio-Rad Laboratories, Hercules, CA) through a 4 % acrylamide stacking gel and a 12 %

acrylamide resolving gel until the tracking dye reached the bottom of the gel. The stacking and resolving gels were run at 10 and 20 mA, respectively. The buffers used were standard Tris-glycine buffers (Laemmli 1970). To visualize the proteins present in the gel, they were either stained with silver or GelCode® Blue stain reagent (Pierce, Rockford, IL).

### 7.1.3 1D SDS-PAGE using Tris-tricine

Prior to loading, 3.0 µL of Douglas-fir ovular secretion was diluted to 15.0 µL with 1X tricine gel sample buffer and immersed in boiling water for 3 min. Electrophoresis was then carried out in a Bio-Rad Mini-Protean 3 Electrophoresis system through a 4 % acrylamide stacking gel and 15 % acrylamide resolving gel at 100 V until the tracking dye reached the bottom of the gel. The buffers used were standard Tris-tricine buffers (Schagger and von Jagow 1987). To visualize the proteins in the gel, they were stained with GelCode® Blue stain reagent.

### 7.1.4 Silver staining

After electrophoresis, the gels were fixed in a 50 % methanol ($CH_3OH$) and 5 % acetic acid ($CH_3COOH$) solution in $dH_2O$ for 20 min. Gels were then washed for 10 min in a 50 % methanol ($CH_3OH$) solution in $dH_2O$ followed by a 10 min wash in $dH_2O$. Gels were sensitized with a 1 min incubation at room temperature in a 0.02 % sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) solution in $dH_2O$. The gels were rinsed twice with $dH_2O$ for 1 min each prior to being incubated for 20 min at 4 °C in a chilled 0.1 % silver nitrate ($AgNO_3$) solution in $dH_2O$. Gels were then rinsed twice with $dH_2O$ for 1 min each. Development of the gels was performed in a 0.04 % formalin (35 % formaldehyde in $dH_2O$) and 2 % sodium carbonate ($Na_2CO_3$) solution in $dH_2O$. The developing solution

was replaced whenever it turned yellow. When bands began to appear, the development was terminated by the addition of a 3 % acetic acid ($CH_3COOH$) solution in $dH_2O$.

### 7.1.5 Staining with GelCode® Blue stain reagent

Gels were first rinsed 3 times with deionized $dH_2O$ for 5 min each. Enough GelCode® Blue stain reagent (Pierce, Rockford, IL) was then added to sufficiently cover the gels and the staining was carried out for at least 1 h at room temperature with gentle agitation. Gels were finally washed 2-3 times with deionized $dH_2O$ until the background was relatively colourless.

### 7.1.6 Bradford protein assay

The Bradford reagent (Sigma, St. Louis, MO) was gently mixed and brought to room temperature prior to the assay. A series of bovine serum albumin (BSA) standards were prepared in $dH_2O$ with concentrations ranging from 2.0 to 10.0 μg/mL. Three μL of Douglas-fir ovular secretion collected from each season were diluted to 1.0 mL with $dH_2O$. The assay was performed by first adding 1.0 mL of each standard and ovular secretion test sample to 1.0 mL of Bradford reagent in separate test tubes. They were then vortexed for approximately 10 s and incubated at room temperature for 30 min prior to measuring the absorbances at a wavelength of 595 nm. Each standard and test sample was performed in triplicate. A BSA standard curve was constructed by plotting the average absorbance values versus amount of BSA protein. The protein concentrations of the Douglas-fir ovular secretions were determined through extrapolation with this BSA standard curve.

### 7.1.7 Reversed-phase high performance liquid chromatography (RP-HPLC)

Protein separation using RP-HPLC was carried out using a BrownLee narrowbore C8 column (Perkin Elmer, Norwalk, CT) attached to a Beckman Gold HPLC and UV detector (Beckman Coulter Inc., Fullerton, CA). Molecules were eluted in a gradient of 0-70 % buffer B in 70 min. Buffer A was a 0.1 % trifluoroacetic acid (TFA) solution in $dH_2O$ and buffer B was a 0.075 % TFA solution in acetonitrile. The eluent was monitored at a wavelength of 220 nm.

### 7.1.8 Electroblotting 1D SDS-PAGE gels

Proteins were transferred from the 1D SDS-PAGE gels onto polyvinylidienedifluoride (PVDF) membranes by applying 90 V for 25 min at 4 °C using a Bio-Rad Trans-Blot® Cell (Bio-Rad Laboratories, Hercules, CA) . The transfer buffer was prepared by dissolving 1.51 g of Tris base and 14.4 g of glycine in 800 mL of $dH_2O$. Once dissolved, the pH of the solution was adjusted to 8.2 with 6 M HCl and the final volume of the buffer was brought up to 1.0 L with $dH_2O$. To visualize the proteins so that they could be excised and submitted for N-terminal sequencing, they were stained with GelCode® Blue stain reagent.

### 7.1.9 N-terminal protein sequencing

The excised PVDF membrane pieces containing the proteins to be sequenced were submitted to the University of Victoria (UVic) Protein Chemistry Center for N-terminal sequencing. Sequencing was performed by Edman degradation using a Perkin Elmer Applied Biosystems 473 pulsed liquid phase protein sequencer or a 475 gas phase protein sequencer (Perkin Elmer, Norwalk, CT).

### 7.1.10 Acetone precipitation of Douglas-fir ovular secretion proteins prior to two-dimensional gel electrophoresis

To precipitate proteins present in 50.0 μL of Douglas-fir ovular secretion, 400.0 μL of ice-cold acetone was added. The solution was then incubated overnight at -20 °C. To pellet the precipitated proteins, they were centrifuged the following morning at 14,000 rpm using an Eppendorf Centrifuge 5415C (Eppendorf, Hamburg, Germany) for 10 min. The supernatant was then carefully removed and the protein pellet left behind was vacuum-desiccated to dryness using a Jouan RC 10.22 Centrifugal Evaporator (Jouan Inc., Winchester, VA).

### 7.1.11 Two-dimensional gel electrophoresis (2D GE)

The acetone precipitated Douglas-fir ovular secretion protein pellet was solubilized in 30 μL of SDS-MIX [a 1 % (w/v) 2-(cyclohexylamino)ethanesulfonic acid (CHES), 2 % (w/v) SDS, 1 % (w/v) dithiothreitol (DTT), and 10 % (v/v) glycerol solution in $dH_2O$]. Prior to electrophoresing the proteins, the isoelectric focusing (IEF) tube gels were first pre-electrophoresed for 1 h at 200 V. Once the protein samples were loaded, they were electrophoresed within the IEF tube gels for 17.5 h at 800 V. Before the proteins were further separated in the second dimension, the IEF tube gels were incubated in equilibration buffer for approximately 15 min. The IEF tube gels were then placed on top of the second dimension gels and overlaid with an agarose solution that polymerized almost immediately. This was done to keep the IEF tube gels on top of the second dimension gels during electrophoresis. The proteins were separated in the second dimension (10 % acrylamide) by electrophoresing them at 1 A until the blue tracking dye reached the end of the gels. To visualize the proteins present in the 2D gels, they were stained using a Colloidal Coomassie protocol (Neuhoff *et al.* 1988).

### 7.1.12 Colloidal Coomassie staining

Gels were slowly agitated in fixing solution (a 50 % ethanol (v/v) and 3 % phosphoric acid (v/v) solution in $dH_2O$) overnight. After fixing, the gels were rinsed 3 times in deionized $dH_2O$ for 30 min each. Gels were then equilibrated in Neuhoff solution (a 16 % ammonium sulfate (w/v), 25 % methanol (v/v), and 5 % phosphoric acid (v/v) solution in $dH_2O$) for one hour. After equilibration, 1 g of Coomassie blue stain (Coomassie Brilliant Blue G250 pure powder, Serva (cat# 17524)) was sprinkled into the Neuhoff solution and staining was carried out for 1-3 d until protein visualization was adequate.

### 7.1.13 In-gel protein digest

The protein containing gel plugs excised from the 2D gels were first cut into 1 - 2 $mm^3$ gel cubes with a razor blade. All gel cubes were placed into a 1.5 mL Eppendorf tube. The proteins were destained with the addition of 200 µL of a wash solution (a 50 % (v/v) methanol and 5 % (v/v) acetic acid solution in $dH_2O$). Destaining was carried out overnight at room temperature.

The following morning, the wash solution was removed from the samples with a plastic pipette and discarded. Another 200 µL of wash solution was added and the gel pieces were rinsed for a further 3 h at room temperature. The wash solution was again removed and the gel pieces were dehydrated by adding 100 µL of acetonitrile and incubating them for 5 min at room temperature. The acetonitrile was removed from the sample and the gel pieces were vacuum desiccated to dryness at ambient temperature in a Jouan RC 10.22 Centrifugal Evaporator.

The proteins were then reduced with the addition of 50 μL of a 50 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate solution and incubating the proteins for 30 min at 56 °C. The DTT solution was removed and the proteins were alkylated with the addition of 50 μL of a 100 mM iodoacetamide in 100 mM ammonium bicarbonate solution and incubating them at 45 °C for 30 min in the dark.

The iodoacetamide solution was removed from the sample and the proteins were dehydrated by adding 100 μL of acetonitrile. The acetonitrile was removed and the gel pieces were rehydrated in 100 mM ammonium bicarbonate. After rehydration, the gel pieces were again dehydrated in 100 μL of acetonitrile. The acetonitrile was removed from the sample and the gel pieces were vacuum desiccated to dryness at room temperature in a Jouan RC 10.22 Centrifugal Evaporator.

The trypsin reagent was prepared by adding 1.0 mL of ice-cold 50 mM ammonium bicarbonate to 20 μg of Sequencing Grade Modified Trypsin (Promega Corporation, Madison, WI). The dehydrated gel cubes were then rehydrated with 30 μL of the trypsin solution on ice for 10 min. The gel pieces were centrifuged to the bottom of the Eppendorf tube and the excess trypsin solution was removed from the sample. Prior to protein digestion overnight at 37 °C, 5.0 μL of a 50 mM ammonium bicarbonate solution was added and the samples were centrifuged to the bottom of the Eppendorf tube.

The peptides were extracted the following morning by incubating the gel cubes in 30 μL of a 100 mM sodium carbonate solution for 1 h at 37 °C.

### 7.1.14 Quadrupole time-of-flight tandem mass spectrometry peptide sequencing

Prior to analysis, the peptide extracts were desalted using glass capillary needles (Protana Inc., Staermosegaardsvej, Denmark) packed with C18 resin. The resin was first equilibrated by rinsing 3 times with 5.0 μL of a 0.1 % formic acid solution in $dH_2O$. The peptide extract was then added to the resin in 5.0 μL aliquots. Once loaded, the peptides were washed 5 times with 5.0 μL of a 0.1 % formic acid solution in $dH_2O$. Finally, the peptides were extracted into sample needles using 2.0 μL of a 50 % (v/v) methanol and 1 % (v/v) formic acid solution in $dH_2O$. Nanospray electrospray ionization (ESI) was used to introduce ions into the Q-STAR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA.). Calibration of the mass spectrometer was performed using CsI with a monoisotopic mass of 132.9 amu and sex pheromone inhibitor iPDI with a monoisotopic mass of 829.54 amu (Bachem #H9985, Bubendorf, Switzerland). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA).

### 7.1.15 Protein identification

The unknown ovular secretion proteins were identified by submitting the amino acid sequences obtained using both N-terminal amino acid sequencing and quadrupole time-of-flight tandem mass spectrometry peptide sequencing to Bork Group's MS blast search at EMBL (http://dove.embl-heidelberg.de/Blast2/msblast.html).

## 7.2 Chapter 4: Safe sex in Douglas-fir

The following methods and materials can be found in the Chapter 3 methods and materials (Section 7.1): Collection of the Douglas-fir ovular secretion, 1D SDS-PAGE, staining with GelCode® Blue stain reagent, electroblotting 1D SDS-PAGE gels, 2D GE, Colloidal Coomassie staining, in-gel protein digest, quadrupole time-of-flight tandem mass spectrometry peptide sequencing, and protein identification.

### 7.2.1 Chitinase activity of the Douglas-fir ovular secretion

Into 100.0 μL of a 0.20 mM para-nitrophenyl-N-acetyl-β-$_D$-glucosaminide [*p*-NP-(GlcNAc), Sigma, St. Louis, MO] solution in $dH_2O$, 1.0 μL of Douglas-fir ovular secretion was added. Samples were vortexed and incubated at 30 °C for 2.5 h. Absorbances were measured at a wavelength of 405 nm every 30 min. The control was 101.0 μL of the 0.20 mM *p*-NP-(GlcNAc) solution.

### 7.2.2 pH dependence of the Douglas-fir ovular secretion chitinase activity

Into 100.0 μL of 0.20 mM *p*-NP-(GlcNAc) solutions of varying pH, 1.0 μL of Douglas-fir ovular secretion was added. The following solutions were used; 50 mM glycine pH 2.5, 50 mM glycine pH 3.3, 50 mM sodium acetate pH 4.0, 50 mM sodium acetate pH 5.1, 50 mM sodium acetate pH 5.5, 50 mM sodium phosphate pH 5.6, 50 mM sodium phosphate pH 5.8, 50 mM sodium phosphate 6.0, 50 mM sodium phosphate pH 6.3, 50 mM sodium phosphate pH 6.6, 50 mM sodium phosphate pH 7.2, 50 mM sodium phosphate pH 8.0, 50 mM glycine pH 9.1, and 50 mM glycine pH 10.0. Samples were vortexed and incubated at 30 °C for 2.0 h. Absorbances were measured at a wavelength of 405 nm.

### 7.2.3 In-gel chitinase assay using 1D SDS-PAGE

Prior to loading, 3.0 μL of Douglas-fir ovular secretion was diluted to 10.0 μL with 2X glycine gel sample buffer (without the reducing agent, 2-mercaptoethanol) and immersed in boiling water for 3 min. Electrophoresis was carried out in a Bio-Rad Mini-Protean 3 Electrophoresis system (Bio-Rad Laboratories, Hercules, CA) through a 4 % acrylamide stacking gel and a 12 % acrylamide resolving gel until the tracking dye reached the bottom of the gel. The concentration of the CM-chitin-RBV reagent (Loewe Biochemica GmbH, Sauerlach, Germany) in the resolving gel was 0.68 g/mL. The stacking and resolving gels were run at 10 and 20 mA, respectively. The buffers used were standard Tris-glycine buffers (Laemmli 1970). After electrophoresis, the gel was incubated in a 50.0 mM sodium phosphate, 0.1 % Tween-20 solution with a pH of 6 until clearance bands could be observed. For protein visualization, the gel was stained with GelCode® Blue stain reagent.

### 7.2.4 In-gel chitinase assay using 2D GE

Ten microliters of Douglas-fir ovular secretion was solubilized in 10 μL of isoelectric focusing (IEF) non-reducing sample buffer (8.0 M urea, 2.0 % v/v NP-40, 2.0 % 3-10 ampholytes). Prior to electrophoresing the proteins, the IEF tube gels were first pre-electrophoresed for 10 min at 200 V, 15 min at 300 V, and 15 min at 400 V. Before the proteins were further separated in the second dimension, the IEF tube gels were incubated in equilibration buffer (with no reducing agent) for approximately 10 min. The IEF tube gels were then placed on top of the 10 % second dimension acrylamide gels and electrophoresed at 20 mA until the blue tracking dye reached the end of the gels. The concentration of the CM-chitin-RBV reagent in the resolving gel was 0.68 g/mL. After

electrophoresis, the gel was incubated in a 50.0 mM sodium phosphate, 0.1 % Tween-20 solution with a pH of 6 until clearance zones could be observed.

## 7.3 Chapter 5: The 28 kDa thaumatin like protein originates in the nucellus

The following methods and materials can be found in the Chapter 3 methods and materials (Section 7.1): Collection of the Douglas-fir ovular secretion, 1D SDS-PAGE, staining with GelCode® Blue stain reagent, electroblotting 1D SDS-PAGE gels, 2D GE, Colloidal Coomassie staining, in-gel protein digest, quadrupole time-of-flight tandem mass spectrometry peptide sequencing, and protein identification.

### 7.3.1 Western blotting with antibodies made against a Douglas-fir thaumatin-like protein

After the proteins were transferred onto the PVDF membrane, the membrane was blocked for 1 h at room temperature in a 0.1 % Tween 20 (ACP Chemicals Inc., Montreal, Que.), 5 % skim milk powder (Difco, Becton Dickinson Microbiology Systems, Sparks, MD) phosphate buffered saline (PBS) solution. The membrane was then washed in buffer (a 0.1 % Tween 20 PBS solution) twice for 1 min each, once for 15 min, and then twice more for 5 min each. After the washing was complete, the membrane was incubated for 1 h at room temperature in a 1 in 7,500 dilution of the primary antibody – a rabbit polyclonal antiserum made against a Douglas-fir thaumatin-like protein. This polyclonal sera was generously donated by Dr. Abul Ekramaddoullah from the Pacific Forestry Center in Victoria, BC. Following this incubation, the membrane was washed in buffer as previously described. The membrane was incubated for 1 h at room temperature in a 1 : 50,000 dilution of the secondary antibody – goat anti-rabbit IgG (H + L) antibodies (Cedarlane Laboratories Ltd., Hornby, Ont.) conjugated to horseradish peroxidase (HRP). Prior to detection, the membrane was washed in buffer for 15 min and then four times for 5 min each.

The detection system used was the ECL+plus Western Blotting Detection System (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, England). The detection solutions were brought to room temperature and mixed in a ratio of 40:1 (1 mL of solution A + 25 μL of solution B). The membrane was removed from the wash solution and incubated in the detection reagent mixture for 5 min at room temperature. Excess detection reagent mixture was removed and the membrane was placed in an x-ray film cassette with a sheet of autoradiography film placed on top of the membrane. The film was exposed until detection was adequate. After detection was complete, the proteins present in the membrane were visualized by staining with GelCode® Blue stain reagent.

### 7.3.2 Electroblotting 2D gels

Proteins were transferred from the 2D gels onto PVDF membranes overnight at 20 mA and 4 °C using a large Bio-Rad Trans-Blot® Cell (Bio-Rad Laboratories, Hercules, CA). The transfer buffer was prepared by dissolving 12.1 g of Tris base, 56.25 g of glycine, 0.5 g of SDS, and 1.0 L of methanol in 3.0 L of $dH_2O$. Once dissolved, the pH of the solution was adjusted to 8.2 and then the final volume of the buffer was brought up to 5.0 L with $dH_2O$. The proteins were visualized by staining them with GelCode® Blue stain reagent.

### 7.3.3 Protein extraction from the integument, nucellus, and megagametophyte tissues

The integument, nucellus, and megagametophyte tissues were dissected from Douglas-fir ovules at the time of secretion production and immediately frozen. The tissues were ground up under liquid $N_2$ separate mortar and pestles and transferred into individual Eppendorf centrifuge tubes. To each tube, 1.0 mL of extraction buffer 1 (a 4

% w/v SDS, 5 % v/v 2-mercaptoethanol, 5 % w/v sucrose solution in $dH_2O$) was added and the tubes were vortexed for 30 min. Cellular debris was pelleted by centrifuging for 10 min at 14,000 rpm in an Eppendorf Centrifuge 5415C (Eppendorf, Hamburg, Germany). The resulting supernatants were transferred to new Eppendorf centrifuge tubes.

The supernatant containing Eppendorf tubes were immersed in boiling water for 3 min and then brought to room temperature. The supernatants were divided into 150 µL portions. To precipitate the proteins, 1.2 mL of ice-cold acetone was added to each tube and they were incubated overnight at -20 °C. The following morning, the proteins were pelleted by centrifuging for 10 min at 14,000 rpm. The supernatants were removed and the pellets were air-dried in the fumehood for 20 min. The dried pellets were then suspended in 50 µL of extraction buffer 2 (extraction buffer 1 with 1 % NP 40) and vortexed. Samples were centrifuged for 10 min at 14,000 rpm and the supernatants were pipetted into new Eppendorf centrifuge tubes. The supernatants were kept frozen at -20 °C until analysis by 1D SDS-PAGE.

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