AN ABSTRACT OF THE DISSERTATION OF

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Title: Genetic and Biochemical Analysis of Victoria Blight: Identification of AFLP Markers and Purification and Characterization of the Oat Saspase.

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Thomas J. Wolpert

Victoria blight of oats (*Avena sativa*) is caused by the fungus, *Cochliobolus victoriae*, which produces the toxin, victorin. Victorin production is required for pathogenicity of the fungus. In oats, sensitivity to the toxin and susceptibility to the pathogen is conditioned by a dominant allele at the *Vb* locus, while oats with a homozygous recessive allele are insensitive to victorin and resistant to the fungus. *Vb* is either tightly linked to or the same gene as *Pc-2*, a gene that confers crown rust resistance in oats. Therefore, the same gene may provide resistance to one pathogen while conferring susceptibility to another. To better understand the interaction of victorin with oats, genetic markers linked to the *Vb* locus were identified and proteases involved in victorin-induced programmed cell death (PCD) were purified and characterized.

Amplified fragment length polymorphism (AFLP) technology was used to identify two genetic markers that flank the *Vb* locus. We produced an F2 population segregating for victorin sensitivity. Over 51,000 markers generated by 512 different AFLP primer pairs were screened for polymorphisms between victorin-sensitive and insensitive genotypes. These results facilitated the production of a genetic map of the *Vb* locus.

Victorin induces a response in oats that has been characterized as a form of programmed cell death (PCD). One biochemical feature of victorin-induced PCD is the proteolytic cleavage of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco). Rubisco proteolysis had previously been shown to be inhibited by general protease inhibitors. Here, we describe inhibition of rubisco proteolysis by caspase-specific inhibitors and present evidence for a protease cascade. The first protease from plants that exhibits caspase specificity and involvement with PCD was purified and characterized. This protease, we have termed a "saspase", contains amino acid sequence homology to plant subtilisin-like serine proteases and is found in the extracellular fluid (ECF) after victorin treatment. Heat shock-induced PCD is also described and displays characteristics similar to victorin-induced PCD, including DNA laddering, rubisco proteolysis, and localization of the saspase in the ECF.

Genetic and Biochemical Analysis of Victoria Blight: Identification of AFLP Markers and Purification and Characterization of the Oat Saspase

by
Warren C. Coffeen

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Warren C. Coffeen, Author

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Chapter 1

Introduction

VICTORIA BLIGHT: BACKGROUND AND SIGNIFICANCE

The overall objective of this research is to further understand the genetic, cellular, and biochemical relationships between a plant pathogen and its host. The disease called Victoria blight of oats is caused by the fungus, *Cochliobolus victoriae*, which is pathogenic because it produces the host-selective toxin, victorin. Only isolates that produce the toxin are pathogenic, while mutants or outcrosses that do not produce victorin are non-pathogenic. Oat genotypes that carry the dominant allele of the *Vb* locus are sensitive to the toxin and thus susceptible to the pathogen, while homozygous recessive alleles confer victorin-insensitivity and pathogen resistance. Therefore, Victoria blight of oats represents a disease where pathogenesis is principally the result of toxin production, and susceptibility in the host is characterized by a single dominant gene that confers toxin sensitivity.

Victoria blight was first described in 1946 (Meehan and Murphy 1946) after the introduction of oat lines carrying Victoria-type resistance to crown rust. Victoria oats were introduced into the USA from Uruguay as a source of resistance to the fungus, *Puccinia coronata*, the causal agent of crown rust (Litzenberger 1949).

Shortly thereafter, because three-fourths of the total oat acreage in the USA was of that variety, Victoria blight became a severe and wide-spread disease of oats.

Resistance to crown rust in Victoria-type oats is conditioned by the *Pc-2* gene. Extensive attempts have been made to separate rust resistance from Victoria blight susceptibility. Two mutagenesis studies were performed in the 1960's to differentiate *Vb* from *Pc-2*, however, both indicated that the two phenotypes were inseperable. Additionally, Rines and Luke (1985), after analyzing somatoclonal variants generated under toxin selection in tissue culture, concluded that all lines that lost victorin-sensitivity also lost crown rust resistance. Recently, Mayama et al. (1995) also tried unsuccessfully to separate *Vb* from *Pc-2*. These results suggest that *Vb*, the gene conferring Victoria blight susceptibility, and *Pc-2*, the gene conditioning crown rust resistance, are either the same gene, or are very tightly linked.

Pc-2 mediated rust resistance is a classical gene-for-gene interaction in which an avirulence factor from the pathogen interacts with a resistance gene product produced in the plant. This interaction activates resistance, which apparently induces many responses, including, but not limited to, production of reactive oxygen species, cell wall fortification and callose production, salicylic acid accumulation, pathogenesis-related protein expression, phytoalexin biosynthesis, and the hypersensitive response (HR; reviewed in Hammond-Kosack and Jones 1996). The possibility that victorin interacts with a gene that activates resistance indicates that victorin may be mimicking an avirulence factor. Physiological studies of victorin support this idea because victorin appears to induce many of the responses associated with resistance, including callose production (Walton and Earle 1985), the respiratory

burst (Romanko 1959), ethylene evolution (Shain and Wheeler 1975), extracellular alkalization (Ullrich and Novacky 1991), phytoalexin synthesis (Mayama et al. 1986), and K^+ efflux (Wheeler and Black 1962). This suggests the possibility that a plant's susceptible response to a necrotrophic pathogen could be similar to a resistance response to a biotrophic pathogen. Therefore, genetic, cellular, and biochemical analysis of the interaction between *C. victoriae* and oats, or more specifically between the response induced by victorin and the product of the *Vb* gene, should provide insight into the relationship and mechanism of both plant disease resistance and susceptibility.

Studies on victorin have revealed that it is composed of a group of closely-related, cyclized pentapeptides with the most prevalent form named "victorin C" (Wolpert et al. 1985). Structural studies led to the development of a biologically active ^{125}I-labeled derivative used for binding studies (Wolpert and Macko 1989). One protein from oats was identified that bound labeled victorin *in vivo* only in susceptible genotypes (Wolpert and Macko 1989). This protein was characterized as the P-protein from the glycine decarboxylase complex (GDC), an important multi-enzyme complex of the photorespiratory cycle (Wolpert et al. 1994). GDC mutants have been shown to be lethal (Somerville and Ogren 1982), and because victorin binds and inhibits the GDC both *in vivo* and *in vitro* (Navarre and Wolpert 1995), it appears likely that victorin inhibition of GDC would also be lethal and thus, causal to symptom development (Navarre and Wolpert 1999). However, characterization of *in vitro* P-protein binding did not reveal any difference between victorin-sensitive and insensitive genotypes (Wolpert and Macko 1989). Also, no differences have been

detected between susceptible and resistant genotypes among the components that comprise the GDC (unpublished data). These data suggest that inhibition of GDC by victorin is a consequence of, and not causal to disease development, and consequently, that neither the P-protein nor any component of the GDC is likely to be the product of the *Vb* gene.

GENETIC ANALYSIS OF THE *VB* LOCUS

Victorin, as a toxin, obviously induces a form of cell death. However, victorin could be mimicking an avirulence determinant and consequently, elicit resistance-associated cell death (the HR). It is currently unknown how victorin induces a cell death response, or how the product of the *Vb* gene facilitates this response. Does the interaction of victorin with the *Vb* gene product lead directly to signaling, or does the *Vb* gene product simply allow victorin access to the cell, where it is toxic? The answer to these questions and others related to the mechanism of the response would be greatly clarified by identification of the *Vb* gene. To facilitate identification, we undertook a genetic approach to discover markers linked to the *Vb* locus. To do this we used a technique called amplified fragment length polymorphism (AFLP; Vos et al. 1995). AFLP is a polymerase chain reaction (PCR)-based technique used to identify genetic markers, and combines both the reliability of restriction endonuclease digestion with the reproducibility of PCR. AFLP provides several advantages over other techniques used to produce genetic markers. Restriction fragment length polymorphism (RFLP) requires more DNA per analysis, is more expensive, and produces only one co-dominant single-locus specific marker per reaction (Lu et al.

1996). Compared to simple sequence repeat (SSR) markers, AFLP is advantageous because no sequence knowledge is required prior to analysis (Jones et al. 1997). Lastly, the reproducibility of AFLP (Jones et al. 1997) and the number of polymorphic markers viewed per reaction (Sharma et al. 1996) is much greater than obtained from random amplified polymorphic DNA (RAPD).

AFLP technology has broad applications, and has been successfully employed in a variety of research projects encompassing a multitude of organisms. One area of interest is in developing markers related to plant genes conferring resistance to plant pathogenic viruses (Jeong et al. 2002), fungi (Pauquet et al. 2001), bacteria (Tai et al. 1999), nematodes (Meksem et al. 2001), and insects (Murai et al. 2001). Furthermore, genetic variation in plant pathogens has also been analyzed by AFLP, including virulence gene mapping in *Cochliobolus sativus* (Zhong and Stafferson 2002), identifying distinctions within Mexican isolates of *Macrophomia phaseolina* (Mayek-Perez et al. 2001), and characterizing differences between pathogenic and non-pathogenic strains of *Xanthomonas axonopodis* pv. *manihotis* (Gonzalez et al. 2002). Another important area of interest is the identification of AFLP markers associated with improving plant fitness and production, such as mapping drought tolerance (Dubos and Plomion 2003) and drought avoidance genes (Price et al. 2002) in maritime pine and upland rice, respectively. Also, the sugar beet genome has been heavily inundated with AFLP markers that are linked to sucrose production (Schneider et al. 2002) and superior quality of individual varieties (Riek et al. 2001). Lastly, the AFLP technique has been utilized in several non-plant systems including: understanding the genetic relationship between different bovine species (Buntjer et al.

2002), finding genetic variations within selective breeds of cattle (Ajmone-Marsan et al. 2002), cloning and sequencing markers from chicken (Knorr et al. 2001), producing a linkage map of black tiger shrimp (Wilson et al. 2002), and evaluating inheritance in clonal rainbow trout (Brown and Thorgaard 2002). Overall, AFLP technology provides a multifunctional and extensive method practical for a variety of applications and systems. Because of this versatility, it was employed in the search of genetic markers linked to the gene conferring victorin-sensitivity in oats.

BIOCHEMICAL ANALYSIS OF VICTORIN-INDUCED CELL DEATH

The proteolytic inactivation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) was identified as a specific victorin-induced event in sensitive oat cells (Navarre and Wolpert 1999). Further characterization revealed that cleavage of the first 14 amino acids (apparently following glutamate-14) of rubisco was prevented by cysteine protease inhibitors; the calcium-channel blocker, lanthanum chloride ($LaCl_3$); and the ethylene inhibitors, silver thiosulfate and aminooxyacetic acid (Navarre and Wolpert 1999). These results suggest that victorin induces a signal cascade that is activated or regulated by calcium and ethylene and involves proteases. Furthermore, $LaCl_3$ provides complete protection against all victorin-induced symptoms in whole leaves (Navarre and Wolpert 1999). This suggests that victorin does not kill the cell directly but rather signals the cell to kill itself.

Because victorin induces a cell death response similar to the HR (see above), and because the HR has recently been characterized as a form of programmed cell death (PCD; Dangle et al. 1996, Mittler et al. 1997, Pontier et al. 1998, Mackey et al.

2002, Abramovitch et al. 2003), it seemed likely that victorin may also induce a form of PCD. This led to the identification of several victorin-induced characteristics that resembled a type of PCD in animals, called apoptosis. These characteristics include cell shrinkage and collapse (Yao et al. 2001), chromatin condensation (Yao et al. 2001), DNA laddering (Navarre and Wolpert 1999, Tada et al. 2001), mitochondrial depolarization and permeability transition (Curtis and Wolpert 2002), and ordered, substrate-specific proteolytic events (Navarre and Wolpert 1999).

PCD is a genetically-controlled, organized form of cellular suicide that functions in eliminating unnecessary or aged cells. It is essential for cellular maturation and morphogenesis and is required to maintain cellular homeostasis in multicellular organisms. In addition, improper regulation of PCD has been implicated in a wide variety of animal diseases (Polverini and Nör 1999, Wang and Wang 1999). Apoptosis, the most characterized form of PCD, has been extensively studied in animal systems and can be distinguished by unique characteristics. Cells undergoing apoptosis display morphological changes including cell shrinkage, chromatin condensation, and apoptotic body formation. Biochemically, apoptotic cells exhibit DNA laddering, and activation of a family of cysteine proteases called caspases (cysteine aspartases; reviewed by Vaux and Korsmeyer 1999, Hengartner 2000).

Therefore, the interaction of victorin with sensitive oats induces a form of PCD that, like animal apoptosis, can be distinguished by discrete morphological and biochemical changes within the cell. Furthermore, victorin-induced PCD is easily initiated, proceeds in a rapid and synchronous manner, and appears to encompass all

leaf mesophyll cells. Thus, oats and victorin are an ideal system in which to study the mechanism and progression of plant PCD.

PCD has also been associated with plant processes other than the HR, including senescence (Bleecker and Patterson 1997, Miller et al. 1999, Schmid et al. 2001), stress (Katsuhara 1997, Solomon et al. 1999), and development (Runeberg-Roos and Saarma 1998, Groover and Jones 1999, Schmid et al. 1999). Currently, very little is known about the fundamental machinery that controls or regulates PCD in plants, except, possibly, for the involvement of cellular proteases (reviewed by Woltering et al. 2002). Proteases have been implicated in several forms of PCD including senescence (Delorme et al. 2000, Schmid et al. 2001, Eason et al. 2002), oxidative stress (Solomon et al. 1999), seed development (Schmid et al. 1998, Schmid et al. 1999, Wan et al. 2002), tracheary element development (Runeberg-Roos and Saarma 1998, Groover and Jones 1999), and the HR (Vera and Conejero 1988, D'Silva et al. 1998, Krüger et al. 2002). Furthermore, enzymes similar to the caspases (the family of proteases involved in animal apoptosis) have been implicated in plant PCD based on inhibition by caspase-specific inhibitors (del Pozo and Lam 1998, Richael et al. 2001, Elbaz et al. 2002, De Jong et al. 2000, Mlejnek and Procházka 2002).

Caspases are cysteine proteases that cleave following aspartate residues, and whose activation during animal apoptosis is critical to the cell death process (reviewed in Stennicke and Salvesen). Caspases specifically process select proteins, either activating or inactivating them, leading to the ordered disassembly of the cell (reviewed by Solary et al. 1997, Nagata 2000). Because of their substrate specificity,

caspases are considered processive proteases rather then degradative proteases, the latter of which typically facilitate extensive protein degradation.

All of the proteases that have been isolated and characterized from plants undergoing PCD have been categorized as degradative enzymes. Although they function differently from caspases, degradative proteases play an important role during animal apoptosis by degrading cellular proteins in the dying cell (Solary et al. 1997). However, degradative proteases do not control or regulate the processes of cell death, as do the caspases. Therefore, in order to better understand the underlying mechanisms of plant PCD, the identification of proteases that function in a processive manner is needed. Unlike degradative enzymes, processive proteases are more likely to initiate and regulate PCD. Because victorin-induced oat cell death presents an ideal system for studying plant PCD, and that cell death implicates protease activation, this study was undertaken to identify possible processive proteases involved in victorin-induced PCD.

Chapter 2

Genetic Analysis of the *Vb* gene in *Avena sativa*

Warren C. Coffeen and Thomas J. Wolpert

ABSTRACT

Cochliobolus victoriae is the causal agent of a disease called Victoria blight of oats (*Avena sativa*), and produces the toxin, victorin. Only isolates that produce the toxin are virulent, while only oats that carry the dominant allele at the *Vb* locus are sensitive to victorin and susceptible to the fungus. The *Vb* locus is also believed to confer resistance to the crown rust pathogen, *Puccinia coronata*. Therefore, identification of the victorin-sensitivity gene may also identify a gene conferring resistance to crown rust. We developed a genetic map of the *Vb* locus to help identify the gene. Amplified fragment length polymorphism (AFLP) technology was used to screen a bulked F2 population segregating for victorin-sensitivity. After analyzing over 51,000 markers produced by 512 different primer pairs, two polymorphic markers linked to and flanking the *Vb* locus were identified. A genetic map of 26.1 cM was developed and will be useful in screening future candidates of the *Vb* gene.

INTRODUCTION

The oat pathogen, *Cochliobolus victoriae*, causal agent of Victoria blight (Meehan and Murphy 1946), produces a host-specific toxin, victorin, which is the primary determinant of pathogenicity for the fungus (Meehan and Murphy 1947, Wheeler and Luke 1954). Oat genotypes carrying the dominant *Vb* allele are sensitive to victorin and thus, susceptible to toxin-producing isolates of the fungus (Litzenberger 1949). Resistance to the fungus and toxin insensitivity is conferred by the homozygous recessive allele of *Vb* (*vb,vb*). Victoria blight appeared in cultivated oats during the 1940s after the introduction of the *Pc-2* gene which confers resistance

to *Puccinia coronata*, the causal agent of crown rust (Litzenberger 1949). Extensive attempts to genetically separate the *Vb* gene from the *Pc-2* gene have failed (Luke et al. 1960, Luke et al. 1966, Rines and Luke 1985, Mayama et al. 1995). These results have led to the assumption that the *Vb* gene and the *Pc-2* gene are identical or very tightly linked. Developing a genetic map of the *Vb* locus could facilitated molecular cloning of the *Vb* gene, and possibly lead to the identification of a gene that confers disease susceptibility to one pathogen while serving as a resistance gene to another.

In order to identify genetic markers linked to the *Vb* locus, a polymerase chain reaction (PCR)-based technique called amplified fragment length polymorphism (AFLP, Vos et al. 1995) was employed. AFLP is a technique that combines both the reliability of restriction endonuclease digestion with the reproducibility of PCR. In comparison to other genetic markers, AFLP provides several advantages. Restriction fragment length polymorphism (RFLP) requires much more DNA per analysis, is more expensive, and produces only one co-dominant single-locus specific marker per reaction (Lu et al. 1996). Compared to simple sequence repeat (SSR) markers, AFLP is advantageous because no sequence knowledge is required prior to analysis (Jones et al. 1997). Lastly, the reproducibility of AFLP (Jones et al. 1997) and the number of polymorphic markers per reaction (Sharma et al. 1996) is much greater than that of random amplified polymorphic DNA (RAPD) markers.

The AFLP procedure can be broken down into four sections: DNA digestion, double-stranded oligonucleotide adaptor ligation, selective amplification, and visualization by gel analysis. Genomic DNA is digested with two enzymes, one recognizing a six-base sequence, called the "rare cutter", and the other recognizing a

four-base sequence, called the "frequent cutter". The restricted DNA is then ligated to a double-stranded oligonucleotide adapter specific to the "sticky ends" produced by the restriction enzymes. During the selective amplification reaction, single-stranded oligonucleotide primers containing complementary sequences to the adapter and restriction site are designed with selective nucleotides added to the 3′ end, thus enabling only DNA fragments that complement the chosen nucleotides to be amplified. The subset of DNA fragments are visualized on denaturing polyacrylamide gel electrophoresis and differences in banding patterns are indicative of AFLPs.

AFLP technology has been successfully employed in a variety of research projects encompassing a multitude of organisms. One of the initial areas of interest was in mapping plant resistance genes. This included AFLP marker development for genes conferring resistance to plant pathogenic viruses (Jeong et al. 2002), fungi (Pauquet et al. 2001), bacteria (Tai et al. 1999), nematodes (Meksem et al. 2001), and insects (Murai et al. 2001). Furthermore, genetic variation in plant pathogens was also analyzed by AFLP, such as virulence gene mapping in *Cochliobolus sativus* (Zhong and Stafferson 2002), identifying distinctions within Mexican isolates of *Macrophomia phaseolina* (Mayek-Perez et al. 2001), and characterizing differences between pathogenic and non-pathogenic strains of *Xanthomonas axonopodis* pv. *manihotis* (Gonzalez et al. 2002). Another important area of interest was identifying AFLP markers associated with improving plant fitness and production, such as mapping drought tolerance (Dubos and Plomion 2003) and drought avoidance genes (Price et al. 2002) in maritime pine and upland rice, respectively. Also, the sugar beet genome has been heavily inundated with AFLP markers linked to sucrose production

(Schneider et al. 2002) and superior quality of individual varieties (Riek et al. 2001). Lastly, the AFLP technique has been utilized in several non-plant systems including: understanding the genetic relationship between different bovine species (Buntjer et al. 2002), finding genetic variations within selective breeds of cattle (Ajmone-Marsan et al. 2002), cloning and sequencing markers from chicken (Knorr et al. 2001), producing a linkage map of black tiger shrimp (Wilson et al. 2002), and evaluating inheritance in clonal rainbow trout (Brown and Thorgaard 2002). Taken together, AFLP technology provides a multifunctional and extensive method practical for a variety of applications and systems. Because of this versatility, it was employed in the search for genetic markers linked to the gene conferring victorin-sensitivity in oats.

We developed an F2 population segregating for victorin sensitivity that encompassed 325 individuals. Bulk segregating populations were made from DNA of victorin-sensitive and victorin-insensitive individuals and AFLP analysis was used to ascertain any genetic difference between the two populations. Over 51,000 markers produced by 512 different primer pairs were screened for polymorphisms. Two polymorphic markers were identified that were linked to the *Vb* locus. A 26.1 centimorgan genetic map was produced, with two markers flanking the *Vb* locus.

MATERIALS AND METHODS

Plant Material

Oat (*Avena sativa*) lines X469 (Vb/Vb) and Rodney (vb/vb) were crossed to produce an F1 line. Selfing of the F1 produced a segregating F2 population consisting of 325 individuals. F2 plants were grown in greenhouses under a 16-hour photoperiod.

Test for Victorin Sensitivity

Seven-day-old leaves (approximately 8 cm long) from Rodney and X469 were detached and assayed in 200 ng/ml victorin in water for 24 hours. The F2 seedlings were grown for 7 days and the top 4 cm were then removed and assayed in 200 ng/ml victorin in water for 24 hours.

DNA Preparation

After testing for victorin-sensitivity, F2 populations were grown for an additional 2 weeks. DNA was isolated from 0.5 grams of leaf tissue as per protocol communicated by Roger Wise at Iowa State University. Briefly, the tissue was ground in liquid nitrogen, and 1 ml 2X CTAB extraction buffer (100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% w/v CTAB, with 2% v/v 2-ME and 0.5% w/v sodium bisulfite) was added to each sample and incubated at 65°C for 60 min. The samples were cooled to 25°C and 1 ml chloroform:octanol (24:1) was added, mixed, and centrifuged for 20 min at 1500 x g. The upper layer was removed and mixed with 1 ml cold isopropanol. The DNA was removed with a glass hook and placed in 76% v/v EtOH, 0.2 M NaOAc for 5 min then placed in 76% v/v EtOH, 10 mM NH_4OAc for 5 min. The DNA was dabbed on a kimwipe to remove excess EtOH and dissolved in

400 µl TE buffer containing 40 µg/ml RNAse. DNA was quantitated spectrophotometrically.

Adapters and Primers

All oligonucleotides were produced by Ransom Hill Bioscience, based on the sequences provided by Vos et al. 1995 (Table 2-1). Oligonucleotides AD-M1 with AD-M2, AD-E1 with AD-E2, and AD-P1 with AD-P2 were used to produce the *MseI*, *EcoRI*, and *PstI*-specific double-stranded oligonucleotide adaptors, respectively. The double-stranded oligonucleotide adapters were made by incubating together the two single-stranded oligonucleotides at 65°C for 10 min, 37°C for 10 min, then 25°C for 10 min. Stock solutions for the *MseI* adaptor were at 50 µM, while the *EcoRI* and *PstI* adaptors were at 5 µM.

DNA Digestion and Ligation of Adapters

DNA digestion and ligation were performed, with a few modifications, as described by Vos et al. 1995. DNA (250 ng) was digested with 5 units (U) of the "rare-cutting" enzyme (either *EcoRI* or *PstI*) and 5 U of the "frequent-cutting" enzyme (*MseI*) in 5 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 25 mM K-acetate, 10 ng/µl BSA for 2 hours at 37°C. Adapter ligation was performed by adding to the digest reaction 50 *p*mol of the *MseI* adapter and 5 *p*mol of either the *EcoRI* or *PstI* adapter (depending on which restriction enzyme was used), 1 U T4 DNA-ligase, and 1 mM ATP. Reactions were incubated 2 hours at 20°C then diluted 1:10 in 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (TE buffer).

Table 2-1 **Primer Sequences used for AFLP Analysis**

A) Adapter-Ligation Primers

Primer Name	Sequence and Orientation of Adapters
AD E1	5′- CTCGTAGACTGCGTACC -3′
AD E2	3′- CATCTGACGCATGGTTAA -5′
AD M1	5′- GACGATGAGTCCTGAG -3′
AD M2	3′- TACTCAGGACTCAT -5′
AD P1:	5′- CTCGTAGACTGCGTACATGCA -3′
AD P2:	3′- CATCTGACGCATGT -5′

B) Pre-Amplification Primers

Primer Name	Sequence with selective nucleotide in bold
E01	5′- GACTGCGTACCAATTC**A** -3′
M02	5′- GATGAGTCCTGAGTAA**C** -3′
P01	5′- GACTGCGTACATGCAG**C** -3′

C) Selective Amplification Primers*

EcoRI Primer	Selective Nucleotides	*PstI* Primer	Selective Nucleotides	*MseI* Primer	Selective Nucleotides
E31	AAA	P30	CAA	M47	CAA
E32	AAC	P31	CAC	M48	CAC
E33	AAG	P32	CAG	M49	CAG
E34	AAT	P33	CAT	M50	CAT
E35	ACG	P34	CCA	M51	CCA
E36	ACC	P35	CCC	M52	CCC
E37	ACG	P36	CCG	M53	CCG
E38	ACT	P37	CCT	M54	CCT
E39	AGA	P38	CGA	M55	CGA
E40	AGC	P39	CGC	M56	CGC
E41	AGG	P40	CGG	M57	CGG
E42	AGT	P41	CGT	M58	CGT
E43	ATA	P42	CTA	M59	CTA
E44	ATC	P43	CTC	M60	CTC
E45	ATG	P44	CTG	M61	CTG
E46	ATT	P45	CTT	M62	CTT

*Only the three selective nucleotides are shown, remainder of primer sequence is identical to their respective pre-amplification primer.

Pre-amplification and Selective Amplification

The two amplification steps were performed as described by Vos et al. (1995) with minor modifications. The pre-amplification reaction consisted of 5 µl of diluted adapter ligation reaction, 75 ng *MseI* pre-amplification primer, 75 ng of either *EcoRI* or *PstI* pre-amplification primer, 0.2 mM each dNTP, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 1 U *Taq* DNA polymerase in a reaction volume of 50 µl. PCR was performed on a GeneMate thermocycler (Genius), with a total of 20 cycles of 94°C for 30 sec, 56°C for 60 sec, and 72°C for 60 sec, then diluted 1:50 in TE buffer. For the selective amplification step (PCR with primers containing three selective nucleotides), the selective oligonucleotide primer matching the adaptor for the "rare-cutting" enzyme (*EcoRI* or *PstI*) was labeled with ^{33}P. 500 ng of either the *EcoRI*- or *PstI*-selective oligonucleotide primer was added to 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, 1 mM 2-ME, 10 µl {$\gamma^{33}P$}ATP (2,000 Ci/mmol), and 20 U T4 polynucleotide kinase in a reaction volume of 50 µl and incubated at 37°C for 1 hour. This produced enough labeled primer for 100 selective PCR reactions. The selective amplification PCR reaction consisted of 5 ng of either the labeled *EcoRI* or *PstI* primer, 50 ng *MseI* primer, 5 µl diluted pre-amplification DNA, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each dNTP, and 1 U *Taq* DNA polymerase in a reaction volume of 20 µl. The first cycle of the PCR protocol was run under the parameters: denaturation at 94°C for 60 sec, annealing at 65°C for 60 sec, and extension at 72°C for 90 sec. The annealing temperature was

decreased 1°C for the next 10 cycle until it reached 56°C. The protocol concluded with 23 cycles of 94°C for 60 sec, 56°C for 60 sec, and 72°C for 90 sec.

Gel Analysis

PCR samples were mixed with 20 µl of formamide dye (98% v/v formamide, 10 mM EDTA, bromophenol blue, and xylene cyanol) and heated 3 min at 90°C. 3 µl of this mixture was then loaded on a 5% denaturing (28 x 40 x 0.19 mm) polyacrylamide gel (5% acrylamide, 0.25% bisacrylamide, 7.5 M urea in 50 mM Tris-HCl, 50 mM boric acid, 1 mM EDTA) and run on a GibcoBRL sequencing system model S2 (Life Technologies) at 55 W until the xylene cyanol band had migrated 2/3 the length of the gel (2-3 hours). The gel was transferred onto 0.19 mm-thick chromatography paper (Fisher Scientific), dried for 90 min at 80°C under vacuum, and placed on Kodak BioMax MS autoradiograph film (35 x 43 cm) with an intensifying screen (BioMax TranScreen-LE, Kodak) for 24-48 hours.

Genetic Map

Mapmaker/exp version 3.0 (Landers et al. 1987) was made available through the Center for Gene Research and Biotechnology at Oregon State University and used to generate a genetic map.

RESULTS

Mapping Population

The parental oat lines used to produce the mapping population were X469, homozygous dominant for *Vb* (*Vb/Vb*), and Rodney, homozygous recessive for *Vb* (*vb/vb*). Figure 2-1 illustrates typical victorin-induced symptoms for seven-day-old Rodney and X469 leaves exposed to 200 ng/ml victorin for 20 hours. The victorin-insensitive Rodney leaves look healthy, retaining their color and turger, while the victorin-sensitive X469 leaves undergo chlorosis and necrosis, and lose their turger. The two lines were crossed and the resulting F1 (heterozygous *Vb/vb*) was selfed to produce a segregating F2 population (Fig. 2-2). Of 325 F2 individuals scored for victorin sensitivity, 243 displayed a sensitive phenotype and 82 an insensitive phenotype, indicating a segregating ratio of 3:1. DNA was isolated from each F2 individual and a portion of each was pooled into two samples; one sample combining all the sensitive individuals and the other, insensitive individuals.

AFLP Analysis of the Bulk Segregating Populations

The AFLP technique is summarized in Figure 2-3. Genomic DNA from the pooled F2 populations was digested with *EcoRI* and *MseI* or *PstI* and *MseI* (Fig. 2-3A). *EcoRI* and *PstI* were used as the "rare cutter" (recognizing 6 nucleotides) while *MseI* was use as the "frequent cutter" (recognizing 4 nucleotides). The digested DNA fragments were ligated to double-stranded oligonucleotide adapters that complemented the "sticky ends" produced by the restriction enzymes (Fig. 2-3B). Table 2-1A contains the sequence of the two oligonucleotides that were annealed together to create the individual adapters.

Figure 2-1 **Victorin Sensitivity in Oats**

Six-day old leaves were incubated for 24 hour in 200 ng victorin. The victorin-insensitive line, Rodney, is on the left and the victorin-sensitive line, X469, is on the right.

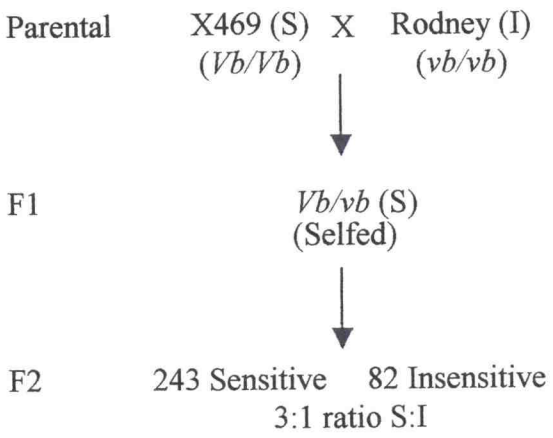
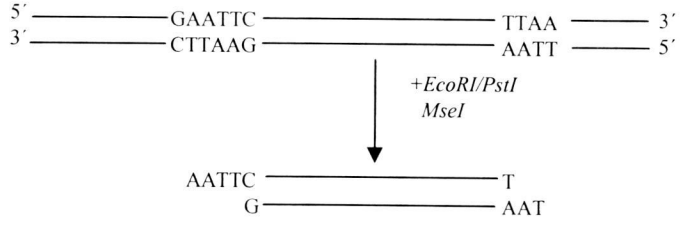


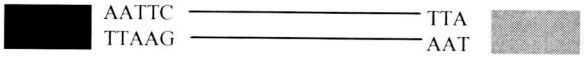
Figure 2-2 **Development of a Segregating F2 Population**

Parental lines X469 and Rodney, homozygous for toxin sensitivity (*Vb/Vb*; S) and insensitivity (*vb/vb*; I) were crossed to produce a heterozygous F1. The F1 was selfed, producing the segregating F2 population. Phenotypic analysis of the 325 F2 individuals gave a 3:1 segregation ratio for toxin sensitivity.

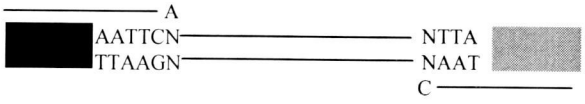
A) Purify DNA and digest with *EcoRI/PstI* and *MseI*. A "rare cutter" (*EcoRI/PstI*) and a "frequent cutter" (*MseI*) are used together.



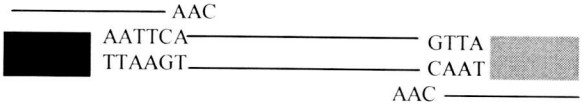
B) Ligate on adapters specific for the "sticky-ends" produced by the enzymes.



C) Perform the pre-amplification PCR. This reaction uses primers specific for the adapters that were ligated onto the fragments, including the restriction site and one selective nucleotide on the 3' end.



D) Perform the selective amplification PCR, each primer identical to the pre-amplification primer with the addition of two more selective nucleotides.



E) The selected subset of DNA fragments is amplified. They are visualized by labeling one of the primers with ^{33}P and running the products out on a denaturing polyacrylamide gel.

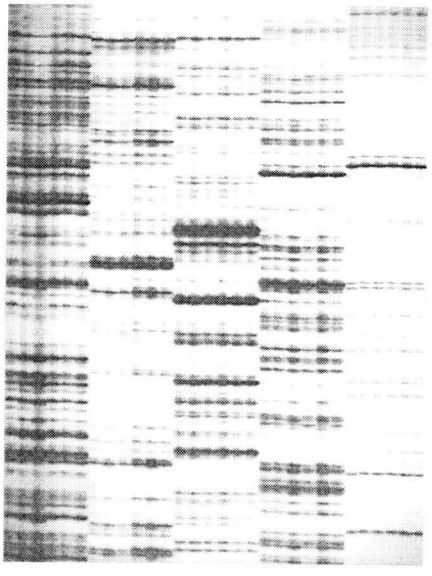


Figure 2-3 **Outline of Amplified Fragment Length Polymorphism Technique**

The pre-amplification reaction aids in reducing the complexity of the digested DNA by amplifying a subset of fragments (Fig. 2-3C), which was necessary when dealing with a genome as large as the allohexaploid oat. The pre-amplification primer (Table 2-1B) was designed with sequence homology to their respective adapter and restriction site, plus each one contains an additional selective nucleotide at the 3' end. Consequently, each pre-amplification primer anneals to 1/4 of the DNA fragments and because of a primer on each end, a total of 1/16 (1/4 x 1/4 = 1/16) of the fragments were amplified. The *EcoRI* pre-amplification primer contained adenine as the selective nucleotide, while cytosine was used in the *PstI* and *MseI* primers.

The primers used for the selective amplification reaction (Table 2-1C) were designed with two additional selective nucleotides, enough to reduce the complexity of the fragments generated so that distinct bands could be visualized by gel electrophoresis. All possible nucleotide permutations were used in designing the primers, thus 16 unique primers were produced, each set complementary to their respective double-stranded adapter. Because the *MseI* primers were used in conjunction with both the *EcoRI* and *PstI* primers (Fig. 2-3D), 512 different primer pair combinations were analyzed.

Approximately 100 markers were produced with each primer pair, ranging in size from 50-300 base pairs (data not shown). After analyzing over 51,000 markers created by the 512 different primer combinations, two polymorphic markers were identified (Fig. 2-4). Primer pairs E37/M48 and E38/M59 produced the polymorphic markers. The selective nucleotides evaluated were ACG for E37, CAC for M48, ACT

for E38 and CTA for M59 (Table 2-1C). *PstI* primers did not produce any polymorphic markers.

AFLP Marker Analysis of F2 Individuals

To identify whether or not the two polymorphic markers were linked to the *Vb* locus, victorin-sensitive and insensitive F2 individuals were scored for the presence or absence of the two markers. A total of 98 F2 individuals were scored; 67 were dominant for the *Vb* allele (victorin-sensitive) while 31 were recessive (victorin-insensitive). Of the 67 victorin-sensitive individuals, marker E37/M48 was present in 66 of them, while the second marker, E38/M59, was found in 60 individuals (9 of each are shown Fig. 2-5A). Marker E37/M48 was present in 12 of the 31 victorin-insensitive individuals while 2 of the 31 tested contained marker E38/M59 (9 of each are shown in Fig. 2-5B).

Development of a Genetic Map

The genetic analysis program Mapmaker 3.0 was used to produce a genetic map of the *Vb* locus and the two AFLP markers (Fig. 2-6). With the LOD score (base 10 logarithm of the likelihood ratio) set at a minimum of 3.0, the two AFLP markers were placed flanking the *Vb* locus. Marker E37/M48 was 16.3 centimorgans (cM) from *Vb* and marker E38/M59 was 9.5 cM away. The total map distance was 26.1 cM.

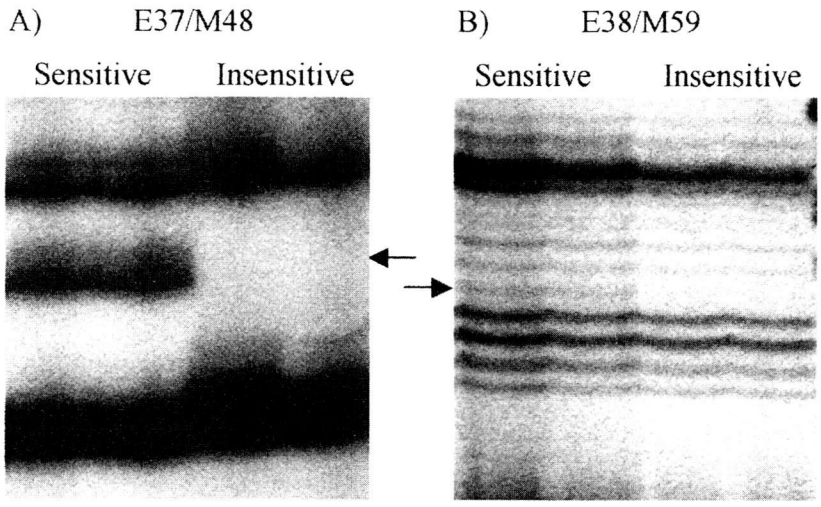


Figure 2-4 **Polymorphic AFLP Markers in Bulk Populations**

Duplicate samples of sensitive and insensitive bulk populations. Arrows indicate polymorphic markers. A) Marker E37/M48 from primer pair E37 and M48 with +3 selective nucleotides of +ACG and +CAC, respectively. B) Marker E38/M59 from primer pair E38 and M59 with +3 selective nucleotides of +ACT and +CTA, respectively.

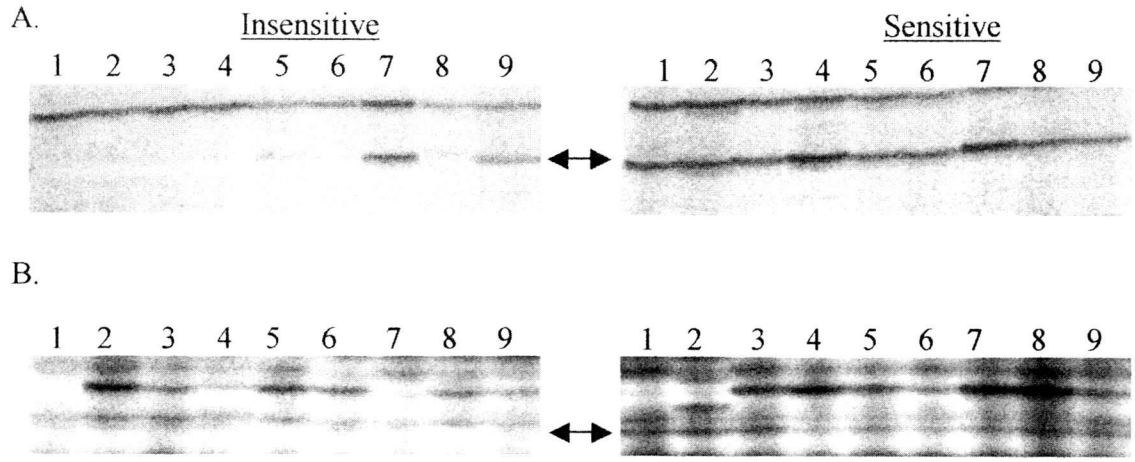


Figure 2-5 **Marker Analysis of Oat Individuals**

A total of 98 oat individuals (67 sensitive and 31 insensitive) were scored for the polymorphic markers E37/M48 and E38/M59. Nine sensitive and nine insensitive individuals are shown A) Marker E37/M48. B) Marker E38/M59.

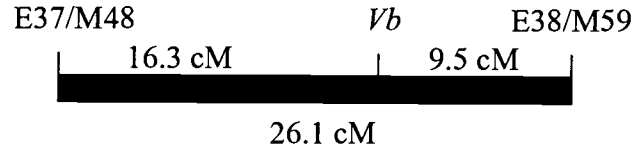


Figure 2-6 **Genetic Map of the *Vb* Locus**

Ninety-eight individuals were genotyped for markers E37/M48 and E38/M59. Of the 98, 67 individuals were toxin sensitive and 31 were toxin insensitive. Mapmaker version 3.0 was used for linkage analysis with a minimum LOD score of 3.0.

DISCUSSION

The AFLP technique was employed to screen an F2 population for polymorphic markers linked to the *Vb* locus. This technique was applied because it is easy to use, generates reproducible markers, is cost efficient, and utilizes PCR. Unlike RFLP, small amounts of DNA are needed per reaction, enabling multiple analyses from small tissue samples. AFLP is advantageous over other PCR-based methods because, unlike SSRs, it requires no prior sequence knowledge (Jones et al. 1997) and is more reproducible and has higher levels of polymorphisms than RAPDs (Sharma et al. 1996, Jones et al. 1997).

After surveying 512 primer pairs only two markers were discovered. Because the primers employed amplified only a small fraction of the total DNA fragments, identifying additional markers should be possible by using primers containing different selective nucleotides. For both the *EcoRI/MseI* and the *PstI/MseI* primer sets, only 1/16 of the possible markers were screened. Digestion of the DNA with different restriction endonucleases should also aid in identifying additional polymorphic markers. The "rare cutters" utilized in this study, *EcoRI* and *PstI*, are the enzymes most commonly used, however *HindIII* (Duim et al. 1999), *BglII* (Kokotovic et al. 2002), *ApaI* (Wong et al. 1999), and *XbaI* (Vos et al. 1995) have been reported as effective for AFLP analysis. Exchanging the "frequent cutter" *MseI* for *TaqI* would probably not be beneficial because most eukaryotic genomes are AT-rich and *MseI* cuts at AATT sites (Vos et al. 1995). *TaqI* has been successfully used for AFLP analysis in genomes that are GC-rich, like *Manihot esculenta* (Wong et al. 1999).

The size of the oat genome may also affect the number of markers identified. Cultivated oat is an allohexaploid (2n = 6x = 42; O'Donoughue et al. 1995) and contains 16,000 mega base pairs of DNA (www.ncbi.nlm.nih.gov). With such a large genome, multiple markers of the same size may be produce by the same primer pair, and could mask or hide a polymorphism. One way to decrease the chance of this occurring is to reduce the complexity of the banding pattern by screening with primers containing four selective nucleotides. The use of four selective nucleotides on one primer has been applied successfully to AFLP analysis in organisms with large genomes like *Pinus sylvestris* (Lerceteau and Szmidt 1999) and *Alstroemeria* species (Han et al. 1999). In organisms with a small genome, like *Arabidopsis thaliana*, typically only two selective nucleotides per primer are used (Peters et al. 2001).

Bulk segregant analysis of an F2 population permitted the identification of AFLP markers linked to the toxin sensitivity gene, *Vb*. We analyzed results from 512 AFLP primer pairs, and because approximately 100 markers are generated per primer pair, over 51,000 markers were evaluated for which only two were polymorphic between the sensitive and insensitive populations. Ninety-eight individuals, 67 sensitive and 31 insensitive, were scored for the presence of the two markers and the results were used to develop a genetic map of the *Vb* locus. Marker E38/M59 was placed 9.5 cM from *Vb*, while the other marker, E37/M48 was 16.3 cM away.

The overall objective of this project was to identify markers tightly linked to the *Vb* locus and produce a high resolution genetic map. However out of 51,000 markers, only two were identified as linked to *Vb* and neither were tightly linked. Expanding these analyses to encompass all selective nucleotides or employ different

restriction enzymes would require considerable labor and time, and yet it is not clear that such work would provide additional, informative data. The parental lines used to create the segregating F2 population were likely too genetically similar to produce sufficient polymorphisms. Creating a new segregating population with genetically diverse parents would likely provide the numbers of polymorphic markers needed for a high resolution genetic map of the *Vb* locus. Nevertheless, the two AFLP markers identified in this study will likely be useful for screening candidates for the *Vb* gene.

Victorin sensitivity has recently been identified in individuals within different ecotypes of *Arabidopsis thaliana* (Lorang et al. personal communication). The *AVb* (Arabidopsis-*Vb*) locus, like *Vb* in oats, conditions a dominant victorin-sensitive phenotype and segregates in the expected 3:1 ratio (sensitive: insensitive) when outcrossed to a victorin-insensitive individual. Compared to oats, genetic analysis in Arabidopsis has several advantages: Arabidopsis possesses a small genome (125 Mb) that has been completely sequenced; extensive genetic and physical maps have been developed for all 5 chromosomes; it has a rapid life cycle (~ 6 weeks to maturity) and produces abundant seed while the size of a mature plant remains relatively small; it is readily transformable by *Agrobacterium tumefaciens* ; there are numerous, available, mutant lines and genomic stocks; and there is a large, multinational research community working with Arabidopsis (www.arabidopsis.org/info/aboutarabidopsis.html). Currently, map-based cloning procedures are facilitating the localization of *AVb* on the Arabidopsis genetic map. It is anticipated that the identification of the *AVb* gene will enable us to locate a similar or homologous gene in oats. The AFLP markers linked to the *Vb* locus that were

identified in this study will enable us to screen any putative *Vb* genes identified in oats.

Chapter 3

Characterization and Purification of a Caspase-Like Serine Protease Involved in the Programmed Cell Death Response in Oats

Warren C. Coffeen and Thomas J. Wolpert

ABSTRACT

Victoria blight of oats is caused by the fungus, *Cochliobolus victoriae*, which is pathogenic due to the production of the toxin, victorin. The victorin-induced response in sensitive oats has been characterized as a form of programmed cell death (PCD) and displays morphological and biochemical features similar to apoptosis including, chromatin condensation, DNA laddering, cell shrinkage, altered mitochondrial function, and ordered, substrate-specific proteolytic events. Victorin-induced proteolysis of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) is shown to be prevented by caspase-specific and general protease inhibitors. Evidence is presented for a signal cascade leading to rubisco proteolysis that involves multiple proteases. Furthermore, a protease that is apparently involved in the rubisco proteolytic cascade was purified and characterized. This protease exhibits caspase specificity but shows amino acid sequence homologous to subtilisin-like serine proteases found in plant. The protease is constitutively present in an active form and is re-localized to the extracellular fluid after induction of PCD by either victorin or heat shock. The role of the enzyme as a processive protease involved in a signal cascade during the PCD response is discussed.

INTRODUCTION

Victoria blight of oats (*Avena sativa*) is caused by the necrotrophic fungus *Cochliobolus victoriae* (Meehan and Murphy 1946) which is pathogenic due to the production of the host-specific toxin, victorin (Meehan and Murphy 1947). Isolates of *C. victoriae* that produce victorin are pathogenic on susceptible oats (Wheeler and

Luke 1954), while mutants or outcrosses that do not produce the toxin are non-pathogenic. Host susceptibility and victorin sensitivity are conferred by a dominant allele at the *Vb* locus (Litzenberger 1949). Homozygous recessive genotypes (*vb/vb*) are victorin-insensitive and resistant to the fungus. Victoria blight first appeared in the USA during the early 1940s following the introduction of oats carrying Victoria-type resistance to crown rust caused by the biotrophic fungus, *Puccinia coronata* (Litzenberger 1949). Crown rust resistance conferred by Victoria type oats is due to the *Pc-2* gene. Extensive attempts to genetically separate the *Vb* gene from the *Pc-2* gene including mutagenic approaches, screening naturally occurring mutants, and analyzing somatoclonal variants generated under toxin selection have failed to separate rust resistance from toxin-sensitivity (Luke et al. 1960, Luke et al. 1966, Rines and Luke 1985). These results suggest that the gene conditioning susceptibility to a necrotrophic fungus, *C. victoriae*, is either very tightly linked to, or is the same gene that confers resistance to the biotrophic fungus, *P. coronata*.

Programmed cell death (PCD) is a genetically-controlled, organized form of cellular suicide that functions in eliminating unnecessary or aged cells. It is essential for cellular maturation and morphogenesis and required to maintain cellular homeostasis in multicellular organisms. In addition, improper regulation of PCD has been implicated in a wide variety of animal diseases (Polverini and Nör 1999, Wang and Wang 1999).

PCD has also been associated with several processes in plants including senescence (Bleecker and Patterson 1997, Miller et al. 1999, Schmid et al. 2001), stress (Katsuhara 1997, Solomon et al. 1999), development (Runeberg-Roos and

Saarma 1998, Groover and Jones 1999, Schmid et al. 1999), and the hypersensitive response (HR) to pathogens (Dangle et al. 1996, Mittler et al. 1997, Pontier et al. 1998, Mackey et al. 2002, Abramovitch et al. 2003). Currently, very little is known about the fundamental machinery that controls and regulates PCD in plants.

Apoptosis, the most characterized form of PCD, has been extensively studied in animal systems and can be distinguished by unique characteristics. Cells undergoing apoptosis display morphological changes including cell shrinkage, chromatin condensation, and apoptotic body formation. Biochemically, apoptotic cells exhibit DNA fragmentation (also referred to as DNA laddering) and activation of a family of cysteine proteases called caspases (cysteine aspartases; reviewed by Vaux and Korsmeyer 1999, Hengartner 2000).

In sensitive oats, victorin induces a response characterized as a form of programmed cell death (Navarre and Wolpert 1999, Tada et al. 2001, Yao et al. 2001, Curtis and Wolpert 2002). This response demonstrates similar morphological and biochemical traits to animal apoptosis including cell shrinkage and collapse (Yao et al. 2001), chromatin condensation (Yao et al. 2001), DNA laddering (Navarre and Wolpert 1999, Tada et al. 2001), mitochondrial depolarization and permeability transition (Curtis and Wolpert 2002), and ordered, substrate-specific proteolytic events (Navarre and Wolpert 1999). Furthermore, victorin-induced PCD in oats is easily initiated, proceeds in a rapid and synchronous manner, and appears to encompass at least all leaf mesophyll cells. Therefore, victorin treatment of oats provides an appropriate system in which to study the mechanism and progression of plant PCD.

The proteolytic cleavage of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) has been identified as a specific PCD-induced event in victorin treated oat cells (Navarre and Wolpert 1999). Rubisco cleavage occurs following the first 14 amino acids (apparently following glutamate-14) and is prevented by cysteine inhibitors (Navarre and Wolpert 1999). Rubisco degradation has been identified as a characteristic of senescence (Ferreira and Davies 1989, Weidhase et al. 1987), a form of PCD, and has been shown to occur in oat chloroplast following oxidative stress (Casano and Trippi 1992), a treatment shown to activate PCD in other systems (Amor et al. 1998, Solomon et al. 1999). Additionally, chloroplast-localized proteases have been reported that appear to recognize rubisco as a substrate (Bushnell et al. 1993, Casano et al. 1994). These data indicate that a specific proteolytic process is required to degrade rubisco, and that this process is common in several types of PCD.

Proteolytic alteration of key cellular proteins is a fundamental characteristic of animal apoptosis, and is executed by a family of enzymes called "caspases". Multiple cellular targets exist for caspases, all of which are directly or indirectly involved in the ordered disassembly of the cell. Two types of caspases exist, initiator and effector caspases. Initiator caspases are activated by autoproteolysis, after which they are able to proteolytically activate effector caspases. Effector caspases target cellular proteins, such as poly(ADP-ribose) polymerase (PARP), which is proteolytically inactivated and forms a signature 89 kDa fragment (reviewed by Solary et al. 1997). In addition, CAD, a caspase-activated DNase, is indirectly activated when an effector caspase cleaves ICAD (inhibitor of CAD), allowing CAD to disassociate and process DNA

into nucleosomal fragments (reviewed by Nagata 2000). Caspase proteolysis typifies the ordered, processive nature of apoptosis; and differs from the degradative role of many other proteolytic pathways (e.g. the proteasome-ubiquitin pathway of protein degradation; Solary et al. 1997), because caspases function with exquisite control, cleaving at limited site(s) found within specifically targeted, key proteins.

Recent research revealing that caspase-specific inhibitors prevent different forms of PCD induced by biotic or abiotic treatments suggests that plant PCD systems are similar to animal PCD systems, and utilize caspase-like enzymes (reviewed by Woltering et al. 2002). The biotically-induced HR in tobacco mosaic virus-infected tobacco leaves was prevented by treatment with the caspase-1 and -3-specific inhibitors, Ac-YVAD-CMK and Ac-DEVD-CHO (del Pozo and Lam 1998). Other forms of PCD in tobacco, including those induced by bacteria (Richael et al. 2001) and treatment with a fungal elicitor (Elbaz et al. 2002) were prevented by caspase inhibitors. Similar results were obtained with abiotically-induced PCD in which caspase inhibitors prevented or reduced cell death after treatment with camptothecin (De Jong et al. 2000), staurosporine (Elbaz et al. 2002), and isopentenyladenosine (Mlejnek and Procházka 2002). In addition, heat shock-induced PCD in tobacco suspension cells stimulated cleavage of PARP and activation of a caspase-3-like protease (Tian et al. 2000). Also, tobacco protoplast treated with menadione displayed PARP cleavage (Sun et al. 1999). However, to date, no protease that recognizes caspase substrates or is inhibited by caspase inhibitors has been identified in plants.

Studies have described several types of proteases associated with plant PCD such as in senescence (Delorme et al. 2000, Schmid et al. 2001, Eason et al. 2002),

oxidative stress (Solomon et al. 1999), seed development (Schmid et al. 1998, Schmid et al. 1999, Wan et al. 2002), tracheary element development (Runeberg-Roos and Saarma 1998, Groover and Jones 1999), and the HR (Vera and Conejero 1988, D'Silva et al. 1998, Krüger et al. 2002). However, further characterization of these proteases reveal they are degradative enzymes, not processive enzymes, like the caspases or other proteases involved in signaling pathways. Their degradative nature suggests that their role in PCD may be associated with the terminal decomposition of the dying cell.

This research describes the purification and characterization of a serine aspartate-specific protease possessing caspase specificity which we have termed a saspase. The saspase, purified from oats, contains amino acid similarities to other subtilisin-like serine proteases from plants. The oat saspase likely functions in a cascade that includes multiple proteases and leads to the proteolytic processing of rubisco. It is also constitutively present in the cell, not being transcriptionally or translationally activated during the response, but released into the extracellular fluid (ECF) upon induction of PCD. Heat shock-induced PCD is also further characterized and displays similar biochemical features as victorin-induced PCD, including DNA laddering and rubisco proteolysis. These results and the implications of numerous subtilisin-like serine proteases involved in plant disease, development, and death are discussed.

MARERIALS AND METHODS

Experimental Materials

Oat (*Avena sativa*) seedlings of the line X469 (victorin-sensitive) and X424 (victorin-insensitive) were grown in a growth chamber for 6-7 days under a 16-hour photoperiod at 24°C. All protease substrates and caspase inhibitors were purchased from Calbiochem (La Jolla, CA). The remaining chemicals and protease inhibitors were purchased from Sigma (St. Louis, MO).

Treatment of Oat Tissue

Oat leaves were treated with victorin by one of two methods. Oat leaves, with their epidermis removed, were either floated on 20 mM MOPS pH 6.5 with 20 ng/ml victorin or intact oat leaves were infiltrated with 1000 ng/ml victorin in water and then cut into 4 cm leaf segments. Epidermal-peeled leaves of oats were treated with victorin for 4 h while the infiltrated leaf segments were treated for 15 h.

For heat shock treatments, 4 cm water-infiltrated leaf segments were incubated for 60 min at 45°C. Leaves were water-infiltrated to facilitate collection of ECF. After heat shock, leaves were incubated at 25°C for the indicated time before ECF, total protein or DNA were collected.

Total protein and DNA were extracted from leaf samples by homogenizing 1 cm of leaf tissue in 200 µl phenol combined with 200 µl TES buffer (50 mM Tris-HCl pH 7.6, 5 mM EDTA, 2% SDS). Following homogenization, the phases were thoroughly mixed and separated by centrifugation for 10 min. The protein (phenol) phase was removed and the proteins were precipitated by mixing with 5x vol. 0.1 M ammonium acetate and 2.5% ß-mercaptoethanol in cold methanol with 2.5% and

incubating the mixture a minimum of 4 hours at 4°C. The DNA (aqueous) phase was precipitated by mixing with 2.5x vol. cold ethanol and 0.1x vol. 3 M NaAcetate and incubating overnight at -20°C.

ECF was collected by cutting the 4 cm leaf segments into two, 2 cm segments and placing them vertically in a 1.5 ml microcentrifuge tube with a pinhole in the bottom. The tubes were placed inside a collection tube and centrifuged for 10 min at 1000 x g in a swinging bucket rotor. A total of 30-40 µl of ECF was typically collected from six, 4 cm leaf segments.

Protease Activity Assays

Substrate hydrolysis was typically measured by adding 10 µl of a sample to 90 µl of 20 mM MOPS pH 6.5, 0.5 M NaCl supplemented with 20 µM of the indicated substrate for a final volume of 100 µl. Samples were incubated at 25°C for 60 minute in 96-well microtiter plates. Fluorescence produced by hydrolysis of the substrates was read with a SpectroMax Gemini fluorometer (Molecular Devices; Sunnyvale, CA).

The optimal pH for oat saspase hydrolytic activity against the substrate, Z-VAD-AFC was measured over the pH range of 5.5-7.5. A 20 mM MES buffer containing 0.5 M NaCl was used for the assays at pH 5.5, 6.0, and 6.5 and 20 mM MOPS, 0.5 M NaCl was used for assays at pH 6.5, 7.0, and 7.5.

Five salts, NaCl, KCl, $MgCl_2$, $CaCl_2$, and $ZnCl_2$ were tested at 10 mM and 100 mM in 20 mM MOPS pH 6.5 for their affect on activity of the oat saspase.

Reversible and Irreversible Binding Experiment

Partially purified proteolytic activity that hydrolyzed the caspase substrate, Z-VAD-AFC, was assayed for inhibition with three inhibitors: Z-VAD-CHO, Z-VAD-FMK, and Ac-VAD-CMK. The inhibitors (200 μM) were each added to 200 μl of sample equilibrated in 20 mM MOPS pH 6.5, 1 mM DTT, and 100 mM NaCl, then incubated for 2 hours. Ten-microliter aliquots from each treatment were assayed for VAD-AFC hydrolysis (see below). Samples were washed with 1 ml 20 mM MOPS pH 6.5, 1 mM DTT, and 100 mM NaCl and concentrated back to original volume with a 2 ml Centricon-30 (30,000 MWCO) (Amicon; Beverly, MA). Ten-microliter aliquots were assayed after the first wash, then washed and assayed a second time with the same technique.

Purification of the Oat Saspase

SOLUBLE PROTEIN PREPARATION AND AMMONIUM SULFATE PRECIPITATION

Oat leaves (200 grams) were sliced into 1-2 mm cross-sectional pieces and incubated 4 hours in water with 100 ng/ml victorin. The slices were then homogenized with a mortar and pestle in cold 20 mM MOPS pH 7.0 with 1 mM DTT. The homogenate was filtered through 4 layers of cheesecloth and centrifuged for 10 min at 10,000 x g. The resultant supernatant was removed and centrifuged for 1 h at 100,000 x g. The soluble protein solution was mixed with 50% (w/v) ammonium

sulfate for 30 min at 4°C, centrifuged for 30 min at 10,000 x g, and the pellet was solublized in 20 mM MOPS pH 7, containing 1 mM DTT, and 1 M ammonium sulfate. The solublized pellet was mixed for 30 min at 4°C, centrifuged for 10 min at 10,000 x g to remove insoluble material, and filtered through a 0.22 micron filter.

HYDROPHOBIC INTERACTIONS CHROMATOGRAPHY

Prior to the establishment of a purification protocol, initial characterization of protease activity was performed on soluble protein extracted from 60 grams oat tissue (prepared as above but without ammonium sulfate precipitation), following separation and concentration by hydrophobic interactions chromatography (HIC). HIC was performed in a 15 mm x 40 cm column packed with Phenol Sepharose High Performance resin (Amersham Bioscience; Piscataway, NJ) equilibrated in 20 mM MOPS pH 7, 1 mM DTT, and 1 M ammonium sulfate. The sample was adjusted to contain 20 mM MOPS pH 7, 1 mM DTT, and 1 M ammonium sulfate, and loaded directly onto the column. The column was then washed in the same buffer and eluted with a 200 min, linear gradient of 1.0-0 M ammonium sulfate. For initial characterization of protease activity, all fractions were assayed with the substrates Z-VAD-AFC and Z-DEVD-AFC. Two proteolytic activities were identified; activity A eluting at ~0.3 M ammonium sulfate and activity B eluting at ~0.45 M. For the purification protocol, fractions were assayed for proteolytic activity only with Z-VAD-AFC.

HEPARIN AFFINITY CHROMATOGRAPHY

Active fractions from the HIC column were pooled and the buffer was exchanged with 20 mM MOPS pH 7, 1 mM DTT, and 50 mM NaCl by filtering through an Amicon Ultra-15, 30,000 MWCO centrifugal filter device (Millipore; Bedford, MA). Following buffer exchange, the sample was loaded onto a 5 ml Heparin HP affinity column (Amersham Bioscience), washed with 20 mM MOPS pH 7, 1 mM DTT, and 50 mM NaCl, and eluted in 20 mM MOPS pH 7, 1 mM DTT, and 2 M NaCl.

ANION EXCHANGE CHROMATOGRAPHY

Active fractions from the heparin column were prepared for anion exchange chromatography by replacing the buffer with 50 mM Tris-HCl pH 8.5, 2 mM EDTA, and 1 mM DTT (by concentration and washing in an Amicon Ultra-15). The resulting sample was loaded onto a Mono Q FPLC 5/5 column (Amersham Bioscience) and eluted with a 200 min linear gradient of 0-0.3 M NaCl.

SIZE EXCLUSION CHROMATOGRAPHY

The active fractions from the anion exchange column were concentrated with an Amicon Ultra-15 into 8 mls with the buffer consisting of 20 mM MOPS pH 6.5, 1 mM DTT, and 150 mM NaCl. Two, 4 ml samples were loaded onto a Sephacryl S-200 (16/60) column (Amersham Bioscience) for size exclusion chromatography. Fractions containing proteolytic activity (Z-VAD-AFC) were tested for purity by silver staining of an SDS-PAGE.

SDS-PAGE and Protein Blotting

SDS-PAGE was based on the system developed by Laemmli (Laemmli 1970). All oat saspase samples were analyzed on an 8% gel. Silver staining was performed following the protocol developed by Blum et al. (1987). Electroblotting was done in a Trans-Blot SD semi-dry transfer cell (Bio-Rad; Hercules, CA) with transfer buffer consisting of 25 mM Tris, 192 mM Glycine, and 0.02% SDS in 20% methanol. Proteins were blotted onto a Protran nitrocellulose membrane with a 0.45 μm pore size (Schleicher and Schuell; Keene, NH).

Membranes were blocked with 1% BSA in TBST (100 mM Tris-HCl pH 7.6, 0.9% NaCl, 0.1% Tween 20) for 20 min. For western blots, the oat saspase polyclonal antibody was added at a 1:2000 dilution in TBST for 1 hour, washed 3x for 15 min each in 1x TBST, then 2° antibody (goat anti-rabbit conjugated to horseradish peroxidase); (Santa Cruz Biotechnology; Santa Cruz, CA) was added at a 1:100,000 dilution for 1 hour. Blots were washed 4x for 15 min each in TBST before development with SuperSignal West Pico chemiluminescent substrate (Pierce; Rockford, IL). Biotin blots were incubated with NeutrAvidin conjugated to horseradish peroxidase (Pierce) at a 1:50,000 dilution for 1 hour, washed 4x for 15 min each, and developed the same as western blots.

Amino Acid Sequencing

Oat saspase-1 and oat saspase-2 (OS-1 and OS-2; see results) were run on SDS-PAGE, blotted onto a PVDF membrane, and stained with 0.1% Coomassie Blue R-250 in 1% acetic acid and 40% methanol. The 84 kDa bands were excised and

destained in 50% methanol. NH_3-terminal sequencing was conducted by Edmonds degradation at The Institute of Molecular Biology, Eugene, OR. The three internal peptides from OS-2 were sequenced by Edmonds degradation at the Stanford PAN Facility (Palo Alto, CA) after in-gel tryptic digest, HPLC, and MALDI-TOF MS analysis.

Polyclonal Antibody Production

Polyclonal antibodies against OS-1 and OS-2 were produced in rabbits by subcutaneous injection at the Laboratory of Animal Research, Oregon State University. Gel-purified OS-1 and OS-2 (~1 μg) were mixed with the adjuvant, TiterMax Gold (Sigma) prior to injection. Booster injections (~1 ug of OS-1 and OS-2 mixed in TiterMax Gold) were administered at week 4 and 8, and final serums were collected at week 11.

RESULTS

Evidence for a Protease Cascade leading to Rubisco Proteolysis

Rubisco proteolysis is a characteristic of victorin-induced programmed cell death in oats (Navarre and Wolpert 1999). Three caspase inhibitors, VAD-CMK, AAD-CMK, and DEVD-CMK, and two general cysteine protease inhibitors, leupeptin and E-64, prevented *in vivo* rubisco proteolysis induced by victorin (Fig. 3-1). The inhibition of rubisco proteolysis by caspase-specific inhibitors suggested that a caspase-like protease(s) might be present in victorin-treated oat tissue. Total soluble

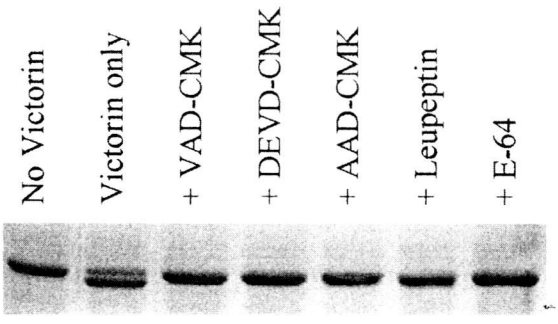


Figure 3-1 **Inhibition of Rubisco Proteolysis with Protease Inhibitors**.

Leaf segments with epidermis removed were pretreated with 200μM of the indicated inhibitor for 2 hrs then with 20ng/ml victorin for 4 hrs. Coomassie blue-stained 12% SDS-PAGE of total protein extract. Only the region containing rubisco is shown.

protein was extracted from 60 grams of victorin-treated oat leaves and a low level of proteolytic activity that hydrolyzed the general caspase substrate Z-VAD-AFC was observed. To concentrate the activity, the soluble protein extract was loaded onto a HIC column. Two distinct proteolytic activities were separated during chromatography: activity A (fractions 76-100) which hydrolyzed Z-VAD-AFC and eluted at ~0.3 M ammonium sulfate; and activity B (fractions 56-67) which hydrolyzed Z-DEVD-AFC and eluted at ~ 0.45 M ammonium sulfate (Fig. 3-2A). The fractions containing each activity were individually combined for further analysis. Activity A hydrolyzed the substrate Z-VAD-AFC and Z-AAD-AFC, but not Z-DEVD-AFC (Fig. 3-2B), while activity B only hydrolyzed Z-DEVD-AFC (Fig. 3-2B). Figure 3-2D illustrates percent inhibition of the two activities by the same protease inhibitors that prevented rubisco proteolysis (Fig. 3-1). Z-VAD-AFC hydrolysis by activity A was completely inhibited by the caspase inhibitors Z-VAD-CMK and Z-AAD-CMK, but was unaffected by Z-DEVD-CMK, leupeptin, and E-64 (Fig. 3-2D; open bars). However, activity B hydrolysis of Z-DEVD-AFC was only inhibited by Z-DEVD-CMK (Fig. 3-2D; striped bars). These data suggest that there are two distinct caspase-like proteases involved in the proteolytic processing of rubisco, because all three caspase-specific inhibitors prevent rubisco proteolysis while two specifically inhibit only activity A and the other specifically inhibits only activity B. Furthermore, because neither caspase-like activity is inhibited by leupeptin or E-64, both of which prevent rubisco proteolysis, a third, non-caspase-like protease is also likely involved. Thus, a proteolytic cascade involving at least three proteases, two

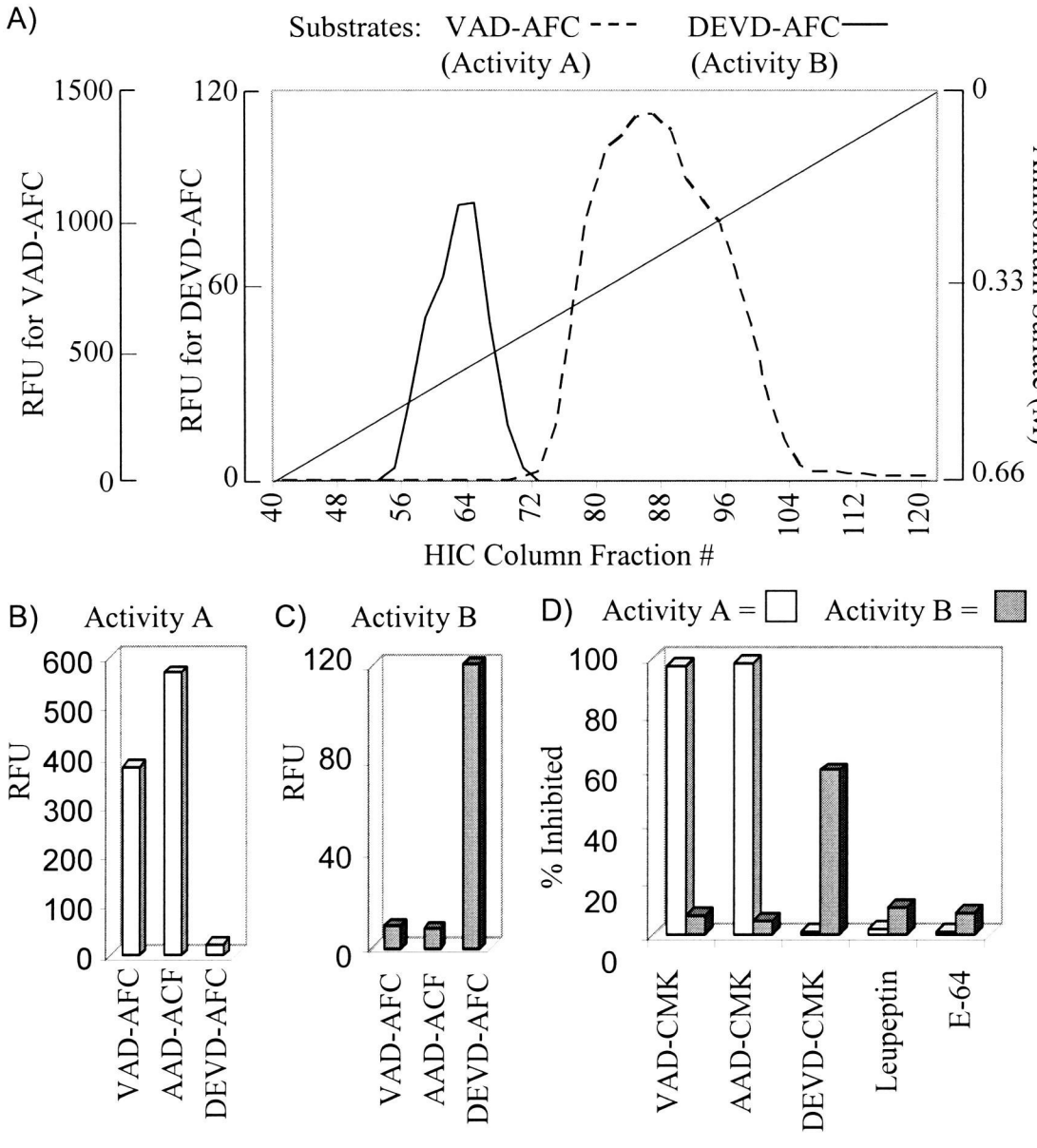


Figure 3-2 **Characterization of Two Caspase-Like Proteolytic Activities.**

A) Activity profile of fractions from a HIC column. Activity A hydrolyzed VAD-AFC and activity B hydrolyzed DEVD-AFC. The protease in activity B was assayed by incubating 50 µl of active fraction with 50 µl 20 mM MOPS (pH 7) with 20 µM substrate for 2 hrs. Activity A assayed as described in materials and methods. B) Activity A assayed for proteolytic activity with indicated substrates. C) Activity B assayed with same substrates as in (B). Assay conditions same as in (A). D) Inhibitor profile of activity A and B. Samples were pretreated with inhibitor (200 µM) for 2 hrs, then assayed for activity with VAD-AFC (activity A) or with DEVD-AFC (activity B). RFU = Relative Fluorescence Units.

caspase-related and one general protease, is activated during victorin-induced PCD and leads to the proteolytic processing of rubisco.

Reversible and Irreversible Inhibition by Caspase Inhibitors

The protease(s) constituting activity A from the HIC column, which hydrolyzed Z-VAD-AFC and Z-AAD-AFC, displayed the highest levels of activity; therefore this activity was selected for further characterization. Initial attempts to visualize the protease(s) with the biotinylated caspase inhibitor, biotin-VAD-FMK, on a biotin blot were unsuccessful. Biotin-VAD-FMK prevented Z-VAD-AFC hydrolysis, but did not covalently label the protein. Three different types of inhibitors with the same tri-peptide recognition motif, Z-VAD-CMK, Z-VAD-FMK, and Ac-VAD-CHO, were tested for the nature of their binding to the protease (Fig. 3-3A). CMK (chloromethylketone)-based inhibitors irreversibly inhibit both cysteine and serine proteases while FMK (fluoromethylketone)-based inhibitors irreversibly inhibit cysteine proteases but reversibly inhibit serine proteases. CHO (aldehyde)-based inhibitors reversibly inhibit both cysteine and serine proteases. The data in Figure 3-3A suggest the protease with caspase-like hydrolytic activity is a serine protease, not a cysteine protease, because only Z-VAD-CMK irreversibly inhibited the protein. The indication that the protease irreversibly inhibited by Z-VAD-CMK is a serine protease was later confirmed by amino acid sequencing (discussed below). Because the protease(s) with caspase-like hydrolytic activity is a serine protease, we referred to this enzyme as a saspase.

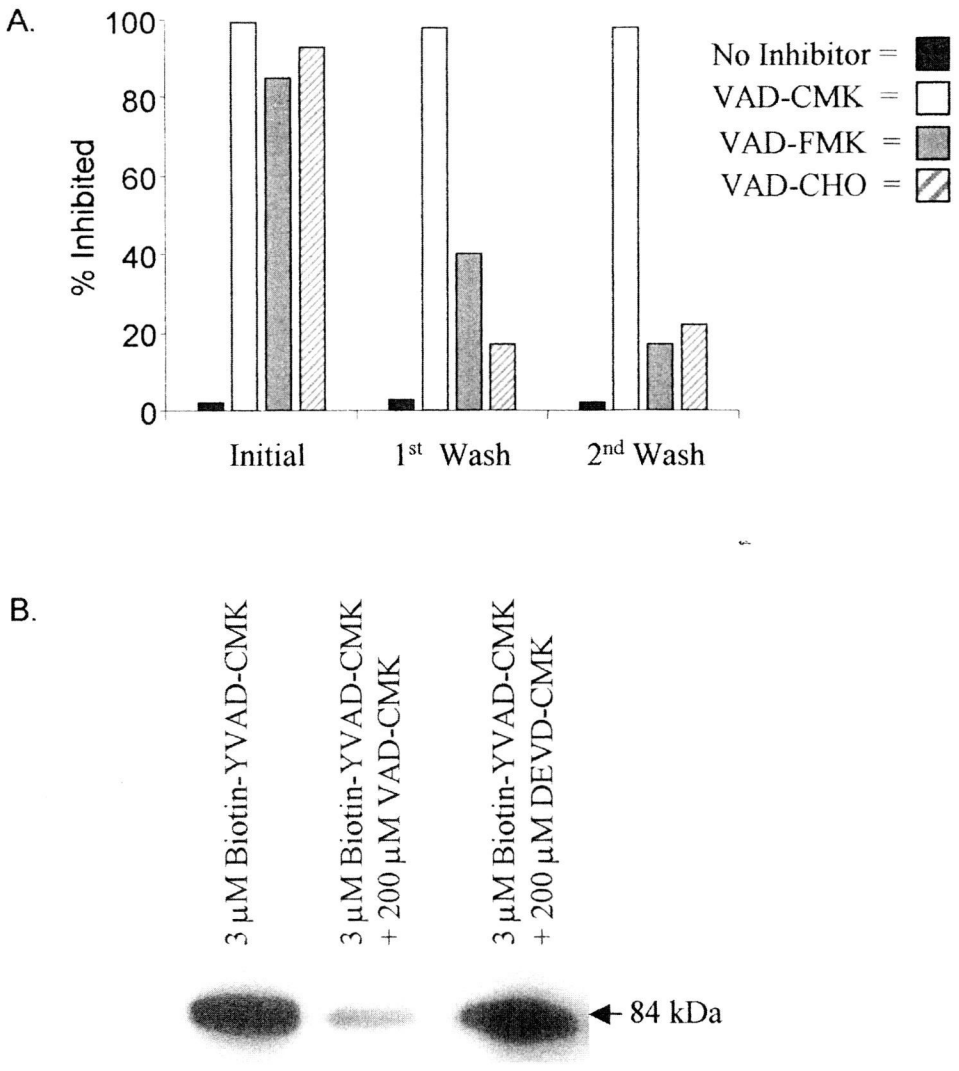


Figure 3-3. **The Caspase-like Protease is an 84 kDa Serine Protease**.

A) Irreversible inhibition of the protease was achieved only with the CMK-based inhibitor. B) The caspase-like protease binds specifically to Biotin-YVAD-CMK, while binding is out-competed by Z-VAD-CMK but not by Z-DEVD-CMK.

The biotinylated inhibitor, biotin-YVAD-CMK, irreversibly binds and inhibits the activity of the saspase. Biotinylated, inhibitor-bound saspase was visualized on a biotin blot, and migrated with an apparent mass of 84 kDa in SDS-PAGE (Fig. 3-3B). Binding of biotin-YVAD-CMK appears to be ligand-specific because pre-addition of Z-VAD-CMK competed with binding of biotin-YVAD-CMK (Fig. 3-3B; lane 2), whereas pre-addition of Z-DEVD-CMK (which does not inhibit activity) had no affect on binding (Fig. 3-3B; lane 3).

Extracellular Localization of the Oat Saspase after Victorin Treatment

Treatment of leaves with victorin caused release of the saspase into the ECF. ECF was collected from victorin-infiltrated leaves at 0-6 hours following infiltration, and the saspase activity was measured by Z-VAD-AFC hydrolysis (Fig. 3-4A). Protease activity was first detected at 1 hour, was maximal at 5 hours, and started to decrease at 6 hours. Portions of the same ECF samples were incubated with 200 μM biotin-YVAD-CMK for two hours, separated by SDS-PAGE, and blotted for biotin visualization. The appearance of the 84 kDa oat saspase band in the ECF (Fig. 3-4B) coincided with hydrolytic activity.

Infiltration of leaves with 400 μM biotin-YVAD-CMK provided *in vivo* labeling of the saspase. In Figure 3-4C, ECF was collected from leaves that were pre-infiltrated with biotin-YVAD-CMK for two hours, then infiltrated with 1000 ng/ml victorin mixed with 100 μM biotin-YVAD-CMK and incubated under the same conditions and for the same times as in Figure 3-4A and B. Accumulation of the saspase in the ECF followed approximately the same pattern as shown in Figure 3-4B,

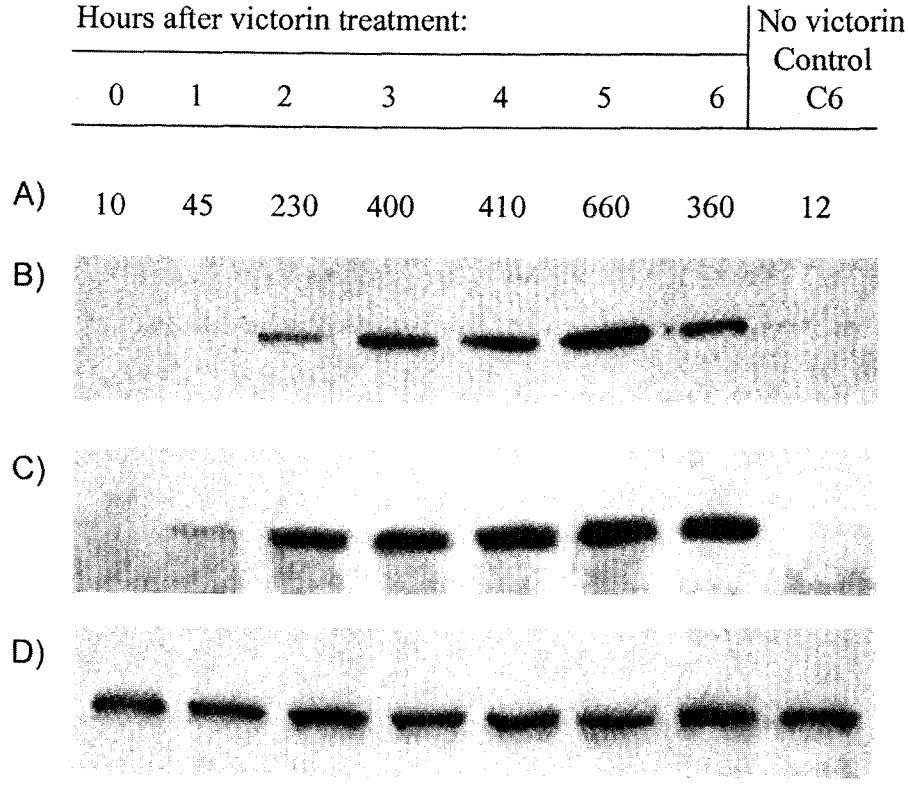


Figure 3-4 **The Saspase is Released into the Extracellular Fluid after Victorin Treatment.**

Leaf infiltration assay. A) Z-VAD-AFC hydrolytic activity, in relative fluorescence units, of the oat saspase in ECF collected from oat leaves 0-6 hour after treatment with 1000 ng/ml victorin. B) Biotin blot of same ECF collected in (A). Samples were labeled with 200 μM biotin-YVAD-CMK for 2 hours prior to electrophoresis. C) and D) Leaves were pre-infiltrated with 400 μM biotin-YVAD-CMK, incubated 2 hour, then infiltrated with 1000 ng/ml victorin mixed with 100 μM biotin-YVAD-CMK. C) Biotin blot of the ECF. D) Biotin blot of total protein extract from same experiment as in (C), note that the saspase is uniformly labeled in all samples including the water only/no victorin control (last lane).

although activity could not be measured because of inhibition by biotin-YVAD-CMK (Fig. 3-4C). Transcriptional or translational activation apparently was not required; results indicate (Fig. 3-4D) a constant level of saspase labeling in total cellular protein throughout all time points, including the 6 hour no-victorin control (Fig. 3-4D; last lane). Treatment of leaves with cyclohexamide prior to victorin treatment also had no effect on the accumulation of the oat saspase in the ECF (data not shown).

Heat Shock-Induced PCD is Similar to Victorin-Induced PCD

Victorin-sensitive and insensitive leaves were treated with water (Fig. 3-5; lanes 1 and 4), 1000 ng/ml victorin (Fig. 3-5; lanes 2 and 5), or heat shock (45° C for 1 hr) (Fig. 3-5; lanes 3 and 6). The ECF was collected 4 hours after start of the treatment and protein and DNA samples were collected after 15 hours. Heat shock induces the typical characteristics of victorin-induced PCD in victorin-sensitive and insensitive oats, including rubisco proteolysis (Fig. 3-5A, lanes 3 and 6), DNA laddering (Fig. 3-5B, lanes 3 and 6), and release of the 84 kDa saspase into the ECF (Fig. 3-5C and D, lanes 3 and 6). The water controls for both sensitive (lane 1) and insensitive (lane 4) leaves did not display any of the characteristics associated with PCD, nor did victorin-treated insensitive leaves (lane 5). Heat shock-induced rubisco proteolysis, like that induced by victorin (Fig. 3-1), was prevented by the caspase inhibitors Ac-VAD-CMK, Ac-AAD-CMK, and Ac-DEVD-CMK (Fig. 3-6).

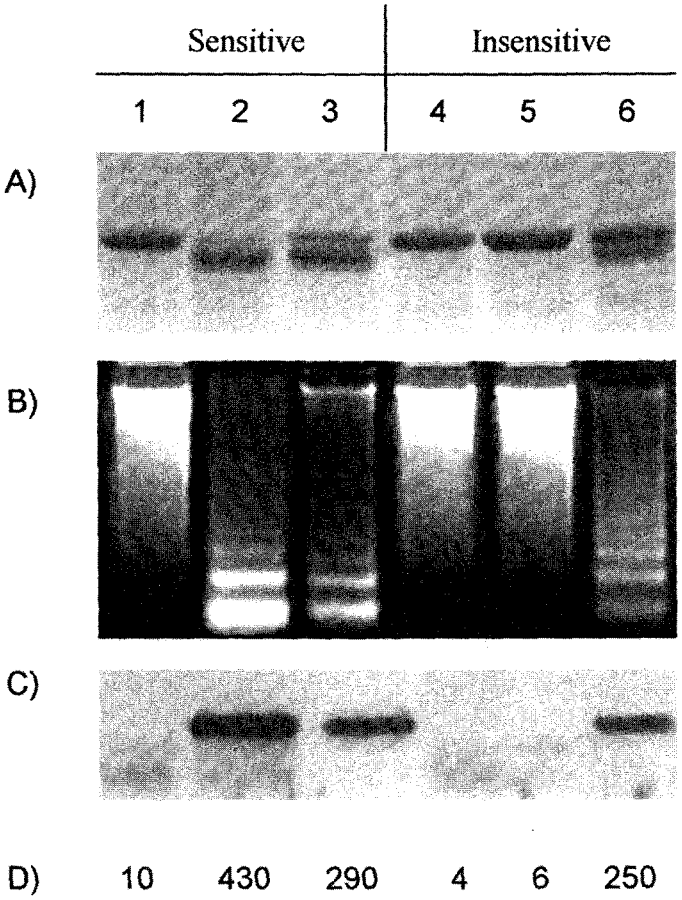


Figure 3-5 **Heat Shock Induced PCD in Victorin-Sensitive and Insensitive Oats.**

Victorin-sensitive and insensitive oat leaves were infiltrated with water (lanes 1, 3, 4, and 6) or 1000 ng/ml victorin (lanes 2 and 5). Water-infiltrated leaves in lanes 3 and 6 were heat shocked for 60 min at 45°C, then incubated at 25°C. A) Coomassie blue-stained SDS-polyacrylaminde gel of total protein extracted 15 hours after treatment. Rubisco cleavage is seen in sensitive leaves treated with victorin and heat and in insensitive leaves treated with heat. B) 1.5% agarose gel of DNA extracted from above samples. DNA laddering induced by treatments that induce rubisco cleavage. C) Biotin blot of ECF collected from above samples 4 hours post treatment and incubated with 200 μM biotin-YVAD-CMK for 2 hours before electrophoresis. Labeling of the 84 kDa oat saspase corresponds to the induction of rubisco cleavage and DNA laddering. D) Z-VAD-AFC hydrolytic activity of the oat saspase in the ECF.

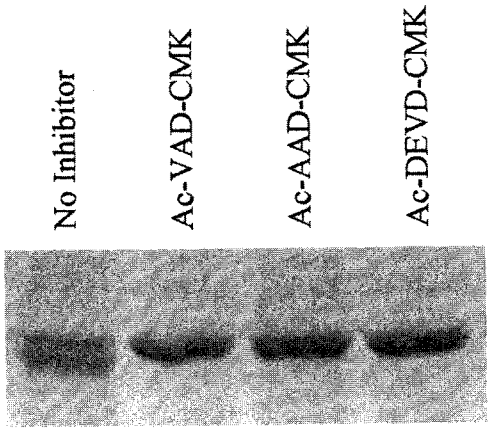


Figure 3-6 **Heat Shock-Induced Rubisco Proteolysis Prevented by Caspase Inhibitors.**

Oat leaves were pre-incubated with 400 μM of indicated inhibitor for 2 hours then heat shocked for 60 min at 45°C. Proteins were isolated 15 hour post heat shock.

Purification of the Saspase

The saspase purification protocol from 200 grams of victorin treated (100 ng/ml) oat leaves is summarized in Table 3-1. The proteolytic activity in the crude extract and the 50% ammonium sulfate fraction was either too dilute to obtain an accurate measurement of activity or contained an inhibitory compound, because total activity (Table 3-1; column 3) more than doubled following the HIC column. Anion exchange chromatography separated the saspase activity into two closely eluting peaks, the first called oat saspase-1 (OS-1) and the second oat saspase-2 (OS-2) (data not shown). Both OS-1 and OS-2 migrated in an SDS-PAGE with an apparent mass of 84 kDa as detected by silver staining or by biotin bloting (biotin blot samples were preincubated with biotin-YVAD-CMK; data not shown). Further characterization of OS-1 and OS-2 reveal similar substrate and inhibitor profiles (Fig. 3-7) and sequence identity between 23 amino acids of their NH_2-termini (discussed below). Because OS-1 and OS-2 possessed extremely similar characteristics, indicating they are the same protein or a highly related protein family, we combined the proteins from the two peaks for some analyses. Combined proteases are referred to as oat saspase-combined (OS-C). After size-exclusion chromatography, a single band migrating at the apparent molecular mass of 84 kDa in SDS-PAGE and corresponding in size to the earlier identified saspase (Fig. 3-3B), was visualized by silver staining (Fig. 3-8).

The Saspase is a Subtilisin-Like Serine Protease

Amino acid sequencing was performed on gel purified OS-1 and OS-2 following anion exchange chromatography. The NH_2-terminal sequence shown for

Table 3-1 **Purification of OS-C from 200 Grams Victorin-Treated Leaves.**

Purification Step	Total Protein (mg)	Total Activity (pmol/min)*	Specific Activity (pmol/min/mg)*	Purification Fold	Yield (%)
Crude Extract	812	1577.6	1.94	1	100
50% AS Precip.	380	1520.0	4.0	2.06	96
HIC Column	45.46	3957.4	87.1	44.8	250
Heparin Affinity	12	2502.4	208.5	107.36	158
Anion Exchange	0.188	1932.0	10304.0	5304.78	122
Size Exclusion	0.096	1313.3	13680.0	7042.83	83

*1 pmol of substrate cleavage = 6.024 fluorescence units

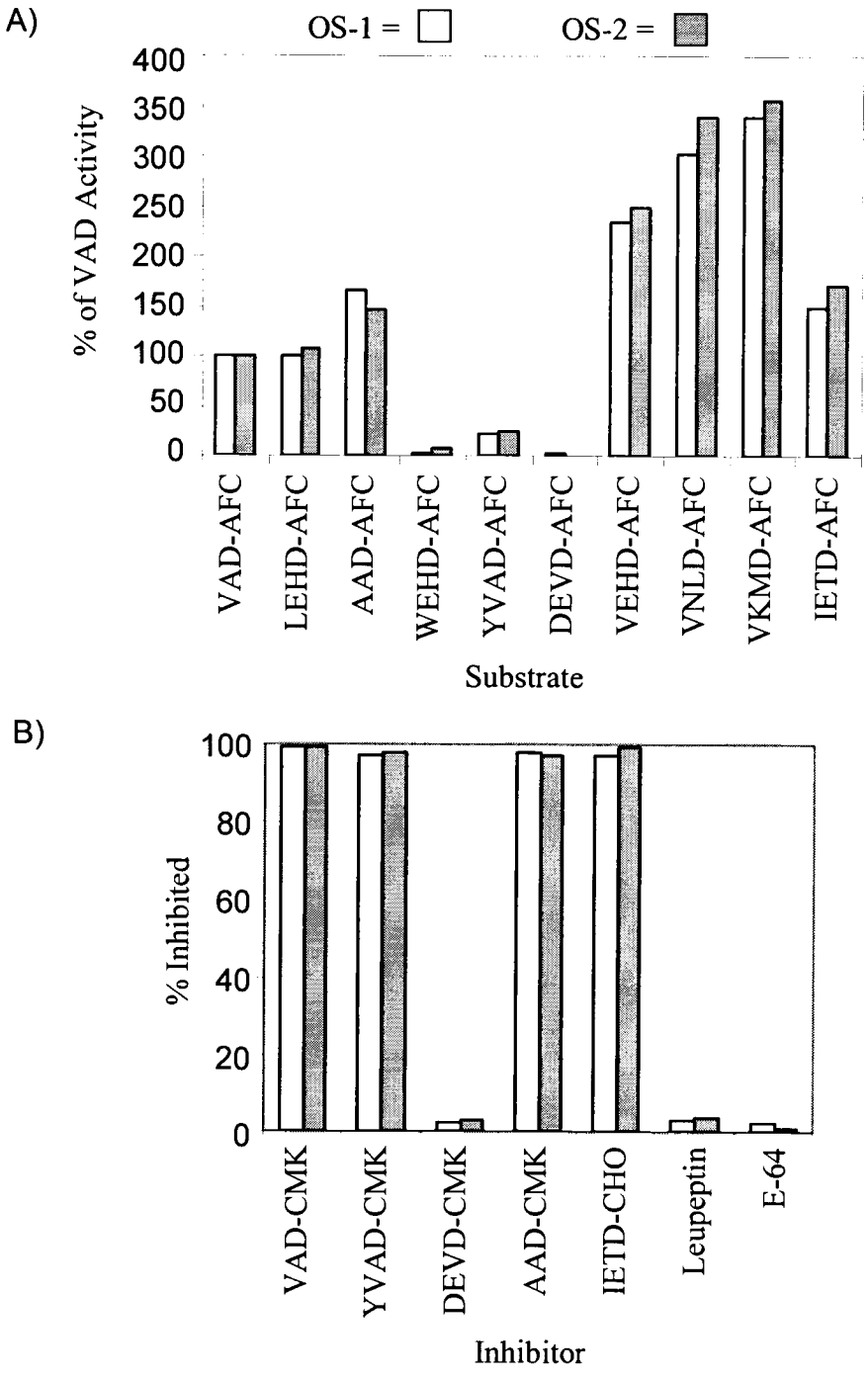


Figure 3-7 **Comparison of OS-1 and OS-2 Substrate and Inhibitor Profile**

(A) OS-1 and OS-2 from AE column were assayed 1 hr for proteolytic activity against indicated substrates. Substrate hydrolysis rate is expressed as percent of VAD-AFC hydrolysis. (B) OS-1 and OS-2 were pretreated with inhibitors (200μM) for 2 hrs then assayed 1 hr for VAD-AFC hydrolysis.

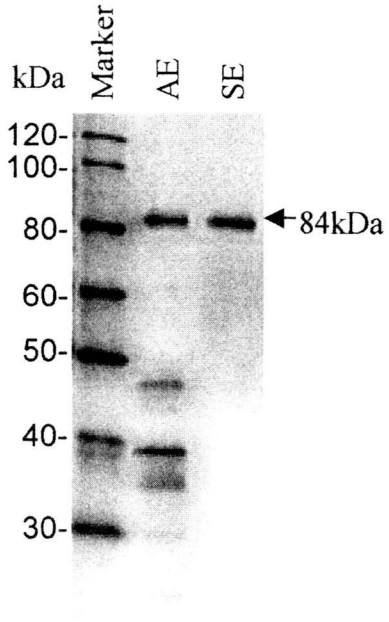


Figure 3-8 **Silver Stained Gel of Purified OS-C**.

Partially purified OS-C from anion exchange chromatography (AE; lane 2), and purified OS-C after size exclusion chromatography (SE; lane 3).

OS-2 (Table 3-2) is identical in OS-1. Consistent with the inhibitor studies, the protein sequence of the NH_2-terminus of both OS-1 and OS-2 were homologous to the mature NH_2-terminus of a number of plant subtilisin-like serine proteases (OS-2 seq. in Table 3-2). Because OS-2 was isolated in higher quantities than OS-1, we performed a partial tryptic digestion of only OS-2. Three internal peptides were sequenced, each showing strong homology to at least one subtilisin-like serine protease (Table 3-2). A putative subtilisin-like serine protease from rice was the most similar protein found (79%; 49 out of 62 amino acids identical) and was the only subtilisin-like serine protease with homology to internal peptide 3 of OS-2.

Characterization of OS-C Hydrolytic Activity

OS-C exhibited maximal hydrolytic activity toward Z-VAD-AFC at pH 6.5 (Fig. 3-9). Less than 50% of the activity remained after a one-pH unit change.

Salts generally enhanced oat saspase activity. The effects of different salts on Z-VAD-AFC hydrolysis by OS-C are presented in Table 3-3. Monovalent salts (i.e., NaCl and KCl) were five to ten times less efficient at stimulating hydrolysis than divalent salts (i.e., $MgCl_2$ and $CaCl_2$). The heavy metal salt, $ZnCl_2$, inhibited OS-C activity.

The specificity of OS-C for commercially available protease substrates is listed in Table 3-4. Like caspases, OS-C apparently requires an aspartate residue at the P1 position of the substrate, but does not cleave all substrates fitting that requirement, indicating a highly specific recognition motif. The highest levels of hydrolysis were observed with the substrates Ac-VEHD-AFC, Ac-VKMD-AFC, Ac-VNLD-AFC, Ac-

Table 3-2 **Comparison of Partial Amino Acid Sequence of OS-2 with other known Subtilisin-Like Proteases.** Rice subtilisin – putative subtilisin-like protease (accession #BAB89803). P69A, P69B, and P69C – subtilisin-like proteases from tomato (accession #'s CAA76724, CAA76725, and T06577 respectively). ARA12 – subtilisin-like protease from *Arabidopsis thaliana* (accession # NP_569048). Cucumisin – subtilisin-like protease form *Cucumis melo* (accession # A55800). Identical amino acids are in bold.

Protein	Amino Terminus	Internal Peptide 1
OS-2	**TTHTPEFLGLSAAGG-LWEAS-EYG**	**VHPDWSPAAVR**
Rice Subtilisin	109-**TTHTPEFLG**VS**GAGG-LWE**TA-S**YG**-133	545-**VHP**E**WSPAA**IR-556
P69A	115-**TTHT**SS**FLGL**QQNM**G**-V**W**KD**S**-N**YG**-137	548-T**HPDWSPAA**IK-558
P69B	115-**TTHTP**S**FLGL**QQNM**G**-V**W**KD**S**-N**YG**-137	547-T**HPDWSPA**VIK-557
P69C	115-**TTHTP**S**FLGL**QQNM**G-LW**KD**S**-N**YG**-137	547-S**HPDWSPA**VIK-557
ARA12	107-**TTRTPLFLGL**DEHTAD**L**FPEAGS**Y**S-131	558-**VHP**E**WSPAA**IR-568
Cucumisin	111-**TT**RSWD**FLG**FPLTVP---RR**S**-QVE-131	541-YN**PTWSPAA**IK-551

Protein	Internal Peptide 2	Internal Peptide 3
OS-2	**EVTNVGDGPASYTAK**	**SPIVATTASSTPF**
Rice Subtilisin	674-V**VTNVGA**GA**ASY**R**AK**-688	748-**SPIVATT**L**SST**RL-760
P69A	667-T**VTNVGD**AKS**SY**KVE-681	----------
P69B	666-T**VTNVGD**ATS**SY**KVE-680	----------
P69C	667-T**VTNVGD**AKS**SYT**VQ-681	----------
ARA12	666-N**V**DG**VG**AYKYTR**T**VT-679	----------
Cucumisin	655-TL**T**S**V**APQAST**Y**R**A**M-670	----------

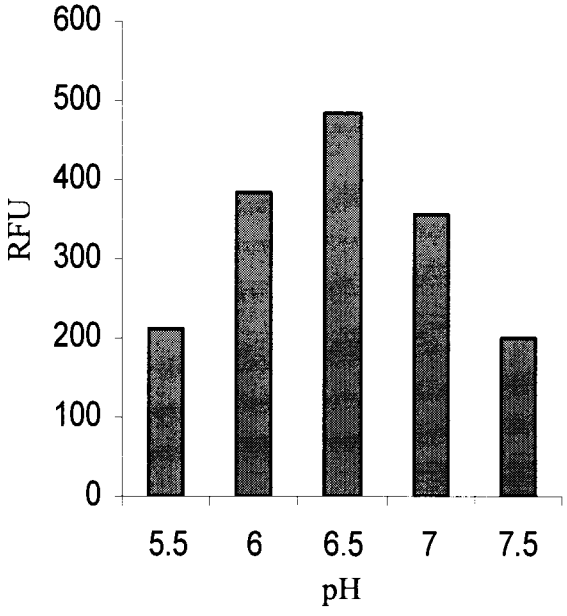


Figure 3-9 **Effects of pH on Proteolytic Activity of OS-C.**

Partially purified OS-C was assayed 1 hr with the substrate VAD-AFC at different pH.

Table 3-3 **Effect of Different Salts on Activity of OS-C.** Partially purified enzyme assayed 1 hr with indicated salt.

Salt	Conc.	Activity
NaCl	10mM	10
	100mM	127
	500mM	625
KCl	10mM	10
	100mM	180
$MgCl_2$	10mM	120
	100mM	675
$CaCl_2$	10mM	116
	100mM	750
$ZnCl_2$	10mM	7
	100mM	30

IETD-AFC, and Z-AAD-AFC. OS-C did not cleave any other protease substrate tested, including the general protease substrate, casein (Table 3-4).

The effect of inhibitors of OS-C hydrolytic activity towards Z-VAD-AFC and Ac-VKMD-AFC (the most preferred substrate) are presented in Table 3-5. Hydrolytic activity of OS-C was affected by the inhibitors in a comparable manner irrespective of the substrate used to test activity (Table 3-5). OS-C appeared to display slightly less specificity towards inhibitors than towards substrates. For example, the recognition motif "VDVAD" and "LEVD" were both potent inhibitors of activity while OS-C poorly recognized them as a substrate. Also, two non-caspase inhibitors were able to inhibit activity of OS-C (Z-GF-NHO-Bz and PMFS); yet no non-caspase substrate was recognized.

Altered Substrate Specificity and Autoproteolysis

Purified OS-C was assayed for hydrolytic activity against several substrates with or without adding 0.5% SDS to the assay buffer (Fig. 3-10). Partial denaturation of OS-C in 0.5% SDS did not appear to significantly change recognition specificity towards peptide substrates, but it did change the hydrolysis rate compared to non-denatured enzyme. For example, substrates not hydrolyzed under non-denatured conditions, such as DEVD-AFC and VDVAD-AFC, were not hydrolyzed under partially denatured conditions (Fig. 3-10). Furthermore, all substrates hydrolyzed under non-denatured conditions were still hydrolyzed in the presence of 0.5% SDS, but the rate of hydrolysis declined by varying amounts. VNLD-AFC and IETD-AFC

Table 3-4 **Substrate Recognition of OS-C.**
The protease in which the substrate was derived from is indicated in parentheses.

Substrate	% of Z-VAD-AFC
Z-VAD-AFC (General Caspase)	100.0%
Z-YVAD-AFC (Caspase 1)	35.0%
Ac-WVAD-AMC (Caspase 1/4)	77.0%
Ac-WEAD-AMC (Caspase 1/4)	1.2%
Ac-DEHD-AMC (Caspase 2)	1.5%
Z-VDVAD-AFC (Caspase 2)	1.6%
Z-DEVD-AFC (Caspase 3)	0%
Ac-LEVD-AFC (Caspase 4)	65.5%
Z-WEHD-AFC (Caspase 5)	10.6%
Ac-VEHD-AFC (Caspase 6)	272.0%
Ac-VEID-AFC (Caspase 6)	10.0%
Ac-VKMD-AFC (Caspase 6)	363.0%
Ac-VNLD-AFC (Caspase 6)	343.2%
Ac-IETD-AFC (Caspase 8)	185.5%
Ac-LEHD-AFC (Caspase 9)	109.2%
Z-AAD-AFC (Granzyme B)	173.0%
AP-AFC (Dipeptidylpeptidase)	0%
GF-AFC (Dipeptidylpeptidase)	0%
GP-AFC (Dipeptidylpeptidase)	0%
PR-AFC (Dipeptidylpeptidase)	0%
SY-AFC (Dipeptidylpeptidase)	0%
Z-AAL-pNA (Subtilisin A)	0%
H-L-AMC (Aminopeptidase)	0%
Z-RR-AMC (Cathepsin B)	0%
Suc-AAPF-AMC (Chymotrypsin)	0%
Z-AKR-AMC (ICRM)	0%
Boc-GGL-pNA (Subtilisin A)	0%
FTC-Casein (General Protease)	0%

Table 3-5 **Inhibitor Profile of OS-C**. Inhibitors were preincubated with enzyme for 2 hrs at 200μM except PMSF (1mM) and Aprotinin (1μg/ml). Substrate hydrolysis was measured for 1 hr with Z-VAD-AFC and Ac-VKMD-AFC.

Inhibitor	% Inhibited with substrate: VAD-AFC	VKMD-AFC
No inhibitor	0.0%	0.0%
Ac-VAD-CMK	99.0%	97.2%
B-YVAD-CMK	98.0%	98.9%
Ac-DEVD-CMK	10.9%	17.9%
Ac-AAD-CMK	99.5%	98.5%
Ac-LEHD-CHO	72.4%	49.0%
Ac-VEID-CHO	99.0%	99.7%
Ac-LEED-CHO	13.5%	18.9%
Ac-WEHD-CHO	90.8%	90.0%
Ac-DMQD-CHO	94.6%	92.9%
Ac-LDESD-CHO	34.0%	41.5%
Ac-VDVAD-CHO	99.0%	98.5%
Ac-LEVD-CHO	96.3%	99.0%
Ac-IETD-CHO	97.7%	99.2%
Pepstatin A	4.5%	3.0%
Leupeptin	0.2%	4.9%
PMSF	58.1%	52.4%
PCMB	1.2%	2.4%
Z-GF-NHO-Bz	98.3%	94.5%
Boc-APF-NHO-Bz	6.8%	6.3%
AEBSF	5.4%	4.3%
H-Glu-(NHO-Bz)-Pyr	1.8%	2.8%
E-64	4.5%	0.3%
Aprotinin	6.3%	4.8%
Antipain	4.5%	6.2%

hydrolysis were inhibited over 50% with SDS, while hydrolysis of LEHD-AFC and VAD-AFC were inhibited by approximately 30% (Fig. 3-10).

Commercially available rubisco (from spinach) was used as a substrate for OS-C (Fig. 3-11A). Rubisco was incubated 12 hours in 20 mM MOPS pH 6.5 without OS-C (Fig. 3-11A; lane 1), with approximately 200 ng OS-C (non-denatured; lane 2), with OS-C in 0.5% SDS (partially denatured; lane 3), or with OS-C in 0.5% SDS and boiled for 5 minutes (completely denatured; lane 4). Degradation of rubisco by OS-C occurred only under partially denatured conditions, while OS-C had no effect on rubisco under non-denatured or completely denatured conditions.

OS-1 and OS-2 were used to produce polyclonal antibodies in rabbits. The antisera produced from OS-1 and OS-2 protein were cross-reactive with each other (data not shown). Because OS-2 antiserum had a higher titer of polyclonal antibodies, it was used for routine western blot analysis. A western blot of purified OS-C probed with OS-2 antiserum is shown in Figure 11B. After addition of SDS-loading buffer to approximately 200 ng purified OS-C, the samples were either boiled 5 minutes (Fig. 3-11B; lane 1) or not boiled (Fig. 3-11B; lane 2) prior to loading on the gel. Autoproteolyitc degradation of the 84 kDa OS-C occurred while electrophoresed in gel, when the protease was not denatured by boiling (compare lanes 1 and 2; Fig. 3-11B). No autoproteolysis was observed when the oat saspase was incubated overnight in SDS-loading buffer prior to boiling and electrophoresis (data not shown) suggesting the elevated concentrations reached during electrophoresis were required for autoproteolysis.

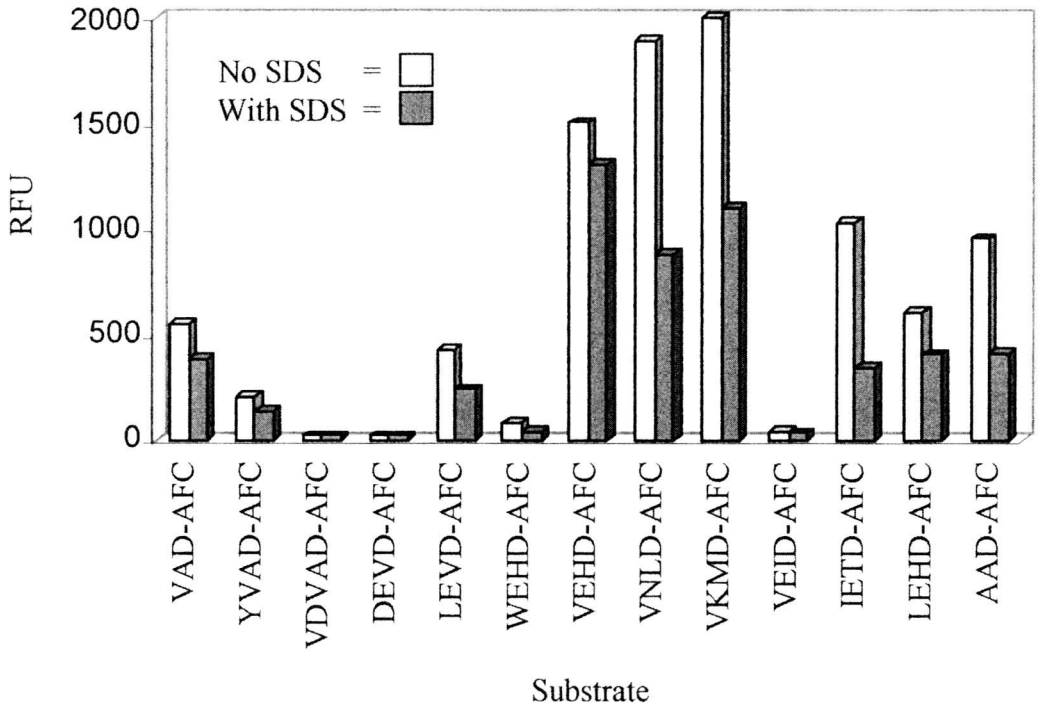


Figure 3-10 **Effects of SDS on Substrate Hydrolysis by OS-C**

Partially purified OS-C (from AE column) was assayed 1 hr for hydrolysis of various caspase substrates with and without 0.5% SDS.

Figure 3-11 **Partially Denatured OS-C is Autoproteolytic and Exhibits Altered Substrate Specificity**.

A) Coomassie blue-stained SDS-polyacrylamide gel of 2 ug rubisco treated with purified OS-C in various states of denaturation. Rubisco only (lane 1), rubisco and OS-C (lane 2), rubisco and OS-C in 0.5% SDS (lane 3), rubisco and OS-C in 0.5% SDS and boiled 5 min (lane 4). B) Western blot of purified OS-C. OS-C was boiled 5 min (lane 1) or not boiled (lane 2) after addition of SDS loading buffer.

DISCUSSION

Characterization of the Saspase

An enzyme with caspase-like hydrolytic activity was purified from victorin-treated oat tissue and identified as a subtilisin-like serine protease and therefore was termed a saspase. Purification revealed two peaks of saspase activity closely eluting from the anion exchange column; referred to as OS-1 and OS-2. The enzymes were undistinguishable by molecular weight (both migrating at ~84 kDa), substrate hydrolysis, inhibitor profile, or NH_2-terminal sequence. Consequently, OS-1 and OS-2 are likely either the same enzyme with different protein modifications (e.g. glycosylation or phosphorylation) that provided a slightly altered elution profile from the anion exchange column, or they are two different, yet extremely homologous enzymes. Several plant species contain families of subtilisin-like serine proteases, including Arabidopsis and tomato, in which four and nine different subtilisin-like serine proteases, respectively, have been identified (see below). Furthermore, an examination of the rice genome database indicated at least seven DNA sequences that have been annotated as subtilisin-like serine proteases (www.ncbi.nlm.nih.gov). One of these was highly homologous to all four peptides sequenced from OS-2 (Table 3-2).

The amino acid sequence from the NH_2-terminus of OS-1 and OS-2 and two internal peptides from OS-2 showed clear homology to previously characterized plant subtilisin-like serine proteases. The characterized proteases include: AIR3 (Neuteboom et al. 1999), SDD1 (Berger and Altmann 2000), ALE1 (Tanaka et al. 2001), and Ara12 (Hamilton et al. 2002) from Arabidopsis; P69A-F (Vera and

Conejero 1988, Tornero et al. 1997, Jordá et al. 1999, Meichtry et al. 1999, Jordá et al. 2000), LeSBT1-4 (Meichtry et al. 1999, Janzik et al. 2000), and TMP (Riggs et al. 2001) from tomato; cucumisin from *Cucumis melo* (Yamagata et al. 1994); Ag12 from alder (Ribeiro et al. 1995); Lim9 from lily (Taylor et al. 1997); RSP1 from rice (Yamagata et al. 2000); SCS1 from soybean (Batchelor et al. 2000); Cg12 from *Casuarina glauca* (Laplaze et al. 2000); and hordolisin (Terp et al. 2000) and SEP-1 (Fontanini and Jones 2002) from barley. However, the third internal peptide showed homology to only a putative subtilisin-like protease from rice (Table 3-2). This putative protease also demonstrates the greatest homology to the other three peptides.

The substrate specificity of OS-1 and OS-2 is distinct from all other known subtilisin-like serine proteases. Among the substrates tested, only certain caspase substrates were hydrolyzed, indicating a requirement for aspartate residues in the P1 position. No other purified protease from plants has been reported to be specific for caspase substrates. Plant subtilisin-like proteases differ widely in their substrate specificity. Cucumisin (Yamagata et al. 1994), macuralisin (Rudenskaya et al. 1995), taraxalism (Rudenskaya et al. 1998), hordolisin (Terp et al. 2000), and Sep-1 (Fontanini and Jones 2002) all have a broad substrate range and are thought to be degradative proteases. Whereas AIR3 (Neuteboom et al. 1999), ALE1 (Tanaka et al. 2001), SDD1 (Berger and Altmann 2000), LeSBT1 (Janzik et al. 2000), SBP50 (Schaller and Ryan 1994), and Ara12 (Hamilton et al. 2002) are described as possessing greater substrate specificity and are thought to function as processive proteases. Until more is known about the biochemical properties of plant subtilisin-like serine proteases, the difference between a degradative or processive function may

prove challenging to distinguish. Because OS-1 and OS-2 do not generally degrade proteins (e.g. casein) and apparently require an aspartate residue in the P1 position of the substrate, it seems evident that they function as processive proteases.

Plant subtilisin-like serine proteases are members of the Pyrolysin family of subtilisin-like serine proteases (Siezen and Leunissen 1997), and are predicted, based on DNA sequence of the cloned proteases, to be expressed as pre-pro-protein precursors. Another family of subtilisin-like serine proteases, the Kexin family, is also expressed as pre-pro-protein precursors. Kex2 from *Saccharomyces cerevisiae*, the prototype member of this family, has been well-characterized (Mizuno et al. 1988). In Kex2, the pre-domain is a signal peptide that directs the enzyme to the endoplasmic reticulum (ER), and is cleaved during import (Van de Ven et al. 1991). The pro-domain is autocatalyically cleaved, releasing the mature, active form of the protease (Gluschankof and Fuller 1994). The pre and pro-domains of plant subtilisin-like serine proteases are thought to each function in a similar manner to the Kex2 enzyme, because several of these enzymes have been localized extracellularly (Vera and Conejero 1988, Yamagata et al. 1994, Taylor et al. 1997) and the NH_2-terminus of mature, active enzymes corresponds to the predicted mature NH_2-terminus with the pro-domain removed (Terp et al. 2000, Popovic et al. 2002). The NH_2-terminus of OS-1 and OS-2 also corresponds in sequence similarity to the mature NH_2-terminus of most plant subtilisin-like serine proteases.

Members of the Pyrolysin family possess a protease-associated (PA) domain, which is also found in zinc peptidases, C-RZF (ring-zinc-finger) proteins, human transferrin receptors, and plant vacuolar sorting receptors (Mahon and Bateman 2000,

Luo and Hofmann 2001). The PA domain is thought to mediate protein-protein interaction and influence substrate determination (Mahon and Bateman 2000). The crystal structure of the human transferrin receptor has been reported and the position of the PA domain characterized (Lawrence et al. 1999). The PA domain sits as a "hinged lid", covering a region with homology to an active site of a remnant protease (Lawrence et al. 1999, Luo and Hofmann 2001). The PA domain of plant subtilisin-like serine proteases may function in a similar fashion; in close association to the active site of the protease, with the domain able to mediate protein recognition and thus substrate specificity. Kexin protease family members contain a region called the P-domain that may function analogously to the PA domain. The P-domain of Kex2 is required for intramolecular maturation of pro-Kex2 and is thought to aid in protein folding and substrate recognition (Gluschankof and Fuller 1994). Because the P-domain is present only in processive proteases, it clearly differentiates processive from other, closely related degradative enzymes (Siezen and Leunissen 1997). However, the presence of the PA domain does not appear to indicate the same distinction, because it is present in all plant subtilisin-like serine proteases, including both degradative and putative processive proteases.

Even though the oat saspase likely functions as a processive protease, it exhibits unusual characteristics when partially denatured in 0.5% SDS or when electrophoresed on SDS-PAGE without complete denaturation. While the rates for substrate hydrolysis were, in most cases, significantly reduced, OS-C retained catalytic activity and hydrolyzed the same peptide substrate in 0.5% SDS. However, OS-C degraded commercially purified rubisco in the presence of 0.5% SDS, a feature it did

not possess when native or when boiled in SDS. Also, OS-C exhibited autodegradation during electrophoresis in an SDS-PAGE. Boiling of OS-C prior to gel loading prevented autodegradation. Autodegradation likely occurred in *trans* and required the highly concentrated protein levels reached while focusing in the stacking gel. If the autodegradative activity was acting in *cis*, then degradation would be expected when the enzyme was less concentrated and partially denatured. However, when purified oat saspase was incubated in SDS-loading buffer overnight and boiled prior to electrophoretic analysis, no autodegradation was observed (data not shown). Altered substrate specificity, hydrolysis rate, and autodegradation of the oat saspase while partially denatured might be caused by an altered association of the PA domain with the catalytic region. Removal of the PA domain changes substrate specificity for the cell-envelope proteinase from *Lactococcus lactis*, a member of the Pyrolysin family of subtilisin-like serine proteases (Bruinenberg et al. 1994). Deletion mutants of the cell-envelope proteinase retain catalytic activity towards normal substrates, but process them at different rates. These data, in conjunction with the PA domain acting as a "hinged lid", covering a remnant protease active site within the transfferin receptor (Lawrence et al. 1999), suggest that the PA domain functions within plant subtilisin-like serine proteases by mediating substrate determination, recognition, and accessibility.

Heat Shock Induced Programmed Cell Death

The cell death program in oats induced by heat shock exhibited similar biochemical features as victorin-induced PCD, including DNA laddering, rubisco

proteolysis, and release of the oat saspase into the ECF. Heat shock-induced PCD has also been characterized in cucumber and tobacco suspension cells. Balk et al. (1999) described internucleosomal cleavage of DNA, 12 hours after heat shock of cucumber cotyledons at 55°C for 10 minutes. In the same experiment, cytochrome C was released from intact mitochondria immediately following heat treatment. Tobacco suspension cells, heat treated for 4 hours at 44°C, underwent PCD characterized by cleavage of poly(ADP-ribose) polymerase (PARP) directly following treatment, while DNA fragmentation and an increase in caspase-3-like activity was observed after 20 hours (Tian et al. 2000). In these experiments oat leaves were heat shocked for 60 minutes at 45°C, which appeared sufficient to induce PCD (Fig. 3-5). It will be of future interest to see whether, after heat shock or victorin-induced PCD, PARP is processed and cytochrome C is release in oats, as in tobacco.

Similarities to other Proteases Involved in Disease, Development, and Death

In the early stages of characterization, and prior to purification and partial sequence analysis, it appeared evident that the saspase was a high molecular weight, extracellular serine protease apparently involved in a protease cascade. That depiction strongly resembled a *Drosophila melanogaster* protease called Gastrulation defective (GD), which is a 72 kDa, extracellular serine protease involved in a protease cascade that leads to dorsal-ventral signaling (Konrad et al. 1998, Han et al. 2000, DeLotto 2001, Dissing et al. 2001, LeMosy et al. 2001). Because GD binds heparin (Dissing et al. 2001), the ability of the oat saspase to bind heparin was tested and established. After purification and partial sequencing, it became clear that GD and the oat saspase

are structurally unrelated, although they may share some similar biological characteristics. The biological significance of GD binding to heparin is not known, but Dissing et al. (2001) suggest that the *pipe* gene, a haparan sulfate 2-*O*-sulfotransferase, might regulate the activity of GD.

A unique characteristic of the saspase is that it seems to be constitutively present in oat cells, and release of active saspase into the ECF is dependent upon induction of PCD but not *de novo* synthesis of the enzyme. This is in contrast to other proteases that have been associated with PCD (Delorme et al. 2000, Eason et al. 2002, Wan et al. 2002), including the hypersensitive response (Vera and Conejero 1988, Krüger et al. 2002), because they are typically regulated at the level of expression. P-69B and P-69C, members of a family of tomato subtilisin-like serine proteases, are two pathogenesis-related proteins (PR proteins) that show increased expression levels during the HR (Jordá et al. 1999). The pathogen-induced P-69 enzymes are also thought to be secreted into the extracellular space (Tornero et al. 1996a). However, unlike the saspase, these proteins are not present in the cell prior to induction of the HR (Tornero et al. 1997).

The question arises as to where the saspase is localized prior to release into the ECF. It could be retained in the ER where it awaits processing and secretion. The Kexin family of subtilisin-like serine proteases is known to autocatalytically remove their pro-domain in the ER prior to moving into the secretory pathway (Gluschankof and Fuller 1994, Hill et al. 1995). A mutation in the Kex2 protease that inhibits removal of the pro-domain stimulates accumulation of proKex2 in the ER (Gluschankof and Fuller 1994). The pro-domain is thought to contain an ER-retention

sequence. If the saspase is stored in the ER, while awaiting processing and secretion, then an inactive form should be identifiable. However, analysis of proteins labeled *in vivo* with biotin-YVAD-CMK and extracted under denaturing conditions (phenol-extracted) from victorin-treated and untreated tissue, revealed similar concentrations of only the 84 kDa (processed) protease (Fig. 3-4D). Soluble protein extracts from victorin-treated and untreated tissue also contained comparable amounts of oat saspase activity in the 84 kDa form (data not shown). These data suggest that the saspase is likely sequestered as an active protease.

Another possible saspase-retention area is the cell exterior, where it could associate with the extracellular matrix or a membrane protein. The ability of the oat saspase to bind heparin may be biologically significant. Heparin or heparin-like molecules are typically extracellular, either membrane bound or soluble (Petitou et al. 1988 and Stringer et al. 2002), and because the oat saspase is ultimately extracellular, it is possible that a heparin-like molecule plays a physiologically significant role, either prior to "release" of the oat saspase or sometime during its function. Whatever its storage location, the fact that the saspase appears to be constitutively present in an active form suggests that the saspase can be rapidly released and a quick response is important for PCD.

Though the physiological substrate(s) of the saspase has not been identified, the data suggest that it activates other proteases. Because the saspase recognizes two of the inhibitors (and their complementary substrates) that prevent rubisco proteolysis, and because victorin treatment affects the localization of the saspase, it appears likely that the saspase is involved in the signal cascade leading to rubisco proteolysis. The

saspase is also likely to function early in the signal cascade because its release into the ECF is not prevented by any of the protease inhibitors that prevent rubisco proteolysis, indicating that these inhibitors function either downstream or after release of the saspase. Furthermore, because multiple proteases appear to be involved in this cascade, the saspase is likely involved in their activation. Are these proteases the direct substrate, or does the saspase interact with an extracellular protein, possibly membrane-associated, which facilitates the activation of intracellular proteases? Several reports have indicated the interaction of extracellular proteases with leucine-rich repeat (LRR) proteins (LRP) during the HR or developmental processes. The pathogen-induced, subtilisin-like serine protease, P-69 from tomato, is able to proteolytically process an extracellular LRP (Tornero et al. 1996b). The LRP, predicted to be soluble in the extracellular matrix, also has increased expression levels during pathogen attack, but the function of the processed or unprocessed form is yet to be determined. Krüger et al. (2002) describe an extracellular cysteine protease, Rcr-3, that is required for Cf-2-dependent HR. Cf-2 is a resistance gene in tomato that encodes a transmembrane LRP with an extracellular LRR domain. Not only is Rcr-3 required for Cf-2-dependent HR, it is also necessary for suppression of Cf-2-dependent autonecrosis. Two other extracellular proteases, SDD1, a subtilisin-like serine protease (Berger and Altmann 2000) and BRS1, a serine carboxypeptidase (Li et al. 2001), have been described as necessary for stomata development and brassinosteroid signaling, respectively. Both proteases function upstream of LRR receptor-like proteins that are required for continuation of their respective signaling pathways (Serna and Fenoll 2002, Li et al. 2001). Whether or not the oat saspase

interacts with another protease, an LRR receptor-like protein, or a ligand for a membrane receptor is currently not known. Identification of the physiological substrate for the oat saspase will be an important step in furthering the understanding of the processes of PCD in oats.

Evidence suggests that the saspase is a component of a PCD-induced protease cascade that ultimately leads to the activation of a protease that is targeted to the chloroplast to cleave rubisco. Although cleavage of rubisco is inhibited by Z-VAD-CMK and Z-AAD-CMK, both of which are recognized by the saspase, and even though partially denatured saspase degrades rubisco *in vitro*, it is not likely that the saspase actually cleaves rubisco *in vivo*. Four lines of evidence suggest an alternative protease cleaves rubisco: 1) E-64, leupeptin, and Z-DEVD-CMK prevent rubisco cleavage but do not affect saspase activity or its release into the ECF; 2) the cleavage site in rubisco (Navarre and Wolpert 1999) does not fit the substrate specificity of the saspase; 3) non-denatured saspase does not cleave commercially purified rubisco *in vitro*; and 4) the saspase appears to function extracellularly.

The saspase is a subtilisin-like serine protease and partial amino acid sequence revealed homology to all characterized plant subtilisin-like serine proteases, with the highest similarity to a putative rice protein. The strict requirement for aspartate residues in the P1 position of the substrate and lack of caseinolytic activity or rubisco degradation *in vitro,* suggest that the oat saspase functions as a processive protease. Release of the oat saspase into the ECF occurs during victorin and heat shock-induced PCD, two treatments that share other characteristics of PCD, namely rubisco proteolysis and DNA laddering. Recently, Woltering et al. (2002) asked the question

"Do plant caspases exist?" This research addresses that question by providing insight into an enzyme that possesses caspase-like activity – the oat saspase. Numerous studies have indicated the involvement of caspase-like proteases in the regulation of plant PCD. Caspase-specific inhibitors prevent PCD in response to bacteria (del Pozo and Lam 1998, Richael et al. 2001), fungal elicitors (Elbaz et al 2002), and various forms of stress (Sun et al. 1999, Tian et al. 2000, Mlejnek and Procházka 2002, Elbaz et al. 2002, Woltering et al. 2002). Although these reports indicate there are proteases in plants that are inhibited by caspase-specific inhibitors and implicated in PCD, no enzyme has yet been identified that pertains to these observations. The saspase is the first enzyme purified from plants that possesses caspase-like specificity and is activated during PCD. Cloning of the saspase gene should be facilitated by the availability of the gene sequence for the homolog in rice, and it will be of interest to see if the putative protein product from the rice gene possesses caspase specificity, and if it is also involved in PCD in rice.

Chapter 4

Conclusions

The interaction between *Cochliobolus victoriae* and susceptible oats induces a cellular response that has been characterized as programmed cell death (PCD; Navarre and Wolpert 1999, Curtis and Wolpert 2001, Tada et al. 2001, Yao et al. 2001). This response is absolutely dependent on secretion of the toxin, victorin (Wheeler and Luke 1954), by the fungus, and the presence of the *Vb* gene in the plant (Litzenberger 1949). The crown rust resistance gene, *Pc-2*, has been described as either tightly linked to, or the same gene as, *Vb* (Luke et al. 1960, Luke et al. 1966, Rines and Luke 1985, Mayama et al. 1995). *Puccinia coronata*, the causal agent of crown rust, and *C. victoriae* are plant pathogens with very different lifestyles. *C. victoriae* is a necrotroph, utilizing dead plant tissue to live on, while *P. coronata* is a biotroph, which completes its lifecycle within living tissue. These observations suggest that one response by the plant (perhaps PCD), induced by two types of pathogens (biotroph or necrotroph), leads to two different outcomes (resistance or susceptibility). Therefore, the interaction between *C. victoriae* and oats provides a unique opportunity to study changes in host physiology during disease and the implications of these changes in determining host resistance and susceptibility.

The first objective of this study was to facilitate the identification and cloning of the *Vb* gene by creating a high resolution genetic map of the *Vb* locus. In order to achieve this goal, a map was constructed with genetic markers derived from an F2 population segregating for victorin sensitivity. Amplified fragment length polymorphism (AFLP) technology was employed to identify two markers that flank

the *Vb* locus. However, neither was tightly linked to *Vb*, and thus, their utility in developing a high resolution genetic map will be limited. Expanding AFLP analyses by employing different selective nucleotides or restriction enzymes would require considerable labor and time, and yet it is not clear that such work would provide additional, informative data. The parental lines used to create the segregating F2 population were likely too genetically similar to produce sufficient polymorphisms. Creating a new segregating population with genetically diverse parents likely would provide the number of polymorphic markers needed for a high resolution genetic map of the *Vb* locus. Nevertheless, the two AFLP markers identified in this study will be useful for screening future candidates of the *Vb* gene.

Recently, victorin-sensitive ecotypes of Arabidopsis were identified. Arabidopsis is a model genetic organism in which the complete genome has been sequenced and multiple mapping lines are available. These resources greatly facilitate the identification of genes in Arabidopsis and they should lead to the identification and cloning of the Arabidopsis gene for victorin sensitivity. Sensitivity in Arabidopsis, as in oats, is conferred by a single dominant gene (Lorang et al. unpublished data) and current data indicate that the physiological responses induced by victorin in Arabidopsis are very similar to those induced in oats (Sweat and Wolpert unpublished data). This suggests that the Arabidopsis gene may be similar to the gene in oats. If this is the case, then the identification of the gene in Arabidopsis will allow for probing of the oat genome for a similar gene. Furthermore, if it belongs to a large gene family, as has been suggested for crown rust resistance genes (Cheng et al. 2002), the AFLP markers developed in this study can be used to determine whether

any of the identified genes are linked to the *Vb* gene. Thus, the AFLP markers will provide a screening procedure for a putative *Vb* gene.

We have purified and characterized the first protease from plants that exhibits caspase-like specificity and is apparently involved in the PCD response. We have referred to it as a saspase, because, unlike caspases, it is a subtilisin-like serine protease. The PCD response in plants is currently under intense characterization by many research laboratories. Because apoptosis is the most well-characterized form of PCD and caspases play such a critical role in the process and, because numerous experiments have shown that caspase-specific inhibitors prevent PCD in plants (del Pozo and Lam 1998, Richael et al. 2001, Elbaz et al 2002, Sun et al. 1999, Tian et al. 2000, Mlejnek and Procházka 2002), it has been speculated that proteases similar to caspases are involved in plant PCD. Until this study however, no protease had been identified that is involved in PCD and recognizes caspase-specific substrates (or inhibitors).

Saspase substrate recognition is highly specific; it only hydrolyzes peptide substrates that have an aspartate residue in the P1 position, and does not cleave or degrade any protein tested. Based on this specificity, the saspase is thought to function as a processive protease. This property is similar to caspases which also function as processive proteases. Caspases cleave at specific sites within limited, key proteins resulting in their activation or deactivation, and the ordered breakdown of the apoptotic cell. However, the only proteases associated with plant PCD have been characterized as degradative enzymes. Protease signaling cascades, such as those

participating in apoptosis, typically involve processive proteases that cleave select proteins at specific sites, facilitating transduction of the signal. Thus it is unlikely that the degradative proteases such as those that have been characterized in association with plant PCD play a role in transduction of the PCD response. However, a processive protease, like the saspase, would be expected to function in a signal cascade, and because inhibition of the saspase prevents PCD-induced rubisco proteolysis, a role within this signal cascade is likely.

Purification of the saspase revealed two distinct peaks of activity eluting from the anion exchange chromatography column, which were called OS-1 and OS-2 (oat saspase-1 and -2). However, OS-1 and OS-2 were undistinguishable by several criteria, including substrate and inhibitor profiles, electrophoretic mobility in SDS-PAGE, and NH_2-terminal sequence. Whether or not OS-1 and OS-2 are the same protein or highly related family members is not known, but the possibility of a family of processive proteases involved in PCD is quite intriguing. There have been families of related subtilisin-like serine proteases identified in Arabidopsis (Neuteboom et al. 1999, Berger and Altmann 2000, Tanaka et al. 2001, Hamilton et al. 2002) and tomato (Meichtry et al. 1999), and at least seven have been annotated from the rice genome. Because our inhibitor studies suggest at least three proteases involved in the signal cascade leading to rubisco proteolysis, it is possible that related saspases with different specificities function in this cascade.

The physiological substrate of the saspase has yet to be identified, but the substrate is likely to be found in the extracellular fluid (ECF). PCD-induction by either victorin treatment or heat shock stimulates the "release" of the saspase into the

ECF. This response does not activate the saspase, because it is constitutively present in an active form within the cell. However, the re-localization of the saspase into the ECF is probably significant, and could likely determine its site-of-action. Two other proteases, SDD1, a subtilisin-like serine protease (Berger and Altmann 2000) and BRS1, a serine carboxypeptidase (Li et al. 2001), are thought to function as extracellular, processive proteases in a signal cascade acting upstream of membrane receptor proteins (Serna and Fenoll 2002, Li et al. 2001). Based on these data, the saspase could function in a cascade by activating a membrane receptor, either directly or indirectly, which could transduce the signal intracellularly. The identification of cellular targets for the saspase is necessary in order to understand the exact involvement of the saspase in PCD. The possible roles describe, such as, activating another protease (e.g. another saspase) in a protease cascade or transducing a signal through a membrane receptor, are both conceivable and with precedent. However, until more is known about the saspase, and possible family members, it will be difficult to establish the exact role of the saspase in PCD.

The saspase exhibits two interesting characteristics that, once better understood, may provide knowledge about the mechanism in which the saspase functions. One characteristic is autodegradation during SDS-PAGE when the saspase is not completely denatured prior to loading. This feature indicates that the saspase can recognize itself as a substrate *in vitro*. Furthermore, this suggests the saspase may possess an auto-regulatory mechanism, which, after a proper stimulus, induces the saspase to process or degrade itself, preventing further activity. Another interesting characteristic of the saspase is its ability to bind heparin. Heparin, a sulfated

glycosaminoglycan, is involved in several biological activities in animals, including binding antithrombin III. Such binding accelerates the rate of antithrombin III-mediated inhibition of serine proteases involved in the blood coagulation cascade (review by Petitou et al. 1988, Capila and Linhardt 2002). Heparin sulfate, a heparin derivative, is ubiquitously distributed on the surfaces of animal cells and in the extracellular matrix where it mediates various physiological and pathological processes (reviewed in Capila and Linhardt 2002). Therefore, the ability of the saspase to bind heparin indicates that a heparin-like molecule, possibly found in the extracellular space (where the saspase is also localized), may be involved in regulating the saspase. Concievibly, the ability of the saspase to bind heparin and its ability to undergo autodegradation, may function in a cooperative manner, in which binding a heparin-like molecule facilitates the eventual inactivation by autodegratation of the saspase.

Cloning the gene(s) that encodes the saspase(s) will be facilitated by identification of the DNA sequence of the homologous protease in rice. It will be of interest to determine if the same putative subtilisin-like serine protease from rice is expressed and, if so, if it functions similarly to the saspase from oats. Heat-shock treatment will be a useful system to induce PCD and to ascertain its characteristics in rice. Identifying the genetic sequence of the saspase (or the family of saspases) will provide useful information and perhaps, insight into the underlying mechanism of saspase function during the progression of PCD.

In conclusion, Victoria blight of oats, the disease caused by the fungus, *C. victoriae*, has been further examined in these studies. Specifically, the interaction

between the toxin, victorin, and the gene conferring sensitivity in oats, *Vb*, has been genetically and biochemically scrutinized. We have identified genetic markers linked to the *Vb* locus in oat that will be useful for screening future gene candidates. A gene that is likely homologous to *Vb* is currently being characterized in Arabidopsis, and once identified, will, in conjunction with the markers linked to *Vb*, facilitate the identification of the *Vb* gene in oats. Cloning the *Vb* gene will provide insight into the mechanism of how PCD is induced by victorin, and could also provide information about the involvement of the saspase in the response. In addition, further characterization of the saspase and its biological function in PCD should supply valuable data pertaining to the underlying mechanism of the disease response in oats.

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