ABSTRACT

Christina D. Fitch. THE SERINE PROTEASE FROM THE VENOM OF *POLISTES DOMINULUS* CONTAINS ALLERGENIC EPITOPES. (Under the direction of Dr. Donald R. Hoffman) Interdisciplinary Program in Biological Sciences, East Carolina University, April 2010.

The protection introduced by immunotherapy to patients with allergies to paper wasps may be partial depending on the sensitizing species. Partial cross-reactivity between major paper wasp groups may be the reason for partial protection. Understanding patient response to venom allergens from the paper wasp groups is important to improve and develop effective treatments. Research has shown that the allergenic serine protease may be important in this partial cross-reactivity. In this study we investigated the importance of epitopes on the allergenic serine protease (Pol d 4) from *Polistes dominulus*. Recombinant Pol d 4 was expressed in prokaryotic and eukaryotic expression systems. The expression products from the eukaryotic system yielded a protein of larger size than predicted size. Two constructs of Pol d 4 were produced in the prokaryotic expression system and were of expected size. Re-folding of the prokaryotic expressed recombinant proteins was not successful. The major amino acid epitopes on Pol d 4 were predicted. Three mutant constructs were chosen, each containing two amino acid substitutions within a predicted epitopes. IgE binding was assayed by immunoblot assays using sera from European and North American patients. The majority of European patient sera had IgE that bound to the recombinant form of Pol d 4. Statistical analysis showed that there was no significant difference in IgE binding to the various proteins. IgE from American patients bound recombinant Pol d 4. Bromelain was used in immunoblot inhibition to assay possible carbohydrate epitopes. The inhibition assay showed evidence of IgE binding. The serine protease

from *Polistes gallicus* was characterized. Identity between the two serine proteases was high. Evidence supports the presence of carbohydrate on the recombinant protease and carbohydrate epitopes may be present. Visually, all three mutant proteins bound IgE; mutant 2 bound IgE from the least number of patients suggesting it may be an important epitope on Pol d 4. Analysis of densitometric data showed no significant difference between the proteins tested. The data suggests that there may be amino acid epitopes present. Further, the high sequence identity between Pol d 4 and Pol g 4 support high cross-reactivity between sister species.

THE SERINE PROTEASE FROM THE VENOM OF *POLISTES DOMINULUS* CONTAINS ALLERGENIC EPITOPES

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Doctor of Philosophy

by

Christina D. Fitch

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DEDICATION

This work is dedicated to my mother and my family for their unwavering support and encouragement.

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I would like to acknowledge my advisor for his support and my committee for their guidance during this process. I would like to thank my family for their willingness to give me support and all the opportunities I needed to finish my work. I am grateful to the laboratory employees who were supportive and helpful throughout the years. They have been invaluable in finishing this project. I would like to acknowledge my fellow students for all the years of thoughtful discussion and moral support. Lastly, I would like to express my gratitude to the Department of Pathology and Laboratory Medicine, the Graduate School, the IDPBS program, and Brody School of Medicine for their support.

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LIST OF ABBREVIATIONS

AcMNPV	Pseudotyping *Autographa californica* multicapsid Nucleopolyhedrovirus
AMP	Ampicillin
ANC	Acetonitrile
ANOVA	Analysis of variance
Api m 7	Allergenic serine protease from *A. mellifera*
BSA	Bovine serum albumin
BCIP/NBT	5 bromo-4-chloro-3-indolyl phosphate
$CaCl_2$	Calcium chloride
CEPS	Conformation epitope prediction server
CCD	Cross-reactivity carbohydrate determinants
CUB	complement, an embryonic sea urchin protein, a bone morphogenetic protein interaction domain
D. arenaria	*Dolichovespula arenaria*
D. maculate	*Dolichovespula maculata*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EAST	Enzyme allero-sorbent test
E. coli.	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FBS	Fetal bovine serum
GCG Software	Genetics computer group software package
High Five™	*Trichoplusia ni* insect cell line
HPLC	High phase liquid chromatography
IgE	Immunoglobulin E
IPI	InterProSurf:protein protein interaction server
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KCl	Potassium chloride
LB	Luria broth media
MALDI-TOF	Matrix-assisted laser desorption/ionization
MCS	Multiple cloning site
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfide
$MnCl_2$	Manganese chloride
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaSCN	Sodium thiocyanate
P. annularis	*Polistes annularis*
P. dominulus	*Polistes dominulus*
P. exclamans	*Polistes exclamans*
P. fuscatus	*Polistes fuscatus*
P. gallicus	*Polistes gallicus*

PBS	Phosphate buffered saline
PCI	Phenol:chloroform:isoamyl
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pol a 1	Allergenic phospholipase A_1B from *P. annularis* venom
Pol d 1	Allergenic phospholipase A_1B from *P. dominulus* venom
Pol d 4	Allergenic serine protease from *P. dominulus*
Pol e 4	Allergenic serine protease from *P. exclamans*
Pol g 1	Allergenic phospholipase A_1B from *P. gallicus* venom
Pol g 4	Allergenic serine protease from *P. gallicus*
PVDF	Polyvinylidene fluoride
OD	Optical density
RAST	Radioallergosorbent test
RPM	Rotation per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf9	*Spodoptera frugiperda* insect cell line
Sf21	*Spodoptera frugiperda* insect cell line
SFM	Serum free media
S. invicta	*Solenopsis invicta*
S. richteri	*Solenopsis richteri*
Sol i 2	*S. invicta* allergen 2
SPDBV	Swiss-Pdb viewer
SV40	Simian vacuolating virus 40
TBS	Tris buffered saline
TFA	Trifluoroacetic acid
Tm	Melting temperature
TRIS-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
V. maculifrons	*Vespula maculifrons*
V. vulgaris	*Vespula vulgaris*
WVE	Whole venom extract

Chapter 1: INTRODUCTION

Allergies affect the lives of millions of Americans (Stempel, 1997). In the United States it is estimated that 50 million individuals suffer from allergic diseases (Gergen *et al.*, 1987). These numbers make allergies the fifth most prevalent chronic disease in adults and the third most common in children under the age of 18 (National Academy, 2000). Approximately 55% of the population has positive skin tests to insect venom (Arbes *et al*., 2005). An anaphylactic reaction occurs in 0.5 to 5% of all individuals allergic to insect venom proteins and causes an estimated 40 deaths per year in the United States (Valentine, 1992). The immunologic reaction to venom protein may be species specific or cross-reactive with homologous proteins of various insect genera. Increasing our understanding of the complex interactions between venom proteins and the immune system will aid in improving patient treatment and care.

The stinging insects that are responsible are the medically important species of the order Hymenoptera, which includes bees, wasps and ants. Paper wasps, belonging to the genus *Polistes*, are separated into groups based on subgenera and geographic location (Pickett and Wenzel, 2004). Testing has shown that the cross-reactivity between different paper wasp subgenera is partial (Pantera *et al.*, 2003 and Hoffman *et al.*, 1990), while cross-reactivity among members within each group is strong. The partial cross-reactivity observed among the paper wasp groups may have an impact on testing and treatment of paper wasp allergies. The allergens present in paper wasp venom have been identified and several have been characterized. Studies with venom serine protease have shown that this allergen may play a role in species-specific immunogenic reactivity. Here, the major allergens found in *Polistes* venom will be discussed in regards to cross-

reactivity, and an examination of the role that the allergic serine protease plays in species-specific IgE reactivity will be investigated.

1.1 Allergens

An anaphylactic reaction was initially reported in dogs reacting to sea anemone toxin by Richet and Portier in 1902. The same reaction was later described in humans against horse serum. Clemons von Pirquet hypothesized that immunity and hypersensitivity reactions would depend on interactions between the human immune system and a foreign substance (Von Pirquet, 1906). These foreign substances would later be identified as antigens and the interaction was mediated by IgE antibody. Antigens that elicit a clinical allergic reaction are typically referred to as allergens.

Interaction between IgE and an allergen causes a cascade of events, which can manifest as a type I hypersensitive or allergic reaction in sensitized individuals. IgE is bound to the surface of effector cells, mast cells and basophils, and the interaction with an allergen causes the release of physiologic mediators. These mediators cause the various physical manifestations (Grammer and Greenberger, 2002). Allergens are typically proteins containing post-translational carbohydrates and can be derived from a variety of sources. Insect allergens are introduced to humans by two main routes. The first pathway is inhalation, and this type of allergen is associated with asthmatic attacks in sensitized individuals (Bernton, *et al*., 1970 and Kang *et al.*, 1979). The alternate route is introduction of the allergen by dermal injection of venom proteins by insects from the order Hymenoptera.

1.2 Epitopes

Binding of IgE is specific for a particular portion of an allergen, referred to as an epitope. Structurally, epitopes can either be conformational or linear. Conformational epitopes consist of amino acids which are in close proximity while the protein is folded correctly but are found on multiple regions of the primary structure. Linear epitopes are amino acids sequentially located on the primary structure. Most allergenic epitopes are conformational. Food allergens consist of both types; linear epitopes are produced during digestion of food products before they interact with the immune system. In insect allergies, interaction of the epitopes with IgE relies heavily on the conformation of the allergen. Therefore, amino acid residues within a particular epitope may span the entire length of the molecule, with the possibility of larger proteins containing multiple epitopes (Kurup *et al*., 1998, Rabjohn *et al*., 1999, Furmonavicience *et al*., 2000, and Ramos *et al*., 2003). Cross-reactivity is the recognition of similar or identical epitopes on proteins from different sources. The presence of cross-reactive epitopes complicates the study and understanding of IgE binding to these proteins.

Clinically, cross-reactive epitopes make identification of the sensitizing species difficult. The presence of the cross-reactive epitopes can make a patient appear allergic to an insect venom protein they have not encountered because of structural homology. Treatment of insect allergies requires the identification of the sensitizing species. Errors in identification of the species could lead to incomplete or partial protection of the patient during treatment.

Cross-reactivity between homologous allergens includes both the recognition of IgE to amino acid and carbohydrate portions of the protein. Characterization of the

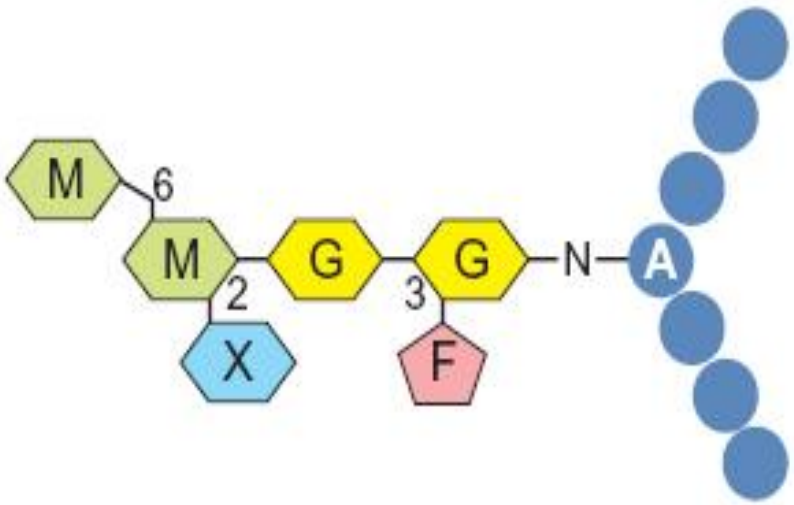
amino acid sequence of various venom proteins has allowed researchers to identify possible homologous cross-reactive allergens. The ability of insect allergens with high sequence identity to cross-react has been extensively documented. The general trend follows that as the sequence homology drops between two allergens, so does the degree of cross-reactivity. This trend is so strong with some allergens that the degree of cross-reactivity decreases as the phylogenetic distance between the insects increases (Hoffman, 1993).

In addition to the amino acid sequence, cross-reactivity has been observed by IgE binding to post-translational carbohydrates on venom proteins. Detectable cross-reactivity between various allergens that lack high amino acid sequence identity can be explained in part by the presence of N-linked carbohydrate groups called cross-reactivity carbohydrate determinants (CCD), shown in Figure 1. First identified in plants and insect glycoproteins, these epitopes typically consist of a 1,3 fucose core (Bencurova, 2004 and Chunsheng, 2008). Research has shown that the CCD has the ability to be a major factor in cross-reactivity between highly diverse groups of glycoproteins from insect and plant sources (Hemmer *et al.*, 2001, Hemmer *et al.*, 2004, and Bencurova *et al.*, 2004). The presence CCD-specific IgE makes identification of sensitizing species difficult. Additional studies have suggested that CCD-specific IgE lack or have very limited capacity to elicit a clinical response in sensitized patients.

1.3 The Order Hymenoptera

The medically important members of the order Hymenoptera can deliver venom allergens by subcutaneous injection. This diverse order includes three major families of social stinging insects; major allergens have been identified and characterized in

Figure 1: The structure of a CCD epitope. The CCD is a 6-sugar chain liked to an asparagine (A). The chain includes two N-acetyl-glucosamines (G), two mannose (M), one 1,3-fucose (F) and a 1,2-xylose (X) (Malandain, 2005).


M
6
M
2
X
G
G
3
F
N
A

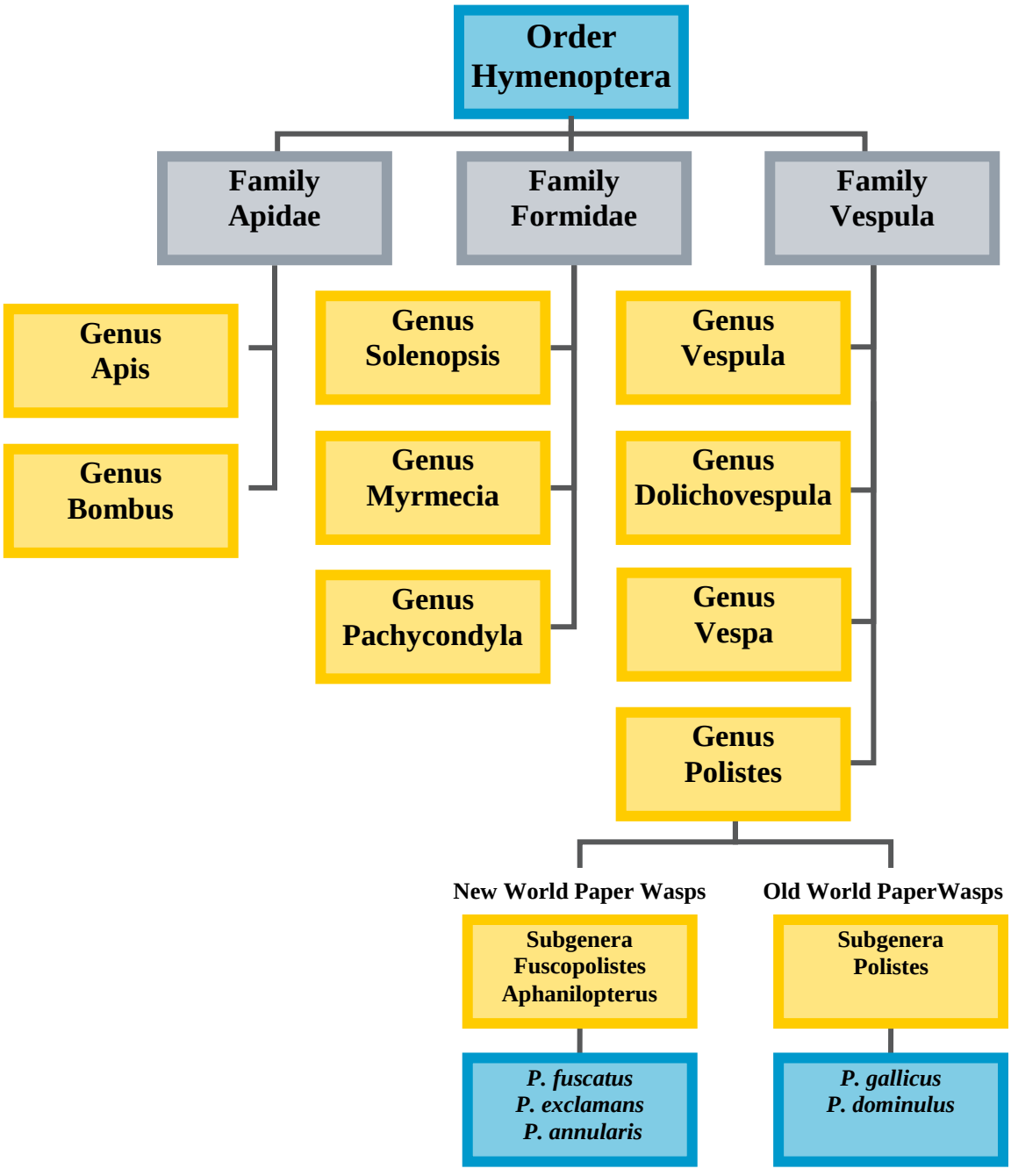
several species groups within these families. These families are: *Apidae* (honey bees and bumble bees), *Vespidae* (wasps, hornets, and yellow jackets), and *Formicidae* (ants) (Figure 2) (Pickett and Wenzel, 2004).

The medically important apids contain two major genera: *Apis* (honey bees) and *Bombus* (bumble bees) (Figure 2). Members of this family are found worldwide in diverse locations. This family has been of particular interest because of their agricultural impact. This family of insects is an economic resource and the occupational hazards that stem from working with members of this group are of great interest.

The members of the family *Formicidae* are commonly referred to as ants (Figure 2). The majority of venom allergens characterized from this family have been from members of the genus *Solenopsis*. The *Solenopsis* ants are found in diverse locations including North America, South America, and islands in South East Asia. Two medically important species *S. richteri* and *S. invicta* inhabit the United States. In addition to *Solenopsis*, allergy inducing members of *Formicidae* includes the *Myrmecia* and *Pachycondyla* genera. *Myrmecia* ants are large primitive ants indigenous to Australia. *Pachycondyla* include ants from Eastern and Western Asia and now found in the United States.

The medically important Vespids are comprised of four genera (Figure 2) which are found in various geographic locations worldwide. The yellow jackets are grouped into the genus Vespula. The various types of hornets are divided into two genera. The first genus, Dolichovespula, includes hornet species, such as Yellow and White faced hornets. The second genus, Vespa, includes European and Mandarin hornets. The last

Figure 2: The medically important Hymenoptera phylogenic tree. The three major allergy causing families of the Order Hymenoptera are shown. The family *Apidae* (honey bees and bumble bees) contains the *Apis* and *Bombus* genera. The *Formicidae* (ants) contains three genera: *Solenopsis, Myrmecia,* and *Pachycondyla*. The *Vespids* (wasps, hornets, and yellow jackets) include four genera; *Vespula*, *Vespa*, *Dolichovespula*, and *Polistes*. Genus *Polistes* is further broken down into two groups, Old World and New World paper wasps. Old World paper wasps include the subgenus *Polistes*. New World paper wasps are composed of two subgenera *Fuscopolistes* and *Aphanilopterus.* Common species of paper wasps are listed for each group. Note, distance between groups does not denote evolutionary distance.


Order Hymenoptera
Family Apidae
Family Formidae
Family Vespula
Genus Apis
Genus Bombus
Genus Solenopsis
Genus Myrmecia
Genus Pachycondyla
Genus Vespula
Genus Dolichovespula
Genus Vespa
Genus Polistes
New World Paper Wasps
Old World PaperWasps
Subgenera Fuscopolistes Aphanilopterus
Subgenera Polistes
P. fuscatus
P. exclamans
P. annularis
P. gallicus
P. dominulus

Vespid genus is *Polistes*; members of this genus are commonly referred to as paper or umbrella wasps.

1.4 *Polistes*

Paper wasps are generally classified into two groups based on geographic origin and subgenus (Figure 2). The first group of paper wasps is New World paper wasps. Members of this group are indigenous to North America. The major allergy-inducing North American paper wasps are represented by two subgenera: *Fuscopolistes*, represented by *Polistes fuscatus*, and Aphanilopterus, which includes two common New World paper wasp species, *Polistes exclamans* and *Polistes annularis*. The second major group of paper wasps is Old World paper wasps. These insects are indigenous to Europe and parts of Asia and Africa. European and North African paper wasps have one major subgenus, *Polistes*. This subgenus contains two common species, *Polistes dominulus* and *Polistes gallicus* (Pickett and Wenzel, 2004). Since the 1970's *Polistes dominulus* has established itself in the Northeastern part of North America.

1.5 Hymenoptera venom

The venom from medically important Hymenoptera insects contains a wide variety of proteins and chemicals with various pharmacological activities. The various components of the venom are intended as a defense mechanism for wasps. The allergic reaction is not intended and does not occur against all venom proteins. The biological purpose for many of these components has been identified, while the function of others has not been resolved.

Formicid venom composition differs from the venom of other Hymenoptera. Fire ant venom is comprised mainly of alkyl and alkenyl piperidine alkaloids (MacConnell *et al*., 1974); these components are responsible for the characteristic sterile pustule that results following injection of venom (deShazo *et al*., 1984 and Javors *et al*., 1993). The allergic reaction from fire ant venom is the result of patient IgE interaction with the small amount of proteins present in the venom. Four major allergens have been characterized in fire ant venom: phospholipase A_1B (Hoffman *et al*., 1988 and Hoffman *et al*., 2005), a 13.3 kDa protein dimer (Hoffman *et al*., 1988, Hoffman *et al*., 1990, Hoffman, 1993, Schmidt *et al*., 1993, Schmidt *et al*., 1996, and Hoffman, 1997), Antigen 5 (Hoffman *et al*., 1988, Hoffman *et al*., 1990, Hoffman, 1993, Hoffman, 1997, and Schmidt *et al*., 2003), and an allergen of unknown function (Hoffman *et al*., 1988, Hoffman, 1993, and Hoffman, 1997) (Table 1).

Apid and Vespid venom have similar compositions. The venom components are grouped into three types of molecules: low molecular weight substances, peptides, and high molecular weight substances (Meier, 1995). The low molecular weight substances include a variety of compounds, such as pheromones, histamine, and serotonin. Histamine makes up a substantial portion of the venom and is one of the major components that facilitate the pain response (Mueller, 1990 and Meir, 1995). Peptides, including kinins, also contribute to the pain response. In addition, various peptides have shown characteristics that aid in the degranulation of mast cells (Meier, 1995). High

Table 1: **List of the major allergens associated with the order Hymenoptera.** The major allergens that have been identified in Apids, Formicids, and Vespids are shown. Honeybee venom contains hyaluronidase, melittin, phospholipase A_2, an allergenic serine protease, a 13 kDa allergen, and an unknown allergen. Formicid venom contains Antigen 5 and Phospholipase A_1B. Vespid venom contains Antigen 5, phospholipase A_1B, and hyaluronidase. Paper wasp venoms also contain an allergenic serine protease.

	Apids	Formicids	Vespids
Antigen 5	Present	Present	Present
Phospholipase	Present, A_2	Present, A_2B	Present, A_2B
Melittin	Present	Absent	Absent
Hyaluronidase	Present	Absent	Present
Serine Protease	Present	Absent	Present in paper wasps
13 kDa Allergen	Present	Absent	Absent
Other allergen	Present	Absent	Absent

molecular weight molecules include a wide variety of proteins, encompassing the majority of enzymes, and contain the major venom allergens (Meir, 1995).

Although the venom composition of Apids is similar to that of the Vespids, the major allergens differ. Honey bees have four identified major allergens: Phospholipase A_2, (King *et al*., 1976, Hoffman and Shipman, 1976, Kuchler *et al*., 1989 Hoffman and Schmidt, 2000 and Li *et al*., 2005), Hyaluronidase (Hoffman, 2006, and Shen *et al*., 2002), Melittin (Habermann *et al*., 1967, Paull *et al*., 1977, and Shi *et al*., 2003), and a CUB Serine Protease (Jeep *et al*., 1996, Kettner *et al*., 1999, Wood and Hoffman, 1983, Winningham *et al*., 2004) (Table 1). The allergens identified in bumble bee venom differ from those found in honey bees. Their venom only contains two major allergens: Phospholipase A_2 (Hoffman and Jacobson, 1996) and a serine protease (Hoffman and Jacobson, 1996). The phospholipases found in honey bees and bumble bees are similar (de Groot *et al*., 1995), however, the serine protease does not cross-react between honeybees and bumble bees (Winningham *et al*., 2004).

1.6 Vespid venom allergens

Venom allergens isolated from Vespids have been extensively studied from a wide variety of these insects found worldwide. A general trend in cross-reactivity is seen when the venoms of these animals are compared. Hornet and yellow jacket venom are partially cross-reactive with one another. The cross-reactivity decreases when the venom allergen from either hornets or yellow jackets is compared to homologous allergens from paper wasp venom. It has been reported that venom of paper wasps within the two major groups is partially cross-reactive, suggesting unique epitopes among the allergens of each group. Three major allergens have been identified in the venom of all

Vespids: Antigen 5 (Fang *et al*., 1987, Hoffman *et al*., 1988, Lu *et al*., 1993, Hoffman, 1993, Hoffman and Schmidt, 1999, Pirpignani *et al*., 2002, Pantera *et al*., 2003, and Fitch *et al*., 2006), Phospholipase A_1B (King *et al*., 1984, Soldatova *et al*., 1993, Hoffman, 1994, Henriksen *et al*., 2001, Pantera *et al*., 2003, Hoffman *et al*., 2005, Moawad *et al*., 2005, and Fitch *et al*., 2006), and Hyaluronidase (King *et al*., 1984, Lu *et al*., 1995, King *et al*., 1996, and Kolarich *et al*., 2005). A fourth allergen, a trypsin-like serine protease, has been isolated from the venom of *Polistes* wasps (Pantera *et al*., 2003, Winningham *et al*., 2004, and Fitch *et al*., 2006).

1.6.1 Antigen 5

The Antigen 5 is one of the most important allergens in yellow jacket and hornet venoms in terms of IgE binding (King and Spangfort, 2000, King and Guralnick, 2004, and Hoffman, 2006). Antigen 5 typically ranges in size from 201-212 amino acids and does not appear to be glycosylated (King *et al*., 1978, Hoffman and McDonald, 1982, Fang *et al*., 1988, and Hoffman and Schmidt, 1999). This protein accounts for approximately 5-10% of the dry weight of the venom (Mueller, 1990). Members of the Antigen 5 family have been found in most eukaryotic organisms and have been characterized in several insect species. Other members of this family are involved in plant pathogenesis (King *et al*., 1990), sexual maturation (Hawdon *et al*., 1996), lizard venom protein (Morrisette *et al*., 1995), and tumor-related proteins in humans (Rich *et al*., 1996 and Hoffman, 2008). The Antigen 5 allergen shows homology with a neurotoxin found in the Asian hornet *V. mandarinia*. However, assays have shown that venom Antigen 5 allergen does not have synaptic toxin activity. Antigen 5 allergen is a

highly conserved molecule in insects; however the biological function in the venom is still not known (King *et al*., 1984, Hoffman and Schmidt, 1999).

Antigen 5 from *P. exclamans* and *P. annularis* (New World paper wasps) were analyzed for sequence identity and cross-reactivity with Antigen 5 from hornets (*D. arenaria* and *D. maculata*) and yellow jackets (*V. maculifrons* and *V. vulgaris*). The Antigen 5 from hornet and yellow jacket venom showed a high degree of cross-reactivity. When cross-reactivity between Antigen 5 proteins from hornets or yellow jackets were compared to the homologous allergen isolated from paper wasp venom, the amount of cross-reactivity decreased to approximately 60% (Hoffman, 1993 and Hoffman, 1997). Comparison ofAntigen 5 from European paper wasps and Formicid venom showed 48% identity (Hoffman *et al*., 1988, Hoffman, 1993, Schmidt *et al*., 2003, and Pantera, 2003). The identity of the Antigen 5 found in venom from *P. dominulus* and *P. gallicus*, European paper wasps, is high at approximately 98% (Lu *et al*., 1993 and Pantera *et al*., 2003). Identity between Antigen 5 from the New World Polistes and Old World paper wasps decreases to 85%. The cysteine residues within Antigen 5 are conserved, suggesting a high degree of structural similarity between molecules from different insect groups (Hoffman and McDonald, 1982, Hoffman, 1985, and Hoffman, 1993). Identity between Old World paper wasps venom allergens and Hymenopteran venom allergens are shown in Table 2.

1.6.2 Phospholipase A_1B

Phospholipase A_1B identified in Vespid venom has the ability to significantly bind IgE. This 42 kDa protein is a member of the lipoprotein lipase superfamily (Hoffman, 2008). This family is responsible for the cleavage of the fatty acid from the 1-

Table 2: **Comparison of percent identity between *P. dominulus* venom allergens and the common Hymenopteran insect allergens.** *P. dominulus* Antigen 5 shared the most identity with the Antigen 5 from the New World paper wasps and the least with the Formicids. The Hyaluronidase sequence from *P. dominulus* has not been identified; therefore a comparison could not be made. The Phospholipase A_1B allergen shared the most identity with the homologous molecule from the New World paper wasps and the least with the Formicids. The serine protease allergen sequence from the New World paper wasp could not be characterized. The serine protease isolated from honey bees shows the highest identity, and the lowest when compared to that from bumble bees.

	Honey Bees	Bumble Bees	Formicids	Hornets Wasps	New World Paper Wasps
Antigen 5	N/A	N/A	48%	57-66%	81-84%
Hyaluronidase	N/A	N/A	N/A	N/A	N/A
Phospholipase A_1B	N/A	N/A	33%	51.7-46.8%	75.4%
Serine Protease	41.5%	31.8%	N/A	N/A	Unknown

position of phospholipids, followed by cleavage of the fatty acid in the 2-position. This enzyme differs from the allergenic phospholipase in Apids, which is a Phospholipase A_2 (King *et al*., 1978, King *et al*., 1983, Hoffman, 1985, and Hoffman, 1994). Cross-reactivity between homologous Phospholipase A_1B allergens in various insect species typically correlates with predicted phylogeny (Hoffman, 1981, Hoffman and McDonald, 1982, Hoffman, 1985, and Hoffman, 1986). The phospholipase A_1B allergen from hornets and wasps show 47-52% identity when compared to the New World paper wasp allergen. The Formicid Phospholipase A_1B has 33% identity. This allergen has been isolated and characterized from *P. dominulus* (Pol d 1) and *P. annularis* (Pol a 1) (Moawad, 2005 and King *et al*., 1984), and a partial amino acid sequence for *P. gallicus* (Pol g 1) phospholipase has been reported (Pantera *et al.*, 2003). Comparison of these amino acid sequences show that Pol g 1, amino acids 1-41, and the derived sequence for Pol d 1 have 95% identity. The identity decreases to 75.4% when Pol d 1 is compared with Pol a 1.

1.6.3 Hyaluronidase

Venom hyaluronidase has been identified as a significant allergen in Vespid venom. This 42 kDa protein (King *et al*., 1984, Lu *et al.*, 1995, King *et al*., 1996, and Kolarich *et al*., 2005) is responsible for hydrolyzing hyaluronic acid, an important component of the extracellular matrix. This cleavage may facilitate the movement of other venom components through the skin.

The hyaluronidase allergen has been isolated and characterized in various insect species, including yellow jackets, hornets, paper wasps and honey bees. Reports suggest that the presence of N-linked carbohydrates (Hoffman, 1994). Sequence identity between

yellow jacket and hornet hyaluronidase is approximately 91.5% (King *et al*., 1996). When compared to hyaluronidase from the paper wasp species, the percent identity decreases to approximately 75% (Pantera 2003). The homologous molecule from honey bees has approximately 50% identity with the Vespids. Cross-reactivity has been reported observed between hyaluronidase from various stinging insects. These studies have shown that high sequence identity is not required for cross-reactivity between hyaluronidase allergen and suggests the cross-reactivity may be due to post-translational glycosylation (Jacobson *et al*., 1992 and Lu *et al*., 1995).

1.6.4 Serine protease

A fourth allergen was identified in the venom of paper wasps (Pantera *et al.*, 2003 Winningham *et al.*, 2004, and Fitch *et al.*, 2006). Analysis of this protein showed it to be a trypsin-like serine protease. A homologous allergen has not been identified in venom of yellow jackets and hornets; however, a serine protease has been isolated from venom of both honey bees and bumble bees (Jeep *et al*., 1996, Kettner *et al*., 1999, Wood and Hoffman, 1983, Winningham *et al*., 2004).

Serine proteases are a well characterized family of molecules due to their importance in a variety of biological processes, such as blood coagulation, digestion, fertilization, extracellular and peptide degradation, and mast cell function. These enzymes cleave the carbonyl side of arginine and lysine. Typically, serine proteases circulate as zymogens, a portion (propeptide) of the enzyme that resembles the tetrahedral intermediate occupying the active site. Cleave of the propeptide activates the enzyme and is irreversible.

Serine proteases have common structural features important for catalytic activity. The catalytic triad, a functional group of histidine, serine and aspartic acid residues, is essential for the cleavage of the substrate. These residues are found closely together while the protease is in proper conformation, however, sequentially they are spaced apart in the amino acid sequence. The cleavage of peptides occurs in the tetrahedral state by forming hydrogen bonds with the negatively charged groups of the substrate, which is stabilized by the histidine residue. These groups form an oxyanion hole, which is important for stabilization In addition to the oxyanion hole, the formation of hydrogen bonds between the loop region of the enzyme and the substrate aid in stabilizing the reaction (Branden, 1999). All proteases have a general cleavage site; however, partial specificity can be inferred by conformation. Specificity is greatly influenced by the binding pocket, which dictates the residues that precede the site of cleavage (Devlin, 1997).

The introduction of the substrate into the binding pocket of the serine protease initiates a complex mechanism which results in the cleavage of the substrate. During stabilization of the substrate in binding pocket the histidine accepts a proton from the serine, allowing nucleophilic attack by the serine on the carbonyl carbon of the peptide bond on the substrate. Next, the histidine donates a proton to the peptide creating a protonated amine on the tetrahedral intermediate, allowing for the release of an amine product. Afterwards, nucleophilic attack by water breaks down the tetrahedral intermediate causing the release of the carboxyl product (Devlin, 1997, Branden, 1999, and Hostrom, 2002).

Serine proteases from paper wasps and honey bees have been characterized and compared. The serine protease allergen from honey bees (Api m 7) has been cloned and characterized. This protease is 245 amino acids in size, with a leader sequence of 35 amino acids, followed by an 88 residue CUB domain (*c*omplement, an embryonic sea *u*rchin protein, a *b*one morphogenetic protein interaction domain), a 31 residue linker, and a 13 amino acid propeptide region. The catalytic triad is present and the entrance to the binding pocket is a methionine, suggesting this molecule may not be a functional enzyme due to the size of the residue. This is supported by the inability of Api m 7 to bind to benzamidine. The mature molecule has four potential N-glycosylation sites, but only one of these appears to be glycosylated (Winningham *et al.*, 2004).

The serine protease isolated from the European paper wasp *P. dominulus* (Pol d 4) has been cloned and characterized. This sequence contained a 19 amino acid leader sequence, a 14 amino acid propeptide, and a mature molecule that was 244 amino acids in size. Analysis of the *P. dominulus* serine protease sequence (Pol d 4) showed it is a trypsin-like molecule with six potential N-glycosylation sites (Fitch *et al*., 2000 and Winningham *et al*., 2004). Initial observation during isolation of Pol d 4 suggest that two to four of these sites may be glycosylated. When compared for identity, Pol d 4 and Api m 7 are 41.5% identical; Pol d 4 and the bumble bee serine protease are 31.8% identical; and the honey bee and bumble bee proteases are 33.1% identical (Winningham *et al*., 2004).

The serine protease from another species of the European paper wasps, *P. gallicus* (Pol g 4) has been isolated and studied. This molecule is approximately 33.5 kDa in size and the first sixteen amino acids of the N-terminus were sequenced (Pantera *et al.*, 2003).

The similarity between Pol d 4 and the N-terminal sequence of Pol g 4 (1-16) shows 92.3% sequence homology (Pantera *et al.*, 2003 and Winningham *et al.*, 2004). The ability of Pol g 4 to specifically bind IgE was assayed using the enzyme allergosorbent test (EAST) and showed that it may be a significant allergen in the venom of *P. gallicus*. RAST performed with Pol g 4 and Pol d 4 demonstrated that these allergens have the ability to bind approximately the same amount of IgE (Pantera *et al.*, 2003). Direct RAST with European patient sera showed more IgE bound the serine proteases from both species than to the Antigen 5 allergen.

The serine protease allergen has been isolated from venom of North American paper wasps *P. exclamans* (Pol e 4). Sera from American patients (sensitized to New World paper wasps) and European patients (sensitized to European and North African paper wasps) were used to assay the cross reactivity between Pol d 4 and Pol e 4. IgE from both groups of patient sera bound to serine protease allergens. RAST showed American patient sera bound more to Antigen 5 than to the serine protease. The IgE from European patients bound both serine proteases during inhibition assays (Winningham *et al*., 2004). The pattern of IgE binding to the allergenic serine proteases suggests that there are shared epitopes. However, inhibition studies suggest species-specific epitopes may have been generated in individuals sensitized by Old World paper wasps. Both proteases have demonstrated the ability to bind benzamidine, suggesting the catalytic portion of both molecules is conserved (Hoffman *et al*., 1998 and Winningham, *et al*., 2003). Differences in amino acid sequence are most likely to occur in the loop framework of the serine protease.

1.7 Treatment of allergic reactions

Treatment for an allergic reaction to insect venom is prescribed by a physician based upon the severity of the patient's physical manifestations. Those individuals who have a normal reaction to the sting are treated with analgesics and cold compresses. Pharmacological treatment of a more severe reaction, resulting in a large local reaction, is used to control swelling and manage pain. Symptoms are managed with antihistamines and analgesics. Steroids may be used if the reaction is extremely wide-spread or disabling (Reisman, 2002).

Patients who experience anaphylaxis in response to an insect sting have short-and long-term treatment options. Immediate treatments can be performed with epinephrine and antihistamines. Antihistamines are taken to combat the effects of histamine delivered into the system. Patients with a severe allergic response have the option of venom immunotherapy. Immunotherapy is an effective means of treatment for patients with a severe reaction to venom allergens (Valentine, 1984 and Reisman and Livingston, 1992). This method involves the introduction of increasing amounts of allergen to patients over time to induce tolerance (Reisman, 2002). Treatment of insect allergies with immunotherapy is approximately 90% effective. The allergens used in this treatment are generally obtained as venom extracts, and the mechanism behind this particular treatment is not well understood.

The treatment of insect venom allergies with immunotherapy can pose some difficulties. The species of the sensitizing insect dictates the type of venom extract used in treatment. Identification of the causative insect can be complicated, some insect species can be mistaken for another or the sensitizing insect was not seen. The

sensitizing species can be determined by a variety of diagnostic assays. However, these assays may not be cost effective, typically resulting in a mix of venom extracts from representatives of stinging insect genera being prescribed. Recent studies show that treatment with venom from one species of one genus may not fully protect from the sting of a different species of the same genus. This could put the patient at risk for an anaphylactic episode when encountering their sensitizing species.

In the United States, the standard practice for immunotherapy is to use venom extracts; however, other countries are beginning to use allergens from alternate sources. The use of recombinant technology to produce large amounts of a given allergen has begun to gain popularity. Recombinant production of the allergen allows for clean samples and control over the exact amount of the identified allergen used in treatment. In addition, allergens can be mass produced, rendering it more cost effective. Recombinant technology also allows for manipulation of the allergen to reduce immunogenicity but still conferring protection, which could prove to be safer for the patient (Grammer, 2002).

1.8 Experimental objectives

The partial cross-reactivity observed between European and American paper wasp venom allergens affect the protection from immunotherapy among patients. There are four medically significant allergens in paper wasps venom and two of these have the potential to contain unique epitopes. These allergens are the venom hyaluronidase and serine protease. Studies have shown hyaluronidase is cross-reactive without having significant identity between venom allergens. This type of cross-reactivity may be due to post-translational carbohydrate and not amino acid epitopes. The lack of sequence identity between hyaluronidase may contribute unique amino acid epitopes. The serine

protease from European paper wasps has been shown to be cross-reactive with the serine protease allergen from American paper wasp venom. However, in the presence of antigen 5, the European paper wasp serine protease continues to bind IgE, while the American paper wasp serine protease IgE binding drops significantly. Characterizing allergenic proteins and their ability to bind IgE will lead to the production of safer and more effective treatments. The use of recombinant protein technology is being used to understand and improve patient treatment. Improvements in patient testing, will lead to more effective immunotherapy using the correct whole venom extract.

We hypothesize that the allergenic serine protease isolated from the venom of *P. dominulus* contains amino acid epitopes which contribute to the species-specific t immunologic response in patients. In order to address this question, the serine protease from *P. dominulus* was expressed in eukaryotic and prokaryotic expression systems. Probable epitopes were predicted and analyzed to produce mutants of Pol d 4. All recombinant proteases were assayed for their ability to bind IgE from sera obtained from patients sensitized to either European or North American paper wasps. Finally, the sequence of the allergenic serine protease from *P. gallicus* was characterized.

1.8.1 Expression of recombinant Pol d 4

The production and characterization of recombinant Pol d 4 is necessary to evaluate IgE binding to the European paper wasp serine protease allergen. Isolation of the native protein is difficult due to contaminating proteins and requires a great deal of time and materials. Using recombinant prokaryotic and eukaryotic expression systems a non-glycosylated and glycosylated form of Pol d 4 can be produced to study IgE binding.

The apparent cross-reactivity between Pol d 4 and Pol e 4 can be a result of two components. The first component may be the similarity between amino acid sequences of both molecules. The second component contributing to the cross-reactivity between these molecules are post-translational N-linked carbohydrates that are added to the proteins. In an effort to determine the effect of these components on IgE binding, the recombinant serine protease was produced in both eukaryotic and prokaryotic expression systems.

The investigation of Pol d 4 in IgE binding is complicated by the incomplete purification of native proteins. Amounts of other allergens, as small as 0.1-1%, can generate significant IgE binding. Several researchers have addressed this by producing recombinant forms of various allergens. Typically, these recombinant proteins are produced in insect cell lines using baculovirus expression systems. These systems produce the native conformation of the protein and add post-translational glycosylation. Sf9 and High Five™ cell lines were evaluated for expression of Pol d 4.

The High Five™ insect cell line was chosen for expression due to two factors. The first factor is the ability of the High Five™ cells to add certain post-translational carbohydrates. The CCD, an important epitope in insect allergens, is mostly composed of an α 1,3 fucose core (Hemmer *et al.*, 2001 Hemmer, *et al*., 2004, and Bencurova *et al.*, 2004). The Sf9 cells appear to lack fucosylase function; however, the High Five™ cells have retained this function (Straudacher, 1992). The Sf9 and High Five™ insect cells lines were evaluated for the type of carbohydrates that are added during glycosylation. Based on this information, the High Five™ cells were chosen for protein production.

The second type of epitope that may be responsible for IgE binding to Pol d 4 involves post-translational glycosylation. As previously discussed, post-translational glycosylation can contribute to cross-reactivity between venom allergens. Examination of the cross-reactivity between Pol e 4 and Pol d 4 suggests that CCD may be involved. Data shows binding of American and European IgE to both Pol e 4 and Pol d 4. Some of the apparent cross-reactivity between the Old World and New World paper wasp allergenic serine proteases may be a result of the carbohydrate present on the molecules.

In order to facilitate understanding of the binding of venom-specific IgE to the carbohydrate on Pol d 4, a recombinant protein was produced in a prokaryotic expression system. This type of system allows for the production of a recombinant protein which possesses a uniform lack of carbohydrate. Epitopes integral to the allergic reaction are typically conformation dependent. Considering this information, attempts were made to fold the recombinant protein into its proper conformation. These proteins were tested for IgE binding by probing with sera obtained from individuals sensitized to Old World paper wasps.

1.8.2 Mutational analysis of Pol d 4

Amino acid epitopes may contribute to the species-specific immune response between Old World and New World paper wasp venoms. This type of antigenic determinant would provide unique epitopes within the venom which may play a role in species-specific reactivity. Determining the major amino acid epitopes on Pol d 4 will further aid in understanding its role in species-specific IgE binding.

The major epitopes on Pol d 4 were identified and mutated to assay for IgE binding. These epitopes can be distinguished by various computational programs.

Epitopes were evaluated as functional epitopes. Specific amino acids in each epitope were mutated and expressed in the baculovirus system. These amino acids were mutated to a neutral alanine. After purification, proteins were assayed for IgE binding using European patient sera.

1.8.3 Analysis of IgE binding to recombinant Pol d 4

The contribution of Pol d 4 to the unique pool of allergenic epitopes can be determined by studying the binding of patient IgE to the potential epitopes found on recombinant forms of Pol d 4. The importance of post-translational glycosylation was evaluated by assaying IgE binding to recombinant Pol d 4 and inhibition studies with Pol d 4. The three mutant forms of Pol d 4 were used to assay potential major epitopes on the surface of Pol d 4.

1.8.4 Analysis of sequence homology among venom serine protease allergens

Data has shown that the allergenic venom serine proteases from *P. gallicus* and *P. dominulus* bind approximately the same amount of IgE (Sanchez *et al*., 1995 and Pantera *et al*., 2004). Further, investigators have shown that Pol g 4 is a major allergen in *P. gallicus* venom. This data is supported by the phylogenic relationship between insects and their allergen homology. The similarity in binding could be due to post-translational carbohydrates or common amino acid epitopes. In order to clarify the relatedness between Pol g 4 and Pol d 4, the nucleic acid sequence of the Pol g 4 allergen was isolated. This sequence will allow for characterization of Pol g 4 and allow for the comparison to Pol d 4. The data will show identity between the two molecules and cross-reactivity between the venom serine protease from *P. gallicus* and *P. dominulus*.

Chapter 2: MATERIALS AND METHODS

2.1 Expression systems

2.1.1 Eukaryotic expression: baculovirus expression system

The baculovirus expression system has been used extensively for the production of recombinant proteins from various organisms. This system has the advantage of being safe, easy to expand, and can express high levels of target protein. In addition to these attributes, this system is optimal for expression of an insect-derived protein because processing of the protein will be similar to that of the native molecule. Among the various baculovirus expression systems, the Bac-to-Bac® system, purchased from Invitrogen (Carlsbad, CA), was chosen for the expression of Pol d 4.

The Bac-to-Bac™ system utilizes the ability to generate recombinant virus by site-specific transposition (Ciccarone *et al*., 1997). A Tn7 transposition is used to insert the gene of interest into a bacmid DNA which is then propagated in *E. coli* (Luckow, 1993). Transposition events can be determined by blue/white colony selection, due to distribution of the lacZα gene during transposition. Host cells are then transfected with bacmid DNA for viral and protein production.

2.1.2 pFastBac™ 1

The pFastBac™ 1 vector (Anderson *et al*., 1996) (Invitrogen) was used for sub-cloning the gene of interest. This vector contains the sequence for the AcMNPV polyhedron promoter followed by a large multiple cloning site. The vector has a SV40 polyadenylation signal and genes for ampicillin and gentamicin resistance. The vector also contains Tn7 transposition sites which correspond to the target mini-attTn7 on the shuttle vector (bacmid) (Barry, 1988).

2.1.3 Insect cell lines

2.1.3.1 Sf9 insect cells

Transfection of recombinant baculovirus was performed using the Sf9 insect cell line (Invitrogen). These cells were derived from the *Spodoptera frugiperda* (fall army worm) pupal ovarian cell culture Sf21. They are adapted for growth in serum-free media and can be grown as semi-adherent monolayer or suspension cultures. Sf9 cells are optimized for transfections, plaque assays, viral production, and protein expression.

2.1.3.2 High Five™ insect cells

The insect cell line High Five™ (Invitrogen) was used for expression of recombinant proteins. This cell line was derived from ovarian cells isolated from the *Trichoplusia ni* (cabbage looper). This cell line was adapted for suspension cultures and growth in serum-free conditions. High Five™ cells have the capability to produce 5-10 fold more recombinant protein than Sf9 cells.

2.1.3.3 Insect cell care and storage

The Sf9 and High Five™ (Invitrogen) cell lines were maintained as suspension cultures. All cultures were continuously incubated at 27°C, shaking at 225 RPM in a Lab-Line® incubator-shaker (Labline, Melrose Park, IL). The Sf9 cells were cultured in Sf-900™ II SFM (Gibco, Carlsbad, CA) containing 50 mg/L of gentamicin (Gibco). Express Five® SFM (Gibco) containing 50 mg/L of gentamicin and 2 mM L-glutamine (Gibco) was used to maintain the High Five™ cells.

Aliquots were stored in liquid nitrogen to re-establish cultures of Sf9 and High Five™ cells. The cellular aliquots contained 1 X 10^7 cells in 1 ml of storage media [45% conditioned media, 45% fresh SFM media, and 10% DMSO]. Conditioned media was

created by culturing cells in fresh media. Aliquots were frozen gradually at -20°C, then -80°C, and finally stored in liquid nitrogen.

2.1.4 Prokaryotic expression: pET expression system

The pET expression system (Novagen, San Diego, CA) is a large group of plasmids intended for use in the prokaryotic expression of target proteins (Studier and Moffatt, 1986, Rosenberg *et al*., 1987, and Studier *et al*., 1990). The gene of interest is cloned into an expression vector under the control of the bacteriophage T7 RNA polymerase. The vector is transformed into host cells with chromosomal T7 RNA polymerase under the control of lacUV5. This control allows the expression of the gene of interest to be silent until induction with IPTG (Stuier *et al*., 1990 and Dubendorff and Studier, 1991).

2.1.5 pET-17b vector

We used the pET-17b (Novagen) expression vector for the production of recombinant Pol d 4. This vector encodes for ampicillin resistance, N-terminal T7 tag, and a multiple cloning site (MCS) consisting of 15 restriction sites. Target genes were ligated into the vector using *Nde* 1 and *Bam* HI restriction enzymes on the 5´ and 3´ends respectively.

2.1.6 Prokaryotic cells

2.1.6.1 Rosetta-gami™ 2 *E. coli* cells

Rosetta-gami™ (DE3) competent *E. coli* cells (Novagen) were chosen for prokaryotic expression mainly for their ability to account for the codon bias between eukaryotes and prokaryotes. These cells were developed from two host strains. The first, Origami 2, are derived from K-12 *E. coli* cells and enhance the production of disulfide bridges (Bessette *et al*., 1999 and Prinz *et al*., 1997). The second strand is the Rosetta

cell line which is derived from BL21 *E. coli* cells (Phillips *et al*., 1984). The Rosetta contains tRNAs for seven of the rare codons in *E. coli*. Rosetta-gami™ cells carry IPTG-inducible T7 polymerase. These cells are chloramphenicol-resistant and kanamycin-sensitive.

2.1.6.2 Preparation of chemically competent cells

Chemically competent *E. coli* cells were made every six months to ensure optimum transformation. Single colonies from frozen stocks were used to inoculate 250 ml of SOB media [20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 0.5 g KCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$] then incubated on a shaker at 225 RPM at 18°C. Incubation continued until absorbance at OD_{600} was 0.6. The culture was placed on ice for 10 min., transferred to centrifuge bottles, and centrifuged for 10 min. at 2500 x g at 4°C. The supernatant was removed and the pellet was resuspended in 80 ml of ice-cold TB [10 mM HEPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, and 250 mM KCl]. After a 10 min. incubation on ice, the pellet was collected by centrifugation as previously described. The pellet was resuspended in 20 ml of TB, and DMSO was added to a final concentration of 7%. The cellular suspension was separated into 100 µl aliquots and frozen in liquid nitrogen. Stocks were maintained at -80°C.

2.2 Primer design

All primers are listed in Table 3. Primers were ordered from Invitrogen (Carlsbad, CA) and were desalted. Distilled water was used to rehydrate all primers to a working concentration of 400 ng/ml.

Table 3: A list of primers used for sub-cloning, insert verification, and DNA mutation. The nucleic acids are represented by one letter designations, adenine (A), Thymine (T), guanine (g) and Cysteine (C). The letter V represents A, G or C and N represents A, C, G or T.

Primer Name	Sequence	Purpose
HINDLD3´	GAGAAGCTTTTATGATGATGATGATGATGATCCGCCTTGCAGTATGTTTCTCC	Cloning 3´end for in baculovirus expression of GrPol d 4
BAMLD5´	GAGGGATCCATGAATTCTGGTAAAATTATTTTTATTATTTA	Cloning 5´end for in baculovirus expression of GrPol d 4
BAM3´	GAGGGATCCTTAATGATGATGATGATGATGATCCGCCTTGCAGTATGTTTCTCC	Cloning 3´end for in *E. coli* expression of NrPol d 41 and NrPol d 42
NDEPRO	GAGCATATGGAAGAAAATTGTAAATGTGGCTGGG	Cloning 5´end for in *E. coli* expression of NrPol d 41
PDPETMAT	GAGCATATGATAGTAAATGGTGTTGAAACAGAAA	Cloning 3´end for in *E. coli* expression of NrPol d 42
PFASTFWD	ATTCATACCGTCCCACCATC	Sequencing the pFastbac 1 expression vector
PFASTREV	CCTCTACAAATGTGGTATGGC	Sequencing the pFastbac 1 expression vector
BVREV	AGCGGATAACAATTTCACACA	PCR verification of viral transposition
BVFWD	CCCAGTCACGACGTTGTAAAA	PCR verification of viral transposition
MUT 1	GCAATTGTTAAAACAAATGAAAGATTTGCATATTCGATGGCAGTTGGACCAGTTTTG	Mutant 1 forward primer
MUT 1 ANT	CGTTAACAATTTTGTTTACTTTCTAAACGTATAAGCTACCGTCAACCTGGTCAAACAG	Mutant 1 reverse primer
MUT 2	GGTTGGGGTAAATTGCGTATAATGGTC	Mutant 2 forward primer
MUT 2 ANT	CCAACCCCATTTAATCGCATATTACCAG	Mutant 2 reverse primer
MUT 3	CGAGAATAGTAAATGGTGTTGCAACAGAAATAAATGAATTTCCCATGGTGGCAC	Mutant 2 forward primer second set of primers
MUT 3 ANT	GCTCTTATCATTTACCACAACGTTGTCTTTATTTACTTAAAGGGTACCACCGTG	Mutant 2 reverse primer second set of primers
MUT 4	GGGTCACCAGTTTTATGGCATTTATTGCTAACG CTACAATCGGAG	Mutant 3 forward primer
MUT 4 ANT	CCCAGTGGTCAAAATACCGTAAATAACGATTGCGATGTTAGCCTC	Mutant 3 forward primer
CDS Primer	TTTTTTTTTTTTTTTTTTTTTTTTVN	cDNA generation primer
FWDPRO	GAGGAAGAAAATTGTAAATGTGGC	Pol g 4 forward cloning primer
3´GENOMIC	GAGCCTCTTTGTATCAGTTCCGCCTAA	Pol G 4 reverse cloning primer

2.2.1 Design of primers for cloning

Primers were designed for sub-cloning and used in both the eukaryotic and prokaryotic expression systems in a similar manner. All primers were designed to have a melting temperature (T_m) of approximately 65°C. A GAG priming sequence was added to the 5´ end and the 3` primer included a hexa-histidine sequence prior to the stop codon.

Primers were engineered with restriction sites to facilitate ligation into the multiple cloning site (MCS) of the expression vector. These included a *Hind* III restriction site on the 5` end primer prior to the DNA sequence and a *Bam* HI restriction site after the stop codon on the 3` end primer. Primers designed for prokaryotic expression were designed for a *Bam* HI restriction sequence on the 5` end primer and a *Nde* I sequence on the 3` end primer.

The eukaryotic primers were designed to begin at the first methionine of the leader sequence isolated from Pol d 4. A second set of eukaryotic expression primers contained the leader sequence for Sol i 2. The primer for this construct was designed to include the Sol i 2 leader sequence and began at the propeptide sequence for Pol d 4 (amino acid 19).

Primers for expression in the prokaryotic expression system were designed to begin amplification at different areas of the Pol d 4 gene. The first 5´ primer began at the Pol d 4 leader sequence (amino acid 19) and the second 5´ primer began with the Pol d 4 mature molecule (amino acid 40).

2.2.2 Primer design for recombination virus verification

Primers were designed to verify the transposition of the gene of interest into the pFastBac™1 vector. Sequences were chosen downstream and upstream of the mini-

attTn7 transposition sites. The forward primer was designed 130 bp upstream of the insertion site, while the reverse primer was 150 bp downstream of the insertion site. Both primers were designed to have a Tm of approximately 68°C.

2.3 Polymerase chain reaction

2.3.1 Cloning reactions

Polymerase chain reaction (PCR) was used for amplification of the construct using Platinum® *Taq* DNA polymerase (Invitrogen). The following reaction mix was used: 1 µl template DNA (900 ng/µl), 5 µl 10X High Fidelity PCR buffer, 1 µl 10 mM dNTP (Invitrogen), 1.5 µl 50 mM $MgCl_2$ (Invitrogen), 1.2 µl of each primer, 38.5 µl sterile water, and 0.5 µl Platinum® *Taq* DNA Polymerase. The reaction mix was overlayed with 50 µl mineral oil. PCR amplifications were performed on a MiniCycler™ (MJ Research, Ramsey, MI). Reactions were cycled 40 times using the following parameters: sample was denatured by heating to 94°C for 5 min., annealing was done at 55°C for 45 sec., extension occurred at 68°C for 7 min., followed by a final 10 min. annealing step at 72°C after the last cycle. An adenine overhang was added to PCR products for ligation into a TOPO® TA Cloning® vector (Invitrogen). This was done by adding 0.5 µl *Taq* DNA polymerase (Invitrogen) to the PCR mix and incubating for 10 min. at 72°C.

2.3.2 Transposition and recombinant virus verification

The transposition of the proper vector:gene construct was verified by PCR. The same procedure was used to verify the production of recombinant baculovirus. A 50 µl reaction was set up using *Taq* DNA polymerase with final concentrations of the following reagents: 1X PCR buffer [200 mM Tris-HCl (pH 8.4), and 500 mM KCl], 0.1

mM dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.5 ng template, and 5 units *Taq* DNA polymerase. Reactions were denatured for 3 min. at 93°C then cycled 40 times using the following parameters: 94°C for 45 sec., 55°C for 55 sec., and 72°C for 5 min. A final annealing of 72°C for 7 min. was performed.

2.3.3 Agarose gel electrophoresis

PCR products were visualized by agarose gel electrophoresis using an Electrophoresis Systems Mini-Horizontal Unit (FB-SB-710, FisherBiotech (Pittsburgh, PA)). Products were separated on a 0.8% agarose gel. Gels were prepared by dissolving 0.4 g Agarose (Invitrogen) in 50 ml 1X TBE [1 M Tris base, 0.5 M boric acid, and 2 mM EDTA], once dissolved, 5 µl 10 mM ethidium bromide (EtBr) was added. The agarose was allowed to solidify at room temperature. PCR products were prepared for electrophoresis by adding 5 µl agarose gel loading buffer [20% Ficoll 400, 0.1 M ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), and 0.25% Bromophenol blue]. A 100 bp ladder (Invitrogen) was prepared for electrophoresis by combining 1.5 µl ladder with 5 µl agarose loading buffer and 5 µl phosphate buffered saline (PBS). Samples were loaded onto the gel and electrophoresed for 45 min. at 200V. Gels were visualized with the ChemiImager™ Ready System (Alpha Innotech Corporation, San Leandro, CA).

2.4 Baculovirus expression

2.4.1 DNA extraction

PCR products were extracted from agarose gels using the MinElute® Fel Extraction Kit (Qiagen, Valencia, CA). The band corresponding to the estimated size of the construct was excised using a sterile razor blade, weighed, and placed in a 1.5 ml

microcentrifuge tube. QG buffer was added at three times the volume of the sectioned agarose sample and incubated at 50°C until the agarose was completely dissolved. One volume of isopropanol was added and then the contents were mixed. The DNA solution was transferred to a MinElute® Spin Column and microcentrifuged for 30 sec. at 13,200 x g. The flow-through was discarded, the column was cleaned by adding 750 µl of PE buffer, and centrifuged as before. The flow-through was discarded. The column was centrifuged again to remove residual ethanol. The column was transferred to a fresh 1.5 microcentrifuge tube, and 50 µl sterile water was applied to elute the DNA. The sample was concentrated to 5 µl using a CentriVap Vacuum Concentrator (Labconco, Kansas City, MO).

The clone was ligated into the TOPO® TA cloning vector (Invitrogen) for amplification and sequencing purposes. The following were added to a 1.5 ml microcentrifuge tube: 3 µl concentrated PCR product, 1 µl undiluted salt solution [1.2 M NaCl and 0.06 M $MgCl_2$], and 1 µl TOPO® vector followed by incubation at room temperature for 15 min.

2.4.2 Transformations

The ligation product was transformed into chemically competent XL-2 Blue *E. coli* cells (Stratagene). Cells were stored in 100 µl aliquots at -80°C. Three microliters of ligation product was added to cells and then incubated on ice for 30 min. The reaction was heat shocked for 30 sec. at 42°C. Transformations were incubated on ice for 2 min. followed by the addition of then 400 µl pre-warmed SOC (Invitrogen). Cells were incubated at 37°C for 1 hr. while shaking at 225 RPM. The transformation reaction was plated on LB + AMP plates [1.0% Tryptone, 0.5% Yeast extract, 1.0% NaCl, pH 7.0, and

1 mg/ml Ampicillin] containing 20 mg/ml IPTG (Invitrogen) and 50 mg/ml BluoGal (Invitrogen). Plates were incubated overnight at 37°C. Positive colonies (white) were used to inoculate 10 ml of LB + AMP media, and then incubated overnight at 37°C while shaking at 225 RPM.

2.4.3 Plasmid DNA isolation

Isolation of plasmid DNA was performed using the QIAprep Spin Miniprep kit from Qiagen following manufacturer's instructions. Cells were pelleted by centrifugation at 3,000 x g for 15 min. The supernatant was discarded and the pellet was allowed to air dry for approximately 2 min. The pellet was resuspended in 250 µl P1 buffer (with RNase A) and transferred to a 1.5 ml microcentrifuge tube. Cells were lysed by adding 250 µl P2 buffer and mixed gently. The solution was neutralized by adding 350 µl N3 buffer. The isolation reaction was centrifuged at 14,000 x g for 15 min. at 4°C. The supernatant was transferred to a QIAprep spin miniprep column containing 500 µl PB buffer. DNA was collected on the membrane by centrifugation at 13,200 x g for 30 sec. The column was washed with 750 µl PE buffer. A dry spin was performed, the column was transferred to a fresh 1.5 ml microcentrifuge tube, and DNA was eluted from the membrane with 50 µl sterile water. Colonies were screened by restriction digest with *EcoR* I. Each restriction digest had 10 µl isolated plasmid DNA, 2 µl REact® 3 buffer, 7 µl sterile water, and 1 µl *EcoR* I (Invitrogen). The reaction was incubated for 3 hrs. at 37°C and then analyzed by agarose gel electrophoresis, as previously described.

2.4.4 Nucleic acid sequencing

Verification of sequence integrity was done by nucleic acid sequencing. The Core Sequencing Facility at East Carolina University and University of Tennessee,

Knoxville were used to perform this service. The M13 forward and M13 reverse sequencing primers (Invitrogen) were used. Sequencing data was aligned and analyzed using the GCG (Genetics Computer Group) Software Package.

2.4.5 Verification of gene ligation

Clones verified for sequence integrity, were removed from the TOPO® vector and ligated into the pFastBac™ donor plasmid (Invitrogen, Carlsbad, CA). The PCR product in the TOPO® TA vector was extracted by restriction digestion with *Bam* HI and *Hind* III (Invitrogen). The same digest was performed on the pFastBac™ vector in preparation for ligation. In a 1.5 ml microcentrifuge tube, 10 µl isolated plasmid DNA or vector was added to 2 µl REact® 2 buffer, 6 µl sterile water, 1 µl *Bam* HI and 1 µl *Hind* III. The reactions were incubated at 37°C for 24 hrs. The pFastBac™ plasmid was phosphatase treated by adding 1 µl thermosensitive alkaline phosphatase (Gibco), incubated at 37°C for one hr., then a final incubation for 10 min. at 70°C. The restriction digestion of the clone and pFastBac™ were electrophoresed on a 0.6% agarose gel and the correct bands were eluted using the MinElute kit as previously described. Once eluted, the pFastBac™ vector and insert were concentrated to 30 µl and 5 µl, respectively.

Ligation of the insert into the pFastBac™ vector was mediated by T4 DNA Ligase (Invitrogen). The ligation reaction consisted of 3 µl concentrated insert, 1 µl concentrated pFastBac™ vector, 1 µl T4 DNA ligase buffer, and 2 µl T4 DNA ligase. The reaction was incubated for 24 hrs. at 15°C. The XL-2 Blue *E. coli* cells were transformed with the ligation reaction, plated, and plasmids were screened by digestion with *Bam* HI and *Hind* III, as previously described. Clones were sequenced with the

PFASTFWD and PFASTREV primers (Table 3). Sequences were evaluated with the GCG software package.

2.4.6 Transformation of DH10Bac™ *E. coli* cells

The pFastBac™ system required the transformation of DH10Bac™ chemically competent *E. coli* cells using the donor plasmid (containing the gene of interest) to produce a recombinant bacmid. After transformation into the XL-2 Blue *E. coli* cells, the pFastBac™ with the gene of interest was isolated with the QIAprep Spin Minprep kit. This plasmid was then used to transform DH10Bac™ cells, as previously described using 1 µl isolated plasmid DNA. Transformations were plated on DH10Bac™ LB plates containing 50 µl IPTG (20 mg/ml) and 50 µl Bluo-Gal (50 mg/ml). Plates were incubated at 37°C for 48 hrs. Positive colonies were streaked to isolate single colonies. DH10Bac™ media was inoculated with isolated colonies and grown overnight at 37°C shaking at 225 RPM. Cellular stocks were frozen at -80°C with 25% glycerol.

The correct transposition event was analyzed using PCR. Bacmid specific primers, BVREV and BVFWD (Table 3), were used with *Taq* polymerase under the following cycling conditions: DNA was denatured at 94°C for 1 min.; annealing of primers for 45 sec. at 55°C, elongation of DNA for 2 min. at 68°C, the reaction was cycled 30 times, followed by a final elongation step at 72°C for 10 min. PCR products were visualized using agarose gel electrophoresis (0.6% gel) as previously described.

2.4.7 Bacmid isolation

The transposition of the gene of interest was verified by PCR, the bacmid was isolated for transfection. A 2 ml culture of DH10Bac™ media was infected with 5 µl frozen cell stock containing the construct. Cultures were incubated overnight at 37°C

shaking at 225 RPM. Cells were pelleted by centrifugation for 15 min. at 3,000 x g and the supernatant was aspirated. The pellet was spun as before and the remaining supernatant was removed. The pellet was resuspended in 300 µl Solution I [50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 100 µg/ml RNase A] and transferred to a fresh 1.5 ml microcentrifuge tube. Cells were lysed by adding 300 µl Solution II [200 mM NaOH and 1% SDS], mixed, then incubated at room temperature for 10 min. The reaction was neutralized by the addition of 300 µl Solution III [3.0 M potassium acetate, pH 5.5]. Samples were centrifuged at 14,000 x g for 10 min. at 4°C after incubating on ice for 10 min. The supernatant was transferred to a new microcentrifuge tube and centrifuged once more to remove all insoluble material. The supernatant was added to 800 µl isopropanol and precipitated at -20°C for 16 hrs. The bacmid was collected by centrifugation at 14,000 x g for 5 min. The supernatant was discarded, the pellet was washed twice with 70% ethanol and allowed to air dry for approximately 5 min. The bacmid DNA was dissolved in 40 µl sterile water and stored at 4°C for use the next day.

2.4.8 Recombinant bacmid transfection

Viral production was facilitated by transfection of the recombinant bacmid into Sf9 insect cells. One day prior to transfection, 3 ml of log phase Sf9 cells, a total cell count of 2 x 10^6 cells, were plated in a 25 cm^2 cell culture flask (Corning, Corning, NY) and incubated at 27°C until cells reached 75% confluence. Five micrograms of isolated bacmid (in 5 µl) was added to 100 µl Sf-900™ II SFM media (Invitrogen) without antibiotics; and 6 µl of Cellfectin® reagent (Invitrogen) was added to 100 µl Sf-900™ II SFM media without antibiotics. Both solutions were combined and incubated at room temperature for 30 min. During incubation, plated cells were washed once with 2 ml Sf-

900™ SFM without antibiotics, and covered with 2 ml Sf-900™ II SFM media. Following incubation the lipid:bacmid solution was added to 2 ml Sf-900™ II media without antibiotics. The transfection mix was added dropwise to the cells and incubated for 5 hrs. at 27°C. The lipid:bacmid mixture was aspirated and replaced with 2 ml Sf-900™ SFM II media without antibiotics. Transfections were incubated for 7 days at 27°C, until cellular infection and death was detected.

After incubation, virus was harvested for further use. The media was removed from the cells and centrifuged at 800 x g for 5 min. The supernatant containing the recombinant virus was collected and 2% fetal bovine serum (FBS) was added to protect against protease activity. All stocks were wrapped with aluminum to protect from light degradation and stored at 4°C.

2.4.9 Isolation of viral DNA

The production of recombinant baculovirus was confirmed by the isolation of viral DNA and amplification with specific primers. All viral isolations were performed in duplicate. Debris was removed from 750 µl viral stock by microcentrifugation at 800 x g for 5 min. The supernatant was collected, combined with 750 µl 20% PEG [20% Polyethylene glycol in 1 M NaCl], and incubated at room temperature for 30 min. After incubation, the isolation was centrifuged at 13,200 x g for 10 min. The supernatant was discarded and the pellet was air dried for approximately 10 min. The pellet was resuspended in 100 µl sterile water and proteins were degraded by adding 5 µl Proteinase K (20 mg/ml), and incubating at 50°C for 1 hr. Proteins and nucleic acids were separated by adding 105 µl PCI [phenol:choloroform:isoamyl alcohol; 24:24:1] extraction buffer to the viral sample. Isolations were centrifuged at 13,000 x g for 10

min. The supernatant was transferred to a tube containing 10 µl 3 M sodium acetate, 5 µl of 2 mg/ml glycogen, and 300 µl 100% ethanol. Viral DNA was precipitated overnight at -20°C. The precipitated DNA was centrifuged at 14,000 x g for 15 min. at 4°C. The pellet was washed with 500 µl 70% ethanol, allowed to air dry for approximately 5 min., and resuspended in 10 µl sterile water.

Viral isolation products were amplified by PCR to verify the presence of the gene of interest. The reaction mix contained the following: 1 µl BVREV primer, 1 µl BVFWD primer, 1 µl 10 mM dNTP, 5 µl 10X PCR buffer, 2 µl 50 mM $MgCl_2$, 34.5 µl sterile water, and 0.5 µl *Taq* polymerase. The same cycling parameters as the bacmid verification were used for identification of the presence of recombinant baculovirus.

2.4.10 Amplification of recombinant baculovirus

In order to obtain enough virus for protein expression, the virus was propagated by several rounds of infection. P2 viral stock was made by infecting 3 ml of log phase Sf9 cells, 2 x 10^6 cells/ml, with 250 µl of the initial P1 stock the initial recombinant transfection. The culture was incubated for 7 days at 27°C. The P2 viral stock was harvested as previously described. P2 viral stock was used to generate high titer stocks with log phase Sf9 cells in suspension, these cultures were inoculated with 100 µl P2 viral stock for every 100 ml of culture. High titer stock was harvested on day seven and stored as previously described in recombinant bacmid transfection.

2.4.11 Small scale expression

Optimum expression conditions were established with small scale expression cultures. An initial 20 ml Sf9 suspension cultures were set up with a cell density of 2 x 10^6 cells/ml in Sf-900™ II SFM media. These cultures were inoculated with 2 ml high

titer viral stock. The expression cultures were incubated for 7 days, with 5 ml samples taken on days 4, 5, and 6; with the remaining culture being harvested on day 7. Each sample was centrifuged at 800 x g for 5 min. The supernatant and cell pellet were stored at -20°C until analyzed.

2.4.12 Expression optimization

After production of Pol d 4 was verified, the expression culture parameters were optimized. To establish the appropriate amount of inoculum for protein production, various amounts of virus (1, 2, and 3 ml) were added to 20 ml Sf9 cultures. Cultures were harvested on day 4 and purified. Once the appropriate incubation time and viral concentration was determined two insect cell lines, Sf9 and High Five™, were inoculated with 3 ml of virus and harvested on day 4. All purification products were analyzed by SDS-PAGE and Western blotting.

2.4.13 Purification of recombinant proteins

Protein production in these initial cultures was assayed by purifying recombinant proteins from expression supernatants and cell pellets by metal affinity chromatography. Talon® metal affinity resin (Clontech, Mountain View, CA) was used as the stationary phase.

2.4.13.1 Small scale purification

The supernatant was dialyzed using SnakeSkin pleated dialysis tubing (Pierce, Rockford, IL) with a molecular weight cutoff of 7,000 Da. The dialysis buffer [50 mM Tris-HCl and 0.5 M NaCl, pH 8.0] was changed 5 times over a 24 hr. period. Cell pellets were thawed at room temperature and 1 ml of 0.7% SDS was added. The pellet was resuspended by vortexing, centrifuged at 14,000 x g for 5 min., and the supernatant was

collected. The Talon® resin for supernatant and pellet purification was equilibrated with the appropriate buffer, either dialysis buffer or SDS buffer, respectively. A bed volume of 50 µl Talon® was equilibrated with 3 ml of buffer by centrifuged for 3 min. at 800 x g. Samples were combined with the resin and incubated at room temperature for 15 min. under agitation. Samples were centrifuged at 800 x g for 3 min. and the supernatant was collected. The resin was washed twice with 1 ml of the appropriate buffer, centrifuged as before, and the supernatant was collected. Elution was performed by adding 60 µl 0.1 M EDTA to the resin and centrifuging.

2.4.13.2 Large scale purification

After expression conditions were optimized, large scale expression was performed using High Five™ insect cells. Expression cultures of 500 ml were inoculated with 50 ml high titer stock. Cultures were incubated for 4 days at 27°C, shaking at 225 RPM. Cultures were harvested by centrifugation at 10,000 x g for 5 min. and the supernatant was collected and filtered with a 0.22 µm polyethersulfone filter (Corning). After filtration, the supernatant was exchanged against dialysis buffer using SnakeSkin pleated dialysis tubing for 24 hrs. with 5 changes of the buffer. The dialysis product was concentrated to a volume of 30 ml with an Amicon 202 (Millipore, Bedford MA) concentrator using YM-10 Ultrafiltration Discs (Millipore). Purification of the recombinant protease was performed using Talon® metal affinity resin. A bed volume of 750 µl was equilibrated with 30 ml dialysis buffer by centrifugation at 800 x g for 5 min. After equilibration, the resin and concentrated supernatant were incubated together, agitating, at room temperature for 45 min. The resin was collected by centrifugation at 800 x g for 5 min. The supernatant was removed and the resin was resuspended in 10 ml

of dialysis buffer before being applied to a polystyrene gravity-flow column (Pierce). Columns were washed twice with 10 mM imidazole [10 mM imidazole, 50 mM Tris-HCl, and 0.5 M NaCl, pH 8.0]. Bound protein was eluted with 6 ml 250 mM imidazole [250 mM imidazole, 50 mM Tris-HCl, and 0.5 M NaCl, pH 8.0] in 1 ml fractions.

2.4.13.3 High performance liquid chromatography of expression products

The expression products from baculovirus expression were further purified by Cation- Exchange HPLC. Elution samples were concentrated to a total volume of 1 ml. The sample was centrifuged at 13,000 x g for 30 min. to remove debris. The sample was loaded onto a Mono S column (Pharmacia). Elution was performed with 0-80% of 1 M NaCl, with 0.05 M sodium acetate, pH 5.2, gradient at 1 ml/min over 65 min. using a Waters 600 Controller. OD_{280} of sample was determined using a Waters Tunable Absorbance Detector and 1 ml fractions were collected every min.

2.5 Verification of recombinant protein expression

2.5.1 Western blotting

2.5.1.1 SDS-PAGE protein separation

SDS-PAGE was used to determine size and purity of all products. Samples were diluted 1:1 with SDS loading buffer [29 mM Tris, pH 6.8, 32.5 mM glycerol, 3 mM SDS, 6 mM dithiothreitol, and 1.7 µM bromophenol blue] and heated at 95°C for 5 min. A Gold Precast 4-20% Tris/Glycine gradient gel (Lonza, Rockland, ME) was loaded onto M1651 700 vertical electrophoresis system (Bio-Rad) with SDS-PAGE electrophoresis buffer [25 mM Tris-HCl, 0.2 M glycine, and 3.5 mM SDS]. BenchMark® Pre-Stained Protein Ladder (Invitrogen) or PageRuler™ (Fermentas, Glen Burnie, MD) was used for size comparisons. Samples were loaded at a volume of 15 µl and electrophoresed for 45

min. at 175V. Once protein separation was complete, the gel was removed from the cassette and washed three times with 25 ml of distilled water while agitating gently. Samples were visualized by staining the gel with 50 ml of Imperial™ Protein Stain (Pierce) overnight. The stain was removed and the gel was destained by soaking in distilled water for two days, with frequent water changes.

2.5.1.2 Protein transfer to PVDF

Western blotting was an initial assay for recombinant protein production in both expression systems. Blots were probed with an Anti-His (C Term) Antibody specific antibody (Invitrogen). The samples were separated on SDS-PAGE as previously described. Protein transfer to PVDF membrane was performed using a Multiphor II Electrophoresis System (Pharmacia Biotechnology, LKB Bromma). The PVDF membrane was soaked in methanol, followed by a 5 min. incubation in water and then placed in transfer buffer [39 mM glycine, 48 mM Tris-HCl, 0.0375% SDS, and 20% methanol]. Whatman filters (18) were soaked in transfer buffer until fully saturated. Nine of the filters were carefully placed onto the anode. The PVDF membrane was placed on top of the filters followed by the gel and finally the last nine filters. The cathode was placed onto the stack and the gel was transferred at 40V for 50 min.

Once transfer was completed the membrane was blocked overnight with 5% BSA in TBS [25 mM Tris-HCl and 0.5 M NaCl, pH 8.0]. The membrane was washed twice with 15 ml of TBS for 10 min. while agitating gently. The PVDF membrane was incubated for four hours with the anti-6 his C terminal antibody diluted 1:5000 in 1% BSA in TBS, pH 8.0. The membrane was washed twice with TBST [TBS and 0.02% Tween 20, pH 8.0] while agitating for 10 min. The secondary antibody, anti-mouse IgG-

alkaline phosphatase conjugate (Sigma), was diluted 1:5000 in 1% BSA in TBS. The membrane was incubated with the secondary antibody for 2 hrs., then washed twice for 10 min. with TBST. The blot was developed with BCIP/NBT from Sigma. The BCIP/NBT tablet was dissolved in 10 ml of sterile water and applied to the membrane for approximately 5 min. or until development was complete.

2.5.2 MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry analysis was used to confirm production of recombinant proteins in both expression systems. Molecular weights of intact proteins were determined. The production of recombinant protein was verified by analyzing the tryptic digestion products and comparing tryptic peptide sizes against the predicted map.

2.5.2.1 Preparation of intact protein

Whole protein and trypsin digestion products were analyzed to verify protein identity. MALDI-TOF was performed on an Applied Biosystems Voyager-DE™ PRO Biospectrometry Workstation (Foster City, CA). All data was collected using the manufacturer's software, Voyager V5.1.

Intact protein samples were analyzed using the ProteoMass® Protein MALDI-MS Calibration Kit (Sigma, St. Louis, MO). Matrix was prepared by dissolving 10 mg matrix (3,5-Dimethoxy-4-hydroxycinnamic acid) in 1 ml 50% Acetonitrile (ANC) and 0.05% Trifluoroacetic acid (TFA) solution. Elution samples were purified using C4 ZipTips® (Millipore), pipette tips containing chromatographic media for sample purification and concentration. Samples were prepared in 2.5% TFA, for a final concentration of 0.1%-1.0%. ZipTips® were wetted three times with 10 µl acetonitrile, then equilibrated three times with 0.1% TFA in distilled water. The sample was loaded

onto the tip by aspirating and dispensing three to seven times. The column was washed five times with 10 µl 0.1% TFA in water. Protein was eluted with 75% acetonitrile and 0.1% TFA in 4 µl, the solution was aspirated and dispensed three times, then collected. Samples were prepared for MALDI-TOF by mixing 10 µl of the matrix solution with 4 µl of filtered protein. After mixing, 2 µl was spotted onto an Applied Biosystems sample plate and allowed to air dry at room temperature.

2.5.2.2 Preparation of tryptic digest and guanidination of recombinant proteins

A more in-depth analysis of recombinant proteins was done using the In-Solution Tryptic Digestion and Guanidation Kit (Pierce), allowing for an accurate identification of proteins by detecting peptides present after cleavage with trypsin. To a tube, 15 µl of digestion buffer [50 mM ammonium bicarbonate] and 1.5 µl of reducing buffer [100 mM tris (2-carboxyethyl) phosphine-HCl] were added to 10 µg sample protein, and the volume was adjusted to 27 µl with water. This was incubated for 5 min. at 95°C. After cooling, 3 µl of alkylating agent [100 mM iodoacetamide] was added and samples were incubated in the dark at room temperature for 20 min. Samples were digested by adding 1 µl of activated trypsin and incubated for 3 hrs at 37°C. An additional 1 µl of activated trypsin was added and incubation continued overnight at 37°C. The digest was split to produce guanidinated and un-guanidinated fractions. Guanidation was performed by adding 16 µl digestion products to 10 µl ammonium hydroxide and 6 µl 0.6 mg/µl O-methylisourea hemisulfate, then incubated at 65°C for 12 min. The reaction was stopped by adding 3 µl TFA. Fractions were purified with C18 ZipTips®. The conditions for whole protein preparation were used as previously described, with the exception of the elution buffer, peptides were eluted with 70% acetonitrile/0.1% TFA. Preparation for

MALDI-TOF continued as previously described for whole protein assays except the matrix was alpha-cyano-4-hydroxycinnamic acid. Generated fragment sizes were used in database searches for protein homology using the MASCOT program (www.matrixscience.com).

2.6 Prokaryotic expression

2.6.1 DNA isolation and ligation

The product from sub-cloning recombinant baculovirus expressed Pol d 4 was used as a template for expression in the *E. coli* system. Two clones were generated for expression. A common 3´ primer, BAM3´ (Table 3) incorporated a *Bam* HI restriction site and a hexa-histidine tag. The 5´ primer for the first construct was designed to begin with the propeptide region and included a *Nde* I restriction site. The 5´ primer for the second construct was designed at the beginning of the mature molecule and included a *Nde* I restriction site. The clones were amplified by PCR under the same conditions described for baculovirus expression. The PCR product was treated similarly to the baculovirus clone: ligated into the TOPO® TA vector, sequenced, and verified with GCG. Confirmed clones were ligated into the pET-17b expression vector. The ligation reaction was performed under the same conditions previously described for baculovirus expression. Selected colonies were screened with restriction digestion using *Nde* I and *Bam* HI.

2.6.2 Transformation into Rosetta Gami *E. coli* cells

Transformations were performed as previously described in baculovirus expression, using 1 µl of ligation. The transformations were plated onto LB + AMP + Chlor [1.0% Tryptone, 0.5% Yeast extract, 1.0% NaCl, pH 7.5, 34 µg/ml

chloramphenicol, 50 µg/ml ampicillin]and incubated overnight at 37°C. Screening of single colonies was done by restriction digestion with *Nde* I and *Bam* HI.

2.6.3 Expression in Rosetta-Gami™ *E. coli* Cells

Positive colonies were used to inoculate 10 ml of LB + Amp + Chlor and incubated overnight at 37°C, shaking at 225 RPM. The cultures were diluted in 240 ml pre-warmed LB + Amp + Chlor media and incubated at 37°C while shaking at 225 RPM until OD_{600} was approximately 0.6. IPTG was added to a final concentration of 0.4 mM and the culture continued to incubate for 3 hrs.

2.6.4 Purification of recombinant proteins from prokaryotic expression

Purification of the Pol d 4 expressed in *E. coli* is based on a protocol described by King *et al*, (1996). This procedure utilizes a denaturing condition, incubation at high temperature, and metal affinity chromatography to isolate and purify the recombinant proteins. After incubation, cells were pelleted by centrifugation at 5,000 x g for 5 min. The pellet was washed with 10 ml wash buffer [0.05 M tris-HCl, 0.1 M NaCl, and 1 mM EDTA] and centrifuged at 5,000 x g for 5 min. After washing, the pellet was resuspended in dissolve buffer [6 M guanidine, 0.1 M tris-HCl, and 5 mM 2-mercaptoethanol] and incubated for 5 min. at 60°C. The clarified protein was collected by centrifugation at 11,000 x g for 20 min. at 4°C. A 1 ml bed volume of TALON® was equilibrated with dissolve buffer, 14 ml were added to the resin then centrifuged at 800 x g for 5 min. Once equilibrated, the resin was added to the clarified protein and incubated for 45 min. at room temperature while agitating. The resin was collected by application to a polystyrene gravity-flow column. The resin was washed twice with 5 ml wash buffer [6 M urea, 50 mM tris-HCl, and 0.1 M NaCl, pH 8.0], and twice with 5 ml 5 mM

imidazole [5 mM imidazole, 6 M urea, 50 mM tris-HCl, and 0.1 M NaCl, pH 8.0]. Bound protein was eluted with 6 ml 250 mM imidazole [250 mM imidazole, 6 M urea, 50 mM tris-HCl, and 0.1 M NaCl, pH 8.0) in 1 ml fractions. Identification of the recombinant protein was performed by SDS-PAGE, Western blotting, and MALDI-TOF.

2.6.5 Protein refolding

The denaturing conditions used in purification produced unfolded recombinant protein. Several methods of refolding were used and correct folding of the recombinant protease was verified using a Benzamidine column.

The first method of refolding used the dialysis of elution products from purification against PBS for 24 hrs. at 4°C with 5 buffer changes. The second method of refolding involved exposure of elution products to decreasing concentrations of urea. The eluted recombinant protease, 3 ml, was dialyzed using SnakeSkin Pleated Dialysis Tubing. Dialysis began in 6 M urea [6 M urea, 50 mM tris-HCl, and 0.1 M NaCl, pH 8.0] for 4 hrs., then the concentration of urea was decreased to 4 M [4 M urea, 50 mM tris-HCl, and 0.1 M NaCl, pH 8.0] and the sample was dialyzed for an additional 4 hrs. The dialysis buffer was changed again to 2 M urea [2 M urea, 50 mM tris-HCl, and 0.1 M NaCl, pH 8.0] and the sample was dialyzed for 4 hrs. The sample was then dialyzed overnight with dialysis buffer [50 mM tris-HCl and 0.1M NaCl, pH 8.0). Lastly, the sample was dialyzed for 4 hrs. in sterile water. The last method used the properties of redox reagents to facilitate the formation of disulfide bonds. Three samples, 3 ml of purification elution each, were dialyzed in Redox Dialysis Buffer [10% glycerol, 5 mM EDTA, 10 mM reduced glutathione, and 1 mM oxidized glutathione] for 12, 18, and 24 hrs at 4°C.

2.6.5.1 Benzamidine purification

Benzamidine is a competitive inhibitor of trypsin, and was initially used to isolate Pol d 4 from insect venom. Since it binds to the folded active site, Benzamidine was used to verify proper folding of the *E. coli* expressed recombinant protein. A gravity-flow column was packed with 0.5 ml p-aminobenzamidine (Sigma). The resin was washed with 6 ml Borate Buffer [0.17 M boric acid and 0.13 M NaCl]. The sample was loaded onto the column and allowed to flow through. The column was washed with 3 ml Borate Buffer. Elution of bound protein was performed with 2 ml of benzamidine Elution Buffer [0.1 M benzamidine, 0.17 M boric acid, and 0.13 M NaCl], collected in 1 ml fractions. Elution fractions were dialyzed overnight against distilled water to remove benzamidine before samples could be used for analysis (Winningham *et al*., 2004).

2.7 Mutational analysis

2.7.1 Identification of major epitopes

Little information is known about the structure of insect serine proteases. There is no known crystal structure for this family of molecules from insects. Further, information about IgE binding to the allergenic serine protease is scarce. Therefore, to better understand binding of IgE to the allergenic serine protease, major amino acid epitopes were computational predicted and mutated to assay IgE binding.

Epitopes selected for mutation were chosen from information compiled from three sources, increasing the probability of correctly predicting the major epitopes. The Pol d 4 sequence was up-loaded onto two prediction servers. The Conformational Epitope Prediction (CEP) Server (http://bioinfo.ernet.in/cep.html) was used to predict the possible allergenic epitopes (Kulkarni-Kale, 2005). The InterProSurf:Protein Protein Interaction

Server (IPI) (http://curie.utmb.edu) was used to determine residues that could be involved in protein-to-protein interaction (Negi *et al*., 2006). The mature molecule sequence was used in SWISS-MODEL (http://swissmodel.expasy.org provided by Swiss Institute of Bioinformatics Biozentrum) to produce a working model of Pol d 4. This model was based on known existing structures that had a 36-48% homology with Pol d 4. The model was used with the SPDBV (http://spdbv.vitel-it.ch/) program to generate a charge distribution map. Epitopes generated from CEPS were compared with residues predicted by the IPI server for commonality. The shared residues were located for position on the residue charge map. Epitopes are typically areas that are protrudent and have an isolated charge. Epitopes which had residues that were generated by CEPS and the IPI server, and met the epitope criteria were chosen for mutation. In total of six residues were chosen for mutation among four epitopes. A neutral alanine residue was substituted into the chosen sites.

2.7.2 Mutation of Pol d 4

The mutation of Pol d 4 was carried out using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene). All mutations were performed on the construct produced for baculovirus expression.

2.7.2.1 Primer design for DNA mutation

To ensure the correct length of the primers and the proper annealing temperature, mutation primers were designed using the QuikChange® Primer Design Program provided by Stratagene (Los Angeles, CA) on-line. Mutation primers (Table 3) were 5´ phosphorylated and cartridge purified (Invitrogen).

2.7.2.2 Mutation

Mutation reactions were performed using the QuikChange® Lightning Site Directed Mutagenesis Kit from Stratagene. This system utilizes PCR to introduce mutations into a target sequence with the use of specific primers. All mutation reactions were performed using thin-walled PCR tubes (Bio-Rad, Hercules, CA) and was run with a MyCycler™ (Bio-Rad). Reactions were set up as follows: 5 µl 10X QuikChange® Lightning Buffer, 50 ng DNA template, 100 ng appropriate primer (Table 3), 1 µl 10 mM dNTP, 1.5 µl QuikSolution™, 39.5 µl sterile water, and 1 µl QuikChange® Lightning Enzyme. The following conditions were cycled 18 times after a 2 min. denaturation at 95°C: 20 sec. at 95°C, annealing for 10 sec. at 60°C and extension at 68°C for 3.5 min. A final extension was held for 5 min. at 68°C.

Methylated DNA was digested with 2 µl *Dpn* I restriction enzyme at 37ºC for 5 min. XL10-Gold® Ultra-competent Cells (Stratagene) were transformed with the digest reaction. Forty five microliters of XL10-Gold cells were thawed on ice and placed into a polypropylene round bottom tube, and then 2 µl β-mercaptoethanol was added. Cells were incubated on ice for 2 min. and 2 µl of digested DNA was added. The transformation reaction was incubated on ice for 30 min. Cells were heat shocked at 45°C for 30 sec., then rested on ice for 2 min. The reaction was diluted with 400 µl of pre-warmed SOC and incubated at 37ºC for 1 hr., shaking at 225 RPM. Transformation products were plated on LB + Amp plates and incubated at 37°C overnight.

Possible mutants isolated from formed colonies were sequenced and aligned to verify mutations and reading frame. Mutant 3 required two rounds of mutation PCR because of the distance between the residues being mutated. The mutation of residue 135

was performed at an annealing temperature of 68°C. Mutants were expressed in the baculovirus system and purified as previously described.

2.8 Patient sera

Patient sera were acquired by Dr. P. Campi of Florence, Italy and Dr. M. Blanca of Malaga, Spain from individuals sensitized to European paper wasps. All samples were collected with informed consent. The use of previously collected deindentified sera from allergic patients in these studies has been determined to be exempt by the UMCIRB.

2.9 Immunoblotting

The ability of patient IgE to bind to recombinant proteins was assayed by immunoblotting. The purified products of baculovirus and *E. coli* expression, and a whole venom extract (Vespa Laboratories Inc., Spring Mills, PA) were used in each assay. The whole venom extract serves as a control for IgE binding. In preparation for blotting, the purified recombinant samples were concentrated 10 times and the whole venom extract was diluted 10 times. Samples were blotted by pipetting 1 µl onto 7 cm by 0.7 cm Transblot Transfer medium pure nitrocellulose membrane (Bio-Rad). A membrane was blotted for each patient sera assayed. Blots were blocked using NAP Blocker (G Biosciences, St. Louis, MO) diluted 1:2 with TBS for 2 hrs. at room temperature. After blocking, the membranes were washed three times with 20 ml of TBST [TBS + 0.005% Tween 20] for 5 min., agitating. Each blot was incubated with a different patient serum, horse serum or negative human serum control. The sera were diluted using 0.2 ml to 1 ml NAP Blocker (diluted 1:5 in TBS). The blots were incubated with sera overnight with continuous rocking at room temperature. The first wash after incubation was performed on each blot separately to avoid mixing of sera. Two

additional washes were done with TBST for 10 min., agitating. A human anti-IgE-streptavidin conjugate (Vector Laboratories, Burlingame, CA) was diluted 1:1,000 in NAP Blocker (1:5 in TBS), added to blots, and incubated for 5 hrs. at room temperature, rocking. The blots were washed three times as previously described. Biotinylated alkaline phosphatase (Vector Laboratories) was diluted 1:3,000 with NAP Blocker (1:5 in TBS) and incubated with nitrocellulose strips for 1.5 hrs. After washing three times, the blots were developed with BCIP/NBT (Sigma) as previously described.

2.9.1 Immunoblotting inhibition

The ability of patient IgE to bind carbohydrate was assayed with immunoblot inhibition assays. This assay was performed under the same conditions as immunoblotting. However, prior to incubation with the nitrocellulose membrane the patient sera were incubated with 1 mg/ml of bromelain (Sigma). Commercial bromelain is a combination of highly glycosylated cysteine proteases isolated from the fruit and stem of pineapple. Both cysteine proteases contain multiple CCD epitopes and are commonly used in the inhibition of CCD specific IgE.

2.9.2 Densitometry

The color intensity caused by the binding of IgE to the recombinant forms of Pol d 4 was measured by densitometry. Immunoblot strips were visualized using a Bio-Rad ChemiDoc™ XRS and measurements and calculations were performed with the Bio-Rad Quantity 1-D Analysis One Software 4.6.5. Measurements were done by selecting individual protein spots with the volume circle tool. The density was calculated with the volume calculation tool. Reports were exported to Microsoft® Excel.

2.9.3 Calculations

All calculations were performed using Microsoft® Excel. The density of the soluble inhibition immunospot were subtracted from the density of the recombinant Pol d 4 immunoblot protein spot on a immunoblot strip probed with the same sera. The resulting number was divided by the density of rPol d 4 and multiplied by 100. The standard deviation of replicates was calculated as a percentage of the values.

Significant differences in IgE binding between the various proteins used were calculated with one-way ANOVA using PAWS. Data obtained from the densitometric analysis of each immunoblot was used for analysis. Replicates were averaged before use in the statistical test. Data was analyzed in PAWS (predictive analysis software) (SPSS, Chicago, IL) using one-way ANOVA using a P=0.05. A Bonferroni corrections test was also performed to address the multiple comparisons.

2.9.4 Visualizing protein concentration on the immunoblots

In order to verify that the same amount of protein was used between each spot on the immunoblots proteins were blotted and then stained with protein stain. Protein was spotted onto a strip of nitrocellulose membrane as previously described in the immunoblotting protocol. The strip was immersed in Imperial™ Protein Stain (Pierce) for 10 min. The strip was rinsed with water overnight and allowed to dry at room temperature.

2.10 Characterization of the serine protease from *P. gallicus*

2.10.1 Specimens

P. gallicus samples were provided by Dr. Stephano Turillazzi from the University of Florence, Italy. Species were captured and placed in 100% Ethanol for shipment. All specimens were stored at -80°C.

2.10.2 RNA isolation

RNA was isolated using the NucleoSpin® RNA II Total RNA Isolation kit from Macherey-Nagel (Bethlehem, PA). In preparation for isolation, *P. gallicus* insects were centrifuged at 14,000 x g for 30 min. at 4°C. The supernatant was discarded and insects were washed with PBS for 5 min. and flash frozen in liquid nitrogen. The anterior portion of the gaster was excised with a sterile razor and placed in 15 ml Falcon tube containing 350 µl lysis Buffer RA1 and 3.5 µl β-mercaptoethanol. Samples were homogenized by adding half the volume of 0.5 mm of glass beads and vortexed in 10 sec. intervals for 2 min. The lysate was placed into a NucleoSpin® Filter Columns and centrifuged for 1 min. at 11,000 x g. The flow-through was prepared for column binding by adding 70% ethanol and mixed. The lysate was loaded onto a NucleoSpin® RNA II Column and centrifuged at 11,000 x g for 30 min. The membrane was desalted by adding 350 µl of Membrane Desalting Buffer (MDB) and centrifuging at 11,000 x g for 1 min. The DNase digestion mixture was prepared by combining 10 µl DNase I (Invitrogen) with 90 µl DNase Reaction Buffer. Ninety five microliters of the DNase reaction mixture was applied directly to the membrane and incubated at room temperature for 15 min. After incubation, 200 µl Wash Buffer RA2 was added to the column. The column was centrifuged at 11,000 x g for 30 sec. The column was washed by adding 600 µl RA3

Wash Buffer Concentrate and centrifuging at 11,000 x g for 30 sec. A second wash with 250 µl RA3 Wash Buffer Concentrate was centrifuged at 11,000 x g for 2 min. RNA was eluted by adding 50 µl of sterile water to the membrane and spinning at 11,000 x g for 30 sec., twice. Samples were concentrated to 25 µl as previously described.

2.10.3 cDNA synthesis

Total RNA isolated from *P. gallicus* was used to make cDNA with the SMART™ RACE cDNA Amplification Kit from Clontech. In a microcentrifuge tube, 1 µg total RNA was mixed with 1 µl 5' CDS primer, 1 µl SMART™ Oligo, and 2 µl water. The reaction was incubated at 70ºC for 4 min., cooled on ice for 2 min., and microcentrifuged at 14,000 x g for 2 min. The following reagents were added to the tube: 2 µl 5X 1st Strand Synthesis Buffer, 20 mM 1 µl DTT, 1 µl 10 mM dNTP mix, 1 µl anti-RNase (Ambion, Austin, TX), and 1 µl PowerScript™ Reverse Transcriptase (BD Bioscience). The solution was mixed, collected by centrifugation, and incubated for 1.5 hrs, at 42°C.

The cDNA reaction was purified using the MinElute® PCR Purification Kit from Qiagen. Five times the sample volume of Buffer PB was added to the mixture and then was transferred to a MinElute® column. cDNA was collected on the membrane by microcentrifugation at 14,000 x g for 30 sec. The column was washed with 750 µl Buffer PE. Remnants of ethanol were removed by microcentrifuging as before. DNA was eluted from the column with 50 µl water. The cDNA was concentrated to 25 µl as previously described.

2.10.4 Cloning and sequencing

The cDNA generated from total RNA isolated from *P. gallicus* was used to amplify the gene for the allergenic serine protease. Primers were designed from the

sequence of Pol d 4. The 5' end primer begins at the propeptide sequence and the 3' primer ended with the stop codon. Reactions were performed using Platinum® *Taq* DNA Polymerase (Invitrogen) and were set up as previously described.

Cycling conditions were altered to accommodate variability between the template and primers. After an initial incubation at 94ºC for 5 min. the following temperatures were cycled 40 times: 94°C for 1 min., 48°C for 5 sec., 58°C for 5 sec., 68°C for 45 sec., and 2 min. at 72°C. A final step at 72°C for 10 min. was performed.

The PCR products were evaluated by agarose gel electrophoresis. Bands corresponding to the calculated size of Pol g 4 were excised and ligated into the TOPO® TA Vector (Invitrogen) as previously described. Clones were screened by restriction digestion with *EcoR* 1 for an insert corresponding to the estimated size of Pol g 4. Plasmids were isolated, as previously described, from the appropriate clones were DNA sequenced.

2.10.5 Analysis of DNA sequences

Sequencing data was up-loaded to GCG (Genetics Computer Group) Software Package. Forward and reverse sequences were compared to verify the integrity of the sequence. The sequence was aligned with the previously cloned Pol d 4 sequence for identity and homology. A BLAST (Basic local search and alignment tool) search was performed via the NCBI (National Center for Biotechnology Information) internet server (http://www.ncbi.nlm.nih.gov/). Nucleotide blasts were performed with the "nucleotide blast" option and amino acid blasts were performed using "protein blast". The nucleotide collection (nr/nt) database was used for nucleotide blast searches and non-redundant protein sequence (nr) database was used for protein blast searches.

Chapter 3: RESULTS

3.1 Production of recombinant Pol d 4

Recombinant Pol d 4 was produced in several systems in order to investigate the epitopes involved in IgE binding. Obtaining natural protein from venom is costly and labor intensive. Eukaryotic expression of Pol d 4 allowed for the production and purification of recombinant protein. Data has shown that certain asparagine-linked carbohydrates have the ability to bind IgE. Protein production in insect cells creates an opportunity to have similar post-translational modifications to those found on native Pol d 4. Since the presence of carbohydrate may play an important role in the binding of IgE, Pol d 4 was also expressed in a prokaryotic expression system, which lacks carbohydrate moieties.

3.1.1 Eukaryotic expression of Pol d 4

The previously characterized Pol d 4 cDNA sequence was used for expression. The molecular structure was shown in Figure 3. The first 19 amino acids make up the leader sequence. This sequence is essential for cellular trafficking and final destination of the protein. Amino acids 20-34 are the propeptide region of the protease. This portion of the molecule maintains the serine protease in an inactive state (Devlin, 1997). The remaining amino acids (35-244) determine the structure of the serine protease and include essential regions for protease activity. The entire native sequence of Pol d 4 was cloned and expressed in the baculovirus expression system. The production of recombinant Pol d 4 was optimized and assayed for yield and purity.

Pol d 4 was cloned into the pFastBac™ expression vector with a hexa-histidine tag incorporated onto the N-terminus to facilitate purification. Nucleic acid sequencing was

Figure 3: Pol d 4 structure and function. Pol d 4 sequence is divided into three distinct regions based on function. The leader, propeptide, and mature molecule are shown. The leader sequence facilitates protein trafficking. The propeptide maintains the serine protease as an inactive zymogen. The mature molecule contains essential regions for serine protease activity.

Residues	Feature	Function
1 – 19	Leader	Protein Transport
20 - 34	Propeptide	Maintain the Serine Protease as a Zymogen
35 – 244	Mature Molecule	Serine Protease Active Site

used to verify sequence integrity and incorporation of the sequence into the correct reading frame. Plasmid DNA, from positive clones, was isolated and transformed into DH10Bac™ *E. coli* cells containing a shuttle bacmid (DH10Bac™). Correct transposition of the gene of interest was verified by PCR with specific primers. Isolated bacmids were transfected into Sf9 insect cells, then incubated for ten days. After incubation the virus was harvested and used to make a large scale viral stock (P2).

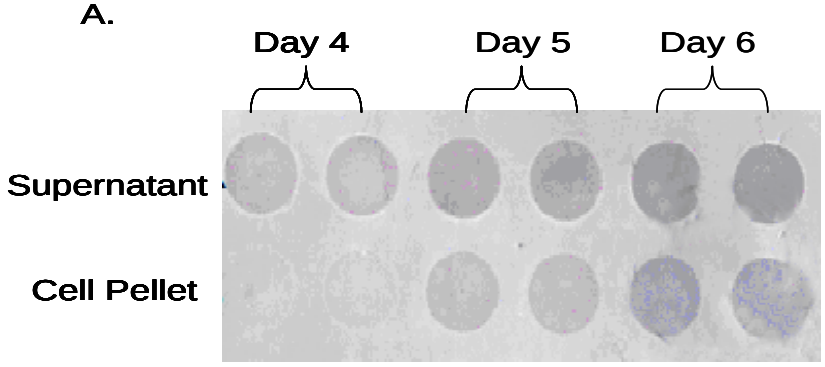
High titer viral stock was used to express a small amount of recombinant protein to facilitate protein identification. Cultures were incubated for 4 days, 5 ml samples taken every 24 hours. Protein was purified from the supernatant and cellular extract of each sample by metal affinity chromatography. These samples were analyzed by immunoblotting using a primary antibody specific for an N-terminal hexa-histidine tag (Figure 4). A protein different from those found in uninfected cells was observed in supernatant 1 (A) and 2 (B) in increasing amounts from day 4 to day 6. Antibody binding was observed in cellular extracts from clone 1 starting on day 5.

The viral clones that were positive for protein in the immunoblot were chosen for further investigation of the identity of expression products. Clone 1 expression products was purified by metal affinity chromatography and prepared for visualization by SDS-PAGE and Western blotting (Figure 5). SDS-PAGE (A) showed a variety of proteins present in the purified protein. A strong band was present at 42 kDa in both clones (lane 1), larger than the calculated size of Pol d 4 (29 kDa). Western blotting (B) showed a 42 kDa protein in lane 1.

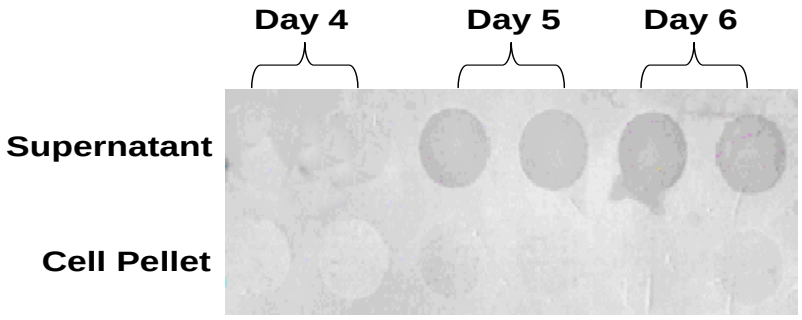
The nonspecific nature of the antibody used for Western blotting and the

Figure 4: Dot blot of initial expression in baculovirus clones. Expression of the baculovirus production of Pol d 4 was initially examined using a dot blot. High titer viral stock was used to infect Sf9 cell cultures and incubated in suspension. Samples of supernatants and cell pellets were collected on days 4, 5, and 6. Protein from supernatants and cells were purified by metal affinity chromatography from two separate clones, 1 and 2. Each sample was applied to the membrane in duplicate. The dot blot was probed with an anti hexa-histidine antibody. The purification products from clone 1 (A), supernatant and pellet from incubation days 4, 5, and 6 were blotted in duplicate. Purification products from clone 1 showed the largest amount of expressed protein on day 6. The purification products from clone 2 (B), supernatant and cell pellet, days 4, 5, and 6 of incubation were blotted in duplicate. The expression products on day 6 showed the greatest amount of antibody binding in clone 2. The purification products from non-infected cells (C), supernatant and cell pellet, were purified and blotted as control. An additional control (D) of a previously verified recombinant protein expression was used as a binding control.

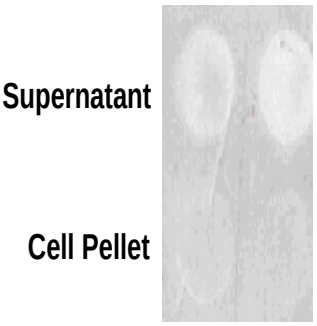
A.


Day 4
Day 5
Day 6
Supernatant
Cell Pellet

B.


Day 4
Day 5
Day 6
Supernatant
Cell Pellet

C.


Supernatant
Cell Pellet

D.

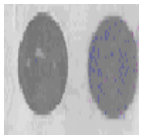
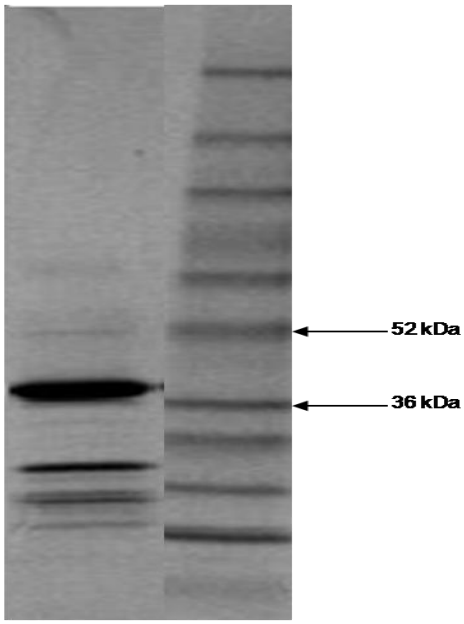
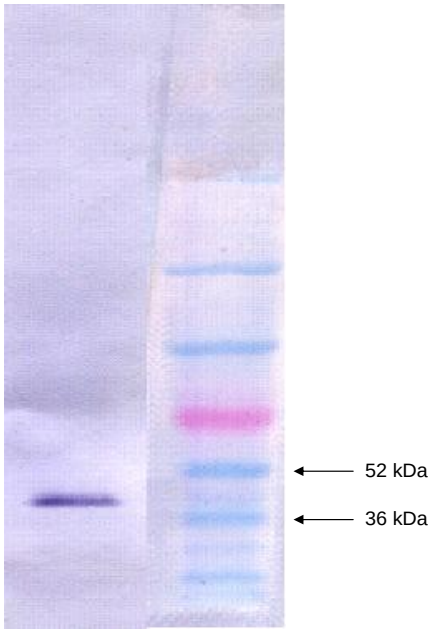


Figure 5: SDS-PAGE and Western blot analysis of purified expression products of recombinant Pol d 4. Expression products were purified using a Ca^{2+} metal affinity column and then assayed by SDS-PAGE and Western blotting. The purification products were concentrated 5X and 15 µl used in SDS-PAGE (**A**). A protein band was visualized at approximately 42 kDa. The same amount of concentrated purified protein was used in Western blotting (**B**) which was probed with the anti-N-terminal hexa-histidine tag antibody. A protein band was identified seen at approximately 42 kDa.

A.



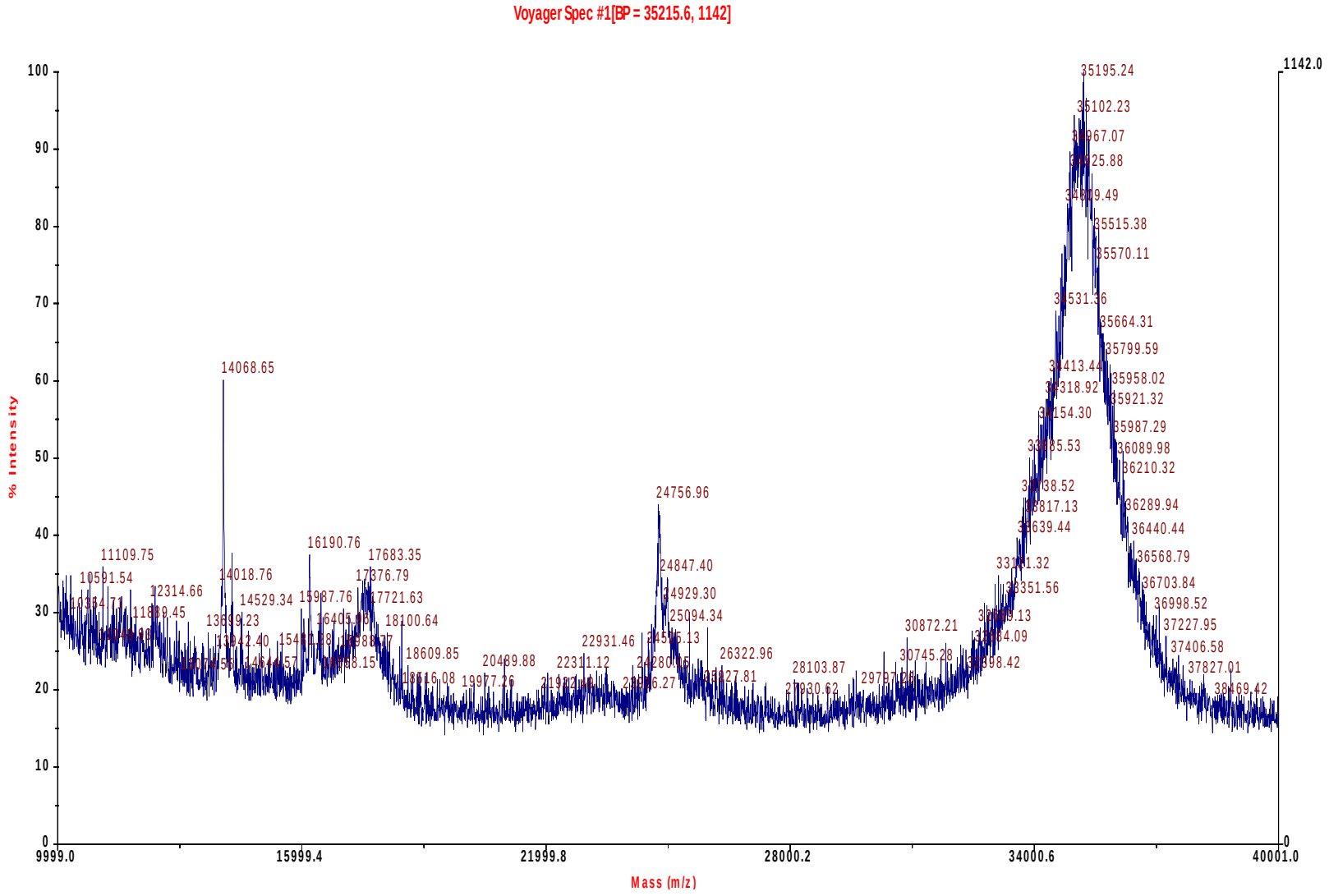
B.



unexpected larger size of the resulting product required further characterization of the recombinant protein. Major proteins purified from the expression cultures were analyzed by MALDI-TOF mass spectrometry (Figure 6). A large, broad peak was observed at 35,195 Da. Secondary peaks were seen at 14,068 and 24,756 Da. The calculated size of Pol d 4 was 29 kDa, which was smaller than the 35,195 Da protein found MALDI-TOF mass spectrometry.

In order to identify the protein observed by MALDI-TOF mass spectrometry, protein finger printing was performed. Purification products were reduced and alkylated, then digested with trypsin and 16 peptide fragments were identified by MALDI-TOF (Figure 7). The peptide fragments that were observed from the trypsin digest of the whole purified protein were compared to the predicted fragments of Pol d 4 (Table 4). Nine of the peptide fragments matched the predicted tryptic peptide fragments of Pol d 4. A homology search using the MASCOT software search engine showed that the fragments had the greatest homology with Pol d 4 and covered 30% of the molecule (Figure 8). Figure 8 shows the Pol d 4 derived amino acid sequence with regions covered by the protein finger printing highlighted in red. Pol d 4 has six potential N-glycosylation sites which are underlined. One of the six sites, N198, was included in a peptide fragments identified by MALDI-TOF. If recombinant Pol d 4 is glycosylated, the presence of these sites during tryptic digest analysis would suggest that they are not glycosylated. The remaining sites, N64, N84, N135, N138, and N246 may be candidates for post-translational glycosylation.

Figure 6: MALDI-TOF mass spectrometry analysis of purified protein from baculovirus expression. Pol d 4 expression products were purified by metal affinity chromatography. Recombinant proteins were purified by metal affinity chromatography and a fraction of 0.1 to 10 µg was prepared for analysis by MALDI-TOF mass spectrometry. A large broad protein peak was observed at 35,195 Da.


Voyager Spec #1[BP = 35215.6, 1142]
% Intensity
Mass (m/z)
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0
9999.0
15999.4
21999.8
28000.2
34000.6
40001.0
35195.24
35102.23
14068.65
24756.96
16190.76
17683.35
11109.75
14018.76
17376.79
10591.54
14529.34
15987.76
12314.66
17721.63
18100.64
24847.40
24929.30
25094.34
18609.85
22931.46
26322.96
30872.21
28103.87
30745.28
35664.31
35799.59
35958.02
35921.32
35987.29
36089.98
36210.32
36289.94
36440.44
36568.79
36703.84
36998.52
37227.95
37406.58
37827.01
38469.42

Figure 7: MALDI-TOF mass spectrometry analysis of tryptic fragments of baculovirus expressed Pol d 4. Recombinant Pol d 4 was purified by metal affinity chromatography. Trypsin was used to digest 10 µg of purified protein. Fragments were prepared and analyzed with MALDI-TOF mass spectrometry. Peaks at were identified at 991.3, 1,080.6, 1,554.5, 1,589.4, and 1,918.5, corresponding with the predicted tryptic fragments of Pol d 4.

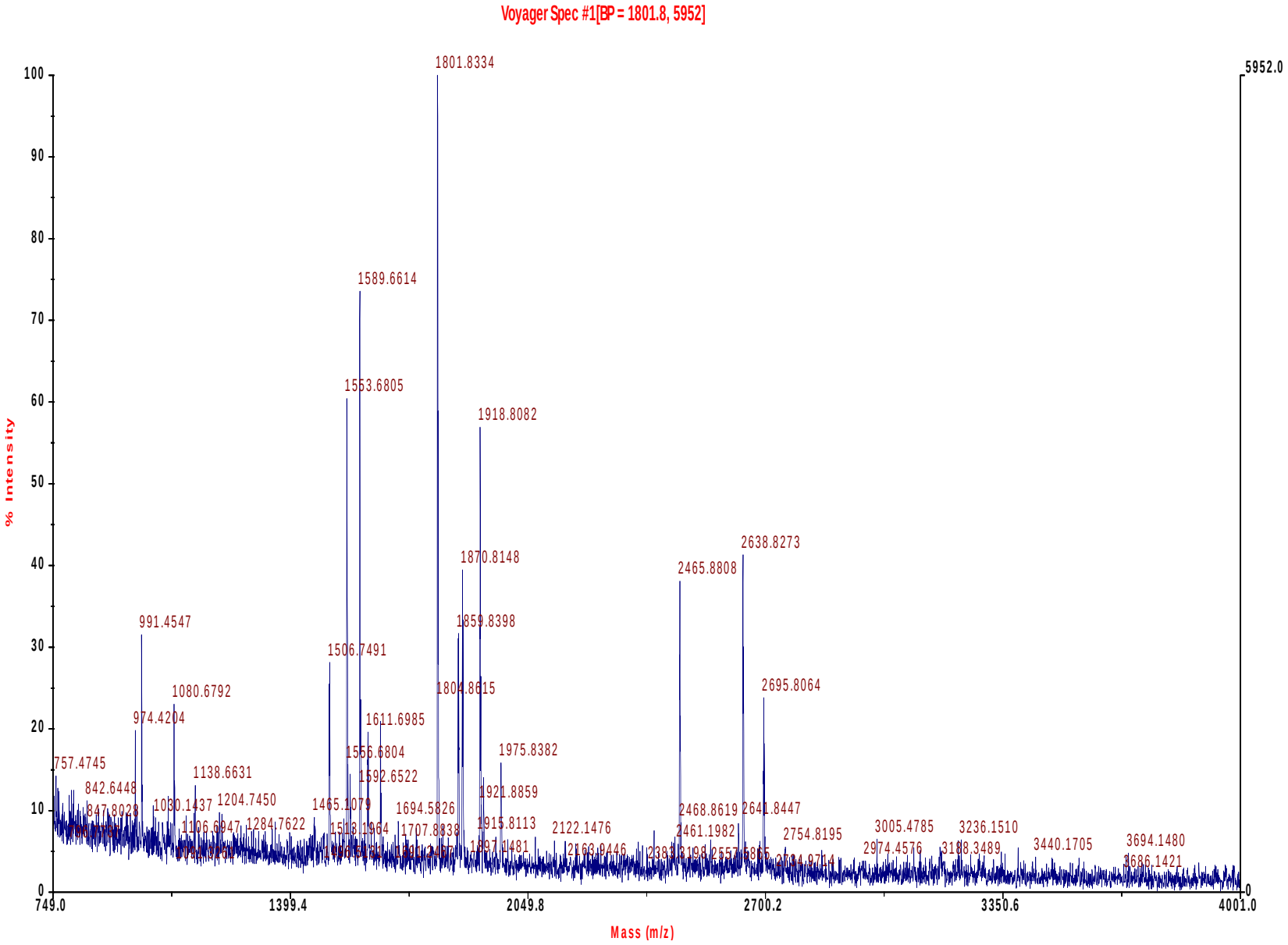

Voyager Spec #1[BP = 1801.8, 5952]
% Intensity
Mass (m/z)
1801.8334
5952.0
1589.6614
1553.6805
1918.8082
1870.8148
2638.8273
2465.8808
1859.8398
991.4547
1506.7491
1804.8615
2695.8064
1080.6792
974.4204
1611.6985
1975.8382
1556.6804
757.4745
1592.6522
1138.6631
842.6448
1921.8859
1030.1437
1204.7450
1465.1079
1694.5826
2468.8619
2641.8447
1915.8113
2122.1476
2461.1982
2754.8195
3005.4785
3236.1510
2974.4576
3188.3489
3440.1705
3694.1480
3686.1421
0
10
20
30
40
50
60
70
80
90
100
749.0
1399.4
2049.8
2700.2
3350.6
4001.0

Table 4: List of predicted tryptic peptide fragments of Pol d 4 and the matching fragments detected by MALDI-TOF mass spectrometry. The derived sequence of Pol d 4 was up-loaded into the MS-Digest search engine to generate a list of predicted peptide fragments. Five peptide fragments identified during MALDI-TOF mass spectrometry were identical to he predicted fragments, their molecular weights were, 991.3, 1,080.6, 1,554.5, 1,589.4, and 1,918.5. The fragments that corresponded with the predicted tryptic fragments are marked in red.

Fragment	Predicted Tryptic Fragments	MALDI-TOF Fragment (Th)
1	[1] EENCKCGWDNPSR [13]	991.3
2	[14] IVNGVETEINEFPMVAR [30]	1918.5
3	[31] LIYPSPGMYCGGTIITPQHIVTAAHCLQK [60]	
4	[61] YKR [63]	
5	[64] TNYTGIHVVVGEHDYTTDTETNVTK [88]	
6	[89] R [89]	
7	[90] YTIAEEVTIHPNYNSHNNDIAIVK [114]	
8	[115] TNERFEYSMK [124]	
9	[125] VGPVCLPFNYMTR [137]	1554.5
10	[138] NLTNETVTALGWGK [151]	
11	[152] LR [153]	
12	[154] YNGQNKVLR [162]	
13	[163] K [163]	
14	[164] VDLHVITR [171]	1080.6
15	[172] EZCETHYGAAIANANLLCTFDVGR [195]	
16	[196] DACQNDSGGPILWR [209]	1589.4
17	[210] SPTTDNLILVGVVNFGR [226]	
18	[227] TCADDAPGGNAR [238]	
19	[239] VTSFMEFIHNATGETYCK [256]	
20	[257] ADHHHHHH* [265]	

Figure 8: Characterization of the derived Pol d 4 amino acid sequence with MALDI-TOF mass spectrometry data. The amino acid sequence for the leader, propeptide, and mature molecule sequence are depicted. Potential N-glycosylation sites are identified by an underscore. Regions that were covered by protein finger printing peptide fragments are highlighted in red.

1 EEN**CKCGWDN PSRIVNGVET EINEFPMVAR** LIYPSPGMYC GGTIITPQHI VTAAHCLQKY

61 KRTNYTGIHV VVGEHDYTTD TETNVTKRYT IAEVTIHPNY NSHNNDIAIV KTNERFEYSM

121 K**VGPVCLPFN YMTR**NLTNET VTALGWGKLR YNGQNSKVLR KVDLHVITRE QCETHYGAAI

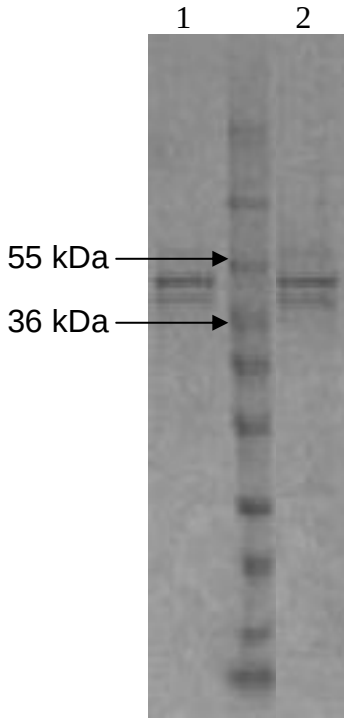
181 ANANLLCTFD VGR**DACQNDS GGPILWR**SPT TDNLILVGVV NFGRTCADDA PGGNARVTSF

241 MEFIHNATIG *ETYCK***ADHHH HHH***

The expression of Pol d 4 in the baculovirus system was confirmed by CTL BIO Services. The intact protein was separated by SDS-PAGE, then digested with trypsin. Resulting peptide fragments were extracted, desalted and concentrated. Fragments were analyzed on a Finnegan LTQ mass spectrometer using a one hour gradient and data dependent MS/MS collection (data not shown). Multiple rounds of peptide sequencing showed the presence of two unique peptides, R.IVNGVETEINEFPMVAR.L and K.TNERFEYSMK.V, which were observed several times. Analysis of these sequences showed that the recombinant protein scored extremely high for the venom serine protease from *P. dominulus*.

Expression was optimized for quantity and purity. The optimal incubation time was 5 days post-inoculation. Varying amounts of virus was added to cultures to determine ideal viral load. Protein production data (not shown) determined that 8 ml of high titer stock per 100 ml of insect cultured yielded the highest amount (0.2mg/L) of recombinant protein. The production of recombinant Pol d 4 in the Sf9 cells yielded a small amount of protein. The production of protein was compared between the Sf9 and High Five™ insect cell lines. Cells were infected with the same amount of virus, incubated the same amount of time, and purified using the same protocol. Protein production was compared by visualization on SDS-PAGE (Figure 9). The production of recombinant Pol d 4 was approximately the same in High Five™ cell but purity was low due to the presence of contaminating proteins. Ultimately, the High Five™ cell line was ultimately chosen for expression based upon its ability to fully glycosylate recombinant proteins with fucoidan polysaccharides.

Figure 9: Visual comparison of recombinant Pol d 4 produced by Sf9 and High Five™ insect cell lines. High titer viral stocks were used to infect Sf9 and High Five™ insect cells. Supernatants from both cell lines were harvested and purified by metal affinity chromatography. Purification elutes were electrophoresed on a 4-20% SDS-PAGE. The recombinant protein was approximately 42 kDa in size. Elution from protein produced in Sf9 cells are shown in lane 1 and those produced from High Five™ cells are in lane 2.


1
2
55 kDa
36 kDa

After purification, several contaminating proteins and peptides were still visible on SDS-PAGE. The purification products were separated by cation exchange HPLC (Figure 10). The recombinant protease was isolated over a large sodium chloride gradient beginning at minute 30 until minute 45 (highlighted). HPLC elution products were visualized by SDS-PAGE and Western blot (Figure 11). The SDS-PAGE (A) and Western blotting (B) had a band at approximately 42 kDa, with secondary bands at 24 and 19 kDa.

3.1.2 Prokaryotic expression of Pol d 4

Expression in a prokaryotic system was performed to generate a recombinant serine protease which lacks post-translational glycosylation. These proteins were used to assay the IgE binding to amino acid allergenic epitopes. Two constructs were generated and expressed, one containing the propeptide and one without the propeptide. Constructs were amplified by PCR, verified by nucleic acid sequencing, and ligated into the pET-17b expression vector.

Positive transformants were analyzed by restriction digestion with *NDE* I and *Bam* HI and visualized by agarose gel electrophoresis. Isolated clones were transformed into Rosetta-gami™ (DE3) competent *E. coli* cells for expression. Expression of non-glycosylated protein containing the propeptide was initially analyzed by SDS-PAGE (A) and Western blotting (B) (Figure 12). A band was visualized in both assays at approximately 31 kDa similar to the calculated molecular weight of the molecule which is 29.6 kDa. The elution products were assayed with MALDI-TOF (Figure 13). The protein size was 29.7 kDa (circled). Trypsin digestion was performed for peptide sequencing.

Figure 10: Elution of recombinant Pol d 4 expressed in baculovirus from cation exchange HPLC. Three liters of baculovirus expression cultures was harvested and recombinant protein was purified by metal affinity chromatography. Purification products were separated by cation exchange HPLC. The recombinant protease was confirmed in collections, by SDS-PAGE, Western blotting and MALDI-TOF mass spectrometry, over a large region of the gradient from minute 30 to minute 45 (boxed area).

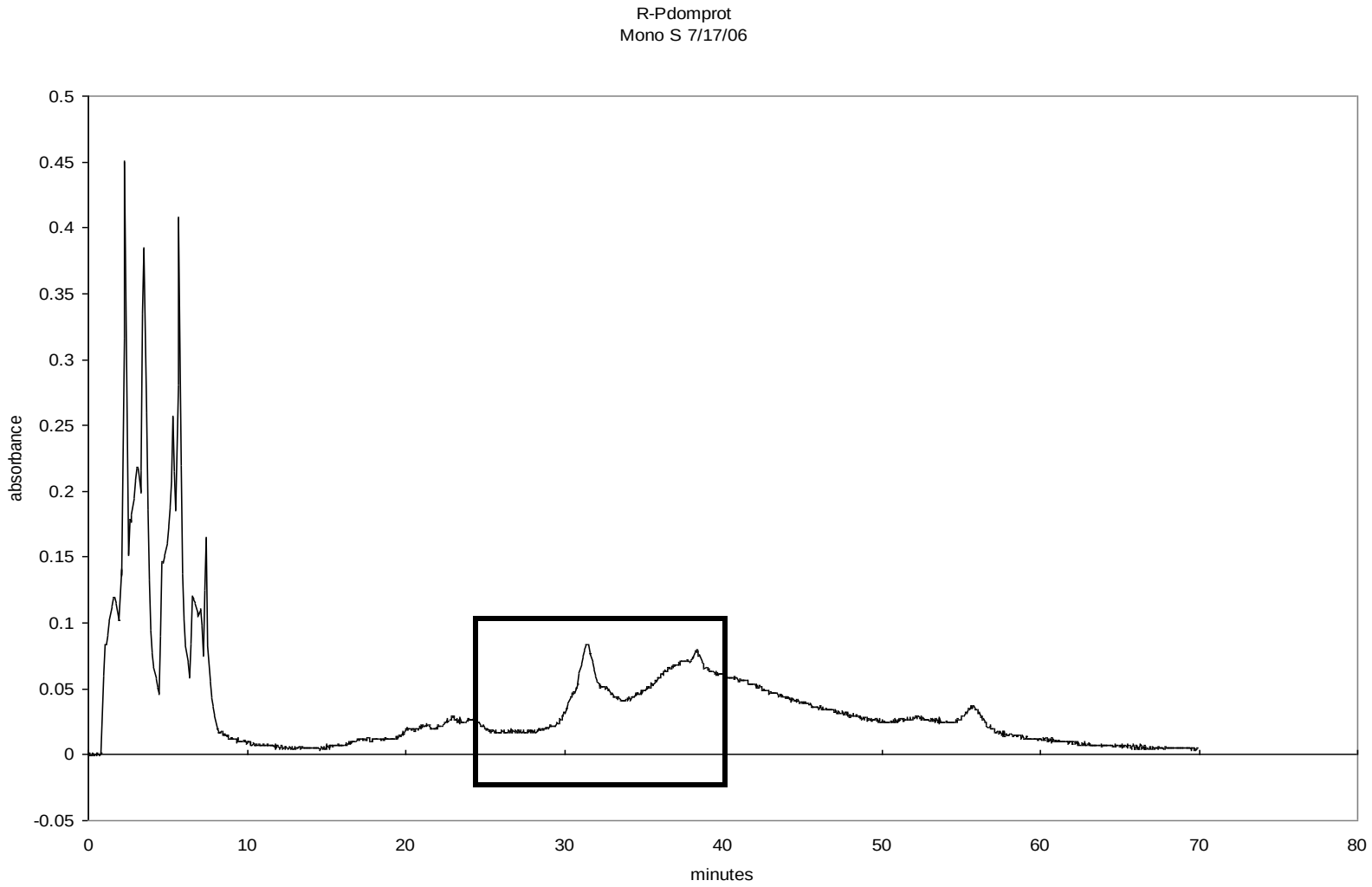
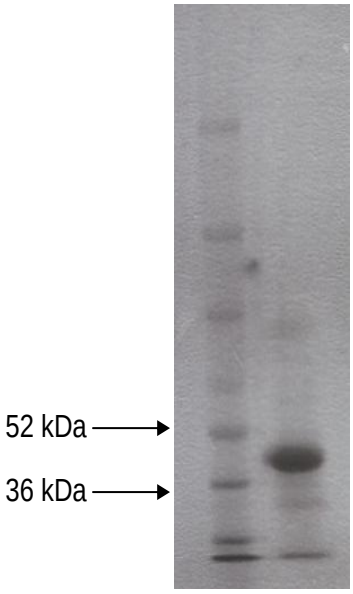

R-Pdomprot
Mono S 7/17/06
absorbance
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
10
20
30
40
50
60
70
80
minutes

Figure 11: SDS-PAGE and Western blot analysis of elution products from cation-exchange HPLC. A. Fractions obtained from cation exchange HPLC were concentrated 5X and 15 µl was used visualized protein by SDS-PAGE (A) and Western blotting (B). A. A protein band at 42 kDa was present. B. The Western blot was probed with an anti C-terminal histidine antibody. A protein band was visualized at approximately 42 kDa.

A.



B.

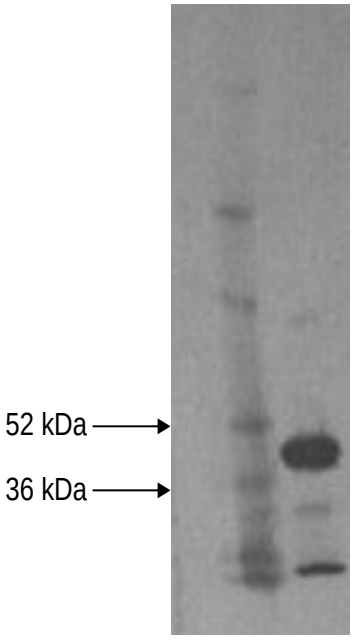
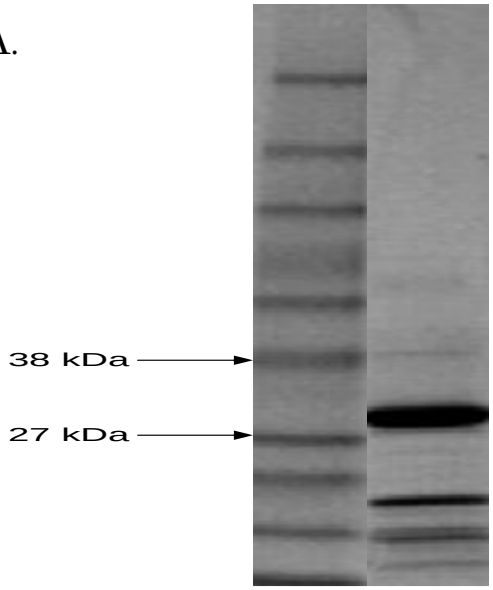


Figure 12: Visual verification of *E. coli* expressed recombinant Pol d 4 by SDS-PAGE and Western blot. Expression of the non-glycosylated protease with the propeptide in *E. coli* cells was induced with IPTG. Supernatant and cell pellets were harvested and protein was purified by metal affinity chromatography. Fifteen microliters of the purification product was used in SDS-PAGE and Western blotting. (A) Purification products were electrophoresed by SDS-PAGE. A protein band was seen at approximately 31 kDa. (B) Western blot analysis was performed on purification elutes probing with an anti-C terminal hexa-histidine tag antibody. A protein band was visualized at approximately 31 kDa.

A.



B.

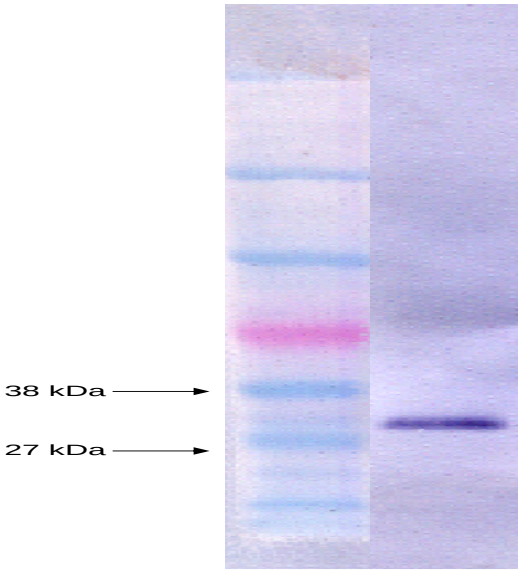
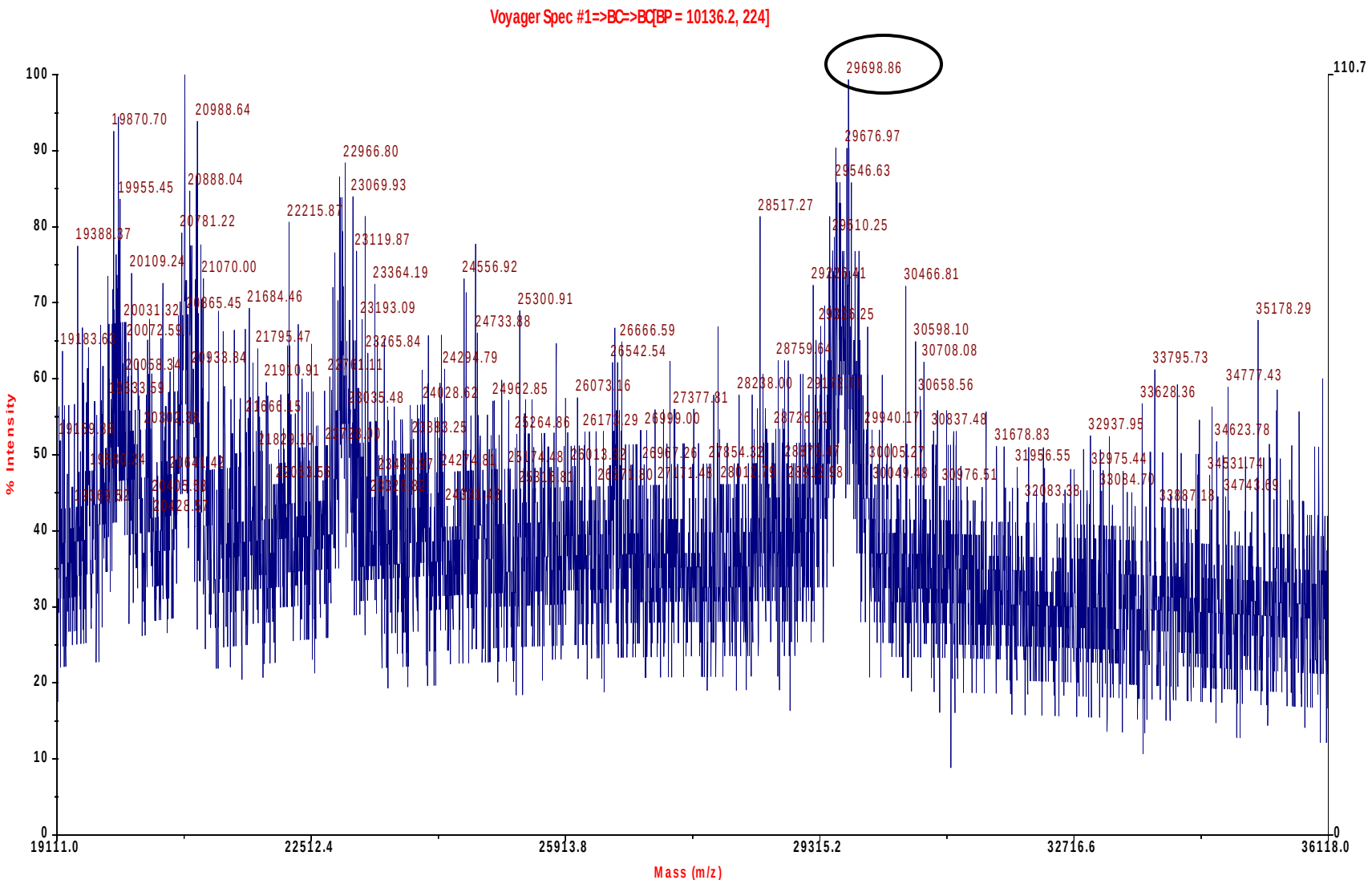


Figure 13: MALDI-TOF mass spectrometry analysis of intact recombinant Pol d 4 with the propeptide expressed in the *E. coli* expression system. Protein was purified from the expression of non-glycosylated Pol 4 with propeptide by metal affinity chromatography. A fraction of 0.1 to 10 µg of purification product was analyzed by MALDI-TOF mass spectrometry and a strong peak (circled) at 29,698.86 Da. The calculated size of Pol d 4 is 29.6 Da.


Voyager Spec #1=>BC=>BC[BP = 10136.2, 224]
% Intensity
Mass (m/z)
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90
80
70
60
50
40
30
20
10
0
19111.0
22512.4
25913.8
29315.2
32716.6
36118.0
110.7
0
29698.86
29676.97
29546.63
28517.27
29610.25
30466.81
35178.29
33795.73
34777.43
33628.36
34623.78
20988.64
19870.70
19955.45
20888.04
20781.22
19388.37
20109.24
21070.00
22966.80
23069.93
22215.87
23119.87
23364.19
24556.92
25300.91
21684.46
23193.09
24733.88
26666.59
26542.54
28238.00
28759.64
30598.10
30708.08
30658.56
30837.48
31678.83
31956.55
32937.95
32975.44
33084.70
33887.18
34531.74
34743.69
30005.27
30049.48
30976.51
32083.38

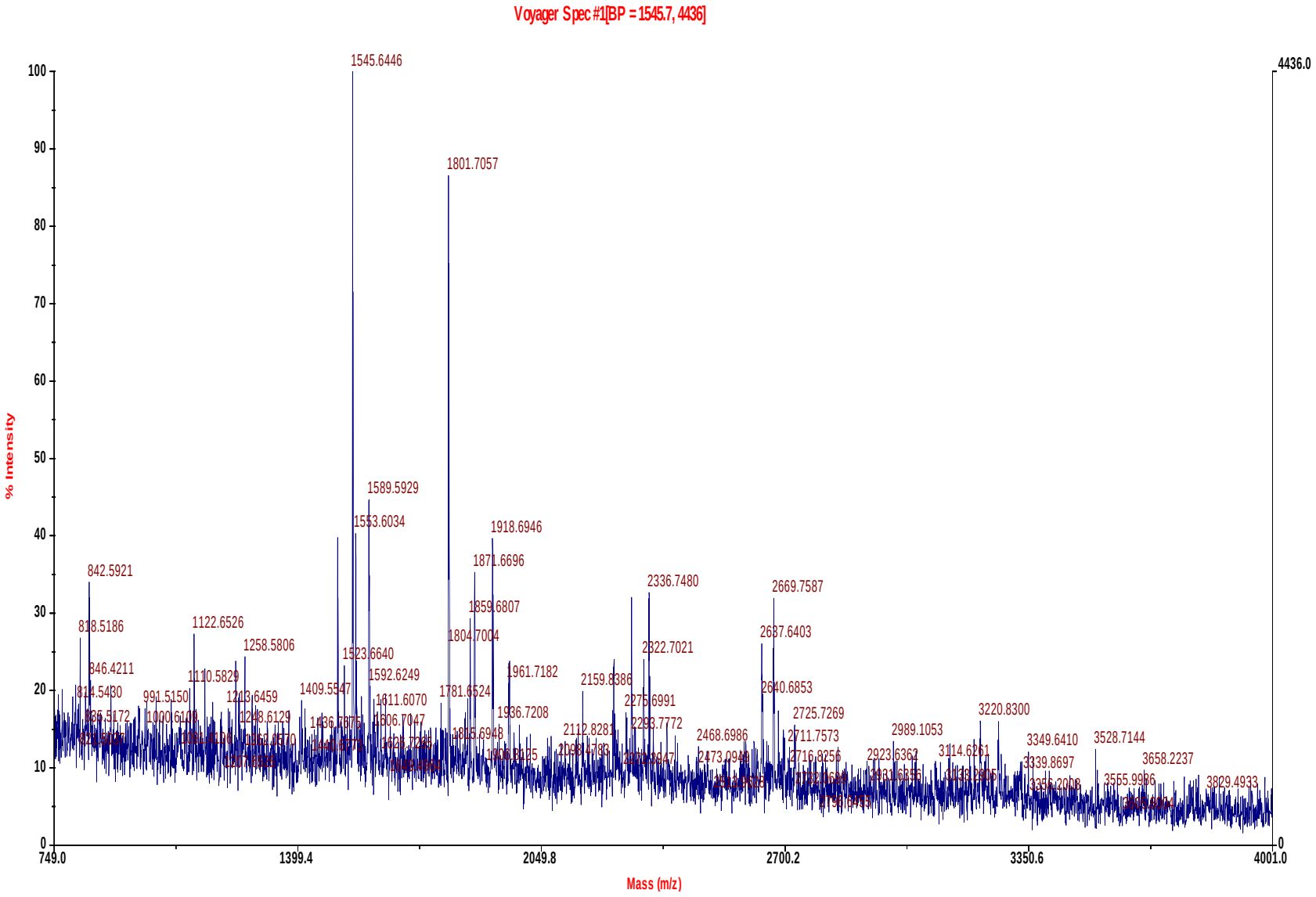
Nine of the predicted Pol d 4 trypsin restriction fragments were represented in peptides identified by MALDI-TOF (Figure 14). The resulting fragments covered approximately 38% of the Pol d 4 sequence (Table 5).

A construct was generated for expression in *E. coli* that did not contain the propeptide sequence, non-glycosylated Pol d 4 without propeptide. This construct was prepared and expressed with the same protocol used in the expression of non-glycosylated Pol d 4 with the propeptide. Expression products were purified by metal affinity chromatography and visualized by SDS-PAGE and Western blotting (Figure 15).

Both assays showed a protein approximately 27 kDa in size, correlating with the calculated size of non-glycosylated Pol d 4 without propeptide (27.5 kDa). Samples were evaluated by MALDI-TOF analysis (Figure 16). A strong peak that represented a protein of 28,149.49 Da in size was observed.

Expression in *E. coli* systems produces protein without the post-translational carbohydrate, additionally, this protein may not be in the proper conformation. Conformation may be lost due to the harsh conditions required for protein isolation and purification from *E. coli*. Three protocols were used in an attempt to refold the recombinant serine proteases, non-glycosylated Pol d 4 with and without the propeptide, into the correct conformation. After each refolding attempt proteins were separated using benzamidine chromatography to isolate correctly folded serine proteases. Elution from benzamidine chromatography was visualized by SDS-PAGE (data not shown). Protein was not absorbed on the benzamidine column following each of the refolding protocols.

Figure 14: MALDI-TOF mass spectrometry analysis of tryptic fragments of *E. coli* expressed Pol d 4. Protein products from *E.coli* expression were purified by metal affinity chromatography. Trypsin was used to digest 10 µg of protein which was then prepared for analysis by MALDI-TOF mass spectrometry. Peaks at were identified at 1554.6, 1553.6, 1589.4, 1801.7, 2290, 1669.7, 3269, and 3535 Da corresponding with the predicted tryptic fragments of Pol d 4.


Voyager Spec #1[BP = 1545.7, 4436]
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Mass (m/z)
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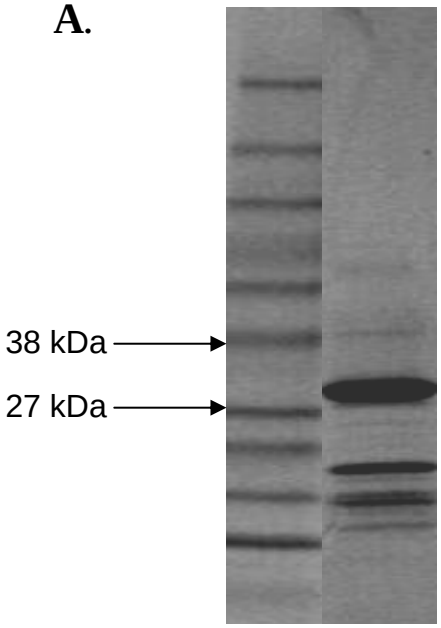
90
80
70
60
50
40
30
20
10
0
749.0
1399.4
2049.8
2700.2
3350.6
4001.0
4436.0
1545.6446
1801.7057
1589.5929
1553.6034
1918.6946
1871.6696
2336.7480
2669.7587
1859.6807
842.5921
818.5186
1122.6526
1258.5806
2637.6403
1804.7004
2322.7021
1523.6640
1961.7182
846.4211
1110.5829
1592.6249
2159.8386
2640.6853
814.5430
1213.6459
1409.5547
1611.6070
1781.6524
2275.6991
2725.7269
991.5150
1248.6129
1606.7047
1936.7208
2293.7772
3220.8300
2989.1053
2112.8281
2711.7573
2468.6986
3349.6410
3528.7144
3114.6261
2923.6362
2716.8256
3339.8697
3658.2237
3555.9986
3829.4933

Table 5: Comparison of the tryptic fragments generated by peptide sequencing of non-glycosylated Pol d 4 with the propeptide to the Pol d 4 amino acid sequence. Non-glycosylated Pol d 4 with the propeptide was expressed and purified by metal affinity chromatography. Protein was digested with trypsin and peptide fragments were separated using MALDI-TOF mass spectrometry. Nine of the 20 predicted fragments were identified from the expression products. The predicted peptides covered by the observed fragments were 3, 7, 10, 16, 17, and 19 (in red). The sizes of the observed fragments are included on the table. Those fragments with more than one peptide mass are the result of incomplete cleavage of the intact protein. Peptides identified by MALDI-TOF mass spectrometry covered 38% of the Pol d 4 sequence.

Fragment	Predicted Tryptic Fragments	MALDI-TOF Fragment (Th)
1	[1] EENCKCGWDNPSR [13]	
2	[14] IVNGVETEINEFPMVAR [30]	
3	[31] LIYPSPGMYCGGTIITPQHIVTAAHCLQK [60]	3269 and 3535
4	[61] YKR [63]	
5	[64] TNYTGIHVVVGEHDYTTDTETNVTK [88]	
6	[89] R [89]	
7	[90] YTIAEEVTIHPNYNSHNNDIAIVK [114]	
8	[115] TNERFEYSMK [124]	
9	[125] VGPVCLPFNYMTR [137]	
10	[138] NLTNETVTALGWGK [151]	1553.6
11	[152] LR [153]	
12	[154] YNGQNKVLR [162]	
13	[163] K [163]	
14	[164] VDLHVITR [171]	
15	[172] EZCETHYGAAIANANLLCTFDVGR [195]	
16	[196] DACQNDSGGPILWR [209]	1589.4
17	[210] SPTTDNLILVGVVNFGR [226]	1801.7
18	[227] TCADDAPGGNAR [238]	
19	[239] VTSFMEFIHNATGETYCK [256]	2290
20	[257] ADHHHHHH* [265]	

Figure 15: Evaluation of *E. coli* expressed Pol d 4 without the propeptide by SDS-PAGE and Western blotting. Expression products of *E. coli* expressed Pol d 4 without the propeptide were harvested and purified with metal affinity chromatography. The same amount, 15 µl, of protein was used in the SDS-PAGE and Western blot. A. Protein samples were prepared and electrophoresed using SDS-PAGE. A protein band was seen at approximately 27 kDa. B. Western blotting was performed using metal affinity purification products. A band was seen at approximately 27 kDa.

A.



B.

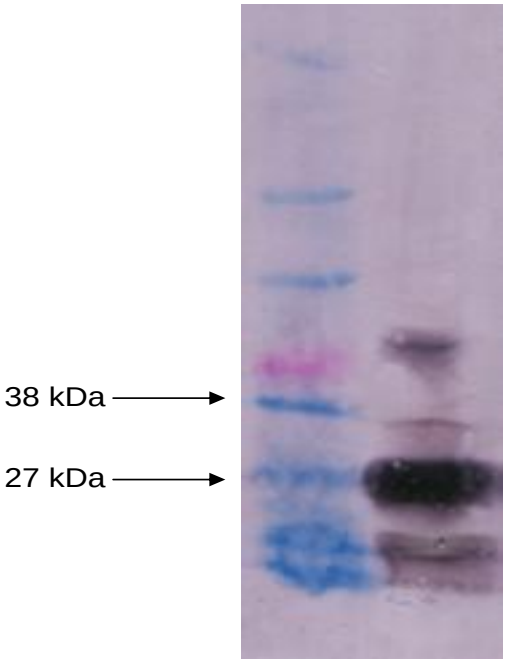
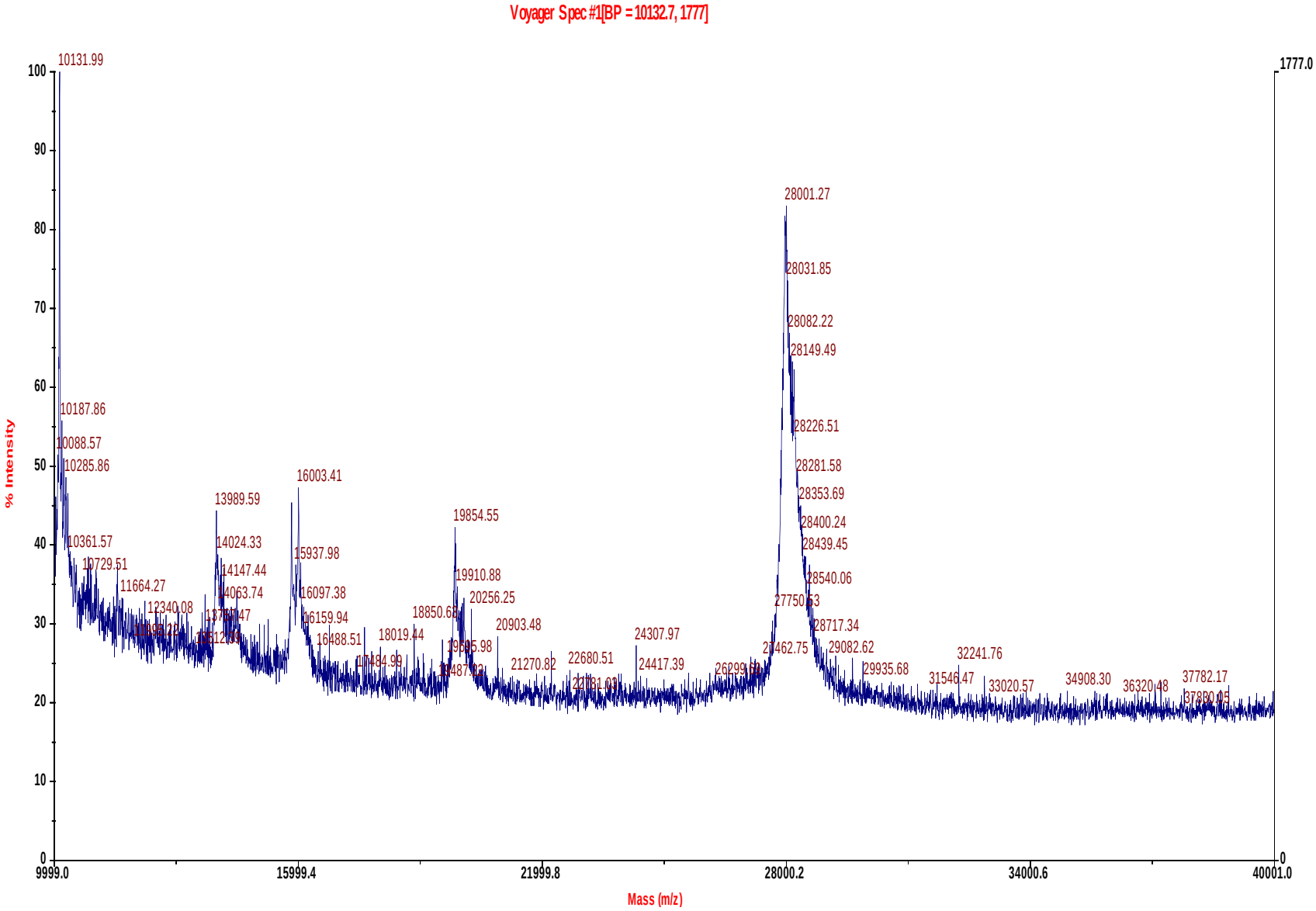


Figure 16: MALDI-TOF mass spectrometry analysis of intact protein from *E. coli* expressed Pol d 4 without propeptide. Harvested protein was purified from the expression of *E. coli* expressed Pol d 4 without propeptide by metal affinity chromatography. A fraction of 0.1 to 10 µg of purified protein was used in MALDI-TOF mass spectrometry, which indicated a protein (circled) at 27,977 Da.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9999.0
15999.4
21999.8
28000.2
34000.6
40001.0
10131.99
10187.86
10088.57
10285.86
10361.57
10729.51
11664.27
12340.08
13989.59
14024.33
14147.44
14063.74
16003.41
15937.98
16097.38
16159.94
16488.51
18019.44
18850.68
19854.55
19910.88
20256.25
20903.48
21270.82
22680.51
24307.97
24417.39
26299.60
27750.63
28001.27
28031.85
28082.22
28149.49
28226.51
28281.58
28353.69
28400.24
28439.45
28540.06
28717.34
29082.62
29935.68
31546.47
32241.76
33020.57
34908.30
36320.48
37782.17

3.2 Mutation of Pol d 4

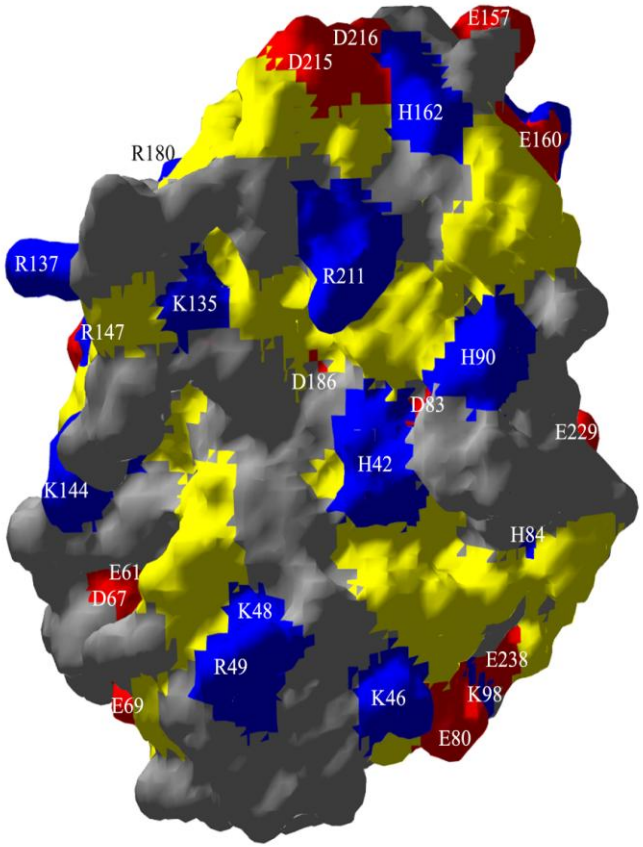
3.2.1 Mutational analysis of Pol d 4

The interaction of Pol d 4 and patient IgE will supply information about the importance of Pol d 4 in the species specific immunologic reaction. The major amino acid epitopes contribute to this type of reaction. The epitopes found on Pol d 4 were predicted using CEPS. The prediction server generated 11 possible allergenic peptides. A second server, IPIS, was used to predict the amino acid residues on Pol d 4 which may be involved in protein-to-protein interactions. The IPIS predicted 34 amino acids play a role in protein-protein interaction. Both data sets were compared and aligned for common amino acid residues (Table 6). The data sets had 23 amino acid residues in common, highlighted in red on Table 4. The data was visualized on model of the charge distribution of Pol d 4 (Figure 17). Three sets of amino acids were chosen for mutation based on their identification with computational models and their position on the charge distribution model. The first construct had a mutational substitution in predicted epitope 6. Glutamine 101 was chosen for mutation and arginine 102, while not identified by the IPS, was also mutated based on its position in on the charge distribution model. The second construct had mutational substitutions in predicted epitopes 3 and 10, within these epitopes histidine 42 and arginine 211 were mutated. The last construct utilized predicted epitopes 1 and 8, arginine 137 and glutamine 6 were mutated to an alanine. Mutation reactions were performed with the QuikChange® Lightning Site-Directed Mutagenesis Kit, allowing for the mutation of amino acid residues in the previously generated

Table 6: The comparison of the amino acid residues predicted by IPIS with conformational epitopes predicted by CEPS. The amino acid sequence of Pol d 4 was up-loaded onto CEP server and 14 potential epitopes were generated. The same information was up-loaded onto the IPIS server and amino acid residues that play a role in protein-protein interaction were predicted. Both data sets were compared for common amino acid epitopes which are labeled in red.

1	3-NgVETEInEfP-13
2	21-PSPGmY-26
3	42-HcLQKYkRTNYTG-54
4	64-YTTdTETNV-72
5	79-AEvTIhPNYNSH-90
6	100-NERfEYSMK-108
7	116-FNYMTRNLTNeTvT-129
8	136-LRYNGQNsKV-145
9	155-TREQcETHyGAAiANA-170
10	209-FgRTcADD-216
11	229-EFiHNaTIGE-238

Figure 17: Model of Pol d 4 depicting surface charges. The molecular model was prepared with the SPDBV program using the previously described Pol d 4 sequence. Basic amino acids are labeled in blue, acidic amino acids are labeled in red, non-polar amino acids are labeled in yellow, and polar amino acids are labeled in grey.


E157
D216
D215
H162
E160
R180
R137
R211
K135
R147
H90
D186
D83
E229
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H84
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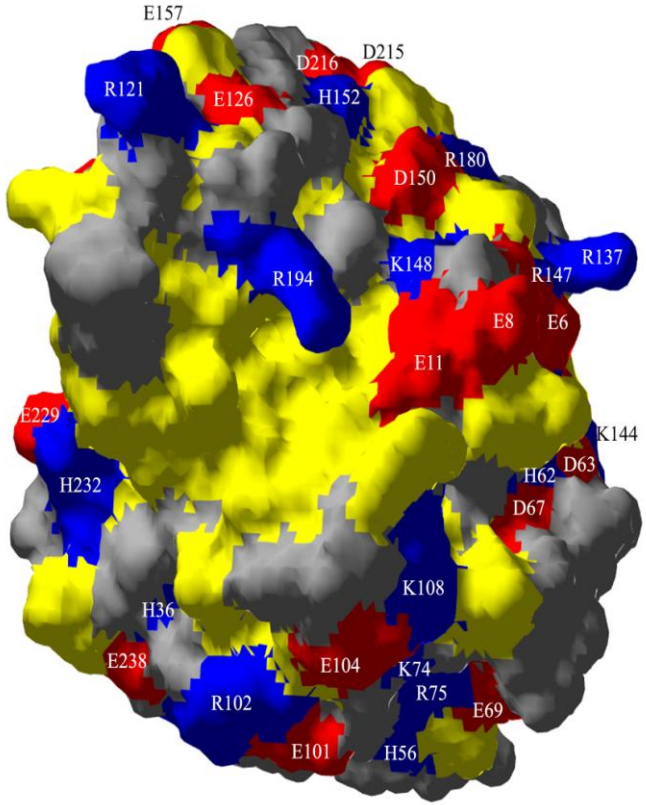
E69
E80


E157
D216
D215
R121
E126
H152
R180
D150
R194
K148
R147
R137
E8
E6
E11
E229
K144
H62
D63
H232
D67
K108
H36
E238
E104
K74
R75
E69
R102
E101
H56

pFastBac:Pol d 4 construct using the Sol i 2 leader sequence. All mutations were verified by nucleic acid sequencing and analysis using the GCG software package.

3.2.2 Expression of Pol d 4 mutations

All Pol d 4 mutants were expressed in the baculovirus expression system to maintain conformation and possible post-translational glycosylation. Expression was carried out in the same manner as previously described. Small amounts of expression culture were purified, and expression was visualized by immunoblot using an anti hexa-histidine tag antibody to probe for protein (data not shown). Expression in each of the mutants was optimal at day 2 post-infection of High Five™ cells.

Expression products from all three mutants were visualized by SDS-PAGE (data not shown). Mutants were seen as a 42 kDa protein band. The calculated size for mutants is approximately 30 kDa. The molecular weights were determined by MALDI-TOF (data not shown). Mut 1 and Mut 2 were seen as a broad peak at approximately 35 kDa. Data from Mut 4 showed a peak that ranged from 30 to 35 kDa. Protein fingerprinting of the mutants showed various degrees of coverage (data not shown). The mutated fragments were observed during analysis. Mut 2 produced 8 tryptic fragments that aligned with the Pol d 4 sequence, representing 49% of the molecule. Mut 2 had 4 fragments which aligned with the Pol d 4 sequence and these covered 14% of the molecule. Mut 4 had 3 fragments which aligned with the Pol d 4 sequence and covered 25% of the entire sequence (Table 7).

Table 7: Evaluation of MALDI-TOF data from the expression of mutant 1, 2, and 4. Whole protein products from mutation expressions were purified by metal affinity chromatography and concentrated 5X. Approximately 5 µg of purified protein was analyzed by MALDI-TOF mass spectrometry. For each mutation, predicted and whole protein sizes are shown. MALDI-TOF data showed that mutants are approximately 30-35 kDa in size with large and broad peaks. Similar to data gathered for recombinant Pol d 4. The purified protein was digested with trypsin for each mutant. The table lists the number of fragments that were matched to the Pol d 4 sequence and the percentage that was covered by the peptides.

Mutant	Predicted Protein Size	Whole Protein Size	Fragments Matched	% Sequence Covered
1	29553 Da	~ 35 kDa	8	49%
2	29553 Da	~ 35 kDa	4	14%
4	29553 Da	~ 30-35 kDa	3	25%

3.3 IgE Binding to recombinant Pol d 4

The involvement of the amino acid sequence and the carbohydrate determinants on IgE binding were assayed by immunoblotting and immunoblot inhibition. These tests utilized sera from individuals who have varying degrees of IgE reactivity to European paper wasp venom, and sera from patients sensitized to North American paper wasps. The amount of protein blotted for each assay was verified by staining with Imperial protein stain.

The recombinant forms of Pol d 4 were purified and bound to nitrocellulose strips at 5 µg per immunospot. Sera were titrated at 1:20, 1:10 and 1:5 to determine the optimum amount needed to visualize IgE binding. Initial assay with the different titrations showed that the minimum amount of sera that could be used was a 1:5 dilution. Prior to the beginning immunoblot assays with all patient antigens, 2 sera was used to assay specific binding of patient sera to recombinant Pol d 4. Patient sera were incubated with 10 µg of recombinant Pol d 4 or whole venom prior to exposure to the membrane bound protein in the immunoblot assay (Figure 18). Visually, binding to membrane bound recombinant Pol d 4 decreased when sera was incubated with recombinant Pol d 4 and was completely missing when sera was incubated with whole *P. dominulus* venom. The different forms of recombinant Pol d 4 were probed with human sera obtained from non-allergic patients to assay non specific binding (Figure 19). There was no significant binding to recombinant Pol d 4 from these patients' sera. Each strip was probed with one of thirteen European patient sera, with varying degree of IgE reactivity to *P. dominulus* venom (Table 8). Sera from a second set of European patients sensitized to paper wasp

Figure 18: Immunoblot inhibition with soluble antigen. European patient serum 3 and American patient serum 2 were used for immunoblot inhibition with soluble antigen. All sera were used at a dilution of 1:5. Recombinant Pol d 4 (A), mutant 1 (B), mutant 2 (C), mutant 4 (D) and whole venom (E) were blotted onto the membrane at a concentration of 5µg. The European patient 3 serum (1), European patient 3 serum + 10 µg recombinant Pol d 4 (2) , European patient 3 serum + 10 µg whole venom (3), American patient 2 serum (4), American patient 2 serum + 10 µg recombinant Pol d 4 (5), and American patient 2 + 10 µg whole venom (6) were the treatments.

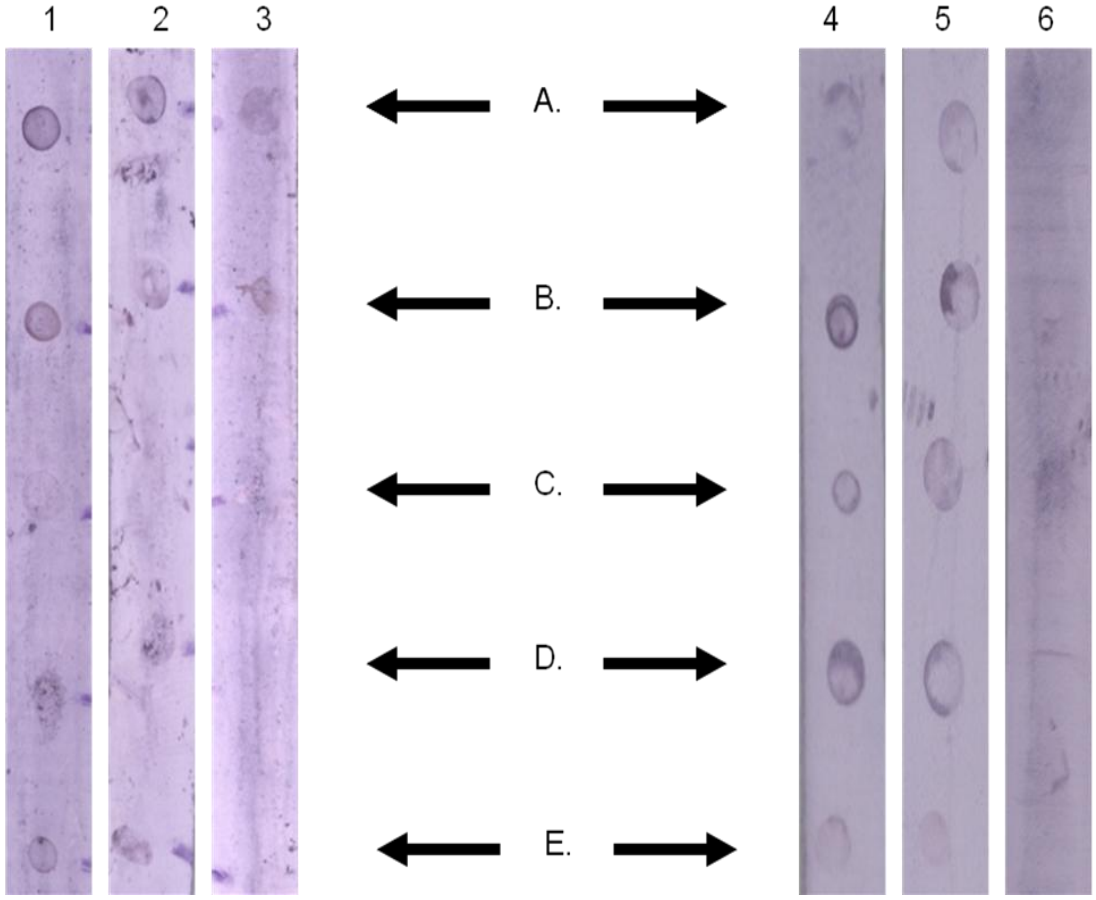

1
2
3
4
5
6
A.
B.
C.
D.
E.

Figure 19: Immunoblot assay with negative patient sera. Negative patient sera 1 and 2 were diluted 1:5 and used for immunoblot. Recombinant Pol d 4 (A), mutant 1 (B), mutant 2 (C), mutant 4 (D) and whole venom (E) were blotted onto the membrane at a concentration of 5µg. There was no significant IgE binding detected.

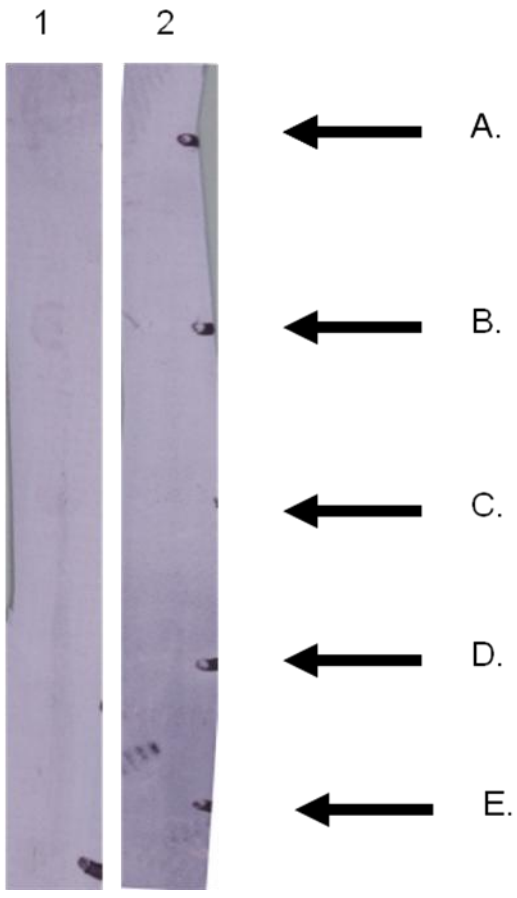

1
2
A.
B.
C.
D.
E.

Table 8: List of IgE antibody levels of European patient sera to venom from *P. dominulus*. Thirteen sera from patients sensitized to European paper wasps were assayed by RAST for IgE specific for the venom components. Patients with RAST score 2 (0.7-3.49 i.v. IgE level) has a moderate level of allergen-specific IgE, RAST score 3 (3.5-17.49 i.v. IgE level) has high level of allergen-specific IgE, RAST score 4 (17.5-49.49 i.v. IgE level) has very high levels of allergen-specific IgE.

Patient Number		IgE Antibody Level (KU/L)
1		**13.6**
2		**9.1**
3		**2.1**
4		**3**
5		**36.6**
6		**49.5**
7		**21.5**
8		**14.7**
9		**47.5**
10		**21.1**
11		**15.5**
12		**40.5**
13		**7.1**

were also assayed. Visually all sera contained IgE that bound to the *P. dominulus* venom extract. Binding of IgE, from the first set of patient sera, to different forms of recombinant Pol d 4 were assayed by immunoblot (Figure 20). IgE bound to the recombinant glycosylated form of Pol d 4 in 61.5% of the sera tested. Mutant 1 and 2 each bound IgE from 54% of patients, while mutant 4 bound IgE from 46% of patients. The whole venom control bound IgE in 11 of the 13 patients (85%) and the native protease bound IgE in a total of 9 patients (69%). A second set of European patient sera contained four patients and were used to assay IgE binding to the various forms of Pol d 4 (Figure 21). Visually the baculovirus expressed form of Pol d 4 bound IgE in 100% of the sera. All three mutants bound IgE from patients 2, 3, and 4 (75%). The whole venom bound IgE in 3 of the 4 sera (75%), the exception being patient 2. The second set of European patient sera had 100% of IgE bind the native protein. Finally, the recombinant proteins were assayed with two sera from patients who had previously tested negative against vespid venom (data not shown). The immunoblot using the negative sera did not have visible IgE binding.

In order to compare IgE binding to the recombinant forms of Pol d 4 a densitometric analysis of each immunospot was performed. Seventeen patients in total were assayed and the data for recombinant Pol d 4 (rPol d 4), mutants 1, 2, and 4, whole venom control and native Pol d 4 is reported in Figure 22. The standard deviation for each protein immunospot was calculated and averaged. The standard deviations are as follows: rPol d +/- 65%, mutant 1 +/- 54%, mutant 2 +/- 64%, mutant 4 +/- 71%, and whole venom +/- 73%. The whole venom control and native Pol d 4 had an average of 1.1×10^6 and 1.3×10^6 INT/mm^2 respectively. The baculovirus expressed Pol d 4

Figure 20: Immunodetection of IgE binding to various forms of recombinant Pol d 4 with sera from patients allergic to European paper wasps. Sera (1:5) from patients previously identified with positive IgE binding reactions to European paper wasps were used to assay IgE binding to recombinant forms of Pol d 4. Two replications were performed with 40% of the patient sera and one with remaining sera. Patients were numbered 1-13. The recombinant forms of Pol d 4 were blotted at a concentration of 5 µg per spot. The proteins blotted were: baculovirus expressed recombinant Pol d 4 (A), mutant 1 of Pol d 4 (B), mutant 2 of Pol d 4 (C), and mutant 4 of Pol d 4 (D). Whole venom (E) and native Pol d 4 (F) were used as controls.

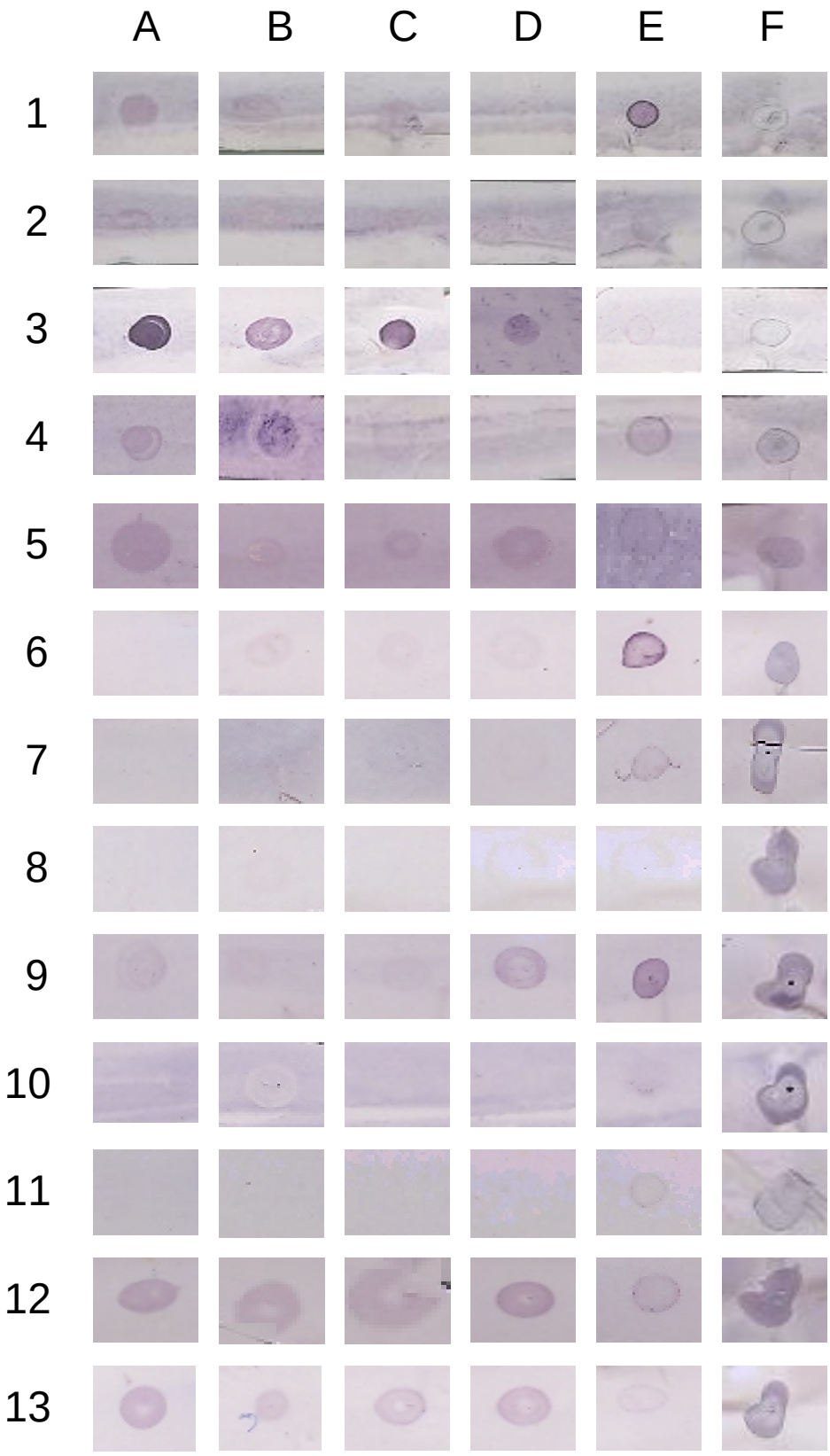

A
B
C
D
E
F
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 21: Immunodetection of IgE binding to various forms of recombinant Pol d 4 with a second set of sera from patients sensitized to European paper wasp. Sera(1:5), from patients previously identified with positive IgE binding to European paper wasps, were used to assay IgE binding. One replication was performed. Patients are numbered 1-4. The recombinant forms of Pol d 4 were spotted at a concentration of 5 µg and are: baculovirus expressed recombinant Pol d 4 (A), mutant 1 of Pol d 4 (B), mutant 2 of Pol d 4 (C), and mutant 4 of Pol d 4 (D). Whole venom (E) and native Pol d 4 (F) were used as controls.

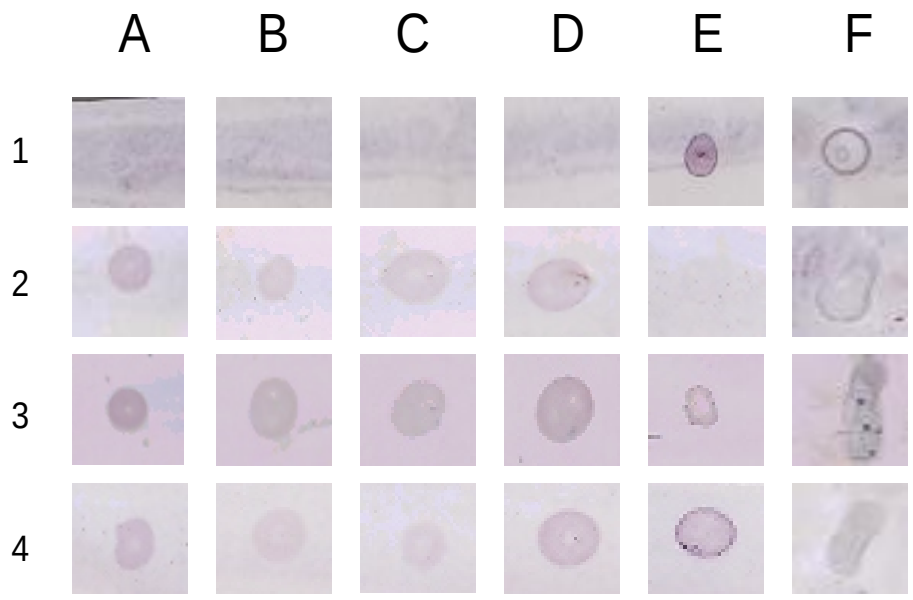
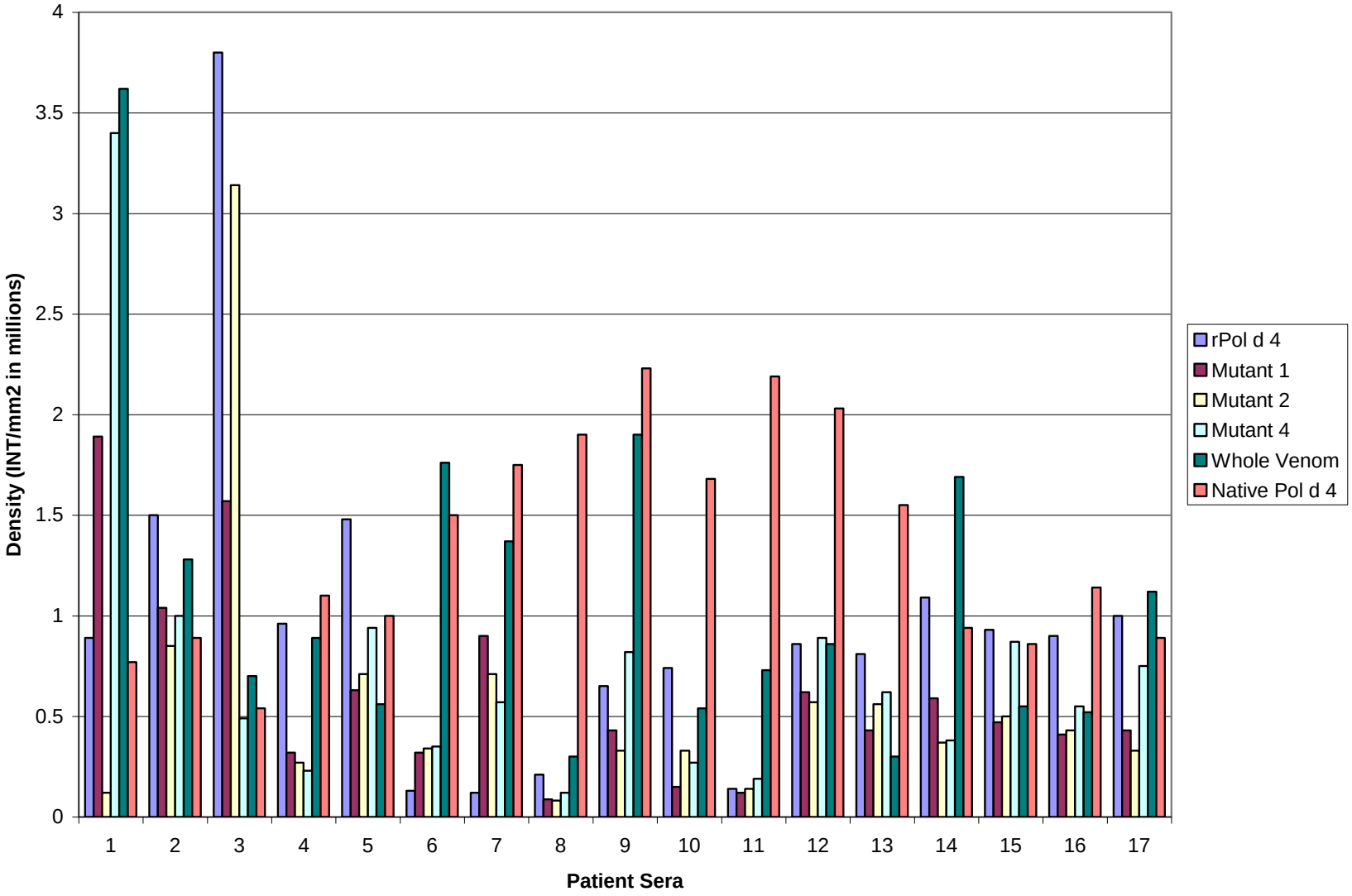

A
B
C
D
E
F
1
2
3
4

Figure 22: Densitometric analysis of the IgE binding of 17 European patient sera to four forms of recombinant Pol d 4. Two sets of patient sera were used densitometric analysis. The first included immunoblots using 13 European patients (1-13), with RAST reactivity recorded, and the second set included four additional European patients (14-17). The densities for baculovirus expressed recombinant Pol d 4, mutant 1 of Pol d 4, mutant 2 of Pol d 4, mutant 4 of Pol d 4, whole venom, and native Pol d 4 were graphed. The standard deviation for the recombinant forms of Pol d 4 are: +/- 65% for baculovirus expressed recombinant Pol d 4, +/- 54% for mutant 1 of Pol d 4, +/- 64% for mutant 2 of Pol d 4, +/- 71% for mutant 4 of Pol d 4, and +/- 73% for whole venom. One-way ANOVA analysis of IgE binding showed that there was no significant difference between each group of protein.


Density (INT/mm2 in millions)
4
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Patient Sera
rPol d 4
Mutant 1
Mutant 2
Mutant 4
Whole Venom
Native Pol d 4

immunospots had an average of 3.8 x 10^6 INT/mm^2 for all 17 sera assayed. Patient 3 had the highest value while patient 7 had the lowest. The highest average density for the mutants was in response to mutant 3 at 7.3 x 10^5 INT/mm^2, while the lowest average density was to mutant 2 at 5.7 x 10^5 INT/mm^2. The average density of mutant 1 was 6.1 x 10^5 INT/mm^2. The lowest density values for all three mutants were observed from patient 8. Patient 1 had the highest density values to mutants 1 and 4, while the highest for mutant 2 was patient 3. The densitometric data obtained from assays with European patient sera was used to assay the differences in IgE binding to the various recombinant proteins by one-way ANOVA. The statistical test showed that there was no significant difference in between patient sera.

Three sera from American patients were used to assay IgE binding to the various forms of recombinant Pol d 4 (Figure 23). These patients have been shown to have reactivity with other Vespids. Visually the baculovirus expressed form of Pol d 4 bound 66% of the sera. Patients 2 and 3 had IgE that bound to mutants 1, 2, and 4. The whole venom and native Pol d 4 bound IgE from all patient sera. The densitometric values were collected for the American patient immunoblots and are shown in Figure 24. The average IgE binding for the controls, whole venom and native Pol d 4, was 6.6 x 10^5 and 8.2 x 10^5 INT/mm^2 respectively. The highest density values for recombinant Pol d 4 and the three mutated forms were seen in response to sera from patient 2. The average binding to rPol d 4 was 1.8 x 10^6 INT/mm^2, mutant 1 was 1.6 x 10^6 INT/mm^2, mutant 2 was 1.67 x 10^6 INT/mm^2, and mutant 4 was 1.8 x 10^6 INT/mm^2

Figure 23: Immunodetection of IgE binding to various forms of recombinant Pol d 4 with sera from patients sensitized to American paper wasp. Sera from patients previously identified with positive IgE binding to American paper wasp venom were used to assay binding to various forms of recombinant Pol d 4. Sera were diluted 1:5. Patients were numbered 1-3. Each protein was spotted at concentration of 5 µg. The recombinant forms of Pol d 4 represented are: baculovirus expressed recombinant Pol d 4 (A), mutant 1 of Pol d 4 (B), mutant 2 of Pol d 4 (C), and mutant 4 of Pol d 4 (D). Whole venom (E) and native Pol d 4 (F) were used as controls. Only one replication was performed using all of the sera.

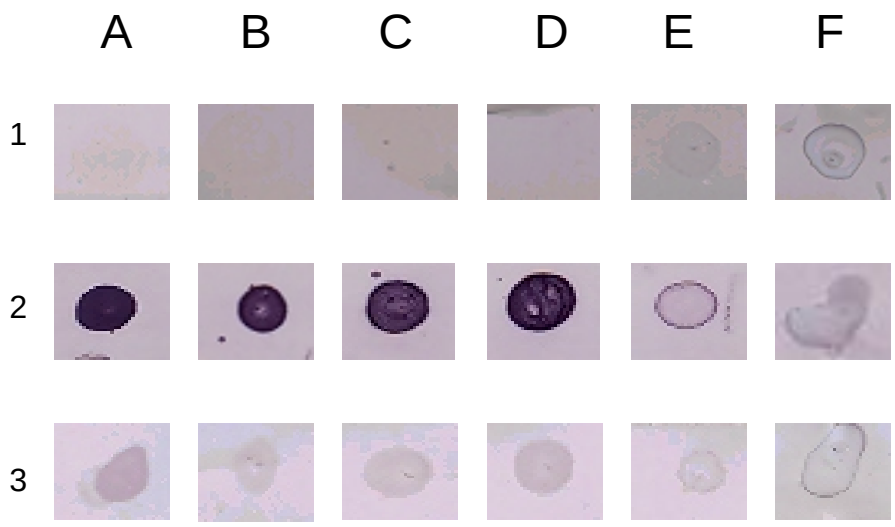
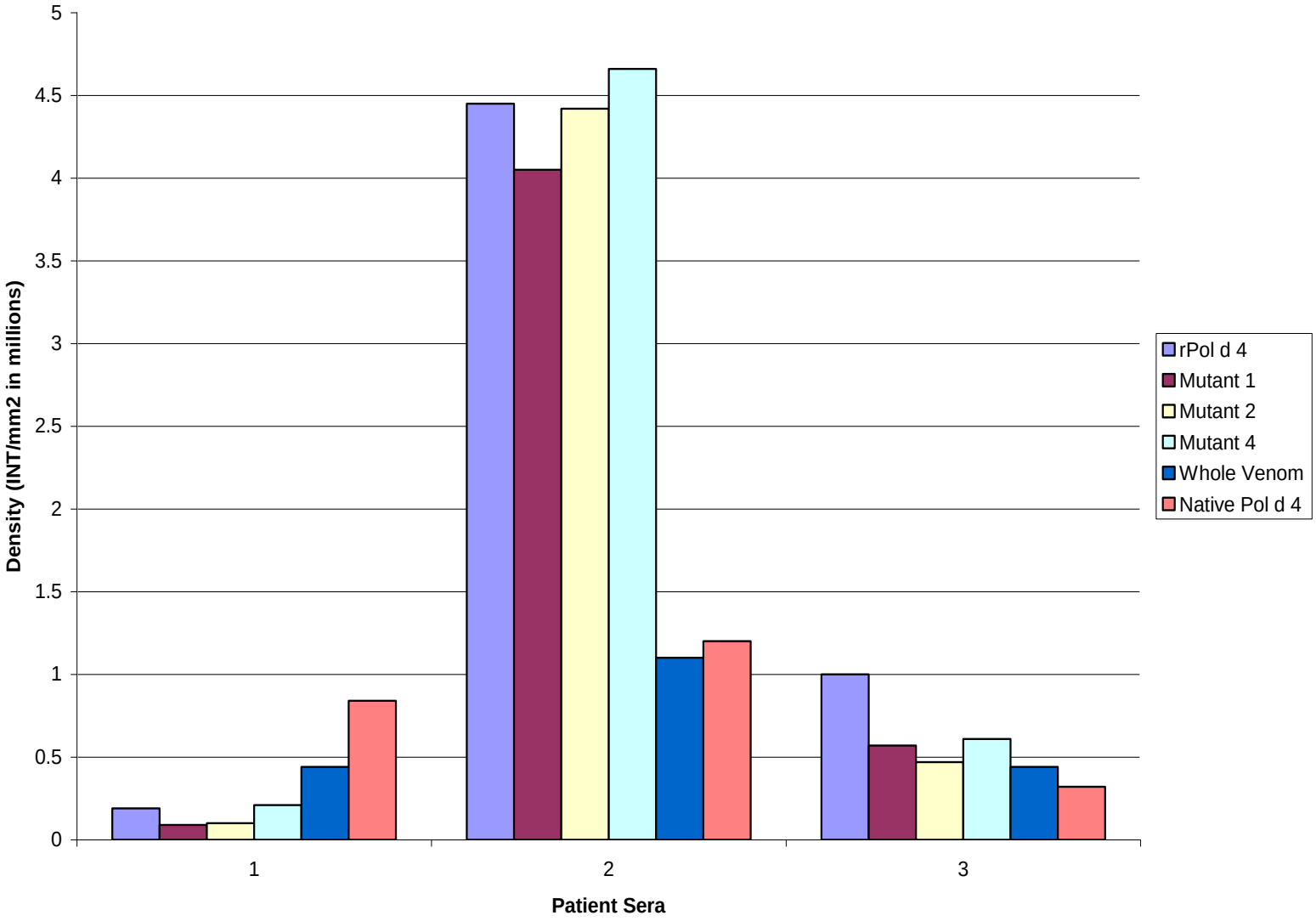

A
B
C
D
E
F
1
2
3

Figure 24: Densitometric analysis of three American sera IgE binding to four forms of recombinant Pol d 4. Three sera from American patients sensitized to American paper wasp venom were used to assay IgE binding to the recombinant forms of Pol d 4. The densities for baculovirus expressed recombinant Pol d 4, mutant 1 of Pol d 4, mutant 2 of Pol d 4, mutant 4 of Pol d 4, whole venom, and native Pol d 4 were graphed. Due to the limited amount sera available only one replication was performed with all the patient sera.


5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Density (INT/mm2 in millions)
1
2
3
Patient Sera
rPol d 4
Mutant 1
Mutant 2
Mutant 4
Whole Venom
Native Pol d 4

In an attempt to assay the presence of CCD in IgE binding from different European patient sera, the carbohydrate specific IgE was inhibited with bromelain. This assay utilizes bromelain as inhibitor, a set of highly glycosylated cysteine proteases isolated from pineapple, to occupy cross-reactive carbohydrate-specific IgE. The sera used in this assay were selected from the results of the previous immunoblot. The patient sera used were; European patients 5, 8, 9, 10, and 11 to assay the change in IgE binding to rPol d 4 during inhibition assays. The sera was incubated with 1 mg/ml of bromelain prior to incubation with membrane bound recombinant Pol d 4. Immunoblots are shown in Figure 25. The strongest IgE binding among the immunoblot inhibition samples was seen in patient 8, while the lowest was seen from patient 11.

3.4 Cloning of Pol g 4

The cDNA of Pol g 4 was cloned and one was used to evaluate identity with the known cDNA sequence and the derived protein sequence of Pol d 4. The resulting sequence consists of 277 nucleic acids. The sequence was obtained from an independent PCR, a second PCR reaction was not obtained to account for possible polymerase mutations. A BLAST search was performed with the Pol g 4 sequence, with the most closely related sequence being the allergenic venom serine protease from *P. dominulus*. The aligned sequences had 96% identity (Figure 26).

The nucleic acid sequence was translated and characterized with the BLAST application on the NCBI website. The amino acid sequence was placed in a trypsin-like superfamily. A 12 amino acid propeptide was identified upstream of the mature molecule. A propeptide cleavage site was identified ending at isoleucine 15. The three

Figure 25: Inhibition immunoblot assay of IgE binding to recombinant Pol d 4. The purified recombinant proteins were spotted at a concentration of 5 µg and all human sera was diluted 1:5. The blots of baculovirus expressed recombinant Pol d 4 (A) and whole venom (B) were probed with sera from European patients (5, 8, 9, 10, and 11). Inhibition assays were used to identify the possible involvement of carbohydrate-specific IgE in IgE binding to recombinant Pol d 4. Prior to incubation with the membrane bound proteins, the sera and a horse sera control (Con) was incubated with 1 mg/ml of Bromelain to compete for CCD specific IgE. The inhibition reaction was used to probe recombinant Pol d 4 (C) and whole venom (D). Only one replication of this assay was performed.

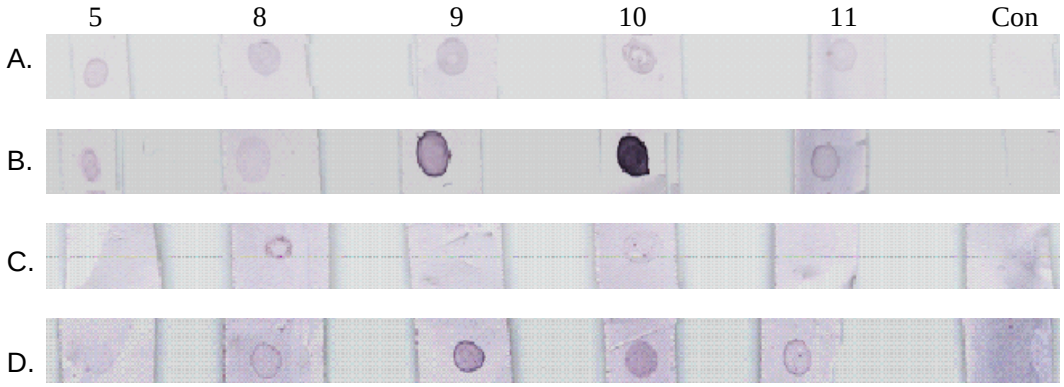
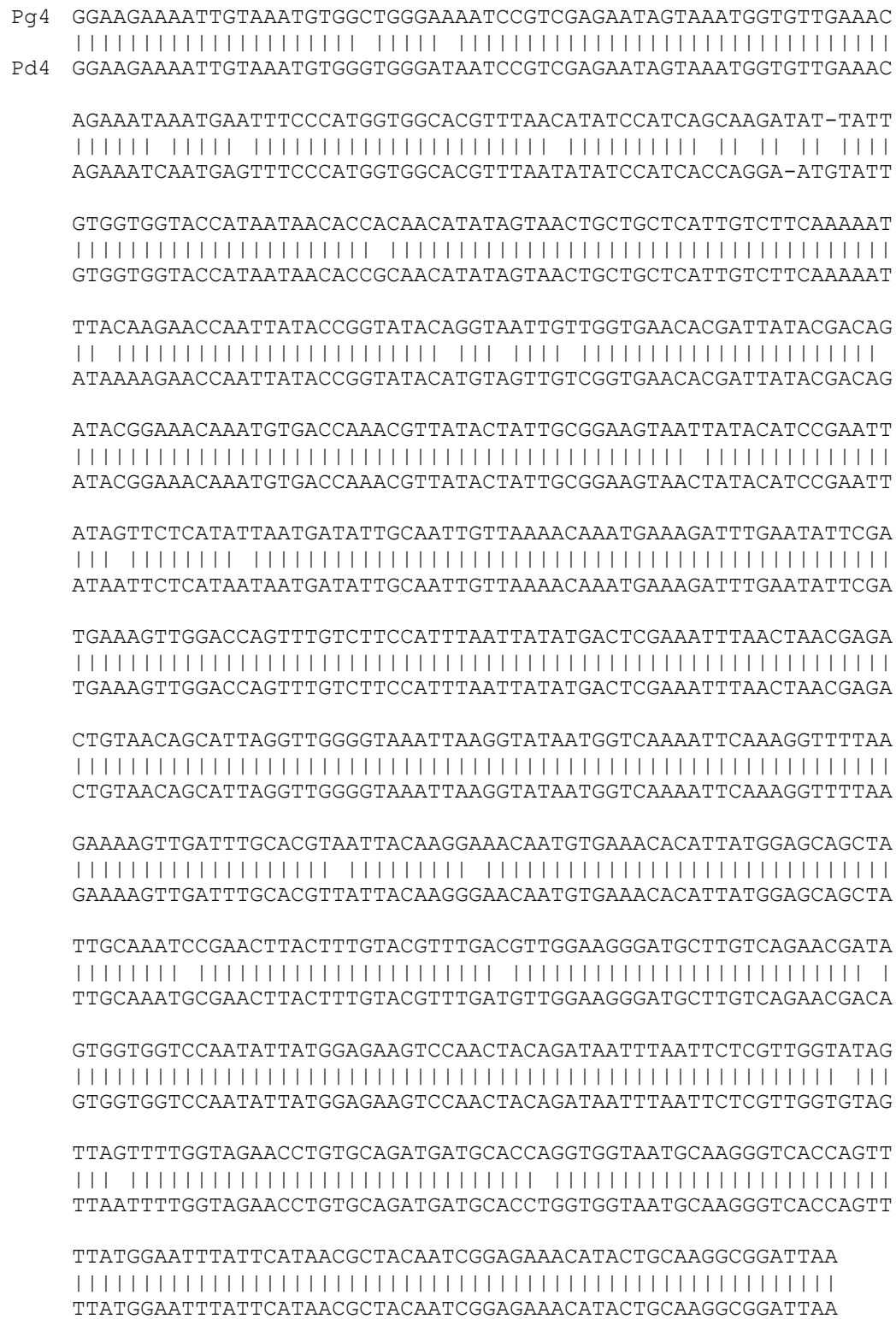

5
8
9
10
11
Con
A.
B.
C.
D.

Figure 26: Alignment of the Pol g 4 cDNA sequence with the Pol d 4 cDNA sequence. The Pol g 4 sequence was cloned from mRNA and characterized by nucleic acid sequence. Only one clone was evaluated. Pol g 4 (top) and Pol d 4 (bottom) cDNA sequences were aligned with BLAST via the NBCI website. Identity is indicated with a line (I). The sequences were 96% identical.

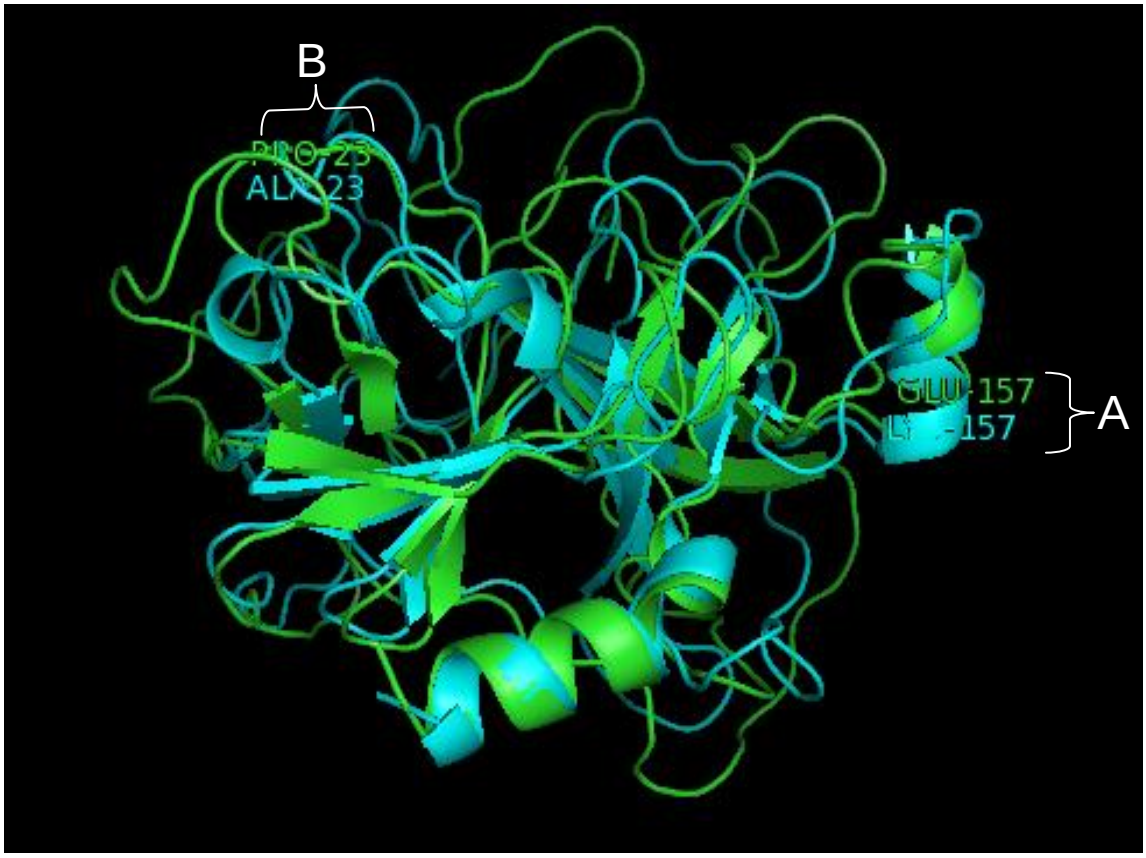

Pg4 GGAAGAAAATTGTAAATGTGGCTGGGAAAATCCGTCGAGAATAGTAAATGGTGTTGAAAC
Pd4 GGAAGAAAATTGTAAATGTGGGTGGGATAATCCGTCGAGAATAGTAAATGGTGTTGAAAC
AGAAATAAATGAATTTCCCATGGTGGCACGTTTAACATATCCATCAGCAAGATAT-TATT
AGAAATCAATGAGTTTCCCATGGTGGCACGTTTAATATATCCATCACCAGGA-ATGTATT
GTGGTGGTACCATAATAACACCACAACATATAGTAACTGCTGCTCATTGTCTTCAAAAAT
GTGGTGGTACCATAATAACACCGCAACATATAGTAACTGCTGCTCATTGTCTTCAAAAAT
TTACAAGAACCAATTATACCGGTATACAGGTAATTGTTGGTGAACACGATTATACGACAG
ATAAAAGAACCAATTATACCGGTATACATGTAGTTGTCGGTGAACACGATTATACGACAG
ATACGGAAACAAATGTGACCAAACGTTATACTATTGCGGAAGTAATTATACATCCGAATT
ATACGGAAACAAATGTGACCAAACGTTATACTATTGCGGAAGTAACTATACATCCGAATT
ATAGTTCTCATATTAATGATATTGCAATTGTTAAAACAAATGAAAGATTTGAATATTCGA
ATAATTCTCATAATAATGATATTGCAATTGTTAAAACAAATGAAAGATTTGAATATTCGA
TGAAAGTTGGACCAGTTTGTCTTCCATTTAATTATATGACTCGAAATTTAACTAACGAGA
TGAAAGTTGGACCAGTTTGTCTTCCATTTAATTATATGACTCGAAATTTAACTAACGAGA
CTGTAACAGCATTAGGTTGGGGTAAATTAAGGTATAATGGTCAAAATTCAAAGGTTTTAA
CTGTAACAGCATTAGGTTGGGGTAAATTAAGGTATAATGGTCAAAATTCAAAGGTTTTAA
GAAAAGTTGATTTGCACGTAATTACAAGGAAACAATGTGAAACACATTATGGAGCAGCTA
GAAAAGTTGATTTGCACGTTATTACAAGGGAACAATGTGAAACACATTATGGAGCAGCTA
TTGCAAATCCGAACTTACTTTGTACGTTTGACGTTGGAAGGGATGCTTGTCAGAACGATA
TTGCAAATGCGAACTTACTTTGTACGTTTGATGTTGGAAGGGATGCTTGTCAGAACGACA
GTGGTGGTCCAATATTATGGAGAAGTCCAACTACAGATAATTTAATTCTCGTTGGTATAG
GTGGTGGTCCAATATTATGGAGAAGTCCAACTACAGATAATTTAATTCTCGTTGGTGTAG
TTAGTTTTGGTAGAACCTGTGCAGATGATGCACCAGGTGGTAATGCAAGGGTCACCAGTT
TTAATTTTGGTAGAACCTGTGCAGATGATGCACCTGGTGGTAATGCAAGGGTCACCAGTT
TTATGGAATTTATTCATAACGCTACAATCGGAGAAACATACTGCAAGGCGGATTAA
TTATGGAATTTATTCATAACGCTACAATCGGAGAAACATACTGCAAGGCGGATTAA

amino acids of the active site were identified, histidine 45, asparagine 140, and serine 260. The substrate binding site consists of asparagine 202, serine 275, and glycine 224. A search of the NCBI protein database showed that the amino acid sequence was most closely related to Pol d 4 (Figure 27). The search demonstrates that the Pol g 4 amino acid sequence was most closely related to the Pol d 4 amino acid sequence (96%) followed by an anticoagulant serine protease from *Vespa magnifica* (52%), venom protein from *Nasonia vitripennis* (44%), venom serine protease from *Apis mellifera* (42%), and a trypsin-like protease from *Anthonomus grandis* (42%). Models of Pol d 4 and Pol g 4 were generated, using SWISS-MODEL which showed similar structure and substrate binding sites (Figure 28). However, there are some notable amino acids changes between the two molecules. The change of alanine (23) to a proline is a significant change within the structure of the molecule. Also, the substitution of a glutamic acid at position 158 (lysine) in Pol d 4 alters the local charge and extends a α-helix in the Pol g 4 model.

Figure 27: Comparison of the derived Pol g 4 and Pol d 4 amino acid sequences. Nucleic acid sequences for Pol g 4 and Pol d 4 were used to obtain amino acid sequences using GCG. The derived amino acid sequence for Pol g 4 (top) was compared with the sequence of Pol d 4 (bottom) using NCBI program BLASTn. The middle sequence shows the amino acids in common between both sequences, the + indicates amino acid similarity and a blank space denotes a difference in charge. These sequences share 96% identity.

```
Pg4  EENCKCGWENPSRIVNGVETEINEFPMVARLTYPSARYYCGGTIITPQHIVTAAHCLQK
     EENCKCGW+NPSRIVNGVETEINEFPMVARL YPS   YCGGTIITPQHIVTAAHCLQK
Pd4  EENCKCGWDNPSRIVNGVETEINEFPMVARLIYPSPGMYCGGTIITPQHIVTAAHCLQK

     FTRTNYTGIQVIVGEHDYTTDTETNVTKRYTIAEVIIHPNYSSHINDIAIVKTNERFEYS
     + RTNYTGI V+VGEHDYTTDTETNVTKRYTIAEV IHPNY+SH NDIAIVKTNERFEYS
     YKRTNYTGIHVVVGEHDYTTDTETNVTKRYTIAEVTIHPNYNSHNNDIAIVKTNERFEYS

     MKVGPVCLPFNYMTRNLTNETVTALGWGKLRYNGQNSKVLRKVDLHVITRKQCETHYGAA
     MKVGPVCLPFNYMTRNLTNETVTALGWGKLRYNGQNSKVLRKVDLHVITR+QCETHYGAA
     MKVGPVCLPFNYMTRNLTNETVTALGWGKLRYNGQNSKVLRKVDLHVITREQCETHYGAA

     IANPNLLCTFDVGRDACQNDSGGPILWRSPTTDNLILVGIVSFGRTCADDAPGGNARVTS
     IAN NLLCTFDVGRDACQNDSGGPILWRSPTTDNLILVG+V+FGRTCADDAPGGNARVTS
     IANANLLCTFDVGRDACQNDSGGPILWRSPTTDNLILVGVVNFGRTCADDAPGGNARVTS

     FMEFIHNATIGETYCKAD
     FMEFIHNATIGETYCKAD
     FMEFIHNATIGETYCKAD
```

Figure 28: Super position of the molecular models of Pol d 4 and Pol g 4. Models of Pol d 4 (green) and Pol g 4 (turquoise) were generated using SWISS-MODEL. The models were superimposed using PVPDB viewer. Two notable substitutions between the two serine proteases are highlighted. The two changes are from (A) glutamic acid (Pol d 4) to lysine (Pol g 4) and (B) proline (Pol d 4) to alanine (Pol g 4).


B
A

Chapter 4: DISCUSSION

The cross-reactivity between European and American paper wasp venom allergens has been shown to be partial. The allergenic serine protease isolated from European paper wasp venom has been identified as an important venom allergen (Sanchez *et al*., 1995, Pantera *et al*., 2003, and Winningham *et al*., 2004). The identification of allergenic epitopes on Pol d 4 will provide data that will be important in determining the role of Pol d 4 in the partial cross-reactivity between paper wasp groups. During characterization of baculovirus expressed recombinant of Pol d 4, evidence indicated that it may be heterogeneously glycosylated. The *E.coli* expressed recombinant form of Pol d 4 was unsuccessfully refolded. Immunoblot assay results of various forms of recombinant Pol d 4 contain amino acid epitopes and possibly CCD. Lastly, the nucleic sequence for Pol g 4 had high identity to the Pol d 4 sequence, supporting the evidence that the two molecules bind the same amount of IgE.

4.1 Analysis of the production of recombinant Pol d 4

In order to evaluate IgE binding to Pol d 4, the protein was expressed in a baculovirus and E. coli system. The previously reported nucleic acid sequence was used for expression.

4.1.1 Eukaryotic expression of Pol d 4

Recombinant protein expression is widely used to produce proteins from a variety of sources. Expression of target proteins allow for the production of large amounts without the laborious tasks of insect collection, protein isolation, and extensive purification. A variety of cell lines, both eukaryotic and prokaryotic are used with associated expression vectors. Typically, proteins derived from insects are expressed in

either a baculovirus or bacterial expression system. Allergens produced in the baculovirus systems can retain native conformation, bioactivity, and IgE reactivity. This is due to similarities in protein processing, such as, glycosylation and conformational folding. In addition, this system allows the use of native leader sequences for the secretion of target proteins resulting in easier purification.

The baculovirus expression system was chosen for expression of a recombinant form of Pol d 4 which would be similar to native Pol d 4. Several laboratories have reported the successful expression of insect venom allergens in this type of system (King *et al*., 1996, Schmidt *et al*., 1996, Schmidt *et al*., 2003, and Soldatova *et al*., 2007). Glycosylation is essential for proper folding of some proteins. Additional research has shown that carbohydrates may have an important role in IgE binding to this venom allergen and investigation of IgE epitopes must include carbohydrate (Hemmer *et al*., 2001, Bencurova, 2004, Hemmer *et al.*, 2004, and Chensheng, 2008).

Analysis of recombinant Pol d 4 produced in the baculovirus system yielded a protein that was larger in size than the calculated size. Mass spectrometry analysis of the intact recombinant protein confirmed the molecular weight observed with SDS-PAGE and western blotting. The difference in size between the recombinant protein and the calculated molecular weight of Pol d 4 could possibly be the result of post-translational modification of the expressed protein.

Evidence obtained during the characterization of the product of expression in Pol d 4 in a baculovirus system may indicate that it maybe glycosylated. An increase in protein molecular weight due to glycosylation has been observed in many proteins. An example of an increase in molecular weight by glycosylation is *Bothrops* (snake) protease

A, a serine protease in the same family as Pol d 4. Studies have shown carbohydrate accounts for 62% of the molecular weight of the native form of *Bothrops* protease A (Nobuhiro *et al*., 2003 and Leme *et al*., 2008). Analysis of the amino acid sequence of Pol d 4 showed the presence of six potential N-glycosylation sites (Fitch *et al*., 2000 and Winningham *et al.*, 2004) and two to four of these sites appear to be glycosylated in the native form. During mass spectrometric analysis of tryptic peptide fragments only three of the six glycosylation sites were present on the observed fragments, suggesting if the recombinant protease is glycosylated it would be limited to three of the sites.

The change in mass and charge maybe contributed to the several post-translational modifications. Recombinant proteins can undergo several modifications, such as, acetylation, phosphorylation, alkylation, and glycosylation which may not mimic modifications observed in the native protein. For example, Du *et al*. (2005) have shown that phosphorylation of serine residues in histidine-tag sequence can cause heterogeneity in mass of an expressed kinases. While our construct does not contain serine residues in the histidine-tag, a phenomenon such as this may affect the size of our recombinant protein and should be investigated to verify glycosylation of the recombinant serine protease.

The difference in size and charge when observing the baculovirus expressed recombinant form of Pol d 4 may be caused by different factors. Differential glycosylation has been observed in proteins expressed in Sf9 (Mannberg *et al*., 1992 and Percival *et al.*, 1997). The initial expression and evaluation of the glycosylated recombinant form of Pol d 4 was performed in Sf9 cells. The recombinant protein expressed in the High Five™ cell line show similar properties suggesting that these

proteins could contain the same type of modification. The differential charge may be attributed to an artifact of expression in the Sf9 cell line. Studies of the enzyme activity of recombinant proteins which have heterogeneous carbohydrates show that this may not affect enzymatic activity. However, the heterogeneous glycosylation of Pol d 4 may affect the binding of carbohydrate-specific IgE.

4.1.2 Prokaryotic expression of Pol d 4

The use of *E. coli*-produced recombinant allergens in immunotherapy has become of interest in the treatment of insect allergy. These expression systems are efficient and cost effective methods of generating large amounts of target protein. An advantage of this system is the ability to manipulate recombinant proteins to be efficient at stimulating the immune system without causing an allergic reaction. However, proteins produced in these systems lack the post-translational modifications typical of eukaryotic expression systems. Without these modifications the proteins could retain or lack proper structure. The binding of IgE to proteins expressed in these systems must be assayed for their ability to bind IgE.

Pol d 4 was produced in the *E. coli* system in an attempt to assay the binding of IgE to a non-glycosylated form of the serine protease. The purification of recombinant proteins showed that *E. coli* expressed recombinant form of Pol d 4 did not retain native conformation. Several protocols were used to re-fold the recombinant proteins into native conformation, however, these attempt failed to yield a protein that bound to benzamidine.

The lack of apparent conformation of the prokaryotic-expressed recombinant Pol d 4 may have been influenced by a number of factors. First, the harsh conditions needed

for purification of proteins within inclusion bodies can have an irreversible effect on disulfide bond formation. The various protocols used in this study to re-fold recombinant Pol d 4 utilized redox reagents to encourage the formation of disulfide bonds. Second, the lack of glycosylation typical of this type of expression system may have an important effect on the conformation of the recombinant protein. King *et al*. (1996) has shown that a recombinant allergen produced and re-folded in a prokaryotic system has the ability to bind IgE from sensitized patient sera. However, hyaluronidase isolated from Vespula genus, lacks post-translational carbohydrates. The absence of carbohydrate may have made the re-folding of this protein difficult due to their influence on protein conformation. Seppala *et al*., (2009) has shown the importance of carbohydrate on the conformation of another allergen, hyaluronidase. Studies with this molecule showed that when carbohydrate was enzymatically stripped it loses native conformation (Seppala *et al*., 2009). The attempts at re-folding both prokaryotic expressed Pol d 4 constructs may have been influenced by the lack of post-translational modifications. Finally, re-folding of recombinant Pol d 4 produced in the prokaryotic system might be possible using a different protocol. Numerous re-folding protocols are available in the literature; the protocols that were chosen may not have been optimal for the re-folding of Pol d 4.

4.1.3 Epitope analysis of Pol d 4

Data suggests native Pol d 4 contains both carbohydrate and amino acid allergenic epitopes. The amino acid epitope most likely confers species-specific immunologic reactiveness and clinical significance. In order to investigate the role of Pol d 4 in the partial cross-reactivity between European and American paper wasp venom allergens, the

potential allergenic amino acid epitopes were identified, mutated, and assayed for IgE binding.

The determination of allergenic epitopes can be accomplished by different means. The first and most reliable is the visualization of IgE binding by x-ray diffraction of crystals. This method shows the structure of the molecule, and where and how IgE binds to that allergen. This technique requires large amounts of pure protein samples, and IgE from sensitized patients, as well as, an array of equipment and expertise. Several laboratories have used peptide fragments which cover the sequence of the protein of interest. These peptides are probed with IgE, and fragments which bind the IgE can be used to aid in determining the major allergenic epitopes. However, allergenic epitopes of venom allergens are largely conformational. Monoclonal antibody inhibition has been used to map allergenic epitopes. The antibodies are generated against major epitopes, then used to assay the binding of IgE to the allergen of interest. This method requires the generation of monoclonal antibodies which can be time consuming and expensive. Further, the identification of epitopes requires a crystal structure or structural information about similar allergens. Finally, epitopes can be predicted by computational analysis. We predicted epitopes by use of a completed molecular model then mutated, expressed, and evaluated IgE binding to the protein.

Predicted epitopes on Pol d 4 were evaluated for potential allergenic IgE epitopes. The prediction servers typically use models to locate the epitopes. There is no crystal structure available for Pol d 4 or a similar insect serine protease. Three constructs were produced, each containing mutations in two epitopes. Studies utilizing mutagenesis of allergenic epitopes have shown that the change of one amino acid can be sufficient to see

a significant drop in IgE binding (Burks *et al*., 1997 and Beezhold *et al*., 2003). Selected amino acids were mutated to alanine. The alanine residue is neutral and supports the formation of α-helices and β-pleated sheets to aid in maintaining protein conformation (Rabjohn *et al*., 1999). The substitution of an amino acid may change the conformation of the protein in areas other than the targeted epitopes. These changes were not assayed and have to be considered when evaluating the IgE binding data for the recombinant mutants of Pol d 4. The changes in the selected epitopes were used to study epitopes that bind IgE on Pol d 4.

4.2 Analysis of IgE binding to the recombinant and mutated forms of Pol d 4

Partial cross-reactivity between venom allergens of European and North American paper wasps has been documented (Winningham *et al.*, 2003). Other studies have shown that immunotherapy with North American paper wasp venom may confer only partial protection to patients sensitized by European paper wasps (Bonadonna *et al*., 2007). The hyaluronidase and phospholipase A_1B have shown high amounts of cross-reactivity, therefore, they may not be important in species-specific immune response (Hoffman, 1981, Hoffman and McDonald, 1983, Hoffman, 1985, Hoffman, 1986, Jacobson *et al*., 1992, and Lu *et al*., 1995). Antigen 5 and the serine protease may play an important role in species-specific immunologic reactions to venom of European and North American paper wasps (King and Spangfort, 2000, Pantera *et al*., 2003, King and Guralnick, 2004, Winningham *et al*., 2004, and Hoffman, 2006).

Studies with the isolated venom serine protease from each species group showed a distinct pattern of IgE binding from patients sensitized to different paper wasp groups. Winningham *et al.* (2003) showed the serine protease from European and North

American paper wasps bind IgE from sera of American and European sensitized patients. However, the presence of a competitor significantly decreased the binding of IgE North American serine protease, while binding the European protease continued to be present. These results suggest carbohydrate and amino acid epitopes may be present on the allergenic serine protease isolated from European paper wasps.

The binding to amino acid and carbohydrate epitopes were evaluated by immunoblotting. This method, while convenient, has several limitations. The immunoblot assay, unlike western blotting, does not require denaturing conditions, allowing the recombinant proteins to maintain conformation. However, the absence of protein seperation makes it difficult to ascertain the size of the protein being bound by IgE and reducing the amount of background binding that may occur because of contaminating proteins. In addition, immunoblotting may result in non-uniform binding of IgE to the recombinant Pol d 4 due to unequal exposure to the sera which may cause false negative results. Also, unlike other means of assaying protein binding to immunoglobulin, such as ELISA, assaying the colometric changes is difficult due to several factors. The different exposure times of the IgE:protein complex to the substrate may result in a higher or lower densitometric reading and an increase in background. In the described assays, a relatively large concentration of sera and recombinant was used. Titration of sera showed that the 1:5 dilution of patient sera was optimal to visualize IgE binding. The amount of protein was chosen due to the presence of impurities consistently found in the purification of Pol d 4 recombinant proteins. Both concentrations were used in order to account for the small amount of IgE, approximately 5×10^{-5} mg/ml found in human sera.

Imumunoblot was used to assay recombinant and three mutants of Pol d 4 for IgE binding. In addition to the recombinant proteins, a whole venom control was used. This control was used to verify the reactivity of patient sera to venom allergens. This control was used to verify the results of the RAST on the European sera and to visualize the binding of IgE in the other sera to something contained within the venom. A second control, the native serine protease isolated from *P. dominulus* venom via benzamidine, was used to visualize the binding of patient IgE to the native Pol d 4 allergen. The native Pol d 4 was several years old and may have been proteolyzed. The degradation of this protein may account for the atypical and inconsistent immunoblot results with this protein.

The two recombinant forms of Pol d 4 produced in the prokaryotic expression system were assayed for IgE binding with sera from patients sensitized to European paper wasps. Initially, assays using the two prokaryotic forms of Pol d 4 showed that patient IgE did not bind to either recombinant protein (data not shown). The lack of IgE binding to both of the prokaryotic proteins suggests two factors may have influenced the epitopes on Pol d 4. The IgE may be binding to carbohydrates present on the recombinant proteins. Prokaryotic expression of both Pol d 4 constructs would lack these modifications. Secondly, amino acid epitopes on insect allergens are typically conformation dependent. The lack of post-translational carbohydrate and the conditions needed for purification may interfere with disulfide bonding and therefore may have interfered with protein conformation.

The glycosylated form of recombinant Pol d 4 and all three mutants were evaluated for their ability to bind IgE from sera of 17 patients previously assayed for

reactivity to European paper wasp venom (Table 9). The majority of patient sera bound to the whole venom control with the exception of one. The lack of binding to the venom control suggests that IgE specific for the CCD may not be present in this serum but binding to native Pol d 4 suggest IgE specific to that molecule. Since the allergenic serine protease is a minor component of the whole venom, approximately 3%, this patient may not have IgE specific for the major allergens in the venom, causing decreased detection of IgE binding to the whole venom control. Sera with IgE that did not bind the baculovirus expressed form of Pol d 4, did have IgE which bound to *P. dominulus* venom. The lack of IgE binding in these patients suggests that IgE from these patients may be specific for other allergens found in the venom. One of these, Patient 6, had a high reactivity to *P. dominulus* venom of 49.5 KIU/ml, however, no binding to the baculovirus expressed recombinant form of Pol d 4, and marginal binding to the three mutant forms. This type of IgE binding indicates that a high degree of reactivity does not predict binding to a given allergen within the venom.

Immunoblots of the mutants were analyzed to evaluate the importance of the predicted IgE epitopes on binding. One patient had IgE bind to mutant 4 but not to the remaining recombinant form of Pol d 4. This type of binding pattern suggests that the mutation may influence protein conformation, exposing additional epitopes.

Evidence of major epitopes within Pol d 4 can be determined by the lack of IgE binding to the mutated forms of recombinant Pol d 4. Visually, mutant 1 bound the least

Table 9: The binding patterns of IgE from *P. dominulus* sensitized sera to multiple recombinant forms of Pol d 4. The reactivity of the first 13 European patients and the second set of European patients (14-17) are listed along with the binding of patient IgE to various forms of recombinant Pol d4 IgE binding was assayed on glycosylated Pol d 4 (Pol d 4), *E. coli* expressed recombinant form of Pol d 4, three mutant forms of Pol d 4 (mutant 1, mutant 2, and mutant 4), whole venom extract, and native Pol d 4. Negative IgE binding (-), marginal binding (+/-), binding (+), and strong binding (++) are indicated for each serum.

Patient Sera	Reactivity	rPol d 4	Mutant 1	Mutant 2	Mutant 4	Whole Venom	Native Protease
1	13.6	+	+/-	+/-	-	++	+/-
2	9.1	+	-	-	-	+	+
3	2.1	+	+	++	-	+	+
4	3	+	-	-	-	+	+
5	36.6	+	-	+	+	+/-	+
6	49.5	-	+/-	+/-	+/-	++	+
7	21.5	-	+/-	-	+	+	+
8	14.7	-	-	-	-	+/-	+
9	47.5	+	-	-	+	+	+
10	21.1	-	-	-	-	+/-	+
11	15.5	-	-	-	-	+	+
12	40.5	+	+	+	+	+	+
13	7.1	+	+	+	+	+	+
14		-	-	-	-	++	+
15		+	+	+	+	-	+
16		+	+	+	+	+	+
17		+	+	+	+	+	+

of the three mutants, the lack of reactivity to mutant 1 suggests that it may play a role in IgE binding in a large group of patient.

Two patients had IgE to bind to all of the recombinant proteins probed. Interestingly, one of these had a relatively high IgE reactivity, 40 KIU/ml, and the other was low at 7 KIU/ml. This showed that both sera contained IgE specific for Pol d 4 however, other antibodies specific for CCD and other venom allergens maybe more abundant in some patients than others.

The recombinant forms of Pol d 4 were probed for IgE binding with sera from three patients sensitized by American paper wasps (Table 10). All three sera showed strong reactivity to American paper wasps venom and patients 1 and 2 showed strong reactivity to yellow jacket venom. Patient 1 showed very little binding to the recombinant form of Pol d 4 and the three mutant forms, however IgE from this patient bound to whole venom and the native protease. This suggests that patients may have IgE specific for a different allergen with in the venom and that the native protease isolation may contain contaminating allergens. Sera from patients 2 and 3 had IgE that bound to all forms of the recombinant serine protease. Patient 2 serum displayed strong IgE binding to the recombinant protease and the three mutants, as well as the native serine protease and the whole venom extract. This type of binding may have been due to the carbohydrate present on the protein. The presence of carbohydrate-specific IgE may account for the binding of IgE from Patient 3 to the recombinant forms of Pol d 4. The densitometric analysis of the immunoblot data showed that patient 2 had the strongest binding.

Table 10: The binding patterns of IgE from American sera to multiple recombinant forms of Pol d 4. Three American patient sera are listed along with the binding of patient IgE to various forms of recombinant Pol d 4 IgE binding was assayed on E. coli expressed recombinant form of Pol d 4 (Pol d 4), three mutant forms of Pol d 4 (mutant 1, mutant 2, and mutant 4), whole venom extract, and native Pol d 4. Negative IgE binding (-), marginal binding (+/-), binding (+), and strong binding (++) are indicated for each sera.

Patient Sera	rPol d 4	Mutant 1	Mutant 2	Mutant 4	Whole Venom	Native Protease
A1	-	-	-	-	+	+
A2	+	++	++	++	+	+
A3	+	+	+	+	+	+

Evaluation of IgE binding from European patient sera to the three mutated forms of Pol d 4 was performed by densitometric analysis. Data showed that the lowest number of patients had IgE to bind to mutant 1. Only 35% of the sera assayed bound mutant 1. An important epitope is defined as one that will bind IgE from at least 80% or more of the patients' sera (Burks *et al*., 1997). The amino acid changes in mutant 2 seem to have decreased the IgE binding by 65%, which indicates this epitope may be important for IgE binding to Pol d 4. The determination of the importance of the epitope being investigated with mutant 2, and possibly mutants 1 and 3, requires the evaluation of a larger population of sera from patients that have been sensitized to European paper wasps.

The importance in post-translational carbohydrate IgE binding to recombinant Pol d 4 may be important in explaining the results reported in Winningham *et al*. (2003) Several pieces of data obtained during the identification of baculovirus expressed Pol d 4 suggests that the recombinant protease may be glycosylated. However, the presence of carbohydrate has not been directly identified. Immunoblot inhibition was performed to investigate if CCD specific epitopes may be present on recombinant Pol d 4.

Bromelain, a highly glycosylated set of proteins, were used as competitors for CCD specific IgE in the Immunoblot inhibition assays. Commercial bromelain mainly consists of two cysteine proteases, stem bromelain and fruit bromelain. The conditions used during the inhibition assays did not meet optimal pH or temperature for the activation of bromelain (Rowan *et al*., 1990) However, it is still possible for the activation of a small percentage of the cysteine proteases, which may affect the binding IgE. The recombinant serine protease from *P. dominulus* does not contain the cleavage sites for either the fruit or stem bromelain protease. However, the involvement of

activated bromelain, even small percentages, may affect proteins present within the sera. Cleavage of IgE may present as a decrease in protein binding. Proteolysis of other proteins present could theoretically expose pseudo-epitopes, either carbohydrate or amino acid, resulting in false positive results.

During the immunoblot assay a visual decrease in IgE binding to recombinant Pol d 4 in the presence of the bromelain competitor. This pattern of IgE binding could suggest the presence of post-translational carbohydrate specific IgE. However, it was previously stated that this type of moiety has not been definitive identified. The decrease in IgE binding in the presence of bromelain may have been a result of non-specific binding the bromelain proteases or the activation of the cysteine proteases. Further study has to be performed the effectively determine the role of IgE binding to the post-translational carbohydrate. Primarily, the presence and composition of carbohydrate on the recombinant protease has to be clarified and characterized. Secondly, an inhibition assay with a competitor that does not have the capacity to disrupt the system would be important. Engineered molecules, such as the BSA neoglycopeptide, produced by Wilson et al. (1998). This molecule allows the presence of the bromelain carbohydrate groups which are conjugated onto BSA removing the possible effects of the cysteine protease activity while maintaining the IgE epitopes.

American patient 2 had IgE that readily bound all mutant forms of Pol d 4. The serine protease from the American *Polistes* is hypothesized to be related to that found in the Old World paper wasps. It is probable that the difference between European and North American paper wasps is not in the active site between these molecules but in the framework or surface regions (Sanchez *et al*., 1995 and Winningham *et al*., 2003). These

regions would be responsible for the generation of specific IgE. The data from the American *Polistes* sensitized patient suggest that the serine protease from Old and New World paper wasp groups may share epitopes.

Knowledge of the specific interactions between allergens and IgE antibody will aid in our understanding of the complex series of events that occur during an allergic reaction. Details about allergens will enable researchers to better identify and characterize epitopes. In addition, the characterization of allergens and their epitopes will allow optimal treatment of patients by immunotherapy using insect venom extract. Identification of species group-specific epitopes will aid in the generation of molecules for immunotherapy that will be hypoallergenic and protective (Maglio *et al*., 2002). Characterization of these molecules will help determine which expression system is optimal for recombinant production and the important components that are required for proper conformation. Epitope mapping will also allow the generation of artificial conjugate molecules for treatment which may lead to better and safer patient care for insect allergies.

4.3 Characterization of the allergenic serine protease from *P. gallicus* (Pol g 4)

Members of the order Hymenoptera are responsible for fatal immunological reactions to insect venom (Valentine, 1992). Cross-reactivity between allergens in the venom of these insects is caused by protein homology. It has been shown that sequence identity correlates with the relatedness between insect species (Hoffman, 1981, Hoffman and McDonald, 1982, Hoffman, 1985, and Hoffman, 1986). Epitopes that define an allergen's immunologic reactiveness typically are composed of amino acid residues. The serine protease found in *P. gallicus* is an important venom allergen and it binds a

similar amount of IgE as Pol d 4 (Sanchez, 1995 and Winningham *et al*., 2004). To gain a better understanding of the apparent relatedness of these molecules, we characterized the cDNA sequence of Pol g 4. The Pol g 4 sequence shared high identity with Pol d 4. Analysis of the sequences showed that both molecules share the same active sites with little variation in the framework. This supports the high cross-reactivity of IgE binding between the venom serine proteases of *P. dominulus* and *P. gallicus*.

4.4 Conclusions

The presence of carbohydrate and amino acid specific-IgE epitopes on the allergenic serine protease (Pol d 4) isolated from venom of *P. dominulus* was investigated. Eukaryotic expression of Pol d 4 had a higher molecular weight than predicted and a heterogeneous charge, suggesting that a post-translational modification may have affected the recombinant protein. Expression of two constructs in a prokaryotic system yielded protein that corresponded with the calculated size of Pol d 4, however, the folding of this protein into proper conformation was not confirmed using the described protocols. Allergenic epitopes were predicted and three were mutated to investigate IgE binding.

Immunoblots showed IgE from the majority of European and American patients bound recombinant Pol d 4, demonstrating that the expressed protein had the ability to be immunologically reactive. Further, assays with the same sera showed that IgE binding to the three mutants decreased by approximately half and this may indicate that these epitopes may be important in IgE binding in some patients. Inhibition immunoblot studies showed a decrease in IgE binding, however, further investigation of the post-

translational modifications of recombinant Pol d 4 needs to be more extensive to determine if carbohydrate are definitively involved.

The molecular identification of the allergenic serine protease isolated from the venom of *P. gallicus* (Pol g 4) showed high identity with Pol d 4, followed by a variety of other insect serine proteases including, the honey bee and bumble bee venom serine proteases. Comparison of the models generated with the sequences of Pol d 4 and Pol g 4 showed structural similarities, suggesting both molecules may share allergenic epitopes. Additional studies of the allergenic serine protease from European and North American paper wasps are needed to fully understand the allergenic serine proteases' role in the allergenic patient response.

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APPENDIX A: UMCIRB APPROVAL LETTER

University and Medical Center Institutional Review Board
East Carolina University, 600 Moye Boulevard
1L-09 Brody Medical Sciences Bldg. • Greenville, NC 27834
Office **252-744-2914** • Fax **252-744-2284** • **www.ecu.edu/irb**
Chair and Director of Biomedical IRB: L. Wiley Nifong, MD
Chair and Director of Behavioral and Social Science IRB: Susan L. McCammon, PhD

Date: March 4, 2010

Principal Investigator: Donald R. Hoffman, PhD
Dept./Ctr./Institute: Pathology & Laboratory Medicine
Mailstop or Address: 642

RE: **Exempt Certification**
UMCIRB# **10--0102**
Funding Source: Pathology & Laboratory Medicine

Title: Studies of Polistes Venom Allergens

Dear Dr. Hoffman:

On 2/27/10, the University & Medical Center Institutional Review Board (UMCIRB) determined that your research meets ECU requirements and federal exemption criterion #4 which includes research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects..

It is your responsibility to ensure that this research is conducted in the manner reported in your Internal Processing Form and Protocol, as well as being consistent with the ethical principles of the Belmont Report and your profession.

This research study does not require any additional interaction with the UMCIRB unless there are proposed changes to this study. Any change, prior to implementing that change, must be submitted to the UMCIRB for review and approval. The UMCIRB will determine if the change impacts the eligibility of the research for exempt status. If more substantive review is required, you will be notified within five business days.

The UMCIRB Office will hold your exemption application for a period of five years from the date of this letter. If you wish to continue this protocol beyond this period, you will need to submit an Exemption Certification Request at least 30 days before the end of the five year period.

Sincerely,

Chairperson, University & Medical Center Institutional Review Board