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Allergen Proteinases and Allergic Airway Inflammation: Signaling via Proteinase-Activated Receptors

by

Daniel Polley

A THESIS

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Abstract

Cockroach and *Alternaria alternata* are common allergens to which sensitization is associated with the development of asthma. Trypsin-like activity that activates Proteinase Activated Receptor-2 (PAR2) has been reported in both allergens, though the cockroach- and *Alternaria*-derived proteinases have yet to be identified and characterized. Trypsin-specific activity-based probe labeling revealed three distinct enzymes in the cockroach allergen (E1, E2, E3), and one in the Alternaria allergen (Alt). Each of the enzymes was isolated and purified with ion-exchange chromatography, and the isolated enzymes characterized by determining their biochemical properties. The enzymes were distinct based on their: (1) selectivities for fluorogenic peptide substrates (Km), (2) sensitivities to serine proteinase inhibitors (Ki), and (3) amino acid sequences (mass spectrometral sequencing). Each enzyme was also assessed for its ability to activate/dis-arm PAR1 and PAR2 by (1) measuring Ca^{2+} signaling, (2) measuring MAPK phosphorylation, and (3) monitoring the cleavage of PAR-linked luciferase constructs. Each of the enzymes was found to activate PAR2-mediated Ca^{2+} and MAPK signaling at equal concentrations. The enzymes were found to regulate PAR1 differentially; cockroach E1 and E3 are biased agonists for PAR1, preferentially activating MAPK signaling over Ca^{2+} signaling, whereas E2 and Alt are non-biased agonists for PAR1, activating both Ca^{2+} and MAPK signaling at relatively low concentrations. Finally, the ability of the allergen-derived enzymes to regulate PAR signaling in airway-derived epithelial cells and mouse bronchial tissues was assessed. Each allergen enzyme activated MAPK signaling in the airway cells, and activation of the cells with E1 and Alt

induced IL-8 expression. Enzymatic regulation of PAR signaling at the tissue level was assessed as the smooth muscle relaxation response in mouse aortic and bronchial tissues. Alt and E1 were able to induce relaxation via PAR2 in tissues from PAR1 knockout mice, but only Alt elicited a relaxation response via PAR1 in PAR2-null tissue. PAR signaling in the airway has been shown to contribute to inflammation and allergic sensitization, so each of the four allergen-derived trypsin-like enzymes are potential therapeutic targets in allergic airway disease.

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Most importantly, I thank my family and friends for providing constant love and support. And Angela, for supporting me, challenging me, listening to me, laughing with me, and being my best friend.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
A	Alanine
F	Phenylalanine
G	Glycine
K	Lysine
P	Proline
Q	Glutamine
R	Arginine
V	Valine
A549	Type II alveolar carcinoma-derived cell line
ABP	Activity-based probe
Alt	Alternaria-derived trypsin-like enzyme
AMC	Aminomethyl coumarin
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BEAS-2B	Transformed bronchial epithelial cell line
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CE	Cockroach allergen extract
CHO	Chinese hamster ovary cell line
DEAE	Diethylaminoethyl
DMEM	Dulbecco's modified Eagle medium
dNTP	deoxyribonuclotide triphosphate
DPP4	Diphenylphosphonate
E1-3	Cockroach-derived enzymes 1-3
ECL	Enhanced chemiluminescent

ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinase
EST	Expressed sequence tag
FBS	Fetal bovine serum
FPLC	Fast phase liquid chromatography
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
HBSS	Hanks' balanced salt solution
HEK	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
IC50	Half maximal inhibitory concentration
Ig	Immunoglobulin
IL	Interleukin
Ki	Inhibition constant
Km	Michaelis constant
KNRK	Kirsten-virus transformed normal rat kidney cells
L-NAME	N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride
MAPK	Mitogen-activated protein kinase
mCh	mCherry red fluorescent protein
Nluc	Nano-Luciferase
PAB	Proteinase activity buffer
PAGE	Polyacrylamide gel electrophoresis
PAR	Proteinase-activated receptor
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.1 % Tween-20
PCR	Polymerase chain reaction

pI	Isoelectric point
Rluc	*Renilla reniformis* luciferase
SBTI	Soybean trypsin inhibitor
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TL	Tethered ligand
TLCK	N-α-tosyl-L-lysinyl-chloromethylketone
YFP	Yellow fluorescent protein

CHAPTER 1 INTRODUCTION

1.1 Asthma

1.1.1 Introduction to asthma

Asthma is a growing concern, representing a substantial worldwide public health and financial burden. Asthma prevalence has been found to range from 1% - 18% in populations around the world, and is increasing on a global level[1]. Asthma accounts for 1% of all work days lost, and is responsible for 250 000 deaths per year worldwide[1]. Despite improvements in preventative care and asthma treatment, costs associated with asthma are still increasing, highlighting the need for additional treatment options[2].

Asthma is characterized by chronic inflammation of the conducting airways with progressive, irreversible changes to the structure of the airway epithelium, smooth muscle and submucosal mesenchymal tissue. Asthmatic inflammation is similar to allergic inflammation in that it is generally characterized by a type 2 T-helper cell (Th2) differentiation phenotype with eosinophilic cellular infiltration playing a prominent role in disease pathogenesis[3], although neutrophils, basophils and mast cells are also common contributors[4]. When recruited to the site of allergen exposure in the airways of sensitized individuals, these leukocytes release inflammatory mediators that, in the chronic inflammatory condition of the asthmatic lung, can contribute to host tissue damage and the resulting structural remodeling that occurs in parallel in the pathogenesis of asthma[4].

This structural component of the disease is characterized by airway obstruction that can be both reversible (contraction of the airway smooth muscle) and irreversible (physical narrowing of the conducting airways). Airway remodeling refers to the

progressive structural changes that occur during the development of asthma. It is characterized by a hypertrophy and thickening of the smooth muscle layer, subepithelial fibrosis, decreased cartilage volume and fibronectin deposition, increased vascularization and microvascular leakage, and changes in the epithelial cellular makeup, including goblet cell hyperplasia[4]. The results of these changes are thicker, less flexible airway walls and excessive mucus production, which result in a chronic and irreversible narrowing of the airway. The structural changes may also contribute to airway hyperresponsiveness, the characteristic exaggerated smooth muscle contractile response to a variety of stimuli that can lead to life-threatening narrowing of the airways during asthma exacerbations[5]. Conventionally, it was thought that the process of airway remodeling occurred secondarily to chronic airway inflammation as a result of the damage incurred by the inflammatory mediators, but there is evidence of airway remodeling early in the pathogenesis of young patients, in some cases prior to overt signs of airway inflammation[5]. This suggests that the two major features of asthma, chronic inflammation and airway remodeling, may develop in parallel, and so the processes underlying each may provide separate targets for therapeutic intervention[6].

1.1.2 Societal burden of asthma

Though substantial research has been conducted with the goal of understanding and treating the disease, asthma still represents a significant, and growing, public health, economic and humanistic burden. In Canada, there are an estimated 3 million asthma patients, representing ~8.5% of the population over the age of 12 and 13% of children[7]. The prevalence of asthma in adults in Canada has increased substantially,

from 2.1% of the population over the age of 15 in 1979 to 8.5% as of 2012. Likewise in the USA, an estimated 1/12 adults and 1/11 children have been diagnosed with asthma[8], and similar prevalence rates have been reported in industrialized nations around the world[1]. Since asthma is characterized by periodic episodes of incapacitating exacerbations, often requiring hospitalization, the economic costs of the disease can be substantial. Total costs of asthma are estimated to be $56 billion per year in the USA , accounting for both direct costs of treating the disease and indirect costs such as lost productivity due to work days missed[9], and substantial costs to the health care system and the economy have also been reported in Canada[10]. Most importantly, the humanistic burden of asthma is substantial. In addition to the estimated 250 000 deaths annually attributed to asthma[1], the quality of life of asthma patients can also be greatly affected. Compounding the physical symptoms, asthma patients report higher than average levels of anxiety and depression[10]. The substantial societal burden imposed by asthma highlights the crucial need to develop better preventative measures and therapeutic treatments, which may be assisted by cultivating a better understanding of the physiological mechanisms underlying asthma pathogenesis.

1.1.3 Asthma treatments and control

The common recommended treatments for asthma, comprising corticosteroids to control airway inflammation with their anti-inflammatory effects[11], and short- or long-acting β2-adrenoceptor agonists to reverse or prevent airway smooth muscle contraction associated with asthma exacerbations[12], aim to control symptoms and to prevent exacerbations, but in some cases are insufficient in doing so[13]. As the global

prevalence of the disease continues to increase, the need to develop a better understanding of the underlying pathogenic mechanisms will assume a vital importance in the development of more effective treatments. As I will discuss later, we propose targeting biologically active allergens, specifically allergens with proteinase activity, which possess the ability to directly influence the host response to the inhaled environment.

1.1.4 Contributing factors to asthma pathogenesis

A major source of difficulty in fully understanding the pathogenesis of asthma is that there are numerous and diverse environmental and genetic factors that contribute to individual cases of the disease. Sensitization to airborne allergens is a particularly significant risk factor; though some asthma cases are not associated with allergic sensitization, a majority of asthma cases are preceded by early-life exposure to inhaled environmental allergens[14,15,16]. There are several lines of evidence pointing to the importance of allergic sensitization in the development and severity of disease in asthma patients. Associations between allergen exposure[15], sensitization status and serum immunoglobulin E (IgE)[17], a hallmark of the adaptive allergic response, and the development of asthma have been reported. Furthermore, the early identification of atopic sensitization in young children has been shown to be predictive of the later development of persistent asthma[18]. In addition to a potential initiating role in asthma pathogenesis, exposure of asthmatic individuals to environmental allergens is also associated with acute exacerbations[19,20], and respiratory arrest[21]. Additionally, allergen avoidance in patients with asthma has been found to decrease inflammatory symptoms

and airway hyperresponsiveness, with a relapse observed upon a return to allergen exposure[22],[23].

However, sensitization to environmental allergens alone does not explain the development of persistent asthma, and many other factors have been found to contribute to the process. Familial and twin concordance studies have provided strong evidence to a genetic component to asthma[24], so genetic relation to asthma patients is another important risk factor for asthma. Identification of asthma-susceptibility genes has proven difficult, though genome-wide association studies have identified several genetic loci that are associated with the development of asthma[25]. The positional cloning of loci associated with asthma susceptibility has given some evidence that it may not be the immune system that is the major location of genetic susceptibility to asthma, but rather the epithelium, mesenchyme and smooth muscle[4]. This has led to the theory that there is an innate structural deficiency in people susceptible to developing asthma such that the airway is vulnerable to insult, which over time leads to the chronic inflammation and tissue damage associated with asthma[26]. Interestingly, different loci associate with different types of asthma; childhood-onset asthma is associated with different loci than adult-onset asthma, and atopy is also associated with distinct loci[24]. These findings suggest that asthma risk-factor genes are independent from those contributing to allergic sensitization, and that different subtypes of asthma develop as a result of different genetic contributions.

Though the genetic component is an important contributor, asthma is a complex condition involving many gene-environment interactions[27]. Several environmental contributors important to the development of asthma, aside from exposure to airborne

allergens, have been described. Rhinovirus infection may play a crucial role in the development of asthma in some patients. Rhinovirus infection has been shown to be associated with the development of asthma; infants that suffer multiple rhinovirus-induced wheezing episodes have a significantly higher risk of developing asthma[28]. Additionally, infections with this virus are associated with exacerbations in asthma patients[28]. Allergic sensitization in young children is associated with rhinovirus infection specifically, and not infections with other viruses[29] and it has been suggested that allergic sensitization has a causative effect on rhinovirus-induced wheezing in young children[30]. There is further evidence that early-life lower airway viral infections interact with airway allergies such that the co-occurrence of both in infancy leads to a significantly increased risk of developing asthma later in life[31]. Other potentially significant environmental risk factors include pre-natal and passive exposure to tobacco smoke, which can increase the incidence of asthma and wheeze by up to 20%[32], and air quality, with the exposure to chemical pollutants and particulate matter in the air possibly contributing to the greater incidence of asthma and asthma-related hospitalizations in urban areas with poor air quality[33,34,35]. Similar to rhinovirus infection, it has been suggested that the inflammatory responses induced by exposure to both airborne allergens and air pollution may synergistically drive airway inflammation in the pathogenesis of asthma[36]. The focus of my thesis is on allergen risk factors, which I will discuss in the following Section.

1.2 Allergen risk factors

A number of allergen species have been identified as strong asthma risk factors, the most prominent among which are the house dust mite, German cockroach, various mould species and animal dander[37]. For my thesis, I have chosen to study two such allergens, the German cockroach and *Alternaria alternata* mould. The rationale behind choosing these two allergens is that: (1) both of these allergens represent significant public health burdens; (2) both allergens have been shown to contain trypsin-like activity, but the specific trypsin-like enzymes have not been fully identified or characterized; and (3) they are taxonomically unrelated; as I will discuss later in the introduction we support a theory that the proteolytic activity in allergens contributes to airway inflammation and asthma pathogenesis, so demonstrating this effect in diverse allergen species supports the generality of this proposed pathogenic mechanism. In the following sections I will discuss this rationale in detail.

1.2.1 German cockroach

The German cockroach (*Blattella germanica)* is a major household pest with a worldwide range. Sources of allergens from the cockroach are primarily the feces (frass), as well as the decomposed bodies and molted skins of cockroaches that incorporate into house dust[38]. Cockroach infestation is particularly prominent in low-income, inner city communities, and exposure to cockroach allergen in the household is associated with the development of allergic sensitization to cockroach[39]. Sensitization to cockroach has long been identified as a major risk factor, and potential causative factor, in the development of asthma[40-42]. Atopic sensitization to cockroach allergen has been

suggested as a potential contributing factor to the higher incidence of asthma observed in these inner city communites[43,44], and to the discrepancy of asthma incidence along socioeconomic and racial lines[45]. However, allergy to cockroach is not limited to low-income communities, as the importance of sensitization to cockroach in the clinical manifestation of asthma has been described in a broad range of socioeconomic conditions[46].

Asthmatic children that are sensitized to cockroach tend to exhibit a more severe asthma phenotype, with increased chronicity and higher plasma IgE levels when compared to children sensitized to other common allergens[44], highlighting the importance of this allergen as an environmental factor in the pathogenesis and management of asthma, particularly in the inner city.

1.2.2 Alternaria alternata mould

Fungal allergens have also been broadly implicated as major asthma risk factors[47]; 3-10% of adults are sensitized to mould allergens and those sensitized individuals have a higher risk of developing asthma[48] . In particular, the common plant pathogen *Alternaria alternata*, found in outdoor and indoor environments around the world, has been implicated as a major health concern. It is a major allergen in hot, dry areas in particular[49]. Sensitization to *A. alternata* allergen is significantly associated with the development of asthma in children[50]. Sensitization to this allergen is also associated with particularly severe asthma phenotypes, characterized by higher rates of hospitalization in intensive care units[51,52] , respiratory arrest[21] and death[53]. In particular, a dose-dependent association between environmental levels of the major immunogenic

allergen protein *Alt a 1* and clinical respiratory symptoms has been described[54]. This mould allergen is also a major contributing factor of the phenomenon of 'thunderstorm asthma,' which is characterized by a spike in asthma exacerbations and hospitalizations following violent storms. It has been reported that patients hospitalized due to asthma exacerbations immediately following thunderstorms are more likely to demonstrate allergic reactivity to Alternaria allergen[55]. This effect may be due to an increase in the allergen load in the air following these storms; the concentration of mould spores in the air has been linked to increased emergency room visits for asthma patients[56]. Like the cockroach, Alternaria mould allergen represents a significant health risk with respect to asthma pathogenesis, so developing new ways to counteract the pathophysiological effects of allergen exposure would be beneficial for public health. One potential target for this therapeutic intervention are allergen-derived serine proteinases; both the cockroach and Alternaria allergens have been found to contain trypsin-like serine proteolytic activity, which I will discuss in the following Section.

1.3 Proteinase allergens

1.3.1 Allergen-derived proteinases as pro-allergenic factors

The involvement of proteolytic enzymes derived from inflammatory cells such as mast cells, macrophages, neutrophils and basophils has long been established in the immune response to pathogens[57]. It was later found in occupational settings that proteinases in the environment, when inhaled, can also contribute to allergic sensitization and airway inflammation[58-60]. With this background, the theory of allergen-derived proteinases that actively influence the host response to allergen exposure was

first proposed in the house dust mite (*Dermatophagoides pternonyssinus*), a common household pest to which exposure and allergic sensitization are major worldwide risk factors for asthma[61]. Cloning cDNA sequences encoding the allergen proteins that were identified by reactivity with IgE isolated from the serum of sensitized patients following an allergen test revealed sequence homology of several potent house dust mite allergens to cysteine (Der p 1[62]) and serine proteinases (Der p 3, a trypsin-like enzyme[63,64], Der p 5, a chymotrypsin-like enzyme[65], and Der p 9, a collagenolytic proteinase[66]). Der p 1 in particular is a major immunogenic allergen in the dust mite, exposure to which is strongly associated with the development of asthma[61].

These findings led to the question of whether the proteolytic activity of the dust mite allergens actively contributes to their allergenicity. It was found that the proteolytic activity of the dust mite allergens increases epithelial permeability[67] and induces the expression of pro-inflammatory cytokines[68], suggesting that allergen-derived proteolytic activity may contribute to airway inflammation and the allergic sensitization process. These studies on the dust mite proteinases identified proteinase-activated receptor (PAR) 2 as a target contributing to the physiological effects observed. I will discuss the PAR family of G protein-coupled receptors in detail in a later Section. These findings then led to the further investigation of whether proteinase activity was a feature specific to the house dust mite allergen, or whether it was common to different airborne allergens, representing a common mechanism contributing to allergic sensitization.

Subsequent work proved the latter case, as proteinase activity was described in a diverse variety of allergen species including: German cockroach[69], American cockroach[70], Alternaria, Cladosporium and Aspergillus moulds[71], *Penicillium citrinum*

mould[72], and a number of pollen species[73], among other less common allergen sources such as bat guano[74], Acanthamoeba[75], and shellfish in seafood processing plants[76]. These findings have led to the theory that allergen-derived environmental proteinases act as adjuvants to Th2-mediated allergic inflammation when inhaled[77]. Such allergens can be classified as biologically active allergens that actively contribute to the pro-inflammatory host response upon antigen sampling by the innate immune system in the airway[78]. There is thus strong evidence that the allergen-derived proteinases contribute directly to the sensitization process that is crucial in the pathogenesis of asthma.

1.3.2 Proteolytic enzymes in cockroach allergen

As mentioned in the previous Section, the German cockroach allergen is known to contain proteolytic activity[69], however the characteristics of the individual enzymes present in the allergen remain largely unknown. The sequence of one trypsin-like enzyme in the German cockroach has been identified by screening an expressed-sequence tag library[79], and the cDNA encoding the active enzyme was later cloned and expressed recombinantly[80]. This recombinantly expressed German cockroach trypsin (Tryp1) displayed reactivity with the sera taken from patients sensitized to the cockroach, which suggests that this enzyme is an immunogenic allergen[80], although it has not been identified in this way in a total body extract used in allergen testing. Other experiments assessing the trypsin-like activity in the German cockroach allergen have revealed physiological effects that may have relevance in airway allergic inflammation and asthma, such as the release of pro-inflammatory cytokines[81] from cultured bronchial-derived epithelial cells, and the release[82] and activation[83] of matrix-

metalloproteinase-9 (MMP-9), a potential contributor to submucosal remodeling. An affinity chromatography approach with an immobilized trypsin inhibitor, benzamidine, was used to purify a trypsin-like enzyme from a cockroach frass extract. The fraction obtained was shown to enhance the production of pro-inflammatory chemokines[84]

Another potent immunogenic protein in the cockroach, Bla g 2, which was identified by IgE reactivity following allergen challenge, was found to have sequence homology to aspartic proteinases[85], although expression of a recombinant Bla g 2 protein revealed very weak activity, leading to the speculation that it is an unusual, inactive aspartic proteinase[86]. In the related American cockroach (*Periplaneta Americana),* a trypsin-like enzyme was identified by IgE reactivity in sensitized patients and subsequently cloned (*Per a 10*)[70,87]. Recombinant *Per a 10* has been shown to augment allergic inflammation in a manner dependent on proteolytic activity in a mouse model when co-administered with ovalbumin[88], and has been shown to bias the dendritic cell response in the airway toward a Th2 phenotype by upregulating CD86, increasing IL6 secretion and decreasing IL12 secretion[89]. I will provide a more detailed description of the physiological effects of cockroach trypsins in subsequent sections of this introduction.

1.3.3 Proteolytic enzymes in Alternaria allergen

The presence of extracellular serine proteinases in *Alternaria alternata* mould has long been established[90]. As with cockroach allergen, several pathophysiological effects of mould-derived proteinases in the airway have been described. In airway-derived epithelial cells, proteinase-dependent cytokine production and morphological

changes[91], ATP release[92], and barrier disruption, which was enhanced in cells cultured from biopsy samples taken from asthmatic patients compared to healthy controls[93], which may indicate that the asthmatic epithelium is more susceptible to the deleterious effects of the Alternaria proteinase activity than the healthy airway. In epithelial cells cultured form nasal polyps, the proteolytic activity in the Alternaria allergen induces factors that drive the recruitment of neutrophils and eosinophils[71]. *In vivo*, Alternaria-derived serine proteinase activity drives the recruitment of eosinophils along with airway fibrosis and increased epithelial thickess[94], as well as smooth muscle exacerbations mediated by the expression of IL-33[95] in mouse models. As with the cockroach allergen, the evidence strongly suggests that the serine proteolytic activity in the Alternaria allergen has a pro-inflammatory and pro-allergenic effect. A common feature of these diverse allergens is the presence of active, pro-inflammatory serine proteinases that may represent potential therapeutic targets. Since these enzymes are present in crude extracts of the total allergens, we used an activity-based probe labeling approach to specifically identify the proteinases, which I will describe in the following Section.

1.3.4 Activity-based detection of proteolytic enzymes

Activity-based probes are a recently developed method of specifically labeling and visualizing active proteinases[96]. The probes consist of a serine proteinase suicide substrate, diphenylphosphonate (DPP4), coupled to biotin by a peptide linker region (illustrated in Figure 1.1). The suicide substrate irreversibly alkylates the active serine in the proteinase, which thus covalently labels the enzyme with biotin. The enzymes can then be labeled with horseradish peroxidase-coupled avidin in a modified western blot

procedure. The specificity of the probe is conferred by the sequence of the peptide linker region; probes containing a lysine or arginine residue upstream of the suicide substrate target trypsin-like enzymes, a probe with phenylalanine targets chymotrypsin-like enzymes and a probe with alanine targets elastase-like enzymes.

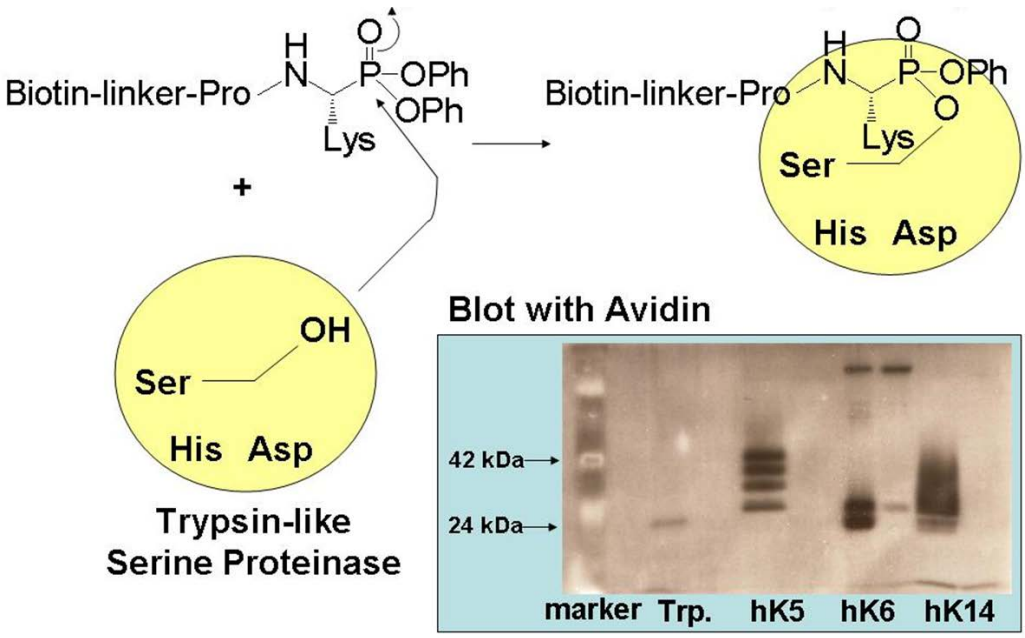


Figure 1.1– Labeling active serine proteinases with activity-based probes.
The probe (upper left corner) consists of a serine proteinase suicide substrate (diphenylphosphonate, DPP4) linked via a peptide to biotin. Enzyme specificity is conferred by the linker peptide sequence (Pro-Lys in this example gives specificity to trypsin-like enzymes). The DPP4 moiety covalently binds and alkylates the active serine residue in the trypsin-like enzyme (lower left) to yield a covalently biotinylated proteinase (upper right). Proteinase samples labeled with the ABP can then be resolved by SDS/PAGE and reacted with horseradish peroxidase (Hrp)-conjugated avidin, a protein which has a very strong and specific affinity to biotin. Activation of the Hrp reveals the labeled active trypsin-like enzymes in a western blot (lower right).

1.3.5 Chromatographic separation of proteins

Since the allergen-derived proteinases are contained within extracts of the total allergen species, a method to isolate and purify the proteinases was necessary for their characterization. Common methods of isolating proteins include size exclusion chromatography, which separates proteins in a mixture based on their molecular weight[97], and affinity chromatography, which, in the case of proteolytic enzymes involves immobilized substrates or inhibitors to isolate active enzymes from a protein mixture[98]. Neither of these approaches would prove useful in separating the allergen-derived proteinases; however, it was possible to take advantage of the different relative charges of the enzymes with ion-exchange chromatography. This type of protein separation involves charged groups immobilized onto an insoluble polymer such as sepharose or cellulose. The charged groups can bind to oppositely-charged proteins in the surrounding solution; columns with negatively charged groups bind positively-charged ions and are referred to as cation-exchange columns, whereas columns with positively-charged groups are referred to as anion-exchange columns. This mechanism allows the separation of proteins based on (1) their overall charge, i.e. proteins that carry a net charge opposite the immobilized charged group will bind to the column while like-charged proteins will pass through, and (2) their relative charge, such that proteins that carry a stronger opposite charge will bind to the column more tightly than more weakly charged proteins. Elution of bound proteins from the column is achieved by passing increasing concentrations of a salt, typically NaCl, through the column. The ion of opposite charge to the immobilized charged group will compete for binding with the

bound proteins, so higher salt concentrations are required to displace more strongly charged proteins[99].

1.4 Proteinase-activated receptors

1.4.1 Introduction to proteinase-activated receptors

As mentioned previously, known targets for the inhaled allergen proteinases are the proteinase-activated receptors (PARs), a family of unusual G protein-coupled receptors comprising four members (PAR1-4)[100]. The most extensively studied members of this family are PAR1, originally identified as the thrombin receptor in the coagulation cascade[101], and PAR2, which is distinguished from PAR1 in that it is traditionally recognized as a trypsin "receptor," and is not activated by thrombin[102]. PAR4 was identified as a "receptor" for both thrombin and trypsin[103]. PAR3 is activated by thrombin, but it appears not to transmit a signal on its own, though it has been found to mediate signaling through PAR1 via receptor dimerization[104].

The PARs are activated by a unique mechanism whereby a masked endogenous tethered ligand sequence is located in the extracellular N-terminal domains of the receptors. Following cleavage by specific agonist proteinases, the tethered ligand sequence is unmasked and signals in an autocrine-like fashion by binding to the extracellular loops of the receptor. This binding induces conformational changes in the receptor that result in the recruitment and activation of G protein signaling complexes[105,106]. Despite the unusual activation method, the PARs transmit their signal as conventional G protein-coupled receptors, with the typical intracellular signaling mechanisms and trafficking dynamics. Activation of the intracellular signaling cascades is initiated by interaction of the receptor with a heterotrimeric G protein, which consists

of an α subunit (Gα) associated with a Gβγ dimer. GPCR-mediated activation of signaling induces the exchange of GDP bound to the Gα subunit for GTP, which results in Gα dissociation from Gβγ. The active, dissociated subunits then interact with downstream effectors and induce the signaling cascade, with different subtypes of Gα subunits interacting with distinct effectors. Signaling is terminated when the GTPase activity of the Gα subunit is activated, returning the subunit to the inactive GDP-bound form and reassociating with the Gβγ subunit. Additionally, the GPCR itself is often modified and internalized to prevent further G-protein-mediated signaling, including trafficking of the receptors to endosomes and lysosomes for degradation[107]. However, PAR signaling in receptors localized to the endosomes can continue through the recruitment of β-arrestins[108] (Signaling mechanisms are illustrated in Figure 1.2).

1.4.2 Activation of PAR1

The conventional activation mechanism for PAR1 involves the cleavage of the extracellular N-terminal sequence by thrombin on arginine (R)41, which reveals the tethered ligand sequence S42FLLR[101]. The efficiency with which thrombin targets PAR1 is thought to involve a hirudin-like domain in the N-terminal region of the receptor, which actively binds thrombin and brings it in close contact with the activation site[101]. Similarly, other proteinase agonists of PAR1 that have been described are localized to the membrane, such as activated protein C (APC) when recruited to the membrane via its receptor endothelial protein C receptor (EPCR)[109], or in a ternary complex at the cell surface like Factor Xa[110], possibly increasing the proximity of the enzymes to the receptor and thus influencing the abilities of the enzymes to target PAR1. A list of

agonist and dis-arming proteinases described for PAR1 is provided in Table 1.1. An additional artificial activation mechanism for PAR1 involves a synthetic peptide representing the endogenous tethered ligand sequence of the receptor. The peptide TFLLR-NH_2 has been identified as a ligand that is selective for PAR1 over PAR2 and PAR4, which activates the receptor in the absence of proteolytic processing of the N-terminal tail[111].

PAR1 transmits its intracellular signal by coupling with the G protein subunits $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$[105]. $G\alpha_q$ activation transduces a signal via phospholipase C, downstream of which the release of Ca^{2+} stores from the endoplasmic reticulum is activated in the cell[112]. Thrombin had also been observed to have a potent mitogenic effect, so its ability to activate MAPK signaling via PAR1 has been explored. In fibroblast cells, PAR1-dependent activation of the ERK1/2, JNK and p38 MAPK pathways was found to be downstream of each of $G\alpha_q$, and $G\alpha_{12/13}$[113], and in astrocytes the MAPK pathways have been shown to be activated by the Gβγ subunit as well as downstream of the $G\alpha_q$ –mediated Ca^{2+} signaling[114]. The G protein specificity of the MAPK pathways activated downstream of PAR1 activation are thus cell-type specific. Other signaling pathways found to be activated downstream of PAR1 activation include the Rho pathway, NF-κB, and transactivation of the HER2/EGFR2 in breast carcinoma cells and EGFR in a number of other cell types[106].

1.4.3 Activation of PAR2

PAR2 has been the most studied receptor in the case of inflammation, and signaling via PAR2 has been linked to a number of inflammatory conditions[106,115]. The

conventional activation mechanism of PAR2 involves the cleavage of R36 by trypsin, which reveals the tethered ligand sequence S37LIGKV (human) or S37LIGRL (murine)[116]. A list of activating and dis-arming proteinases described for PAR2 is provided in Table 1.1. The unmasked tethered ligand sequence then interacts with the extracellular domains to induce the activating conformational change of the receptor[117]. As with PAR1, synthetic peptides representing the tethered ligand sequence can also activate the receptor in the absence of proteolytic processing of the endogenous tethered ligand sequence. The peptide SLIGRL-NH_2 was identified as a PAR2-selective agonist[111]. An altered form of the PAR2 activating peptide with a furoyl group replacing the N-terminal serine (2-furoyl-LIGRL-NH_2; 2fLI) has also been identified as a highly selective PAR2 agonist with ~10x the potency of the SLIGRL peptide[118],[119].

The intracellular signaling pathways activated downstream of PAR2 activation are similar to those activated by PAR1. As with PAR1, PAR2 interacts with $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$[105]. The downstream effects of PAR2 activation likewise include Ca^{2+} signaling via interaction with Gq[120], activation of the MAPK pathways, including ERK 1/2[121], c-Jun N-terminal kinase (JNK), and p38 MAPK[122,123], and activation of the Rho/Rho-associated protein kinase (ROCK) pathway[124]. PAR2 activation also involves the association of the β-arrestin scaffold proteins, which mediate endocytosis and trafficking of the receptor[125]. The internalized PAR2- β-arrestin complex can also mediate and prolong ERK 1/2 signaling[126,127].

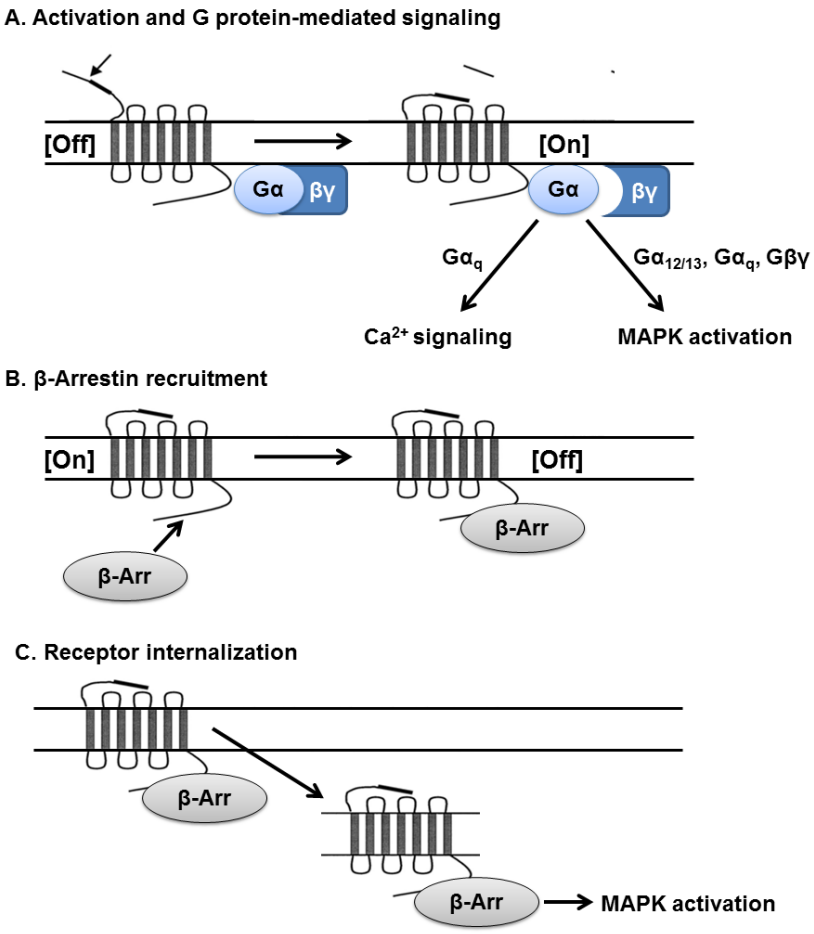


Figure 1.2 – PAR signaling mechanisms.

The events involved in PAR signaling are illustrated using PAR2 as an example **(A)** PARs are activated by proteolytic unmasking (indicated by the arrow) of the tethered ligand (TL) sequence (thick black line). The TL sequence interacts with the extracellular domains of the receptor to induce the activation of G proteins. Two major signaling pathways activated are the Ca^{2+} signaling pathway, mediated by $G\alpha_q$, and the activation of MAPKinases, activated by $G\alpha_{12/13}$, $G\alpha_q$, and $G\beta\gamma$. **(B)** The activation of PAR2 results in the recruitment of the β-arrestin scaffold proteins, which assist in desensitization of the receptor. Activation of PAR1 does not appear to induce the recruitment of β-arrestins, though desensitization and internalization are induced through different pathways. **(C)** Desensitization occurs through receptor internalization. Once internalized, the PAR2-β-arrestin scaffold complex can regulate signaling via the activation of MAPK.

1.4.4 Activation of PAR3 and PAR4

Human PAR4 is cleaved on R47 to reveal the tethered ligand sequence G48YPGQV, which is regulated by both thrombin and trypsin at similar concentrations[103]. Additionally, PAR4 is activated by the peptides GYPGKF-NH_2 (murine) or GYPGQV-NH_2 (human), although a more potent peptide, AYPGKF-NH_2 has also been developed[128]. PAR3 is cleaved by thrombin on K38 to reveal the tethered ligand sequence T39FRGAP[129], however there has been no specific peptide sequence identified as a selective agonist of PAR3[106]. As previously mentioned, it is unknown whether PAR3 is able to transmit an intracellular signal on its own[104], however it has recently been reported that a possible non-canonical cleavage of PAR3 by the coagulation cascade proteinase Factor Xa has an effect in promoting vascular integrity[130].

Protease agonists and dis-armers of PARs.

	Activating protease	Disarming protease
PAR1	Thrombin	KLK1, 14
	Meziothrombin/meziothrombin desF1	ADAM17 or MMP
	Granzyme A	Protease 3
	Plasmin	Trypsin
	APC-EPCR	Cathepsin G
	Trypsin IV	Elastase
	Factor Xa	Plasmin
	KLK1, 4, 5, 6, 14	
	MMP-1	
	Cathepsin G	
	Proatherocytin	
	Pen C 13	
PAR2	Trypsin	Plasmin
	Mast cell tryptase	Protease 3
	TF:Factor Xa: Factor VIIa	Calpain
	Acrosin	Cathepsin G
	Matriptase/MT-SP1	Elastase
	HAT	
	Trypsin IV	
	Granzyme A	
	TMPRSS2	
	Chitinase	
	KLK2, 4, 5, 6, 14	
	Bacterial gingipains	
	Der P1, Der P2, Der P3	
	Pen C 13	
PAR3	Thrombin	Cathepsin G
PAR4	Thrombin	KLK14
	Trypsin	
	Cathepsin G	
	Trypsin IV	
	MASP-1	
	Plasmin	
	Factor Xa	
	Bacterial gingipains	
	KLK1, 14	

Table 1.1– List of described activating and disarming proteinases of PARs 1-4

Table adapted from Adams et al[106]

1.4.5 Biased signaling via PARs 1 and 2

An interesting recent development in PAR signaling mechanics involves the selective activation of specific intracellular signaling pathways at the exclusion of others. Enzymes that induce this type of selective signaling have been termed 'biased agonists,' in that they are biased for one signaling pathway, typically the MAPK signaling arm downstream of the PARs, over another, typically Ca^{2+} signaling[131]. PAR2 displays biased signaling with mutated synthetic peptide agonists; the non-mutated agonist representing the endogenous tethered-ligand sequence SLIGRL activates both Ca^{2+} and MAPK signaling downstream of PAR2 activation, whereas the mutated peptide SLAAAA activates MAPK signaling but not Ca^{2+} signaling[132]. This finding indicates that the sequence of the tethered ligand is important in determining which signaling arms are activated downstream of PAR2. A similar effect was demonstrated with neutrophil elastase, which cleaves PAR2 downstream of the canonical tethered ligand sequence, traditionally referred to as dis-arming the receptor since subsequent exposure to trypsin does not activate PAR2-mediated Ca^{2+} signaling. However, though elastase does not activate Gq-mediated Ca^{2+} signaling, it does activate MAPK signaling via PAR2, representing a distinct mode of PAR2 activation involving cleavage at a non-canonical site[133].

Similar biased signaling via PAR1 has also been described. Activated protein C (APC), a serine proteinase involved in the regulation of blood coagulation, has been shown to have a protective role in the vasculature by cleaving PAR1 in endothelial cells, which prevents subsequent activation of the receptor by thrombin[134]. It was later shown

that APC exerts its effect by cleaving PAR1 at R46, downstream of the canonical thrombin cleavage site R41, acting as a biased agonist distinct from thrombin[135]. Additionally, the neutrophil-derived serine proteinases neutrophil elastase and proteinase-3 were shown to activate biased signaling in PAR1, activating MAPK signaling but not Ca^{2+} signaling[136]. Taken together these results suggest a mechanism of PAR activation whereby certain proteinases cleave the receptors at non-canonical sites, revealing different tethered ligand sequences which preferentially activate one downstream signaling arm over another. Since trypsin has been described to regulate PAR1 by dis-arming the receptor, we considered the possibility that the allergen-derived trypsin-like enzymes may induce biased signaling via PAR1. The proposed mechanism of biased signaling is illustrated in Figure 1.3.

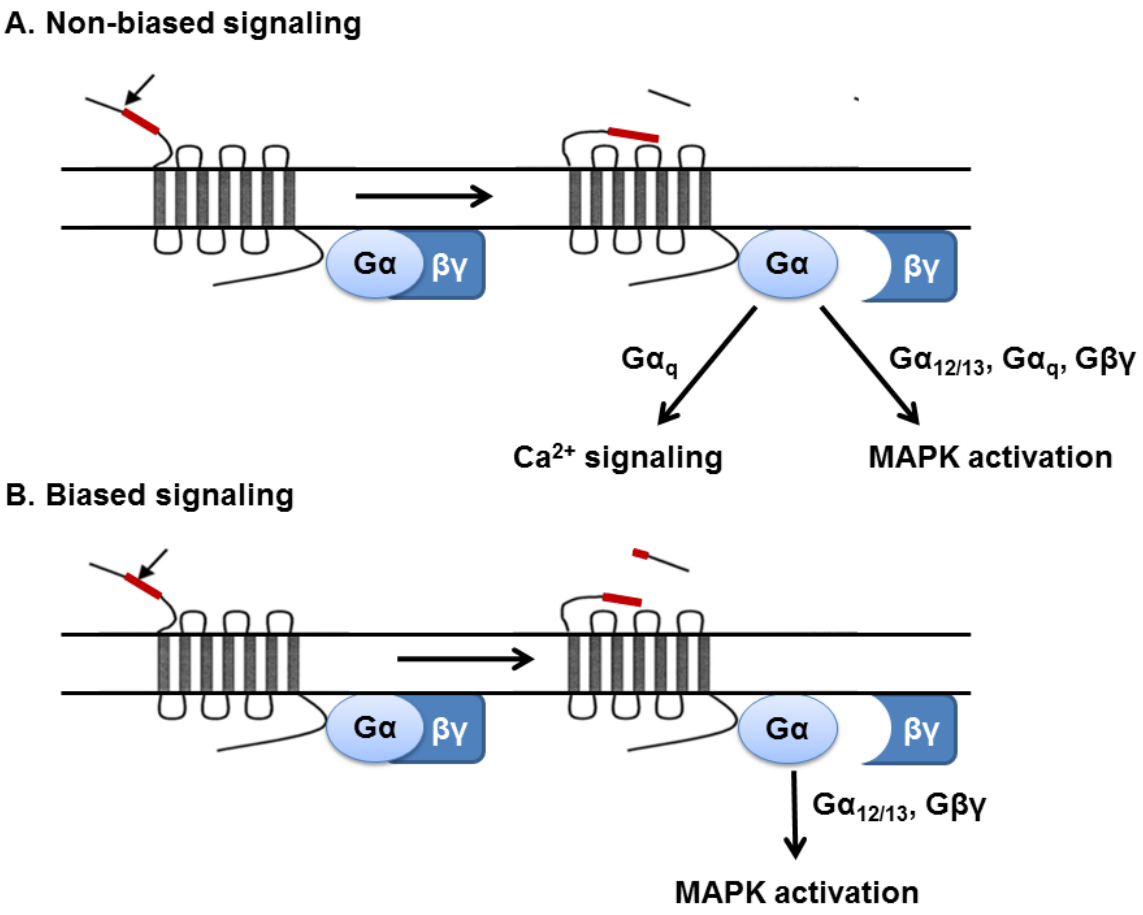


Figure 1.3 – Biased signaling by the PARs.

(A) Proteolytic unmasking of the tethered ligand sequence (red bar) by cleavage at the canonical residue results in non-biased activation of the receptor. **(B)** Cleavage of the receptor at a non-canonical residue results in a distinct unmasked tethered ligand sequence that preferentially activates G protein-mediated MAPK signaling and not Ca^{2+} signaling. In addition, neither the recruitment of the β-arrestin scaffolds nor the internalization of the receptor has been found to occur following activation of the PARs by biased agonists.

Receptor/gene designation	Canonical TL (human)	Receptor-selective PAR-activating peptides	Non-canonical TL	Biased PAR-activating peptides
PAR1/F2R	--//SFLLRN--	TFLLR-NH_2	MMP1: --//PRSFLLRN-- APC: --//NPNDKYEPF-- NE: --// RNPNDKYEPF-- PR3: --//TLDPRSF--	PRSFLLRN; NPNDKYEPF; RNPNDKYEPF; TLDPRSF; YFLLRN
PAR2/F2RL1	--//SLIGKV--	SLIGRL-NH_2; 2-furoyl-LIGRL-NH_2	NE: --//VLTGKL--	SLAAAA-NH_2
PAR3/F2RL2	--//TFRGAP--	TL-derived peptides activate PARs 1 and 2	Not known	Not known
PAR4/F2RL3	--//GYPGQV--	AYPGQV-NH_2; AYPGKV-NH_2	Not known	Not known

Table 1.2 - Canonical and non-canonical PAR cleavage sites and tethered ligand sequences.

Known endogenous tethered ligand (TL) sequences and synthetic agonist peptide sequences for PARs 1-4 are presented. The canonical TL sequences are unmasked by thrombin (PAR1) and trypsin (PAR2). The non-canonical TL sequences presented are unmasked by : matrix metalloproteinase-1 (MMP1), activated protein C (APC), neutrophil elastase (NE) and proteinase 3 (PR3). (Table adapted from Hollenberg et al[131])

1.5 PAR signaling in the airway

1.5.1 Activation of epithelial PAR2 is pro-inflammatory in the airway

Of the PARs, PAR2 has been of particular interest in a number of inflammatory diseases, including inflammatory airway disease[137]. PAR2 is highly expressed in the airway in several cell types, including the epithelium, fibroblasts, smooth muscle and resident immune cells[138]. In the context of airway inflammation, higher expression levels of PAR2 have been reported in both airway epithelial cells[139] and bronchial smooth muscle cells[140] taken from asthma patients compared to those taken from

healthy individuals, suggesting a potential role for the receptor in airway disease. There have since been a number of studies elucidating the role that PAR2 plays in airway inflammation. In *in vitro* studies using airway-derived cells, activation of PAR2 appears to have a pro-inflammatory effect. A number of pro-inflammatory cytokines and chemokines have been found to be up-regulated following activation of PAR2 in airway-derived cell lines, including IL-6[141], IL-8[142], thymic stromal lymphopoietin (TSLP)[143] , which is a potential key regulator of airway Th2-mediated allergic inflammation[144], prostaglandin E2[145] and granulocyte macrophage colony-stimulating factor (GM-CSF), which is associated with the recruitment and survival of macrophages and eosinophils, from both epithelial[146] and fibroblast-derived cell lines[147]. Activation of PAR2 in airway-derived fibroblasts also drives expression of IL-8 and the adhesion molecule VCAM-1 following PAR2 activation[147].

In addition to increasing the expression of pro-inflammatory cytokines, PAR2 signaling may enhance the recruitment of circulating immune cells to the airway by other mechanisms. Activation of PAR2 in epithelial cells increases adhesion of neutrophils to the epithelium, presumably enhancing recruitment of neutrophils to the site of inflammation[124]. PAR2 knockout mice have been found to have diminished eosinophilia in an ovalbumin airway allergic inflammation model, with a reduction in eotaxin detected, although whether eotaxin expression is a direct downstream effect of PAR2 activation was not shown[148]. Additionally, activation of PAR2 in airway epithelial cells has been found to decrease epithelial barrier function by interrupting E-cadherin adhesion[149]. Inhibition of the serine proteinases in an American cockroach allergen extract reduced the morphological changes and detachment of cultured bronchial

epithelial cells, in addition to reducing the expression of pro-inflammatory cytokines IL-8, MCP-1, GM-CSF and CCL20[150]. Activation of PAR2 in airway-derived epithelial cells has also been shown to be a weak enhancer of mucus production, which may play a role in the airway remodeling process[151].

Taken together these data suggest that the activation of PAR2 in the airway epithelial and mesenchymal tissues enhances the sensitization and airway inflammation processes that arise in response to concomitantly presented immunogenic allergens. This enhancement involves inducing factors that recruit leukocytes to the airway and prime the leukocytes to induce type 2 T-helper cell polarization, as well as increasing allergen flux across the epithelium by interrupting the barrier function of the epithelium. The pro-inflammatory response of PAR2 activation in epithelial cells may be particularly significant for environmental, inhaled airborne proteinases since that is presumably the first cell type to which they have access. The disruption of the epithelial barrier may subsequently allow the allergen proteinases access to subepithelial cell types such as fibroblasts, smooth muscle and resident immune cells. Such cells may also, in addition to the epithelial cells, have PAR-mediated pro-inflammatory effects, which may drive the further recruitment of immune cells to the airway. The effects of PAR signaling in immune cells are discussed in the following Section.

1.5.2 Effects of PAR signaling in inflammatory cells

In addition to its involvement in the recruitment of inflammatory cells, PAR2 activation has also been shown to contribute to the activation and maturation of cultured immune cells. Dendritic cells are important contributors to the sensitization process, and

recent evidence suggests a substantial role of PAR2 signaling in driving the dendritic cell response toward allergic sensitization. Activation of PAR2 in airway-derived dendritic cells has been shown to increase dendritic cell trafficking to lymph nodes and subsequent T-cell activation[152]. Additionally, PAR2 signaling in dendritic cells has also been shown to induce the release of TNF-α, which affects the epithelial response to allergen challenge by increasing the release of pro-inflammatory cytokines[153], as well as biasing the dendritic cells to release Th2/Th17-associated cytokines[154], thereby promoting the process of allergic sensitization. PAR1 may also play a role in dendritic cell function, as LPS-matured dendritic cells have been shown to express functional PAR1, the activation of which by the PAR1 activating peptide or thrombin drives a concentration-dependent chemotactic response[155]. PAR1 activation in these cells also induced the release of the CCL18 chemokine, which drives recruitment of lymphocytes and immature dendritic cells, as well as driving the proliferation of fibroblasts[155].

Eosinophils also express both PAR1 and PAR2, although the activation of PAR1 by thrombin had no discernable effect on eosinophil function[156]. By contrast activation of PAR2 by the activating peptide and trypsin[156,157], as well as cockroach[158] and Alternaria[159] allergens results in activation and degranulation of the eosinophils with the associated release of pro-inflammatory and cytotoxic factors, including leukotrienes and reactive oxygen species. The reactive oxygen species released may then feedback and influence the response from the epithelium, dendritic cells and CD4+ T cells to further promote the sensitization process[160]. Neutrophils also express PAR2, which has been shown to be upregulated following allergen exposure resulting in an increased PAR2-dependent release of TNF-α[161] as well as enhancing neutrophil adhesion to lung

epithelial cells[124]. Finally human monocytes and monocyte-derived cells have also been shown to express PAR2. Monocytes express mainly PAR1, but differentiation into macrophages induces the upregulation of PARs 1, 2 and 3[162]. Alveolar macrophages[163], as well as mast cells[164] also contribute to the sensitization process and release pro-inflammatory cytokines following PAR2 activation.

Taken together these results further support the pro-inflammatory function of PAR activation in the airway, and specifically that the activation of PARs in immune cells likely enhances the inflammatory response induced by allergen exposure. Since, as mentioned in the above Section, activation of PARs in the epithelium results in the recruitment of immune cells to the airway, these results suggest a plausible mechanism whereby trypsin-like proteinases in the airway act as adjuvants to the innate inflammatory response induced following allergen exposure in the airways.

1.5.3 Effects of PAR2 signaling by cockroach and Alternaria allergens

Along with the effects alluded to in the previous sections, other consequences of PAR2 signaling by cockroach and Alternaria allergen-derived trypsin-like enzymes have been described. The trypsin-like activity in the cockroach allergen has been shown to activate both Ca^{2+} signaling and MAPK activation via PAR2[165]. Additionally, a number of studies using a cockroach frass extract have outlined several physiological effects of proteinase activity and PAR2 activation. These include a synergistic increase of TNF-α-mediated IL-8 expression in cultured bronchial epithelial cells[166], an increase in CXCL1 and TNFα secretion in the airway of mice following a single allergen exposure, and the recruitment and maturation of myeloid dendritic cells in the airway, along with the

induction of Th2 and Th17-associated cytokines[167]. Trypsin-like activity in the Alternaria allergen has also been shown to induce the release of GM-CSF, IL-6 and IL-8 in airway-derived epithelial cells via the activation of PAR2[168]. These results suggest that the enzymes present in the two allergens that I have studied contribute to airway inflammation via the activation of PAR2. I will discuss further evidence obtained in *in vivo* experiments in the following Section.

1.5.4 In vivo effects of PAR2 signaling in the airway

In addition to the *in vitro* work, a number of *in vivo* allergic sensitization models have recently been published demonstrating the enhancing effect of the activation of PAR2 by allergen proteinases in allergic airway inflammation. Both cockroach frass (feces)[169] and a total body cockroach extract, in a study to which we contributed[170], were found to induce airway inflammation, which was diminished when either the cockroach enzymes were neutralized with a trypsin-specific inhibitor, or in the absence of PAR2 in the airway. The most pronounced effect of these conditions was in the recruitment of leukocytes, particularly eosinophils, to the airway. Neutralizing the trypsin-like enzymes in the cockroach extract resulted in a diminished recruitment of total cells present in a bronchoalveolar lavage (Figure 1.4 A), with eosinophil recruitment in particular diminished substantially (Figure 1.4 B). The use of PAR2 null mice gave similar results, with total cell (Figure 1.5 B) and eosinophil (Figure 1.5 C) significantly reduced. In a similar study neutrophil recruitment was diminished in PAR2-null animals following challenge with the cockroach frass[161], suggesting a common mechanism of allergen-derived PAR2 signaling-dependent leukocyte recruitment to the

airway. Other effects that we observed when PAR2 signaling was blocked or absent in the mice were decreased airway hyperresponsiveness to a methacholine challenge (Figure 1.5 A) and diminished production of the Th2-associated immunoglobulin subtype IgG1 (Figure 1.5 B). However, directly blocking the cockroach trypsin-like enzymes in our study did not reduce airway hyperresponsiveness or IgG1 production (Figure 1.4 C). A similar study using the PAR2 activating peptide SLIGRL in an ovalbumin sensitization mouse model demonstrated enhanced airway hyperresponsiveness, as well as airway inflammation, due to PAR2[171]. Another ovalbumin sensitization study using PAR2 knockout and PAR2 overexpressing animals found a similar PAR2-dependent enhancement of airway hyperresponsiveness[172], so the role of PAR2 in the development of airway hyperresponsiveness in these models may not depend on the activity of exogenous, allergen-derived enzymes. However, it has been shown that primary cultured bronchial smooth muscle cells express PAR2, which induces constriction when activated[173], and *in vivo* it has been shown that bronchial smooth muscle responds directly to the inhaled environment, including the ability to detect proteinases via PAR2[174], so the ability of allergen-derived proteinases to affect the bronchial smooth muscle by direct interaction is a plausible mechanism contributing to the development of airway hyperresponsivness.

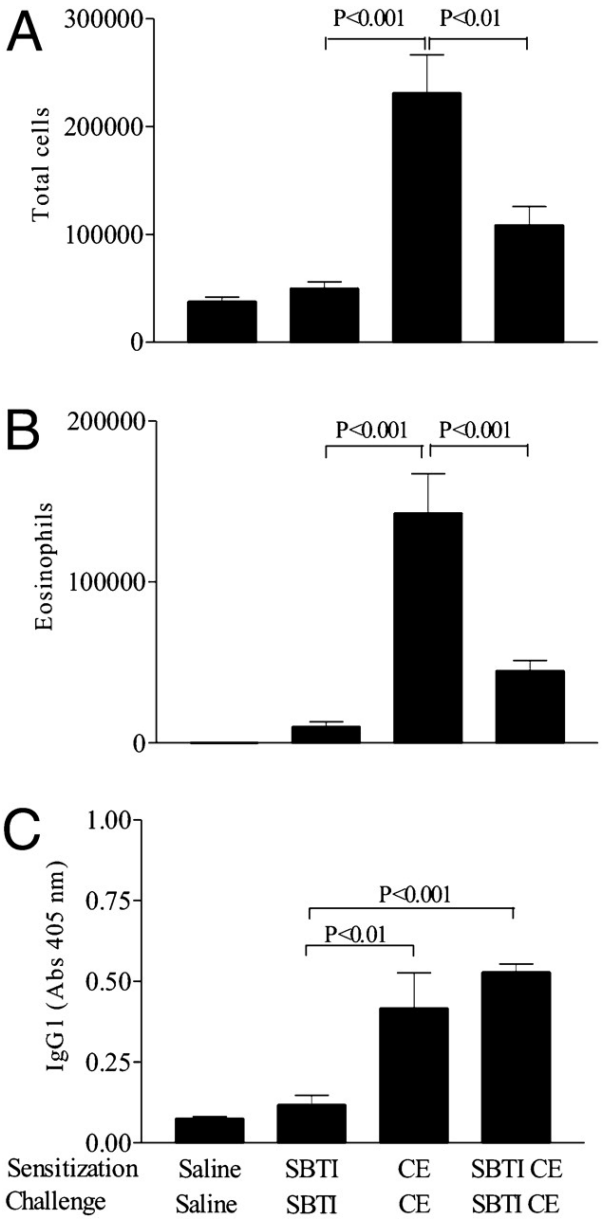


Figure 1.4 – Cockroach extract-mediated eosinophil recruitment is enhanced by proteinase activity.

Mice sensitized and challenged with a cockroach allergen extract (CE) demonstrate robust increase in cell number (A) and eosinophil number in particular (B) in bronchoalveolar lavage fluid. Neutralization of the trypsin-like activity in CE with SBTI prior to administering to the animals resulted in a significant reduction in cellular infiltration to the airway. The treatment had no effect on the production of IgG1 (C) (Figure adapted from Arizmendi et al[170])

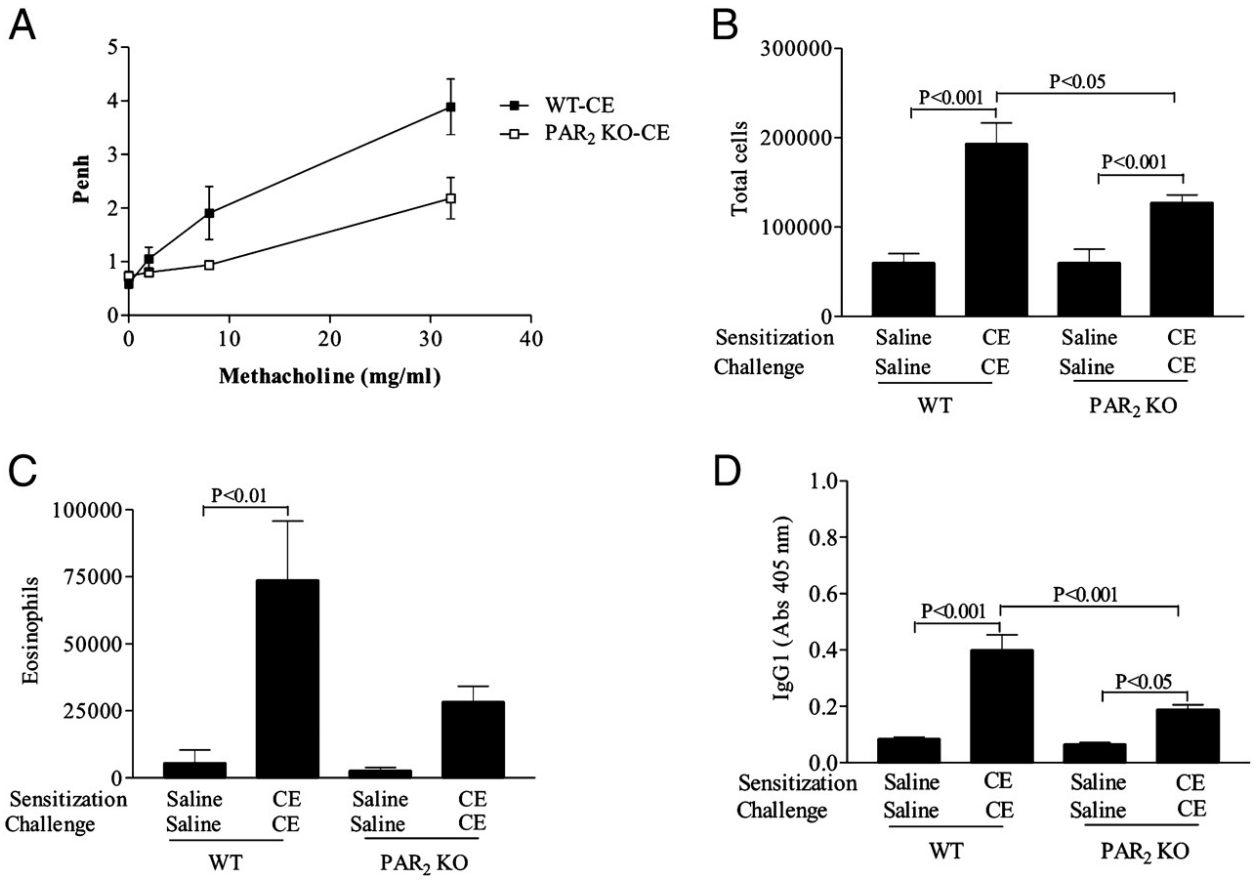


Figure 1.5 - Cockroach extract-mediated eosinophil recruitment is diminished in the absence of PAR2.

PAR2-null mice sensitized to CE had significantly lower cellular recruitment to the airway (B, C) as compared to wildtype mice. In addition, both the airway contractile response to a methacholine challenge (Penh; A) and IgG1 production (D) were significantly lower in the PAR2-null mice than wildtype controls. (Figure adapted from Arizmendi et al[170])

Our results describing the contribution of cockroach-derived trypsin-like enzymes signaling via PAR2 and contributing to airway inflammation are supported by a number of other studies. Similar results showing PAR2-dependent enhancement of the recruitment of immune cells to the airway have been reported with extracts of *A. alternata*[175], house dust mite[176,177] and *Curvularia lunata* mould[178]. Additionally, sensitization to house dust mite in PAR2 null mice resulted in less production of allergen-specific IgG1[177] and IgE[179], as well as reduced lung damage and protein leak across the epithelial barrier[180]. PAR2 null mice sensitized to house dust mite are also found to have lower levels of several pro-inflammatory, Th2-associated cytokines and chemokines such as IL-4[177], IL-5, IL-13, eotaxin-1, IL-17, CXCL1, CCL17 and TSLP[179]. Finally, supplementing ovalbumin challenge with the PAR2-activating peptide SLIGRL prevented the development of tolerance and promoted allergic sensitization through tumour necrosis factor (TNF) signaling[181]. These results suggest that the enhanced airway inflammation induced by the allergen-derived proteinases signaling via PARs in the airway may represent a common mechanism in the process of sensitization to diverse allergens.

1.5.5 PAR1 and PAR4 in the airway

The other two signaling members of the PAR family, PARs 1 and 4, are also expressed in the airway. PAR1 is expressed in airway epithelial tissue[145], fibroblasts[182], and smooth muscle cells[183]. PAR4 expression has also been shown in airway epithelial cells[184] and endothelial cells[183]. The role of PAR4 signaling in the airway is less well understood than for PAR1, but there is evidence that it may promote epithelial to

mesenchymal transition in airway epithelial cells[185]. Whether or not PAR1 and PAR4 play a role in allergic airway inflammation has not been demonstrated. However given that trypsin is a conventional agonist of PAR4[103], and can target (disarm) PAR1[186], it is likely that allergen-derived trypsin-like enzymes target these receptors expressed in the airway concomitantly with allergen exposure in the sensitization process. By extension, PARs 1 and 4 may, in addition to PAR2, represent therapeutic targets for allergic diseases and asthma.

1.6 Hypothesis and aims

There is considerable evidence demonstrating the presence of trypsin-like proteolytic enzymes in a diverse range of common airborne allergens to which sensitization is a major risk factor for the development of asthma. Inhaled allergen-derived trypsin-like proteinases have been shown to target PAR2 in the airway and in doing so contribute to allergic sensitization and airway inflammation. I hypothesized that the cockroach and Alternaria allergens contain distinct trypsin-like enzymes that can signal via both PAR1 and PAR2. Previous studies have demonstrated that this signaling can contribute to airway sensitization and inflammation, so these allergen-derived enzymes may thus represent therapeutic targets in the prevention or amelioration of allergy and asthma symptoms. To explore this possibility it was important to characterize the enzymes present in the allergens and to assess their potential physiological targets.

To test this hypothesis, my aims were:

1. Biochemically characterize, isolate and identify the trypsin-like proteinases in extracts of cockroach and Alternaria allergens

2. Assess each enzyme for its ability to regulate signaling via PAR1 and PAR2, and identify the intracellular signaling mechanisms induced downstream of PAR activation

3. Assess the ability of the enzymes to signal via the PARs *in vitro* in airway-derived cells and tissues

CHAPTER 2 – MATERIALS AND METHODS

2.1 Allergen materials and PAR agonists

2.1.1 Allergen extracts

Total body German cockroach extracts were purchased from Greer Labs (Lenoire, NC catalog #XPB46D3A4). A total *Alternaria alternata* extract (catalog # XPM1D3A2.5) was also purchased from Greer Labs. A mould culture media filtrate was produced by our collaborators at the University of Arizona, Tucson. Both of the commercial extracts are supplied as lyophilized powders. The cockroach extract was re-constituted in 5 ml of 20 mM Tris-HCl, pH 7.2, and the Alternaria total mould extract was re-constituted in 3 ml of the same buffer. The re-constituted extracts were then dialyzed against 1 L of 20 mM Tris, pH 7.2 for 2 h at 4C in a Novagen D-Tube Maxi dialyzer (EMD Millipore Darmstadt, Germany) with a molecular weight cut-off of 6-8 kDa to remove small molecule contaminants in the extract. The extracts were then stored in 250 µl aliquots at -80°C.

Frass of the speckled cockroach (*Nauphoeta cinerea*) was supplied by Dr. Samantha Ross of the Defense Science and Technology Organisation; Melbourne, Australia. 5 g aliquots of the dry frass were extracted in 20 ml volumes of water by shaking for 4 h at 4°C. The supernatant was collected and passed through a DEAE sepharose column at 4°C to remove insoluble contaminants and to crudely purify the trypsin-like enzymes.

2.1.2 Enzymes and peptides

Porcine trypsin (Type IX-S; Sigma, St Louis, MO; catalog # T0303, 16 000U/mg) stock solutions were prepared at a concentration of 0.625 mg/ml in distilled water, representing 10 000 BAEE units/ml (1 BAEE unit = change in absorbance at 253 nm of 0.001 per minute at 25°C and pH 7.4 using benzoyl arginine ethyl ester (BAEE) as a substrate) as quantified by Sigma. Trypsin stock was stored in 15 µl aliquots at -80°C. Thrombin was purchased from Sigma (catalog # T7513) and 2500 U/ml stock solutions were prepared in distilled water. Aliquots were stored in -20°C.

All PAR agonist peptides were synthesized by the Peptide Synthesis Facility (University of Calgary). Peptides were prepared in 25 mm HEPES buffer, pH 7.4, and were standardized by quantitative amino acid analysis to confirm peptide concentration and purity.

2.2 Methods for experiments described in Chapter 3

2.2.1 Enzyme activity assay

To quantify the trypsin-like activity in the allergen extracts, the fluorogenic peptide substrate Boc-Glutamine-Alanine-Arginine (QAR)-aminomethylcoumarin (AMC) (Bachem) was used. The cleavage of the substrate was compared to a trypsin standard curve with known BAEE Unit (U)/ml concentrations. The 10 000 U/ml trypsin stock solution was serially diluted in proteinase activity buffer (PAB; 50 mM Tris-HCl, pH 8; 0.2% NP40; 1.5 mM $CaCl_2$) to final concentrations of: 0.3 U/ml, 0.2 U/ml, 0.1 U/ml, 0.05 U/ml and 0.02 U/ml. 50 µl of each concentration of trypsin was loaded in triplicate to a 96 well black plate (Greiner Bio-One; Kremsmünster, Austria). Crude allergen extracts

with unknown proteinase activity levels were serially diluted in PAB from a 10X dilution factor to 10 000X dilution factor by increments of 10 to determine the approximate dilution necessary for detection of the linear phase of substrate cleavage within the range of the trypsin standard curve. 50 µl of each concentration of the crude extracts was also loaded in triplicate to the 96 well black plates. The QAR-AMC substrate was diluted from its 100 mM stock to 150 µM in proteinase activity buffer prior to addition to the enzymes. 50 µl of the 150 µM substrate working solution was added to each enzyme-containing well and fluorescence was detected with an excitation wavelength of 360 nm and an emission wavelength of 450 nm with the Victor X4 2030 Multilabel plate reader (Perkin-Elmer, Waltham, MA). A kinetic trace was recorded by taking fluorescence measurements every 2 min for 10 min. The slopes of the linear portion of each curve of the trypsin standard were calculated (increase in fluorescence/time) and a curve was generated to compare known trypsin-like U/ml to cleavage of the substrate. The slope of this second curve was then used to extrapolate the concentrations of trypsin-like activity in the crude allergen extracts based on their fluorescence/time curves.

The activity assay was repeated using fluorogenic peptide substrates with different peptide sequences to determine the presence or absence of other types of serine proteinases. Substrates included: Phenylalanine-Arginine (FR)-AMC (serine and cysteine cathepsin substrate; Bachem), Phenylalanine-Valine-Arginine (FVR)-AMC (thrombin substrate; Calbiochem), Alanine-Alanine-Proline-Phenylalanine (AAPF)-AMC (chymotrypsin substrate; Calbiochem), and Alanine-Alanine-Proline-Alanine (AAPA)-AMC (pancreatic elastase substrate; Calbiochem). To express the cleavage of the

substrates in terms of pmol of substrate cleaved per minute, a standard curve of fluorescence/concentration of free AMC (emission at 530 nm) was generated, yielding a conversion factor of 126 fluorescence units measured by the Perkin-Elmer plate reader per pmol of free AMC.

Inhibition of substrate cleavage by treatment with the soybean trypsin inhibitor (SBTI; Sigma) and the trypsin-specific (ABP-PK) and chymotrypsin-specific (ABP-F) activity based probes was performed to verify the ability of the trypsin-like enzymes in the allergen extracts to cleave the above substrates. The allergen extracts were diluted to a concentration of 0.2 U/ml in 200 µl volumes. These aliquots were then incubated with SBTI (10 µM, 10 min incubation) or the activity-based probes (100 µM, 1 h incubation) and 50 µl volumes were added to a 96-well black plate in triplicate. The substrates were then added and fluorescence was monitored as described above.

2.2.2 Bicinchoninic acid protein assay

Protein levels of each of the extracts were measured using a bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Scientific; Rockford, IL). Protein concentrations were determined using a bovine serum albumin (BSA; aliquots provided in the kit) standard curve with known protein concentrations. A standard curve of BSA was prepared with concentrations of: 5 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml. 50 µl of each standard concentration was added in triplicate to a 96-well clear plate (Greiner Bio-One; Kremsmünster, Austria). The allergen extracts were diluted 1000x, 5000x and 10000x and 50 µl of each dilution was added in triplicate to the 96-well plate. The working reagent was prepared as a 50:1 mixture of component A (containing bicinchoninic acid):

component B (containing cupric sulfate). 50 µl of the working reagent was added per well to the BSA standard and allergen extracts and the samples were incubated for 1 h at 37°C. Colourimetric measurements of absorbance at 562 nm were taken with the Perkin-Elmer plate reader. Using the BSA standard curve, a plot of absorbance at 562 nm on the Y-axis over protein concentration on the X axis and the slope of the curve was used to extrapolate the concentration of protein in the allergen extracts.

2.2.3 Activity-based probe labeling

Active serine proteinases in the allergen extracts were labeled with activity-based probes (ABP). The probe consists of a covalent serine proteinase inhibitor (diphenylphosphonate; DPP4) conjugated via a peptide sequence to biotin, allowing active proteinases to bind to the inhibitor moiety resulting in a covalently biotinylated enzyme[96]. The enzyme specificity of the probes are conferred by the peptide linker sequence, with probes specific for trypsin (proline-lysine (PK) or arginine (R) in the peptide sequence directly upstream of the DPP4), chymotrypsin (phenylalanine (F)), or pancreatic elastase (alanine (A)). The crude allergen extracts were diluted in PAB to a final concentration of 1.5 U/ml (15 mU total in 10 µl). The diluted extracts were incubated with 100 µM of each of the activity-based probes for 2 h at room temperature. The reaction was terminated with the addition of 10 µl 2X SDS sample buffer containing β-mercaptoethanol and the reaction products denatured by heating at 92°C for 3 min. 15 µl of the denatured reaction product were loaded into Novex 4-20% polyacrylamide gradient tris-glycine gels (Life Technologies) and resolved by SDS/PAGE using the XCell Sure lock Novex mini-cell system (Life Technologies). Gels were immersed in

running buffer (1X tris-glycine, 0.1% SDS) and a 10 mA current was passed through the gel for 1.5-2 h with a maximum voltage of 140 V. Once resolved, the protein was transferred to a Hybond-P PVDF membrane (GE Healthcare) in the Transblot SD semi-dry transfer cell (Bio-Rad) by passing a 100 mA current through the system for 1 h. Following the transfer the membranes were blocked overnight at 4°C by shaking in 1% ECL prime blocking agent (GE Healthcare) in phosphate-buffered saline (PBS) with 0.1 % Tween-20 (PBST). Blocked membranes were then treated with horseradish peroxidase (Hrp)-conjugated streptavidin (Extravidin peroxidase, Sigma) in a 1:10000 dilution in PBST for 30 min and washed in PBST for 2h with a buffer change every 15 min. The washed membranes were then treated with ECL Select reagents (GE Healthcare; Little Chalfont, UK) and luminescence was recorded with the Kodak Image Station 4000 MM Pro gel doc.

2.2.4 Total protein staining

Allergen extracts were diluted to a final concentration of 5 µg protein/ml (50 µg total in 10 µl) and10 µl volumes of 2X SDS sample buffer containing β-mercaptoethanol were added. The protein samples were denatured by heating for 3 min at 92°C. The samples were resolved by SDS/PAGE as above, but following resolution the gels were washed in fixing solution (10% acetic acid, 15% methanol) for 1 h at room temperature. The fixed gels were then stained overnight in SYPRO ruby fluorescent protein stain (Bio-Rad) by shaking at room temperature in the dark. The following day the stained gels were washed in fixing solution for 2 h and protein was visualized in the Kodak Image Station gel doc as UV light-activated fluorescence.

2.2.5 Ca^{2+} signaling assay

2.2.5.1 Cell suspension Ca^{2+} signaling assay

The activation of PAR2 was assessed with the Kirsten-virus transformed normal rat kidney (KNRK) cell line stably transfected with rat PAR2. Cells were grown to 90% confluence in Dulbecco's modified Eagle medium (DMEM; Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco) and plasmocin antifungal agent (Life Technologies) in 75 cm^2 T-flasks and lifted by incubating in Hanks' buffered saline solution (HBSS, pH 7.4) containing 1 mM EDTA. Cells were centrifuged and the pellet was taken up in 1 ml of a solution of the no-wash Ca^{2+}-sensing dye Fluo-4 NW (Life Technologies) dissolved in Ca^{2+} assay buffer (1 X HBSS, pH 7.4, 10 mM HEPES, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$) at a final concentration of 25 mg/ml, along with 2.5 mM probenecid (Life Technologies). The cell suspension was shaken for 30 min at room temperature in the Fluo-4 solution and 100 µl volumes were diluted in 2 ml in Ca^{2+} assay buffer in 4 ml cuvettes with magnetic stir bars. The Aminco Bowman Series 2 Luminescence spectrometer (Thermo Spectronic, Madison, WI) with Ab2 software was used to detect fluorescence and generate the Ca^{2+} tracings. The cockroach extract was applied directly to the 2 ml cell suspensions for a final concentration of 1 U/ml of trypsin-like activity as calculated above and fluorescence was detected with an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

A desensitization protocol was used to assess the ability of the allergen extracts to activate Ca^{2+} signaling via PAR2. Saturating concentrations of the PAR2 agonist

enzyme trypsin (5 U/ml) were added to the cell suspensions to induce internalization of each respective receptor. A second dose of trypsin was added after the baseline was re-established (5-10 min after the first dose) to confirm desensitization of the cells. Following another 5-10 min period, the cockroach extracts was added. Alternatively, the soybean trypsin inhibitor (SBTI) was added to the cell suspension at a concentration of 10 µM 5 min prior to applying the cockroach extract. Disappearance of the Ca^{2+} transient following trypsin-induced desensitization or SBTI pre-treatment was taken as confirmation of receptor activation by the enzymes in the extract.

2.2.5.2 Monolayer Ca^{2+} signaling assay

PAR2-expressing KNRK cells were plated at 50% density in 96-well black, clear bottom cell culture plates and allowed to grow to confluence overnight. To load the Fluo-4 dye, culture media was replaced with 50 µl of Fluo-4 solution for 45 min at 37C. The Perkin-Elmer plate reader was used both to deliver PAR agonists to the cells using the machine's injector system and to record the resulting fluorescence. The agonist delivery injection system was flushed with 20 mM citric acid and then 20 mM Tris-HCl, pH 9, to remove bound protein. The line was then flushed with 1% bovine serum albumin to prevent agonist-enzyme binding to the tubing. Agonists to be used were diluted in Ca^{2+} signaling buffer (1X HBSS, 10 mM HEPES, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$) to twice the final concentration to be added to the cells in a 2 ml volume. Agonists were then loaded into the delivery system by flushing the injection tubing with 1 ml of agonist volume. After loading the cells with the dye, the plate was loaded into the plate reader. A program for agonist delivery and fluorescence reading was

designed using the plate reader software. The program consisted of: delivery of 50 µl of agonist to each well, a brief shake of the plate to evenly distribute the agonist in the well, and a kinetic fluorescence reading (excitation wavelength of 480 nm and emission wavelength of 530 nm) consisting of 120 0.1 s recordings taken every 0.5 s for a total duration of 1 min. Each agonist was delivered to three separate wells and the average peak of the Ca^{2+} transient taken as a measure of the magnitude of the Ca^{2+} response. Initial concentrations of 0.5 U/ml of the Alternaria filtrate were applied to the dye-loaded cell monolayers. To confirm that the signal was induced via PAR2, the cells in the following wells were desensitized by pre-treating with 5 µM 2fLIGRL for 10 min to allow the intracellular Ca^{2+} concentration to return to baseline. After 10 min the Alternaria filtrate applied to the de-sensitized cells and the fluorescence monitored. To verify that the enzyme activity itself was responsible for the observed Ca^{2+} transients, the proteolytic activity of the fractions was neutralized by pre-treating with a 50 µM concentration of the soybean trypsin inhibitor (SBTI) for 10 min at room temperature. The inhibitor-treated fraction was then applied to the cells and fluorescence monitored.

2.3 Methods for Chapter 4

2.3.1 High S cation-exchange chromatography

An initial isolation of the cockroach enzymes was performed using High S cation exchange chromatography. 5 ml pre-packed High S columns (Bio-Rad Bio-Scale Mini Macro-Prep High S Cartridge) were connected to the fast-phase liquid chromatography system (FPLC; Pharmacia; Stockholm, Sweden). The buffer used for this column was a 20 mM acetate-acetic acid buffer, pH 5, (437 µl glacial acetic acid, 4.23 g sodium

acetate trihydrate, in 1 L of water). Half of the 1 L buffer volume was used as a low-salt buffer containing no NaCl, and the other half was used as a high-salt buffer containing 1 M NaCl (29.23 g NaCl in 500 ml). The column was equilibrated with the following protocol; 10 ml of low-salt buffer at 2 ml/min; 50 ml of high-salt buffer at 6 ml/min; 50 ml of low-salt buffer at 6 ml/min. Following equilibration, one aliquot of the crude cockroach extract (250 µl) was diluted to 1 ml in the low-salt acetate buffer and loaded into the FPLC system. The elution was carried out at 1 ml/min with a NaCl gradient, and 1 ml fractions were collected. The first 10 ml of elution volume was a flow-through with the low-salt buffer. After 10 ml, a NaCl gradient was initiated and continued for 30 ml, reaching 1 M NaCl at 40 ml elution volume. A wash of 5 ml of the high-salt buffer was performed to end the elution protocol. As with the DEAE sepharose column, enzyme activity in each fraction was assessed using the microtiter plate activity assay and active fractions were labeled with the trypsin-specific ABP to identify the enzymes present. This elution procedure was repeated with aliquots of the crude cockroach extract until at least 15 ml of fractions containing each isolated enzyme were collected. The pooled fractions containing each enzyme were then concentrated to ~1 ml as above using centrifugal concentration units with a molecular weight cut-off of 10 kDa. For optimal performance the column was periodically regenerated by washing with 50 ml of 1 M NaOH at 1 ml/min, followed by 50 ml of distilled water at 6 ml/min and then equilibrated with the low- and high-salt elution buffers as above. The trypsin-like activity in the concentrated fractions was quantified by comparing to a trypsin-standard curve, and the protein content assessed with a BCA kit, as described above. Sypro ruby total protein stains (10 µl of each fraction per well) and ABP labeling (15 mU of each fraction

per reaction volume) were then performed on the concentrated fractions to visualize the enzyme purity. Concentrated fractions containing each cockroach enzyme were split into 100 µl aliquots and frozen in -80°C to be stored for future use.

2.3.2 High Q anion-exchange chromatography

A High Q strong anion-exchange column was used to further purify the concentrated cockroach fractions containing each of the isolated enzymes obtained with the High S column, and as an initial purification step for the crude Alternaria extract. 5 ml pre-packed High Q columns (Bio-Rad Bio-Scale Mini Macro-Prep High Q Cartridge) were attached to the FPLC system as with the High S columns. The buffer used for elution from the High Q column was 20 mM Tris-HCl, pH 7.8, with the high-salt wash buffer containing 1 M NaCl. The column was equilibrated with the high- and low-salt Tris buffers using the same protocol described above. Prior to loading the concentrated High S fractions, 1 ml volumes of each were first dialyzed against the low-salt starting buffer for 2 h at 4°C to ensure the appropriate pH for the column run. Alternatively, 250 µl aliquots of the Alternaria extract were diluted to 1 ml in the column starting buffer. After loading the allergen samples, the elution was performed with a NaCl gradient and fractions collected using the same protocol as with the High S column. The resulting fractions were assessed for trypsin-like activity, total protein concentration, labeled with the ABP and stained for total protein as above, and fractions containing the purified enzymes were stored at -80°C for future use.

2.3.3 ELISA assay to quantify the concentration of the Alt a 1 allergen protein

Fractions collected from the High Q chromatographic fractionation of the Alternaria preparations were assessed for the presence of the major allergen protein *Alt a 1* with an ELISA kit (Indoor Biotechnologies, Charlottesville, VA). A 96-well plate was coated with the α-*Alt a 1* antibody by incubating overnight with 100 µl volumes of a 1/1000 dilution of the antibody in a 50 mM carbonate-bicarbonate buffer, pH 9.6. The following day the wells were washed 3X with 100 µl of PBST, followed by a 30 min incubation of the wells in 100 µl of PBST supplemented with 1% bovine serum albumin (BSA). A standard curve was generated using ten doubling dilutions of a recombinant *Alt a 1* protein in the PBST-BSA solution, ranging from 100 – 0.2 ng/ml *Alt a 1*. 100 µl volumes of each *Alt a 1* solution were added in duplicate to the plate antibody-coated plate. The Alternaria allergen samples and fractions were diluted 1/10, 1/20 and 1/40 in the PBST-BSA solution, and 100 µl volumes were applied in duplicate and incubated for 1 h. The wells were then washed 3X with PBST and a biotinylated α-*Alt a 1*antibody was applied to the wells containing the Alternaria samples in a 1/1000 dilution in PBST-BSA and incubated for 1 h at room temperature. The wells were washed again and 100 µl of a 1/1000 diluted solution of streptavidin-conjugated horseradish peroxidase in PBST-BSA and incubated for 30 min at room temperature. After a final wash of the wells, peroxidase activity was activated by adding 100 µl of a 1 mM solution of the peroxidase substrate 2,2'-azino-bis (ABTS) in 70 mM citrate phosphate buffer, pH 4.2, containing a 1/1000 dilution of H_2O_2 to each well, and absorbance at 405 nm was recorded. The

standard curve of A405/*Alt a 1* concentration was used to extrapolate the *Alt a 1* concentrations of the Alternaria samples.

2.3.4 DEAE sepharose ion-exchange chromatography

High concentration fractions containing each enzyme were required for mass spectral analysis of the enzymes, so a rapid step-wise elution protocol was employed, using DEAE sepharose weak anion exchange column (DEAE Sephacel, Pharmacia) was performed. 5 ml of DEAE sepharose slurry was poured into a column (1.2 X 2.4 cm) and allowed to settle. 20 ml of starting buffer, 50 mM Tris-HCl, pH 7.2 containing 50 mM NaCl, was passed through the column to equilibrate, following which 5 ml volumes of the crude extracts were applied. A low-salt wash was collected by applying 1 ml volumes of the 50 mM NaCl buffer until 15 1 ml fractions had been collected. This step was repeated for intermediate and high salt elutions by applying 15 1 ml volumes of the Tris buffer containing 250 mM NaCl and 500 mM NaCl respectively. The fractions were tested for trypsin-like activity with the QAR-AMC substrate and active fractions were labeled with the activity-based probe. Active fractions containing the same enzymes were pooled and concentrated to ~1-1.5 ml in 15 ml Amicon Ultra centrifugal filter devices with a molecular weight cut-off of 10 kDa as described above. Trypsin-like activity in the concentrated fractions was assessed as above by comparing to a trypsin standard curve, and the fractions were frozen at -80°C and stored for future use.

2.3.5 Activity-based probe-mediated affinity chromatographic purification of the allergen-derived enzymes

High-activity, concentrated fractions obtained from the DEAE sepharose column were used to purify each allergen enzyme for mass spectral analysis. All buffers used were passed through a 0.2 µm filter and all work done in a fume hood to reduce contamination of the samples with keratin, which would skew mass spectral sequencing results. A volume of each fraction corresponding to 16 U total of trypsin-like activity (estimated to be ~1 µg mass of each enzyme) was incubated with 100 µM of the activity-based probe for 2 h at room temperature to biotinylate the enzymes. To purify the biotinylated enzymes, Streptavidin-conjugated, magnetic sepharose beads (Streptavidin MagSepharose; GE Healthcare) were used. 20 µl of the 10% bead slurry (2 µg of sepharose beads) was washed five times in 50 µl of binding buffer (**BB**; 50 mM Tris-HCl, pH 7.5, with 150 mM NaCl) to remove the ethanol of the slurry storage buffer and to equilibrate the beads. Following equilibration, the ABP-treated fractions containing biotinylated enzymes were applied to the beads and incubated at room temperature for 2 h while being shaken to avoid the beads settling to the bottom of the tubes. After the 2 h incubation, the beads were pelleted in a magnetic microcentrifuge tube rack. The supernatant was discarded and the beads were washed five times in 50 µl of washing buffer (**WB**, 50 mM Tris-HCl, pH 7.5, with 150 mM NaCl and 2 M urea) to remove non-specific contaminants, following which the beads were washed five times in 50 µl binding buffer to remove the urea from the wash buffer. Washed enzyme-bound beads were resuspended in 10 µl of 2% SDS and boiled at 92°C for 5 min to break the

biotin-avidin bond and elute the purified enzyme. 10 µl of 2X SDS sample buffer was added to the eluted fractions and the total volume resolved by SDS/PAGE as described previously. Gels were washed in distilled water for 15 min to remove SDS, and then stained with BioSafe Coomassie blue (Bio-Rad) non-colloidal protein stain to visualize the total protein contained in the column eluate. Bands visible to the naked eye in the molecular weight range of the ABP-labeled allergen enzymes (20-25 kDa) were excised from the gel with sterile razor blades and stored at -20°C in 1.5 ml microcentrifuge tubes prior to being shipped on dry ice to the proteomics facility at the University of Arizona, Tucson for mass spectral analysis.

2.4 Methods for Chapter 5

2.4.1 Determination of Km values

Fractions obtained from the High Q column containing highly purified cockroach enzymes were used to determine the enzyme kinetics of each enzyme using the fluorogenic peptide microtiter plate assay. Fractions containing each enzyme were diluted such that 50 µl volumes loaded into the plate contained 10 mU of trypsin-like activity (0.2 U/ml concentration). The enzymes were then incubated with increasing concentrations of three serine proteinases substrates, QAR-AMC, FVR-AMC, and GGR-AMC. A range of substrate concentrations was designed to include at least four points in the first order portion of the kinetic curve and at least two points in the zero order portion of the curve with respect to the substrate concentration. The respective concentration curves for each substrate were as follows:

- QAR-AMC; 0, 1.25, 2.5, 5, 10, 25, 50, 75 mM
- GGR-AMC; 0, 2.5, 5, 10, 25, 50, 75, 150 mM
- FVR-AMC; 0, 2.5, 5, 10, 25, 50, 75, 150 mM

Km was determined by plotting the slope of enzyme velocity (fluorescence/time; Y-axis) determined for each substrate concentration over the substrate concentration (X-axis) in GraphPad Prism software. The Michaelis-Menten non-linear regression function included in the software was used to calculate the Michaelis constant (Km-concentration at which recorded enzyme activity is half maximal) and maximal enzyme velocity (Vmax) values for each substrate.

2.4.2 Determination of inhibitor Ki values

Each High Q fraction was added in triplicate to a 96-well black plate at a concentration of 10 mU/well as described above. SBTI and TLCK were added to the enzymes in a range of concentrations and incubated for 10 min (SBTI) or 1 h (TLCK), following which 50 µl of a solution containing 75 mM of the QAR-AMC substrate was added to each well, and fluorescence was monitored as described previously. The curves for each inhibitor were as follows:

- SBTI; 0, 1.25, 2.5, 5, 10, 25, 50, 75 mM
- TLCK; 0, 2.5, 5, 10, 25, 50, 75, 150 mM

The resulting curves were plotted as fractional activity (observed activity at each inhibitor concentration/uninhibited activity). The IC50 (inhibitor volume at which enzyme activity is half of that in the uninhibited condition) was determined with the GraphPad

Prism software's Dixon equation non-linear regression, and the inhibitor constant (Ki) was calculated with the following equation:

Ki = IC50/(1+Km/[S])

2.4.3 Identification of trypsin-like sequences in a cockroach expressed-sequence tag library

The preiously reported annotated cockroach EST library[79] was generated and provided to us by Dr. Mee Sun Ock (Kosin University College of Medicine, South Korea). Putative serine proteinases were identified by searching the database for sequences that were described to be homologous to trypsins and/or serine proteinases. The contig sequences identified in this way were theoretically translated using expasy software (web.expasy.org/translate/), and the open-reading frames corresponding to complete proteins were selected. The translated sequences were then run through the MEROPS proteinase database BLAST program (http://merops.sanger.ac.uk/cgi-bin/blast/submitblast/merops/advanced) to confirm homology to trypsin-like enzymes. The trypsin-like sequences that were identified, as well as the sequence of the cloned cockroach trypsin (Tryp1) identified by Ock et al[80], were then used in the mass spectral analysis of the excised gel bands.

2.4.4 Tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS):

Mass spectral analysis was performed by the staff at the Arizona Proteomics Consortium at the University of Arizona, Tucson, under the direction of our collaborators, Michael Daines and Scott Boitano. Excised coomassie-stained protein gel bands following 1D SDS-PAGE were digested with chymotrypsin (10 µg/mL) at 37^{o}C

overnight. LC-MS/MS analysis of in-gel chymotrypsin digested-proteins[187] was carried out using a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an Advion nanomate ESI source (Advion, Ithaca, NY), following ZipTip (Millipore, Billerica, MA) C18 sample clean-up according to the manufacturer's instructions. Peptides were eluted from a C18 precolumn (100-µm id × 2 cm, Thermo Fisher Scientific) onto an analytical column (75-µm ID × 10 cm, C18, Thermo Fisher Scientific) using a 5-35% gradient of solvent B (acetonitrile, 0.1% formic acid) over 50 min, followed by a 35-45% gradient of solvent B over 9 min, and finally a 95% increase and hold over 0.1 and 5 min, respectively, all at a flow rate of 400 nl/min. Solvent A consisted of water and 0.1% formic acid. Data dependent scanning was performed by the Xcalibur v 2.2 SP1.48 software[188] using a survey mass scan at 60,000 resolution in the Orbitrap analyzer scanning *m/*z 400-2000, followed by collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) of the six most intense ions in the linear ion trap analyzer. Precursor ions were selected by the monoisotopic precursor selection (MIPS) setting with selection or rejection of ions held to a +/- 10 ppm window. Dynamic exclusion was set to place any selected *m/z* on an exclusion list for 45 seconds after a single MS/MS. A "blank" gel piece was digested and subjected to LC-MS/MS in order to generate an exclusion list of ion masses from common contaminants (eg., human keratins) and bovine chymotrypsin peptides that were identified following a search of the MS/MS spectra against the most current version of the Uniprot Sprot protein database (http://www.uniprot.org/downloads; at time of search this database contained 452,768 protein entries), using Thermo Proteome Discoverer 1.2 (Thermo Fisher Scientific). The MS/MS spectra of visible protein bands processed for LC-MS/MS as above were

searched against a custom database made of (1) NCBI proteins that contained the word “trypsin” in their annotation entries and (2) proteins that contained the word “trypsin” found at http://arrakis.vbi.vt.edu:8080/Alternaria_alternata/Info/Index (for analysis of the Alternaria enzyme) or proteins that contained the word “trypsin” found in the cockroach EST library as described above. Variable modifications considered during the search included methionine oxidation (15.995 Da), cysteine carbamidomethylation (57.021 Da), as well as adduction of lysine or cysteine residues by 4HNE (156.115 Da). Proteins were identified at 95% confidence with XCorr scores[189] as determined by a reversed database search.

2.5 Methods for Chapter 6

2.5.1 Monitoring cleavage of synthetic PAR tethered ligand peptides

With the assistance of Dr. Bernard Renaux, the allergen enzymes were assessed for their ability to cleave synthetic peptides representing the tethered ligand sequences of PAR1 and PAR2 as described previously[190]. The following synthetic sequences derived from human PAR1 and rat PAR2 were used (tethered ligand-activating sequences are underlined (*1)* hPAR1, NATLDPRSFLLRNPNDKYE and *(2)* rPAR2, GPNSKGRSLIGRLDTP. These peptides at a concentration of 100 μM were incubated with the allergen-derived enzymes (2 U/ml) for various times up to 30 min at 37 °C. Reactions were terminated by the addition to the proteolysis sample of 2 volumes of a “stop solution” comprising 50% acetonitrile and 0.1% trifluoroacetic acid in water. Samples were either subjected immediately to HPLC analysis, with collection of the E_{214} peak fractions, or were stored at –80 °C for further processing. The cleavage products

were identified by HPLC separation and isolation of the proteolysis products followed by mass spectral MALDI identification of the peptide fragments in the quantified HPLC peaks.

2.5.2 Monitoring PAR cleavage with N-luciferase-tagged PAR constructs

Chinese hamster ovary (CHO) cells were stably transfected with PAR1 or PAR2 constructs with Nano-Luciferase (Nluc; Promega) inserted in the N-terminal region upstream of the receptor activation cleavage site. Upon cleavage with an activating enzyme, the Nluc-containing N-terminal peptide is released from the receptor and the lucifease signal can be measured in the supernatant. Cells containing either the PAR1 or PAR2 construct were grown to 90% confluence in a 24-well culture plate. Cells were washed 3X with 100 µl PBS prior to treatment to remove peptides cleaved by endogenously-expressed proteinases. After washing, 100 µl of samples containing each of the three cockroach enzymes and the Alternaria enzyme, as well as thrombin and trypsin controls were added to the cells in duplicate at a concentration of 1 U/ml. Cockroach E1 and E3, as well as porcine trypsin, were also applied at a concentration of 2 U/ml to the PAR1-Nluc-expressing cells, and thrombin applied at 2 U/ml to the PAR2-Nluc-expressing cells. The cells were incubated with the enzymes for 15 min at room temperature, and then 50 µl of the supernatant was collected and applied to a 96-well black plate. Luciferase activity in the supernatants was activated with the Nano-Glo Nluc substrate (Promega) and luminescence detected with the Perkin-Elmer plate reader. Receptor cleavage was assessed as the ratio of N-luciferase luminescence of the treated cells to an untreated control.

2.5.3 Ca^{2+} signaling assay

Kirsten virus-transformed normal rat kidney (KNRK) cells, which naturally express very low levels of PARs, were used as a background to express PAR1 or PAR2 individually. KNRK cells had been transfected with a PAR1-YFP or PAR2-YFP construct inserted into a pcDNA 3.1(+) vector (Life Technologies) and grown in G418-supplemented Dulbecco's modified eagle medium (DMEM;, Gibco, Life Technologies, Carlsbad, CA) with sodium pyruvate and Plasmocin treatment (Life Technologies) as described previously[191]. A pcDNA vector-only transfected cell line was used as a control. PAR1 or PAR2-expressing KNRK cells were plated at 50% density in 96-well black, clear bottom cell culture plates and the protocol used was identical to that described in Section 2.1.7.2.

Each allergen enzyme was initially applied to both the PAR1 and PAR2-expressing cells at a final concentration of 1 U/ml. In the event that this concentration did not evoke a Ca^{2+} transient, increasing concentrations of enzyme were applied up to 16 U/ml for the allergen-derived enzymes, or 64 U/ml for trypsin. As a negative control, volumes corresponding to concentrations of 4 U/ml of each allergen-derived enzyme, as well as trypsin and thrombin controls, were applied to non-PAR-expressing cells transfected with the pcDNA vector only. A lack of signal at this concentration was taken as confirmation of the PAR-dependence of transients evoked in the PAR-expressing cells.

2.5.4 MAPK activation assay

PAR1 or PAR2-expressing KNRK cells were grown to confluence in 24-well cell culture plates. The culture media was replaced with serum-free DMEM and the cells serum-

starved overnight. Fresh serum-free medium was added the following day for 4 h prior to the application of PAR agonists and allergen enzymes. Enzymes and agonists to be used were diluted in water to 100X final concentration, following which 4 µl of each was added to the 400 µl volume of serum-free medium in each well. Cells were incubated for 15 min with the agonists, then cells were washed in 1X PBS and incubated in 100 µl of cold lysis buffer containing 5 µl of the Protease Inhibitor Cocktail set III (Calbiochem) for 10 min to collect proteins. The supernatant of the lysis step was centrifuged for 5 min at 15000 rpm at 4°C to remove cell debris and then the supernatant of the centrifugation step was collected in microcentrifuge tubes. The supernatants were combined with an equal volume of 2X SDS sample buffer containing β-mercaptoethanol and then denatured by boiling for 3 min. Samples were resolved by gel electrophoresis in SDS-containing polyacrylamide gels and transferred to a PVDF membrane as described for the ABP labeling procedure. Membranes were treated overnight in phosphate-buffered saline with 0.1% Tween-20 (PBST) supplemented with 0.2% NaN_3, 1% ECL prime blocking agent (GE Healthcare), and containing mouse monoclonal α-phosphorylated ERK 1/2 (T202/Y204) antibody (Cell Signaling Technologies) at a 1:10000 dilution factor. The following day the membranes were washed twice in PBST to remove residual NaN_3 and then treated for 30 min with a horseradish peroxidase-conjugated α-mouse IgG (Cell Signaling Technologies) diluted in PBST without NaN_3 at a 1:10000 dilution factor. Membranes were washed again in PBST without NaN_3 for 2 h and then treated with the ECL Select solution and bioluminescence was recorded with the Kodak Image Station gel doc. Membranes were then washed with PBST with NaN_3 to neutralize horseradish peroxidase activity in the α-mouse antibodies, and then

treated overnight with a monoclonal rabbit α-GAPDH antibody (Cell Signaling Technologies) at a 1:10000 dilution. The GAPDH blot signal was used as a loading control. Membranes were then treated for 30 min with a horseradish peroxidase-conjugated α-rabbit IgG (Cell Signaling Technologies) and luminescence recorded as above. Band intensities were quantified using the ImageJ quantification software (http://rsbweb.nih.gov/ij/), and data were expressed as the ratio of p-ERK 1/2 signal to GAPDH signal, normalized to the signals yielded by untreated control cells.

Similar to the previous Section, PAR non-expressing cells transfected with empty vector were also assessed for MAPK activation with 2 U/ml concentrations of each enzyme. A lack of signal was taken as confirmation of the PAR-dependence of the observed phosphorylation of ERK 1/2 in the PAR-expressing cells.

2.5.5 BRET-based detection of β-Arrestin recruitment to PAR2

HEK293 cells were transfected with the assistance of Dr. Rithwik Ramachandran with PAR2-YFP and β-Arrestin-1 or β-Arrestin-2-*Renilla* luciferase (Rluc) and prepared as previously described[133]. Final concentrations of 1 U/ml of each of the cockroach enzyme-containing fractions were added to the cells, followed by a 15 minute incubation period. The Rluc substrate coelenterazine (5 μM; Promega, Madison, WI) was then added to the cells, and both the luciferase-induced luminescence and the YFP fluorescence were monitored. The interaction of PAR2 and β-Arrestin-1 or β-Arrestin-2 was assessed as an increase in the ratio of the YFP signal over the luciferase signal as compared to untreated control cells, indicating the activation of the YFP fluorophore due to close proximity of the β-Arrestin-tagged luciferase to the PAR2-tagged YFP.

2.5.6 Cleavage and internalization of dual-fluorescent PAR constructs

The A549 cell line, derived from type II alveolar carcinoma cells, was used to express the dual-fluorescent PAR1 construct (described by Mihara et al[136]). Cells were grown to 50% confluence in 24 well culture plates, at which point they were supplied with fresh media. The mCherry-PAR1-YFP construct DNA was diluted in 200 µl of OPTIMEM media (Gibco; Life Sciences) to a final concentration of 1 µg/ml with 2 µl of Lipofectamine LTX reagent (Life Technologies) and 1 µl of the Lipofectamine Plus reagent (Life Technologies). The DNA and transfection reagents were mixed by vortexing and then incubated for 20 min at room temperature to form complexes. 100 µl volumes of the DNA-transfection reagent complexes in the OPTIMEM media were applied to the A549 cells and left for 24h at 37°C for the transfection reaction to take place. Transfection success was assessed by visualizing the cells in the 24 well plates in a wide-field microscope by visualizing both the YFP and mCherry signals. Presence of both fluorophore signals in over 30% of the cells was taken as positive transfection. The transfected cells were then lifted out of the 24-well plate by incubating for 10 min in 1X PBS containing 1mM EDTA at 37°C, then the lifted cells from each well were diluted in 4 ml of DMEM culture media supplemented with 10% fetal bovine serum (FBS; Gibco) and plasmocin antifungal agent (Life Technologies). 400 µl of the cell suspension were added to glass-bottom culture plates and left for 2 days to grow to confluence.

After growing to confluence, the cells were treated with the following PAR agonists: thrombin (2 U/ml), trypsin (4 U/ml), and the cockroach enzymes (E1 and E3 separately

at 4 U/ml). Agonists were diluted to their final concentration in serum-free DMEM. The cell culture medium in the glass plates was aspirated and replaced with 100 µl of the agonist-containing serum-free DMEM solutions. After either 3 min or 30 min of incubation with the PAR agonists, the cells were fixed by incubating in buffered formalin for 15 min at room temperature. The formalin solution was then aspirated and the fixed cells were washed twice in 1X PBS containing 0.1% NaN_3 and stored in the dark at 4°C. To activate and visualize the fluorophores in the constructs, the Olympus FV1000 confocal microscope was used. Images of each flourophore were taken separately and combined using the Olympus Fluoview Version 3.0 software.

2.6 Methods for Chapter 7

2.6.1 Ca^{2+} signaling

The type II alveolar carcinoma-derived A549 cell line was used to assess the presence and effects of PAR signaling in an airway-relevant context. The cells were assessed for PAR expression and Ca^{2+} signaling in suspension, as described in Chapter 2. The cells in a confluent 75 cm^2 T-flask were detatched by incubating in 1X PBS with 1 mM EDTA for 20 min at 37°C. The disaggregated cells were pelleted by centrifugation (1000 rpm for 3 min) and the pellet resuspended in 1 ml of Fluo-4 dye solution. Ca^{2+} signaling was monitored as described in Section 2.1.7.1.

In addition, the bronchial epithelium-derived transformed cell line BEAS-2B cells were assessed for PAR expression in the same manner as described above for the A549 cells. As above, PAR1 and PAR2 expression were assessed by treating the cells with 25 µM TFLLR and 5 µM 2fLI respectively.

2.6.2 MAPK signaling

A549 cells were grown to confluence in 24-well plates and serum-starved overnight as described previously with the PAR-expressing KNRK cells. Fresh serum-free medium was applied to the cells for 4 h prior to administering the agonists. Agonist application, cell lysis and the western blotting protocol were performed as described in Section 2.3.3.

2.6.3 Activation of IL-8 expression by the allergen-derived enzymes

2.6.3.1 Isolation of RNA from A549 cells

A549 cells were grown to confluence in 6-well plates. The PAR agonists were diluted to the appropriate concentration in serum-free DMEM and applied to the cells. The cells were incubated with the PAR agonists for 1 h, following which RNA was extracted using the RNeasy Mini Kit (Qiagen; Venlo, Netherlands). Prior to the RNA extraction procedure, all surfaces and materials were washed with 70% ethanol containing 1% H_2O_2 to prevent contamination of the samples with environmental RNase. Cell culture media were aspirated and the cells were lysed in 350 µl buffer RLT containing 0.1% β-mercaptoethanol. 350 µl of RNase-free 70% ethanol was added directly to the RLT buffer in the wells and the total 700 µl volume was loaded into a spin column and centrifuged at 130 000 rpm for 1 min. The flow-through fractions were discarded and the columns were washed with 700 µl of the first wash buffer RW1 and spun. The flow-through was discarded and the columns washed twice in 400 µl of the second wash buffer RPE containing four volumes of 100% ethanol. After discarding the flow-through from the second wash, the column was centrifuged again at 130 000 rpm for 2 min to

remove any ethanol from the column. 30 µl of RNase-free water was added to elute bound RNA from the column, and the column centrifuged at 80 000 rpm for 3 min in a clean microcentrifuge tube. The RNA content of the eluate was verified and roughly quantified by measuring the absorbance at 260 nm with the Nanodrop 2000 UV-Vis spectrophotometer (Thermo Scientific). RNA concentrations estimated to be in excess of 100 ng/µl were determined to be sufficient for reverse-transcription. The RNA was then either used immediately for the reverse transcription reaction or frozen at -80°C for future use.

2.6.3.2 First strand cDNA synthesis

First strand cDNA templates were produced with a reverse transcription reaction using the SuperScript III reverse transcriptase (Life Technologies). Volumes of the samples collected in the previous Section corresponding to 5 µg of RNA were mixed with 5 ng/µl of random hexamer primers and 1 mM dNTP mix in a 10 µl reaction volume, then incubated at 65°C for 5 min, following which they were placed on ice. A cDNA synthesis mix was prepared consisting of: 2X RT buffer (Life Technologies), 5 mM $MgCl_2$, 50 mM dithiothreitol (DTT), 4 U/µl RNaseOUT (Life Technologies), and 20 U/µl SuperScript III reverse transcriptase. 10 µl of the cDNA synthesis mix was added to the 10 µl RNA/primer mixtures and incubated in the Perkin-Elmer GeneAmp PCR System 2400 thermal cycler for 10 min at 25°C, followed by a 50 min incubation at 50°C. The reaction was terminated by incubating for 5 min at 85°C. The cDNA mixtures were incubated at -20°C for use in the PCR reactions.

2.6.3.3 PCR amplification of IL-8 cDNA

Primers specific for human IL-8 were designed using the Uniprot.com primer design software for the human IL-8 cDNA sequence. The primers were: forward – 5'-CCACCGGAAGGAACCATCTC-3'; reverse – 5'-TTCCTTGGGGTCCAGACAGA-3', yielding a reaction product 279 base pairs in length. The amplification reaction was performed using Platinum Taq DNA polymerase (Life Technologies). A PCR master mix consisting of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, and 2 U of Platinum Taq DNA polymerase was prepare, to which 0.2 µM of each of the forward and reverse primers and ~500 ng of the template DNA were added in a reaction volume of 50 µl. The reaction mixtures were then incubated in a thermal cycler with an initial denaturation step of 2 min at 94°C, followed by 30 PCR cycles consisting of a denaturation step of 30 sec at 94°C, an annealing step of 30 sec at 60°C, and an extension step of 1 min at 72°C.

The reaction products were then analyzed with agarose gel electrophoresis. 10 µl of the PCR reaction products were mixed with equal volumes of loading buffer and loaded into 3% agarose gels containing 0.5 µg/ml ethidium bromide. The gels were submerged in tris-acetate-EDTA (TAE) buffer and a current of 100 V was applied for 30 min, following which the PCR products were visualized by UV trans-illumination using the Bio-Rad Chemidoc MP gel doc. A densitometric analysis of the PCR products was performed using Image J software, and the values measured for each treatment were normalized relative to the signal for a β-actin control (forward primer, 5′-

CGTGGGCCGCCCTAGGCACCA-3′, reverse primer, 5′-TTGGCCTTAGGGTTCAGGG GG-3′, product size 237 bp)

2.6.4 Smooth muscle relaxation bioassays

2.6.4.1 Aortic relaxation assay

Vascular bioassays were done with the assistance of Dr. Mahmoud Saifeddine.Aortic tissues were harvested from PAR1- or PAR2-null C57/BL6 mice and prepared for the bioassay following a protocol similar to that described by El-Daly et al[192]. Briefly, segments of arteries were isolated from the mice and tissues were continuously maintained in a standard physiological salt solution buffer containing 114 mM NaCl, 4.7 mM KCl, 0.8 mM KH_2PO_4, 1.2 mM $MgCl_2$, 11 mM D-glucose, 25 mM $NaHCO_3$, and 2.5 mM $CaCl_2$ that was bubbled with a 95%/5% O_2/CO_2 gas mixture to maintain the buffer at pH 7.4. Rings of mouse aorta were suspended vertically by two metal hooks; the upper hook was connected to an isometric force transducer, and the lower hook was connected to an immovable support in 5 ml of cuvettes containing the standard physiological solution. Agonists were added directly to the chambers that contained the vessels. Isometric force-displacement transducers (MLT0201/D; ADInstruments, Sydney, NSW, Australia) connected to a Powerlab/8S data acquisition system and Chart® software (Chart 5 for Windows©, ADInstruments Pty Ltd, Sydney, NSW, Australia) were used to measure changes in tension (g).

Tissue functionality was verified by monitoring the contractile response stimulated by 80 mM KCl. The viability of the endothelium in the preparations was assessed by pre-contracting the tissues with 2.5 μM of the α 1-adrenergic receptor agonist,

phenylephrine (Sigma), followed by monitoring relaxation induced by 1 µM acetylcholine (Sigma). Tissues were washed and then pre-contracted again with phenylephrine, following which either 0.6 U/ml of the Alternaria enzyme or 1 U/ml of cockroach E1 were applied to the tissues. In the PAR2-null tissues, the PAR1-mediated relaxation induced by Alternaria was verified by pre-treating the contracted tissues with PAR agonists. 1 µM 2fLI was added to confirm the absence of PAR2 in the tissue, and 25 µM TFLLR was added to desensitize the PAR1 in the tissue. Following the PAR peptides, the Alternaria enzyme was applied as above.

The role of endothelial nitric oxide synthase (NOS) in mediating the allergen enzyme-induced responses was assessed by pre-treating tissues with 100 µM of the NOS inhibitor N^5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride (L-NAME) >10 min prior to application of the allergen enzymes.

2.6.4.2 Bronchial relaxation assay

Bronchial tissues were harvested from PAR1- or PAR2-null C57/BL6 mice by Dr. Mahmoud Saifeddine and prepared for the bioassay similarly to the protocol described by Koetzler et al[193]. Briefly, the lungs were removed and bronchial rings (2 mm × 3 mm) were dissected free from surrounding tissue. Left and right first generation bronchial rings were mounted in plastic organ baths containing 4 ml Krebs-Henseleit buffer (pH 7.4), and gassed (95% O_2 and 5% CO_2) at 37°C. Four tissues from two animals were mounted for each experiment. Tissues were allowed to equilibrate for 60 min before being exposed to agonists. Changes in isometric tension were measured with a

Statham force-displacement transducer (Gould Statham Instruments Inc., Cleveland, OH)

Tissues were tested for responsiveness by exposure to KCl (50 mM) followed by tissue wash. A period of 20–30 min between the applications of agonists was allowed for the tissues to recover. Tissues were pre-contracted with 1 µM of the cholinergic receptor agonist carbachol (Sigma) and 20 nM substance P (Sigma) was added to the bath to monitor relaxation as a sign of the presence of viable epithelium. Bronchial rings were washed, and subsequently pre-contracted with carbachol. After ~ 5 min (when stable contraction was reached), the DEAE sepharose fraction containing the Alternaria enzyme was added to the organ bath at a final concentration of 0.6 U/ml. To assess the mechanism of Alternaria-induced relaxation, tissues were pre-treated with 3 µM of the COX inhibitor indomethacin (Sigma) >10 min prior to the administration of the enzyme.

2.7 Statistical analysis

All data are presented as means ± SEM. Comparisons of two or more conditions to an untreated control were performed using a one-way ANOVA test, with Dunnett's test to correct for multiple comparisons. Comparisons of two conditions were performed using an unpaired t-test. Significance was assumed with a p value ≤ 0.05.

CHAPTER 3 CHARACTERIZATION OF THE PROTEINASES IN COCKROACH AND ALTERNARIA MOULD ALLERGENS

3.1 Introduction

As mentioned in the introduction, both of the German cockroach and Alternaria allergens are known to contain trypsin-like activity that can activate PAR2 signaling. The materials typically used experimentally to assess the allergen-derived proteinases are extracts used in the clinical setting to test for sensitization to the allergens. These extracts are necessarily crude so that they include all potential immunogenic antigens to which patients may be sensitized. Because of the crude nature of the extracts, specific characterization of the proteinases contained therein has been difficult, so the trypsin-like enzymes have not been quantified or specifically characterized in either allergen. My aim in this Chapter was to perform such a characterization, for which I used activity-based probes, which I discussed in the introduction, to label the serine proteinases in the extracts. I also used fluorogenic peptide substrates to assess the substrate specificity of the trypsin-like enzymes, and to assess the presence of non-trypsin-like enzymes in the crude extracts. Finally, I assessed both allergen extracts to amplify and extend previous work indicating that the crude allergen enzymes can regulate PAR signaling.

3.2 Characterization of the proteolytic activity in crude allergen extracts

3.2.1 Quantification of trypsin-like specific activity in the allergen extracts

To begin studying the enzymes in the crude allergen extracts, it was necessary first to quantify the unit/ml concentration of trypsin-like activity as a way of standardizing the activity between the extracts. To do this, the increase of fluorescence following

cleavage of the trypsin-specific fluorogenic peptide glutamine-alanine-arginine aminomethyl coumarin (QAR-AMC) by each allergen preparation was compared to a trypsin standard curve of increase in fluorescence/min on the y axis and known U/ml concentrations of trypsin on the x axis. The three lots of the cockroach extract used contained between 184 U/ml and 287 U/ml, with protein concentrations of 7 - 15 mg/ml as determined by the BCA reaction. The specific activity of the extracts was consistently between 18 - 25 U/mg across every lot that was used. The total Alternaria extract purchased from Greer labs contained 2.5 U/ml of trypsin-like activity with 1.5 mg/ml of protein for a specific activity of 1.7 U/mg. The filtrates supplied by Dr. Boitano and Dr. Daines of the University of Arizona, Tucson, contained between 0.5 - 1.3 U/ml and between 1.2 - 1.5 mg/ml for specific activities between 0.4 - 1.1 U/mg (summarized in Table 3.2.1).

	Trypsin-like activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)
Cockroach extract	184 - 278	7-15	18 - 25
Alternaria extract	2.5	1.5	1.7
Alternaria filtrate	0.5 - 1.3	1.2 - 1.5	0.4 - 1.1

Table 3.2.1 – Specific activity of allergen preparations.
The trypsin-like activity was quantified by comparing the cleavage of the QAR-AMC substrate by each extract to a trypsin standard curve. Protein concentration was determined with a BCA reaction. Specific activity was determined a Units of trypsin-like activity per mg of protein in the extracts.

3.2.2 Labeling of active serine proteinases with activity-based probe (ABP)

Until now the trypsin-like activity in the crude allergen extracts in the literature has been assumed to represent a single enzyme, so to quantify the number of distinct enzymes in each allergen the active proteinases were labeled using a serine proteinase-specific activity-based probe. In contrast with the total protein stain of the crude extract which shows a large number of proteins (Figure 3.2.1, left blot), labeling of the cockroach extract with the trypsin-specific probe (lysine in the P1 position adjacent to the suicide substrate diphenylphosphonate) revealed three distinct bands between ~23-28 kDa, labeled as enzymes 1-3 (E1, E2, E3) in ascending order of molecular weight (Figure 3.2.1, right blot). There was no labeling observed using either the chymotrypsin-specific probe (phenylalanine in the P1 position) probe or the elastase-specific probe (alanine in the P1 position; Figure 3.2.2), indicating that there are no other major serine proteinases in the extract detectable by these activity-based probes.

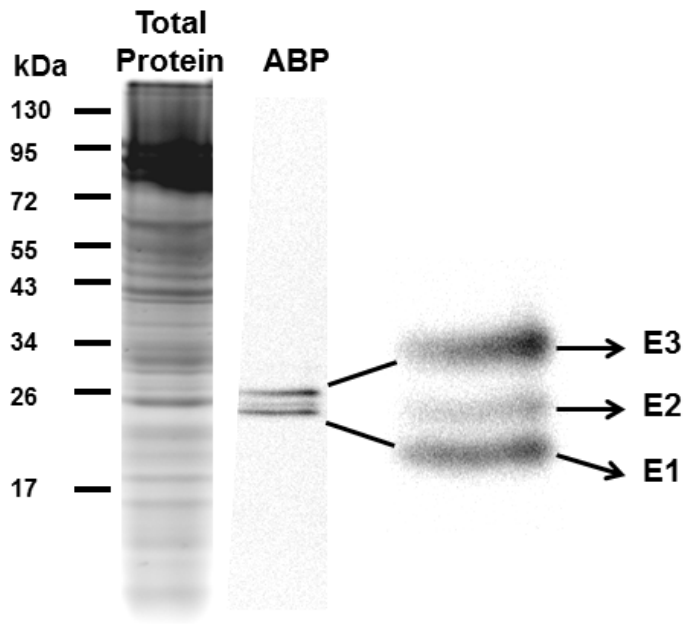


Figure 3.2.1 – Activity-based probe labeling reveals three trypsin-like enzymes in the cockroach extract.

Labeling the active trypsin-like proteinases in the crude cockroach extract with the activity-based probe reveals three distinct bands (right blot). I have labeled the bands enzyme 1-3 (E1-3) in ascending order of molecular weight. A total protein SYPRO ruby stain of the extract (left stain) reveals a large number of contaminating proteins, highlighting the specificity of the probe.

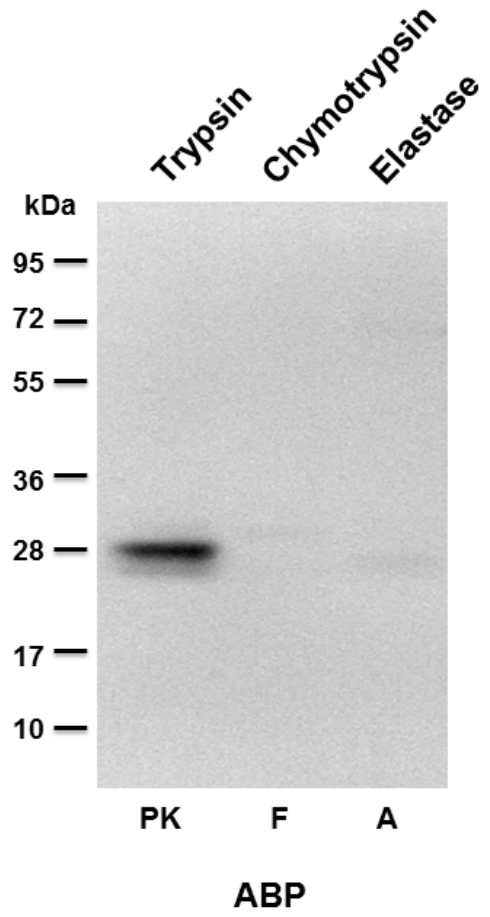


Figure 3.2.2 – Cockroach extract does not contain chymotrypsin- or elastase-like enzymes.

Labeling the crude cockroach extract with activity-based probes specific for chymotrypsin (F – phenylalanine) and pancreatic elastase (A – alanine) does not reveal any additional bands. The only enzymes in the cockroach extract detectable by the serine proteinase-specific activity-based probes are labeled by the trypsin-specific probe (PK – proline-lysine).

Labeling of both the commercial total Alternaria extract and the University of Arizona filtrate with the trypsin-specific (ABP-PK) probe revealed one major band (Alt) of ~23 kDa in both preparations (Figure 3.2.3). Using the chymotrypsin (ABP-F)- and elastase (ABP-A)-specific probes labeled several bands with identical molecular weights ranging from 10-130 kDa in the commercial total mould extract (Figure 3.2.3, left blot). With the latter two probes, the wide range of molecular weights in which the labeled bands were observed, as well as the nearly identical labeling pattern, suggests the possibility of non-specific interaction of the probes with non-proteolytic proteins. This possibility is explored in the following Section using the probes as inhibitors in a fluorogenic substrate cleavage assay. By contrast, there was no labeling observed in the University of Arizona filtrate with the chymotrypsin- and elastase-specific probes, indicating that the proteins labeled in the commercial extract are absent in the in-house produced filtrate (Figure 3.2.3, right blot).

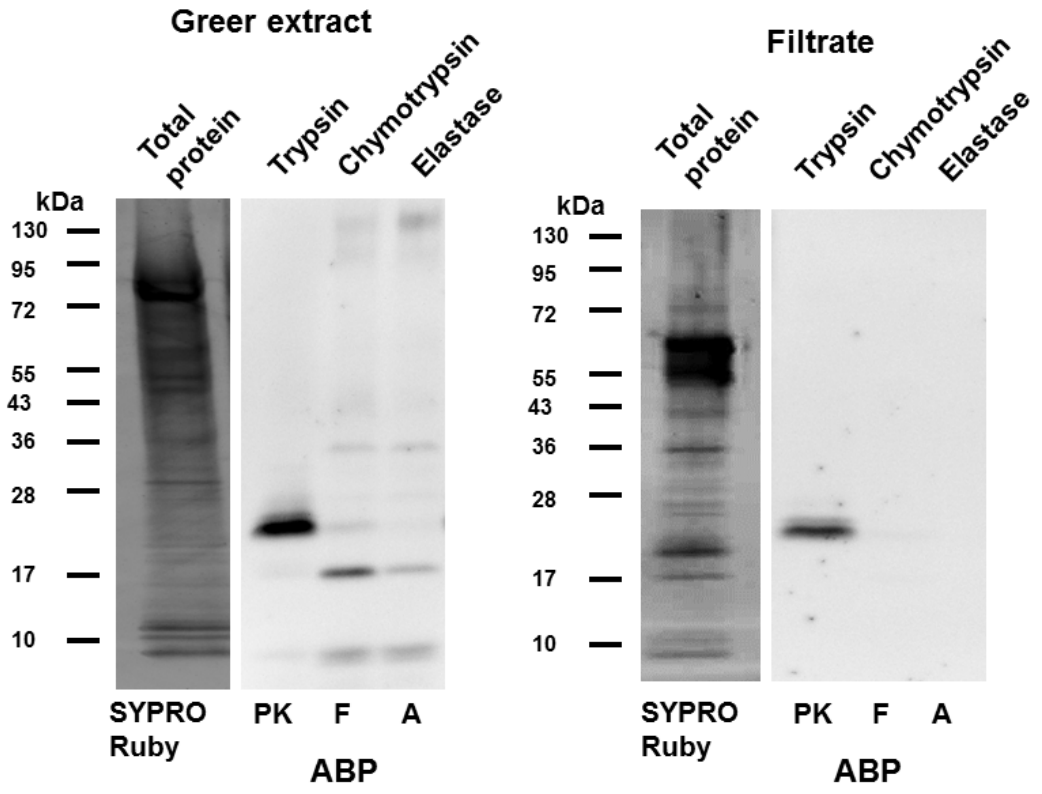


Figure 3.2.3 – Alternaria allergen contains one trypsin-like enzyme.

Two Alternaria allergen preparations, a commercially available total mould extract (left) and a mould culture media filtrate (right) were labeled with three activity-based probes. Labeling the preparations with the trypsin-specific (PK) probe reveals a single major band with the same molecular weight in both. Treating the preparations with the chymotrypsin (F)- and elastase (A)-specific probes reveal several bands in the total mould extract but no labeling in the filtrate. This result suggests the presence of non-trypsin-like enzymes in the extract that are absent in the filtrate.

3.2.3 Cleavage of fluorogenic peptide substrates by the cockroach extract

To explore the possibility of non-trypsin-like serine proteinases in the allergen extracts further, a number of fluorogenic peptide substrates with different enzyme specificities were used. For the cockroach extract, four substrates with positive cleavage were used: QAR-AMC (trypsin substrate), GGR-AMC (urokinase substrate), FR-AMC (serine and cysteine cathepsins substrate) and AAPF-AMC (chymotrypsin substrate). The slope of each fluorescence curve generated after adding each substrate to the cockroach extract revealed different efficiencies with which the enzymes in the extract cleave each substrate: the curve generated with the QAR-AMC substrate had the highest slope (64 pmol substrate cleaved/min), and so was cleaved the most efficiently, followed by FR-AMC (30 pmol/min), GGR-AMC (4.3 pmol/min) and AAPF-AMC (0.6 pmol/min; Figure 3.2.4, right graph). By contrast, equivalent concentrations of porcine trypsin cleaved the QAR-AMC (56 pmol/min) and GGR-AMC (4.3 pmol/min) substrates with similar kinetic slopes, but the curves generated with the FR-AMC and AAPF-AMC substrates had negative slopes, indicating that trypsin did not cleave those substrates (Figure 3.2.4, left graph).

To determine whether the trypsin-like enzymes in the cockroach extract cleaved all of the substrates used, the trypsin-like activity in the extract was neutralized with 10 μM of the soybean trypsin inhibitor (SBTI) prior to adding the substrates. As shown in Figure 3.2.5, cleavage of each of the four substrates was completely blocked by pre-treatment with the inhibitor, suggesting that the trypsin-like enzymes in the extract are responsible for all of the enzymatic activity detected in these assays.

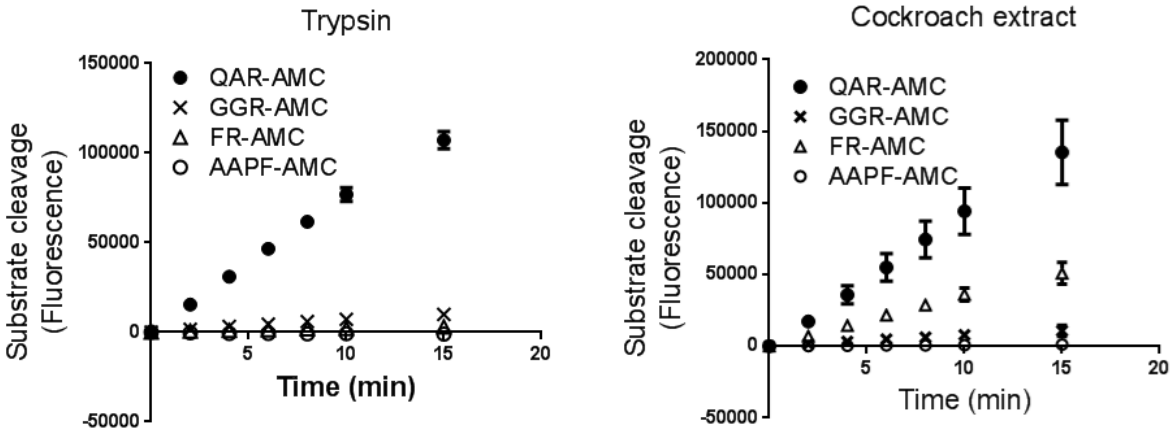


Figure 3.2.4 – Cleavage of fluorogenic peptide substrates.

Porcine trypsin (left) and the cockroach extract (right) were incubated with four fluorogenic peptide substrates with differing enzyme specificities: QAR-AMC (trypsin-specific); GGR-AMC (urokinase); FR-AMC (serine and cysteine cathepsins); and AAPF-AMC (chymotrypsin). The cockroach extract cleaved all four substrates, whereas trypsin only cleaved QAR-AMC and GGR-AMC.

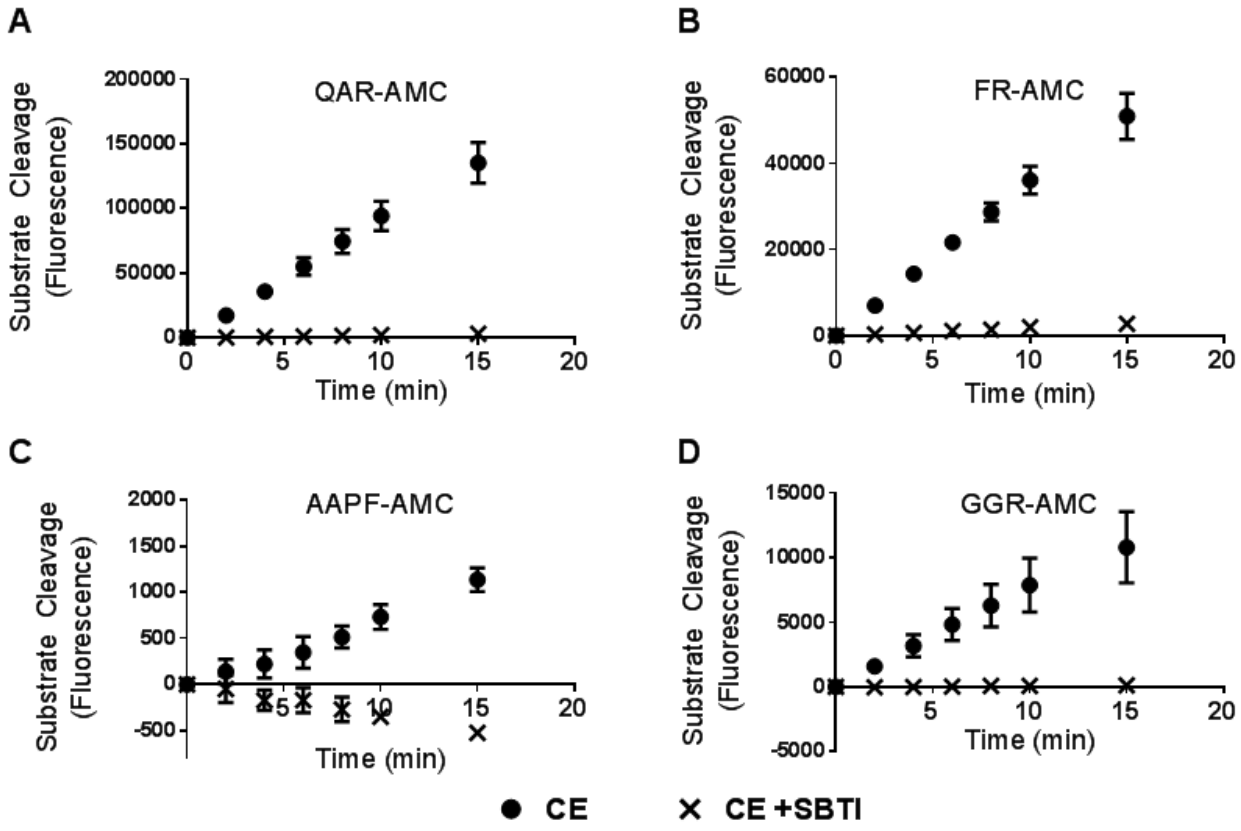


Figure 3.2.5 – All substrate cleavage observed by CE is sensitive to SBTI inhibition

The trypsin-like enzymes in the cockroach extract were neutralized with 10 µM SBTI prior to incubating with the fluorogenic peptides in Figure 3.2.4. Cleavage of all of the substrates (QAR-AMC **(A)**, FR-AMC **(B)**, AAPF-AMC **(C)** and GGR-AMC **(D)**) was completely blocked by SBTI treatment, indicating that the trypsin-like enzymes in the cockroach extract cleave all four substrates.

3.2.4 Cleavage of fluorogenic peptide substrates by the Alternaria allergen

As above with the cockroach allergen, the presence of different types of serine proteinases in the Alternaria allergen preparations was assessed using different peptide substrates. Five substrates were found to be cleaved by the commercial total mould extract: FVR-AMC (thrombin substrate), VPR-AMC (trypsin substrate), AAPA-AMC (pancreatic elastase substrate), as well as AAPF-AMC and FR-AMC. The efficiency with which the enzymes in the total mould extract cleaved these substrates as determined by the slopes of the curves generated was as follows: FVR-AMC had the highest slope (47 pmol substrate cleaved/min), followed by VPR-AMC (18 pmol/min), AAPF-AMC (15 pmol/min), FR-AMC (8 pmol/min) and finally AAPA-AMC (6.5 pmol/min; Figure 3.2.6). In contrast with the in-house produced Alternaria filtrate, only FVR-AMC, VPR-AMC, and FR-AMC generated curves with positive slopes, indicating that neither AAPF-AMC nor AAPA-AMC were cleaved by the enzyme present in the filtrate.

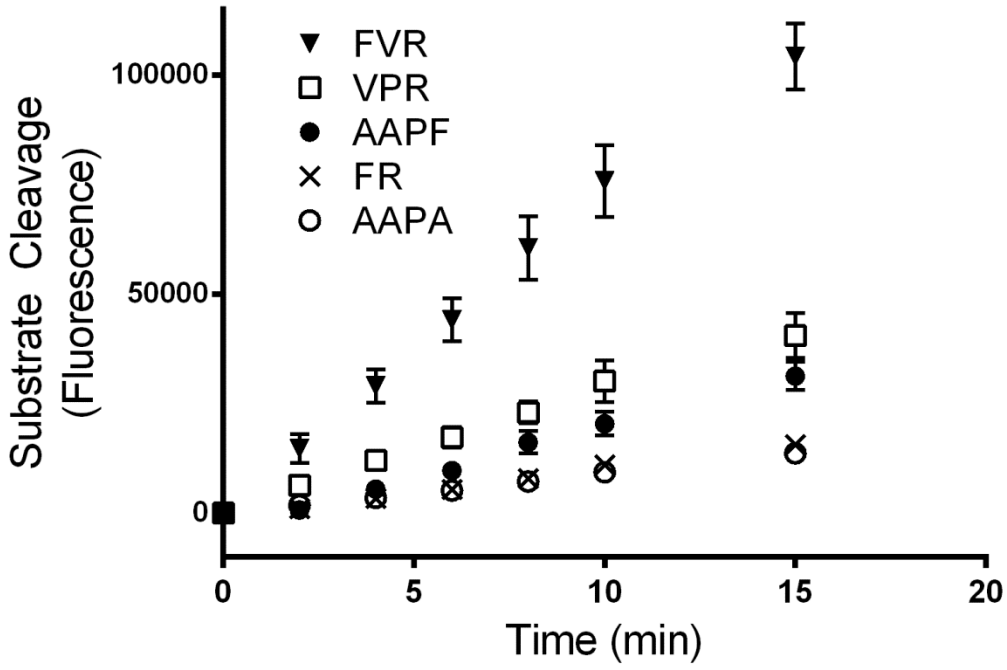


Figure 3.2.6 – Fluorogenic peptide substrate cleavage by the Alternaria-derived enzymes.

The commercial Alternaria total mould extract was incubated with five fluorogenic peptides and fluorescence monitored in a kinetic trace. The enzymes in the extract cleaved each substrate: FVR-AMC (thrombin specific), VPR-AMC (trypsin), AAPF-AMC (chymotrypsin), FR-AMC (serine and cysteine cathepsins) and AAPA-AMC (pancreatic elastase)

To determine whether other, non-trypsin-like enzymes labeled with the chymotrypsin- and elastase-specific probes were responsible in part for the substrate cleavage mediated by the enzymes in the commercial total mould extract, two activity-based probes, the trypsin-specific (ABP-PK) and the chymotrypsin-specific (ABP-F) probes, as well as the soybean trypsin inhibitor, were incubated with the extract prior to the addition of the substrates (Figure 3.2.7 A). Cleavage of the trypsin substrate, VPR-

AMC, was blocked by pre-treatment with both SBTI (~3% activity remaining following inhibitor treatment compared to uninhibited control) and ABP-PK (~15%), but there was no inhibition observed for ABP-F. This result indicated that only the SBTI-sensitive trypsin-like enzyme in the extract that is labeled with ABP-PK cleaves the VPR-AMC substrate. Cleavage of the cathepsin substrate, FR-AMC, was also blocked by ABP-PK (~5%) and SBTI (~2%), but unlike the trypsin substrate was partially blocked by ABP-F (~35%). Cleavage of the chymotrypsin substrate was not blocked effectively by ABP-PK (~91%) or ABP-F (~82%), but interestingly was partially blocked by SBTI (~54%). Finally, cleavage of the elastase substrate, AAPA-AMC, was most effectively blocked by ABP-F (~40%), whereas neither ABP-PK (~95%) nor SBTI (~88%) blocked cleavage of that substrate. These results strengthen the conclusion suggested by the ABP labeling that there are non-trypsin-like enzymes present in the crude total Alternaria extract that are visualized by labeling with ABP-F, which are outside the scope of this thesis but are discussed later. In comparison, the cleavage of the VPR-AMC and FR-AMC substrates by the Alternaria filtrate was completely blocked by SBTI. This result suggests that the *only* active proteinase detected in this filtrate preparation is the single trypsin-like enzyme (Figure 3.2.7 B).

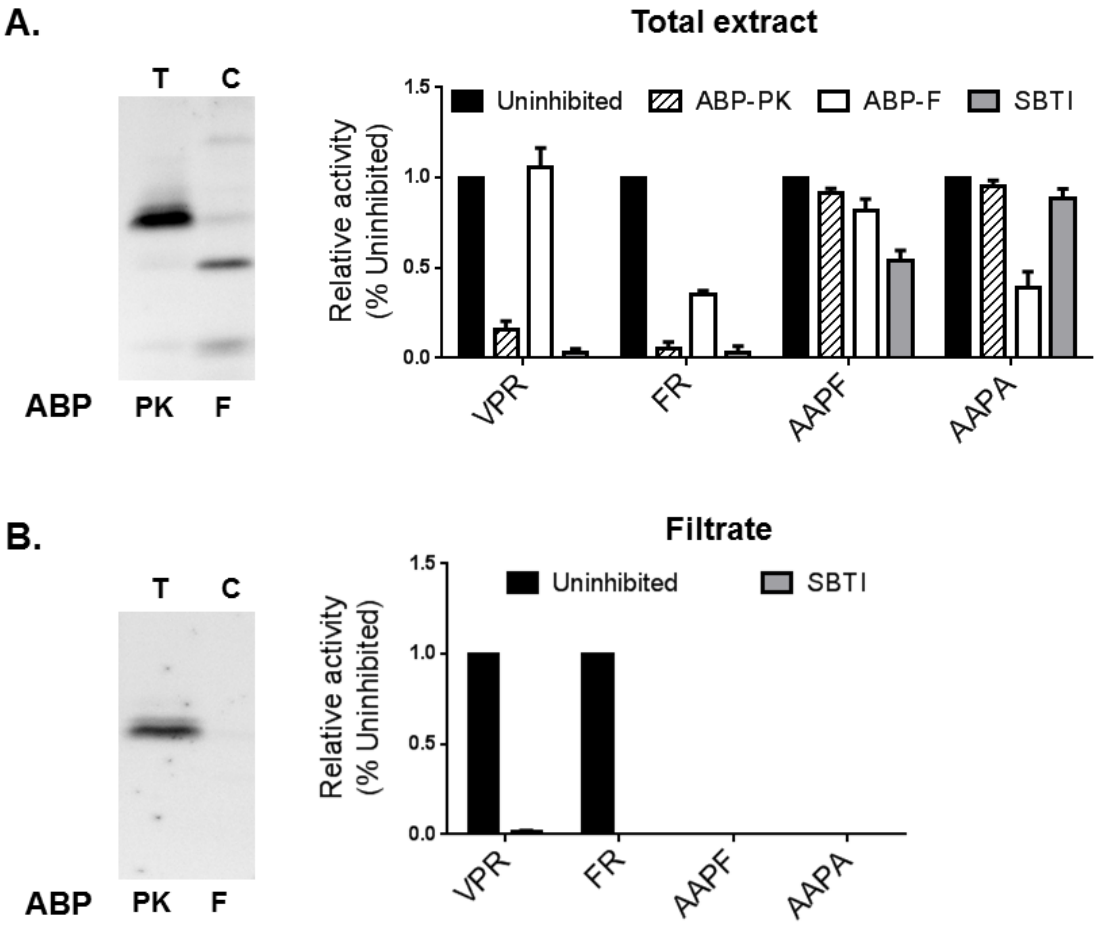


Figure 3.2.7 – Inhibition of Alternaria enzyme-mediated fluorogenic substrate cleavage

To examine the presence of non-trypsin-like enzymes in the total mould extract, the trypsin-specific (ABP-PK) and chymotrypsin specific (ABP-F) activity-based probes (blots on the left), as well as soybean trypsin inhibitor (SBTI), were used as inhibitors in the substrate cleavage assay. **(A)** Cleavage of the VPR (trypsin) and FR (cathepsin) substrates were blocked by both ABP-PK and SBTI, and cleavage of AAPF was partially blocked by SBTI. Cleavage of AAPA was partially blocked by ABP-F, indicating the presence of non-trypsin-like enzymes. **(B)** The Alternaria filtrate, which does not label with ABP-F (left), only cleaves VPR and FR, both of which are completely blocked by SBTI treatment. This result indicates that the filtrate contains only the trypsin-like enzyme.

Peptide	Enzyme specificity	Cockroach extract	Alternaria extract	Alternaria filtrate	Porcine trypsin
QAR-AMC	Trypsin	+	+	+	+
VPR-AMC	Trypsin	+	+	+	+
FVR-AMC	Thrombin	+	+	+	+
FR-AMC	Cathepsins	+	+	+	-
AAPF-AMC	Chymotrypsin	-	+	-	-
AAPA-AMC	P. elastase	-	+	-	-
AAPV-AMC	N. elastase	-	-	-	-

Table 3.2.2 – Summary of substrate cleavage by each allergen preparation

3.3 Activation of PAR2-mediated Ca^{2+} signaling by the allergen extracts

3.3.1 Activation of PAR2 by the cockroach allergen

Having identified potential trypsin-like enzymes in each of the crude allergen extracts, we next aimed to confirm the ability of these crude enzyme preparations to signal via PAR2. Using a KNRK cell line stably transfected with rat PAR2 (the KNRK background cell does not express detectable levels of either PAR1 or PAR2 so each receptor can be expressed individually), the release of internal Ca^{2+} stores following PAR2 activation was monitored using the cell permeant, fluorescent Ca^{2+}-sensitive dye, Fluo-4. Treating the cells with trypsin demonstrates a typical PAR2 response: a brief Ca^{2+} transient followed by a return to baseline (Figure 3.3.1 A). Treatment with the cockroach extract resulted in a brief Ca^{2+} transient, similar to that induced by trypsin, followed by a slow, persistent increase in cytosolic Ca^{2+} levels (Figure 3.3.1 B). To determine whether the trypsin-like enzymes in the cockroach extract were responsible for the observed response, the enzymes were neutralized with the addition of SBTI prior to administering to the cells. Following this treatment, the initial sharp Ca^{2+} transient was eliminated, but the slow persistent increase in cytosolic Ca^{2+} remained (Figure 3.3.1 C). This result indicates that the response induced by the cockroach extract consists of two separate components. To determine whether the initial peak Ca^{2+} signaling response was due to the activation of PAR2 by the cockroach enzymes, the cells were first desensitized by pre-treating with saturating concentrations of trypsin prior to administering the extract. As above, this treatment blocked the first Ca^{2+} response but not the second, indicating that the trypsin-like enzymes in the cockroach extract signal via PAR2 to elicit a Ca^{2+} transient similar to that induced by trypsin (first

peak of Ca^{2+} signaling), and secondarily there is a non-PAR2-mediated increase in cytosolic Ca^{2+} levels induced by a non-specific factor in the extract (Figure 4.3.1 D).

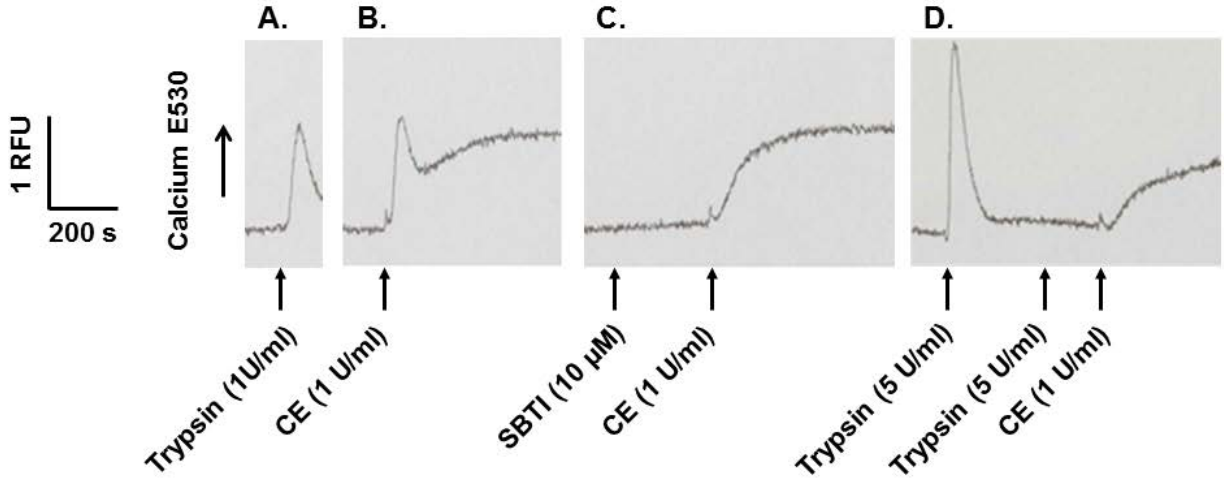


Figure 3.3.1 – Cockroach extract activates Ca^{2+} signaling via PAR2.
(A) A typical PAR2-mediated Ca^{2+} response evoked by 1 U/ml of trypsin. **(B)**The cockroach extract, at a concentration of 1 U/ml, evokes a biphasic Ca^{2+} response, consisting of an initial transient followed by a persistent increase in cytosolic Ca^{2+} levels. **(C)** Neutralizing the trypsin-like activity in the extract with 10 µM SBTI or **(D)** desensitizing the cells with a saturating 5 U/ml concentration of trypsin blocks the first response (indicating that it is PAR2-dependent), but not the second (suggesting that it is non-specific to PAR2).

3.3.2 Activation of PAR2 by the Alternaria allergen

A similar Ca^{2+} signaling assay was performed using the crude Alternaria filtrate. However, due to a limited concentration of trypsin-like activity in this preparation, the assay was performed in cell monolayers in a 96-well microtiter plate, rather than in suspension as above, since smaller volumes of agonists can be used. Treating the cells with the filtrate (0.5 U/ml) resulted in a similar response to that induced by the cockroach extract, consisting of a brief Ca^{2+} transient followed by a slow increase in cytosolic Ca^{2+} levels (Figure 3.3.2, black line). Both pre-treatment of the filtrate with SBTI prior to administering to the cells (Figure 3.3.2, red line) and pre-desensitizing the cells with a saturating concentration of the PAR2 agonist peptide 2fLIGRL (Figure 3.3.2, blue line) resulted in the elimination of the initial Ca^{2+} signal but not the second. This result indicated a similar mechanism as observed in the cockroach extract of an initial, PAR2-mediated Ca^{2+} transient followed by a persistent, non-specific increase in cytosolic Ca^{2+} levels induced by a contaminating factor.

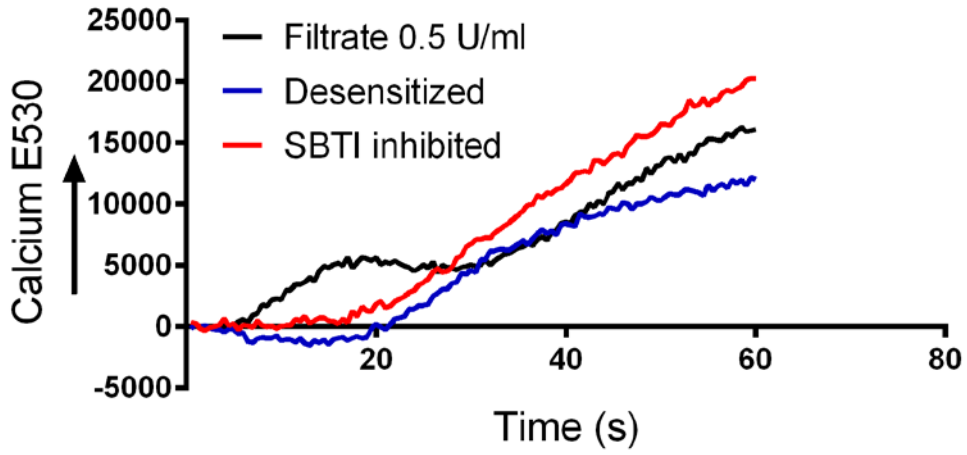


Figure 3.3.2 – Alternaria filtrate activates Ca^{2+} signaling via PAR2.
Alternaria filtrate, at a concentration of 0.5 U/ml, evokes a biphasic Ca^{2+} response, consisting of an initial transient followed by a persistent increase in cytosolic Ca^{2+} levels (black line). Neutralizing the trypsin-like activity in the filtrate with SBTI (red line) or desensitizing the cells with the PAR2 agonist peptide 2fLIGRL (blue line) blocks the first response (indicating that it is PAR2-dependent), but not the second (indicating that it is non-specific to PAR2)

3.4 Discussion

In this Chapter the proteolytic enzymes present in the cockroach and Alternaria allergens were assessed. Three potential trypsin-like enzymes were identified in the cockroach extract, whereas there was no evidence of non-trypsin-like enzymes. ABP labeling and substrate cleavage data revealed that a commercially available total mould extract of the Alternaria allergen has one trypsin-like enzyme and provided evidence for the presence of one or more non-trypsin-like enzymes in the extract. A filtrate of the Alternaria mould culture media, containing factors secreted by the mould but not the mould material itself, was also found to contain a single trypsin-like enzyme with the

same molecular weight as the enzyme labeled in the total mould extract, indicating that of a common enzyme could be present in the two preparations. However, there was no evidence of non-trypsin-like serine proteinases present in the Alternaria culture media filtrate, distinguishing the total mould material from the filtrate. This distinction will be an important consideration for future studies since the non-trypsin-like enzymes could potentially influence the biological response initiated by the Alternaria allergen. Characterizing the non-trypsin-like enzymes in the Alternaria extract was beyond the scope of this thesis, so a purification step was therefore required to separate the trypsin-like enzyme from the other enzymes, which I will discuss in Chapter 4.

These data therefore suggest the presence of multiple serine proteinases in both the cockroach and Alternaria allergens. The cockroach allergen contains three trypsin-like enzymes, and collectively they display broader substrate specificity than porcine trypsin; there were two substrates cleaved by the cockroach extract (FR-AMC and AAPF-AMC) that were not cleaved by porcine trypsin. Of note, this cleavage was sensitive to inhibition by SBTI, indicating that the trypsin-like enzymes are responsible. Likewise, the trypsin-like enzyme in the Alternaria allergen displayed broader substrate specificity to porcine trypsin, although it did not cleave the AAPF-AMC substrate. These indicate that a more broad consideration of possible physiological targets beyond those expected to be targeted by mammalian trypsin-like enzymes may be necessary to fully assess the effects of allergen-derived trypsin-like enzymes in the airway.

Finally, as has been demonstrated in previous of studies, the trypsin-like enzymes in both the cockroach and Alternaria allergens can activate Ca^{2+} signaling via PAR2. Both allergens induced Ca^{2+} transients that were sensitive both to SBTI inhibition

and to PAR2 desensitization of the cells. However, both crude allergens evoked a biphasic Ca^{2+} response comprising an initial brief, PAR2-dependent transient that returns toward baseline before the second persistent increase in the intracellular Ca^{2+} level. Following treatment with the allergen extracts, the cells were no longer responsive, so the ability of the enzymes to disarm PAR1 could not be explored. Furthermore, given the PAR2-unrelated signal induced by the allergen extracts, the activation of MAPK signaling via the PARs could not be specifically assessed with the enzymes in the crude extracts. Again, these findings necessitated a purification step to separate the enzymes in the extracts from the factor(s) responsible for the secondary Ca^{2+} response in order to fully characterize the ability of the enzymes to activate PARs 1 and 2. In the following Chapter, I will discuss the procedures developed to achieve this aim.

CHAPTER 4 CHROMATOGRAPHIC ISOLATION AND PURIFICATION OF THE ALLERGEN-DERIVED PROTEINASES

4.1 Introduction

In the previous Chapter, activity-based probe labeling revealed three trypsin-like enzymes in the cockroach allergen and one in the Alternaria allergen, and the commercially available Alternaria total mould extract was found to contain non-trypsin-like enzymes. Additionally, there were factors in both of the crude allergen extracts that induced a PAR-unrelated, persistent Ca^{2+} signal, preventing a complete characterization of the ability of the allergen enzymes to regulate the PARs. Together, these findings provided the aims of (1) isolating each of the cockroach enzymes in order to characterize and identify them individually, (2) separating the Alternaria trypsin-like enzyme from the non-trypsin-like enzymes, and (3) separating the active trypsin-like enzymes in the allergens from the factors that evoked the secondary, non-PAR2-mediated Ca^{2+} signaling responses. To accomplish these aims, I developed a series of procedures using ion-exchange chromatography, to isolate the active enzymes for use in subsequent experiments, and affinity chromatography to purify the enzymes for mass spectral sequencing.

4.2 Isolation and purification of allergen enzymes with ion-exchange chromatography

4.2.1 Isolation of cockroach enzymes with High S cation-exchange column

Previously I identified three potential trypsin-like enzymes in the cockroach extract and one in the *Alternaria* extract, so my next goal was to describe and identify

each enzyme individually. To assess each cockroach enzyme individually, they first had to be isolated from one another. This isolation was accomplished using ion-exchange chromatography, taking advantage of different isoelectric points for each of the three enzymes. The first purification step involved resolving the crude extract using a High S cation-exchange column at a pH of 5 with a NaCl gradient from 10 mM to 1 M (Figure 4.2.1 A, dashed black line). Each 1 ml fraction collected was analyzed for its proteinase activity (cleavage of the QAR-AMC peptide substrate; Figure 4.2.1 A, black line) and its estimated protein concentration (absorbance at 280 nm; Figure 4.2.1 A, dashed red line). Though most of the protein was found to pass through the column in the low-salt, nonbinding fractions (fractions 4-10), there were two distinct peaks of enzyme activity, one that co-eluted with the majority of protein in the non-binding pass through (fractions 4-8), and one that was eluted in the NaCl gradient at ~250-300 mM NaCl (fractions 19-22).

To identify the enzymes in the eluted activity peaks, the fractions were labeled with the activity-based probe, which revealed that E2 and E3 eluted in the pass-through fractions, with enzyme E3 eluting first in fraction 4, a co-elution of E2 and E3 in fraction 5, and enzyme E2 eluting alone in fractions 6-8. Labeling of the second peak of enzyme activity revealed the presence of enzyme E1 in all of fractions 19-22 (Figure 4.2.1 B, left blot). The total protein in the fractions containing each isolated enzyme was analyzed by SDS/PAGE and gels were stained with SYPRO Ruby for a qualitative estimate of the purity of each enzyme. This staining revealed a partial purification of each enzyme, but with several contaminating proteins (Figure 4.2.1 B, right blot).

This chromatography step was repeated with small aliquots of the crude extract, and fractions containing each isolated enzyme were pooled and concentrated. Since each fraction contained a single isolated enzyme at a sufficient concentration, these fractions were used in subsequent assays to determine the ability of each enzyme to regulate the PARs (Chapter 5). However, since the first chromatography step accomplished only a crude purification of each enzyme, a second chromatography step was performed to purify the enzymes further to allow a more ideal preparation to assess the biochemical properties of each enzyme with enzyme kinetics.

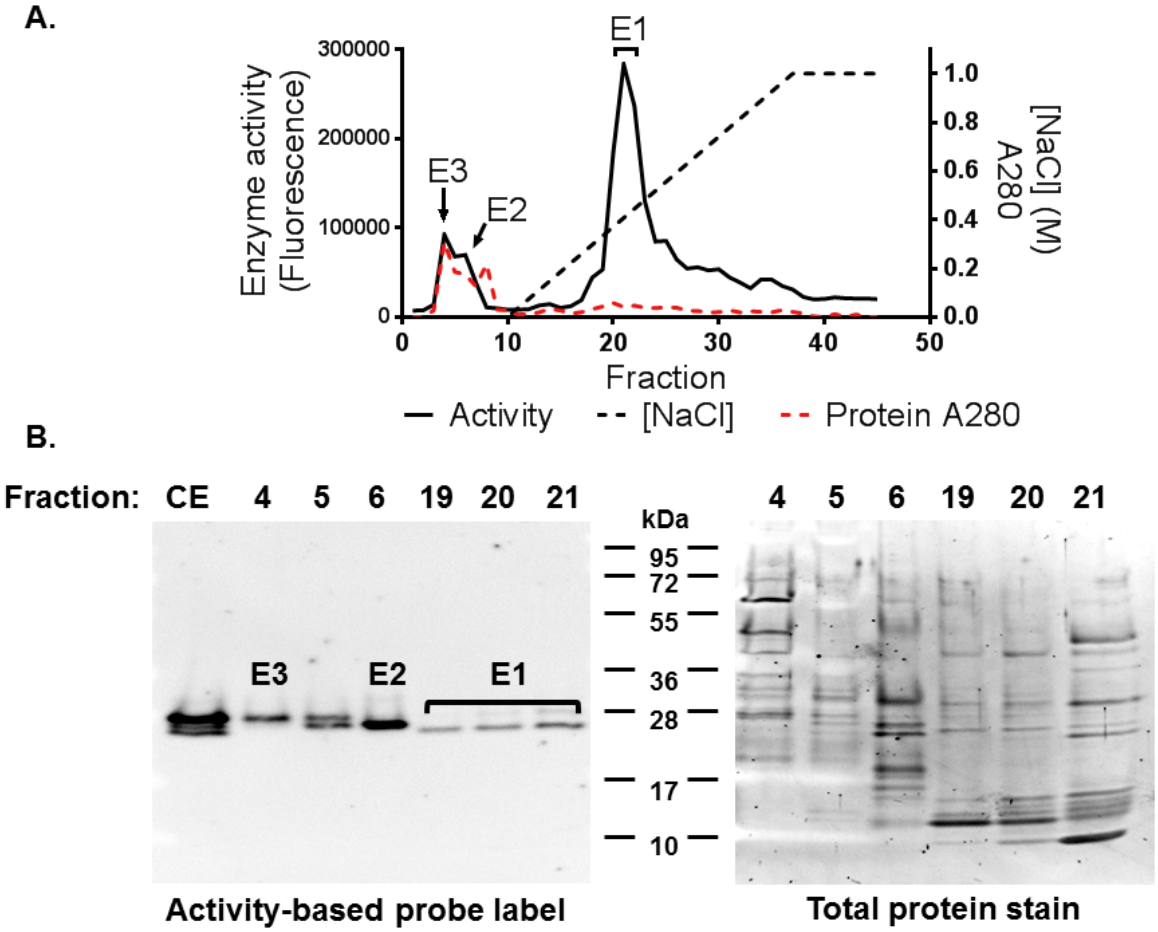


Figure 4.2.1 – Cation-exchange chromatographic isolation of cockroach enzymes. **(A)** The crude cockroach extract was applied to a High S cation-exchange column. Monitoring enzyme activity in the fractions collected reveals two peaks: one in the non-binding flow-through fractions and one in the NaCl elution gradient (dashed black line). **(B)** Labeling the active fractions with the ABP reveals a separation of the three enzymes, with E3 eluting in fraction 4, E2 in fraction 6 and E1 in fractions 19-21 (left blot). A total protein stain of the active fractions reveals several contaminating proteins (right image).

4.2.2 Purification of cockroach enzymes with High Q anion-exchange chromatography

To achieve greater purity for enzyme kinetics experiments, aliquots of the concentrated fractions eluted from the High S column containing each isolated cockroach enzyme were prepared for a secondary chromatography step using the High Q anion-exchange column. It was determined that the ideal pH for purification in this column was 8, so the buffer of each 1 ml fraction volume was replaced by dialyzing against the High Q chromatography starting buffer (10 mM Tris-HCl, pH 8, 10 mM NaCl). Following this dialysis step, each fraction was applied to the column and a NaCl elution gradient was applied similar to the High S gradient (Figure 4.2.2 A,-C, dashed black line in all chromatograms). As above with the High S chromatography, each 1 ml fraction collected was assessed for cleavage of the QAR-AMC substrate (Figure 4.2.2, A-C; black lines). Protein A280 was also measured, but the fractions eluted from the column did not contain sufficient protein concentration to register a positive result, so those data are not presented. For each of the three fractions, there was one major peak of activity. The enzyme activity from the E1-containing fraction did not bind the column and was collected in the low-salt flow through fractions (fractions 4-6; Figure 4.2.2 A), and reacting these active fractions with the ABP labeled one band at the expected molecular weight (Figure 4.2.2 D, right blot). Staining the total protein in these E1-containing fractions with SYPRO Ruby revealed fewer contaminating proteins in the fractions, indicating a higher degree of purity. The active fractions collected from the E2-containing sample were eluted in the NaCl gradient at ~300 mM (fractions 21-23; Figure 4.2.2 B). Labeling these fractions with ABP likewise confirmed the presence of a single band at the expected molecular weight (Figure 4.2.2 E, right blot). The total protein

stain revealed some protein contaminants between 50-70 kDa, so although the fractions are not ideally pure, they appear to be further purified from the High S fractions (Figure 4.2.2 E, left blot). Finally, the activity eluted from the E3-containing fraction was also eluted in the NaCl gradient at ~300 mM, with the peak between fractions 22-24 (Figure 4.2.2 C). The ABP label of these fractions corresponded to a single band at the expected molecular weight (Figure 4.2.2 F, right blot), and the SYPRO Ruby stain confirmed an increased purity of the fractions (Figure 4.2.2 F, left blot). These higher-purity fractions were used for subsequent enzyme kinetics experiments.

Additionally, the elution pattern of each enzyme from the anion- and cation-exchange columns can be used to infer the relative pI values of each enzyme. This information was used as a characteristic to differentiate the enzymes from one another. E1 binds to the negatively charged resin of the cation-exchange column and passes through the positively charged resin of the anion-exchange column, indicating that the enzyme has a net positive charge. By contrast, both E2 and E3 bind to the positively charged anion-exchange column and pass through the negatively charged cation-exchange column, indicating that both of these enzymes may have a net negative charge. These patterns were consistent over the range of pH in the buffers used, from 5-8, indicating that the net charges of each are relatively stable over that range of pH.

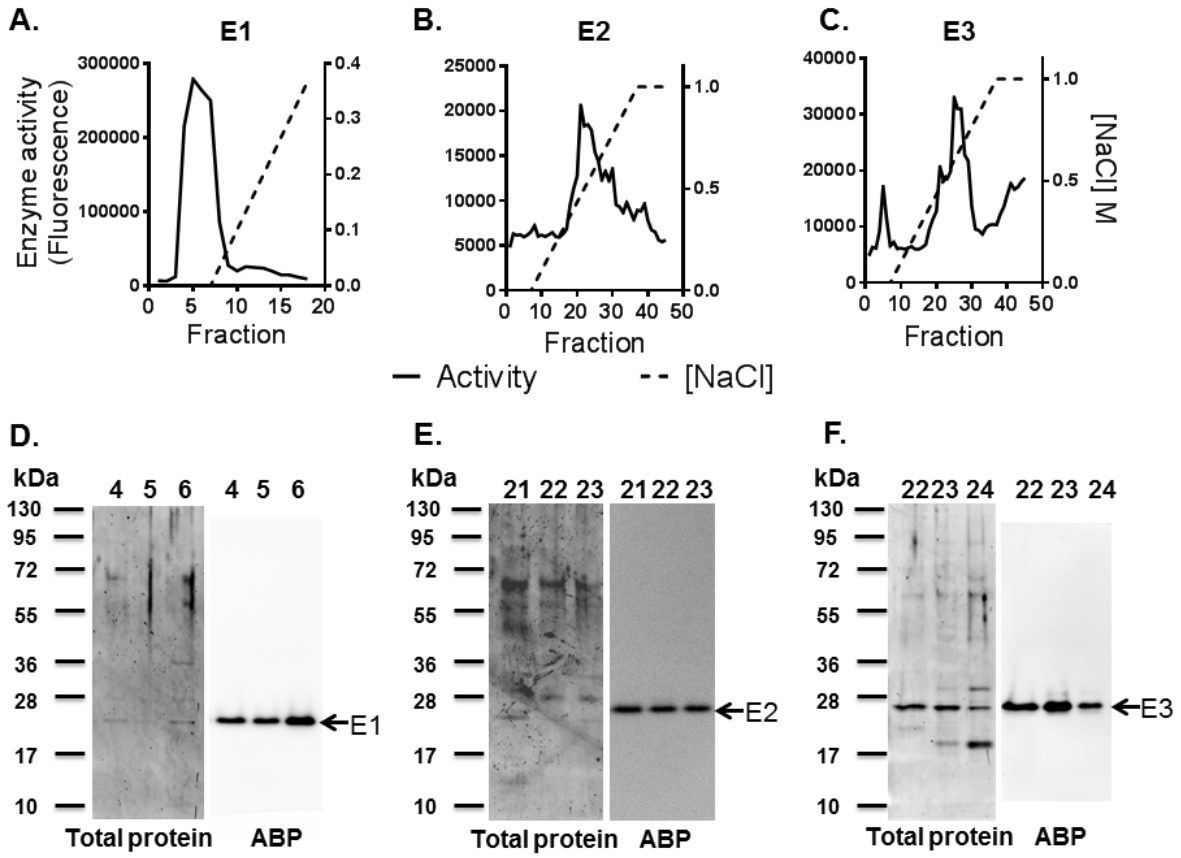


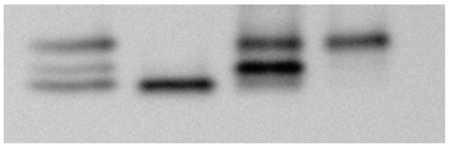
Figure 4.2.2 - Anion-exchange chromatographic purification of isolated cockroach enzymes.

The active fractions from the High S chromatography step (Figure 4.2.1) containing each isolated cockroach enzyme were separately applied to a High Q anion-exchange column for further purification (A-C). Activity-based probe labeling reveals single bands corresponding to each distinct cockroach enzyme (right blots) with reduced contamination by non-specific proteins (left blots; D-F).

4.2.3 DEAE sepharose weak anion-exchange chromatography yields crude, high activity fractions for affinity chromatography

Since ion-exchange chromatography alone did not yield fractions with sufficient enzyme concentration or purity for mass spectral analysis of the enzymes' amino acid sequences, a separate approach involving a crude, high activity isolation of the enzymes followed by an affinity chromatography step was necessary. The initial crude isolation was performed with DEAE weak anion-exchange chromatography. A NaCl step gradient was used to elute bound proteins. Fractions collected with the initial low salt step (50 mM NaCl) contained isolated E1, fractions collected with the intermediate salt step (250 mM NaCl) contained a mixture of E2 and E3, and fractions collected from the high salt step (500 mM NaCl) contained isolated E3 (Figure 4.2.3 A). The activity (U/ml) and specific activity (U/mg protein) of each DEAE sepharose fraction are summarized in Figure 4.2.3 B, revealing high-activity but low-purity fractions. The enzymes in these DEAE sepharose fractions were biotinylated and purified using a streptavidin-conjugated sepharose column in an affinity chromatography step, which is discussed in Section 4.3.

A.



ABP

B.

Fraction	[NaCl] elution (mM)	U/ml	mg protein/ ml	U/mg protein
DEAE 1	**50**	252	3.6	69.9
DEAE 2	**250**	115	13	8.8
DEAE 3	**500**	98	3	32.3

Figure 4.2.3 – Weak anion-exchange chromatographic generation of high-activity fractions.

The crude cockroach extract was applied to a DEAE sepharose weak anion-exchange column. A batch elution method yielded three fractions (DEAE 1-3). **(A)** Labeling of the three fractions revealed isolated E1 in DEAE 1, and isolated E3 in DEAE 3. E2 co-eluted with E3 in DEAE 2. **(B)** A summary of the specific activities in each of the DEAE sepharose fractions. Each fraction has high trypsin-like activity (U/ml), but contains substantial non-specific protein contaminants.

4.2.4 Purification of Alternaria enzyme with High Q anion-exchange chromatography

Since labeling of the *Alternaria* extract with the trypsin-specific ABP revealed only one major band, chromatography was not required to isolate distinct trypsin-like enzymes as with the cockroach allergen. However, as with the cockroach enzymes, a higher degree of enzyme purity was desired for optimal determination of the enzyme's biochemical properties and enzyme kinetics. Furthermore, separation of the active enzyme from the major immunogenic allergen protein in *Alternaria* (*Alt a 1*) was desired for *in vivo* experiments planned by our collaborators at the University of Arizona. To

those ends, the crude Alternaria extract was resolved chromatographically using the strong anion-exchange High Q column, using a similar NaCl gradient elution protocol as with the cockroach enzymes (Figure 4.2.4 A, dashed black line). Measuring proteinase activity in the collected fractions reveals that all of the detectable enzyme activity is present in the non-binding, pass-through fractions (fractions 3-5, with the majority of activity in fraction 4; Figure 4.2.4 A, black line). Protein A280 measurements show a diffuse elution of total protein off the column (Figure 4.2.4 A, dashed red line). ABP labeling of fraction 4 with the trypsin-specific ABP probe confirmed the expected presence of the single trypsin-like enzyme, which had been separated from the non-trypsin-like enzyme labeled with the chymotrypsin-specific probe (Figure 4.2.4 B, left blot). Staining the total protein of the fraction with SYPRO Ruby shows a small number of contaminating proteins, with a major visible band that aligns with the ABP-labeled band and could represent the enzyme (Figure 4.2.4 B, right blot). This more pure fraction was subsequently used in enzyme kinetics and PAR activation assays.

We then aimed to determine if this chromatographic approach to purifying the trypsin-like enzyme in Alternaria could separate the enzyme from the major immunogenic antigen protein in Alternaria*, Alt a 1.* To this end an ELISA method was used to assess each fraction for its concentration of *Alt a 1* allergen following an adaptation of the above High Q chromatographic protocol using a batch elution method (Figure 4.2.5, dashed black line). As above, the enzymatic activity was collected in the pass-through fractions (Figure 4.2.5, black line). The *Alt a 1* ELISA signal was contained exclusively in the NaCl elution gradient (fraction 13-16; Figure 4.2.5 dashed red line),

completely separate from the enzyme. These enzyme-containing fractions purified from *Alt a 1* were then used in *in vivo* experiments by our collaborators at the University of Arizona.

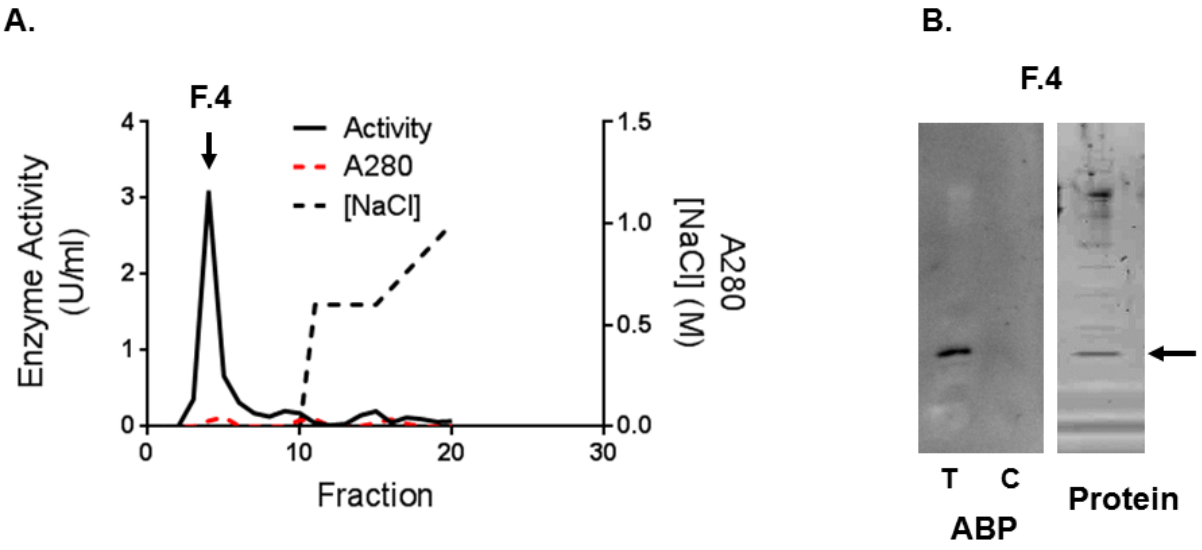


Figure 4.2.4 – Anion-exchange chromatographic purification of the Alternaria enzyme.

(A) Tracking enzyme activity (black line, left Y axis) in the fractions collected from the column reveal a single peak of activity in the low-salt pass through fractions 3-5, with fraction 4 (F.4) containing the majority of the enzyme activity. **(B)** The single trypsin-like enzyme, and none of the non-trypsin-like bands, label with the activity based probes in fraction 4 (left blot). A total protein stain reveals some contaminating proteins with a major band that aligns with the ABP-labeled enzyme, identified with an arrow (right blot).

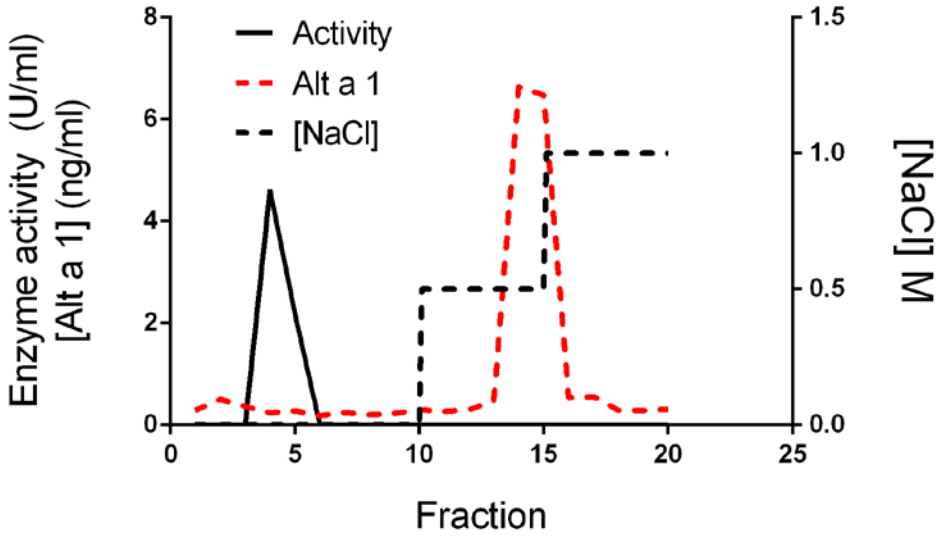


Figure 4.2.5 – Separation of the Alternaria enzyme from the *Alt a 1* allergen protein.

The High Q column presented in Figure 4.2.2 was repeated and the fractions assessed for the concentration of the *Alt a 1* allergen protein using an ELISA kit. The enzyme activity was detected in a single peak in the flow through fractions (black line), whereas the *Alt a 1* ELISA signal (dashed red line) was detected in a separate peak in the NaCl elution (dashed black line).

4.2.5 Partial purification of the Alternaria enzyme with DEAE sepharose weak anion-exchange chromatography

The strong ion-exchange chromatography step described in the previous Section yielded a highly pure fraction, but the enzyme concentration was not sufficient for the streptavidin affinity chromatography step described in the next Section. As with the cockroach extract, the purification method that yielded the most concentrated enzyme activity was a crude purification step using a DEAE sepharose column. The commercially available Greer total mould extract was used as a source of the enzyme

and applied to the column. Bound proteins were eluted using a batch NaCl elution gradient method similar to that used for the cockroach extract (Figure 4.2.6 A, dashed black line). Monitoring the trypsin-like activity passing through or eluting off the column revealed that the majority of enzyme activity is collected in the non-binding, low salt fractions 4-10 (Figure 4.2.6 A, black line). Measuring protein A280 shows that all fractions collected contain a roughly consistent concentration of proteins (Figure 4.2.6 A, dashed red line). The active fractions were combined and concentrated, and labeling of this concentrated active fraction with the trypsin-specific ABP confirms the presence of the single trypsin-like enzyme labeled in the crude extract. The non-trypsin-like enzymes identified by labeling with the chymotrypsin-specific ABP were also found to be present in this fraction (Figure 4.2.6 B, right blot). A SYPRO Ruby total protein stain of this concentrated fraction shows an incomplete purification of the enzyme with several contaminating proteins, although in comparison to the crude extract an increase in purity was observed (Figure 4.2.6 B, left blot). Fractions obtained by repeating this purification step contained the enzyme at a sufficient concentration (20-40 U/ml) for subsequent use in examining the ability of the Alternaria enzyme to signal via PARs 1 and 2 in whole tissue preparations discussed in Chapter 7.

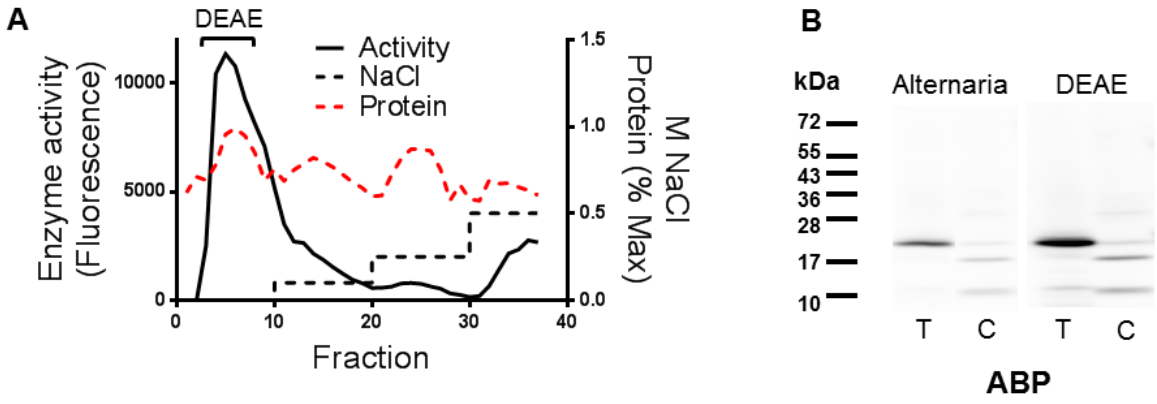


Figure 4.2.6 – Weak anion-exchange chromatographic generation of high-activity Alternaria fractions.

(A) The Alternaria enzyme was crudely purified using DEAE sepharose chromatography. A step gradient of NaCl was used to elute bound proteins (dashed black line). One peak of enzyme activity (black line) was observed in the flow-through fractions (DEAE), while a consistent protein concentration was observed in all fractions collected (dashed red line). **(B)** ABP labeling of the active fractions (DEAE) with the trypsin (T) and chymotrypsin (C)-specific probes (right blot) revealed a similar staining pattern to the total Alternaria extract (left blot).

4.3 Affinity chromatographic purification of each allergen-derived proteinase

To achieve sufficient purity of the allergen enzymes for sequencing, an affinity chromatography method was developed using ABP labeling to biotinylate the active enzymes in the DEAE sepharose fractions, and purifying the ABP-biotinylated enzymes with a streptavidin-conjugated magnetic sepharose column. For a more robust mass spectral analysis, an estimated mass of 1 µg of each enzyme, E1, E2, E3 and Alt (isolated from both the commercial total mould extract and from the in-house produced culture media filtrate) was collected for sequencing. To estimate the mass of of the the enzymes, the specific activity of porcine trypsin (16 000 U/mg) was used as a

benchmark: for each enzyme 1 µg protein/16 U of activity was assumed and volumes of each high-activity fraction containing 16 U of total trypsin-like activity were reacted with the ABP and then applied to the streptavidin column. SYPRO Ruby total protein stains illustrate the process (Figure 4.3.1): The left-most lane for each enzyme represents the starting material; the next lane to the right represents the non-binding fraction collected prior to washing the enzyme-bound column. Finally, the right-most lane for each enzyme represents the bound proteins that were eluted by boiling the column in 2% SDS to break the biotin-avidin bond. The elution reveals a high degree of purity, with prominently visible bands in the expected molecular weight range of each enzyme, with varying degrees of contamination at higher molecular weights. Each visible band corresponding to the expected molecular weight of the enzymes was excised from the gel and sent for chymotryptic digestion and mass spectral analysis by the proteomics facility at the University of Arizona. The images of the stained gels presented in Figure 4.3.1 are representative of the experimental procedure. To avoid contamination of the samples the procedure was performed in a fume hood and thus the gels from which the enzymes were excised were not used for imaging. Additionally, the elution products were not resolved in gels containing the crude fractions, and were stained with the colourimetric Coomassie blue protein stain rather than the fluorescent SYPRO ruby stain presented in the Figure.

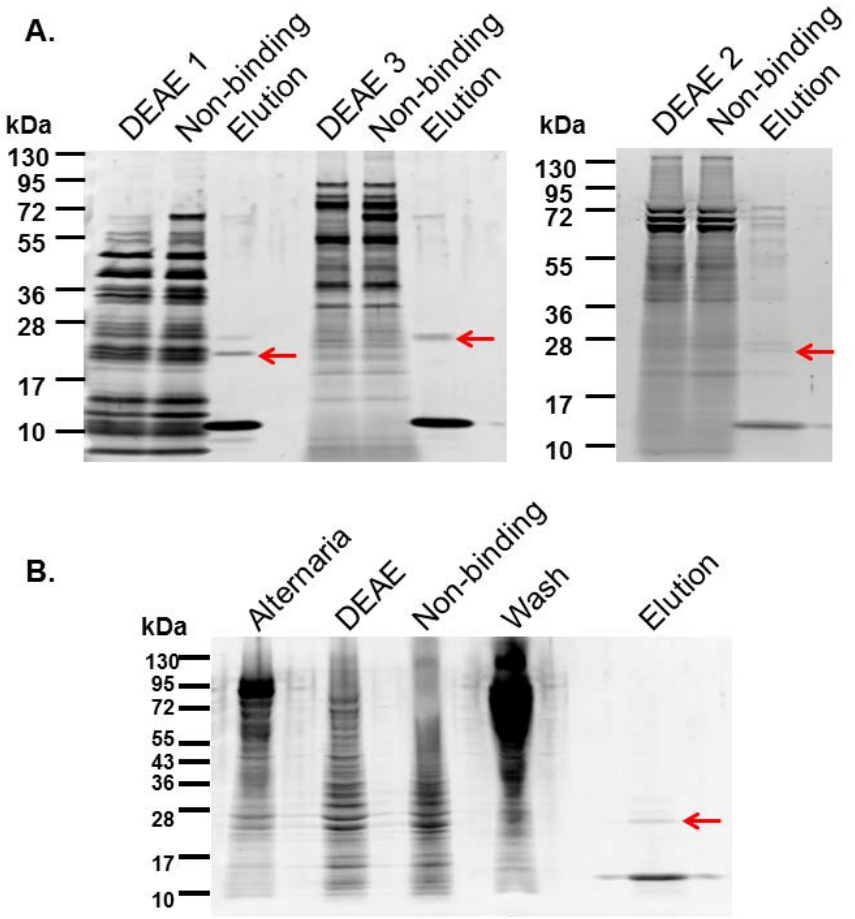


Figure 4.3.1 – Affiinity chromatographic purification of the allergen-derived enzyme.

The ABP-labeled trypsin like enzyme in the active fractions collected from the DEAE sepharose chromatography step were purified using a streptavidin affinity column. **(A)** A total protein SYPRO ruby stain reveals that the proteins present in each fraction containing the isolated cockroach enzymes (DEAE 1-3), the non-binding supernatant fractions from the streptavidin column (Non-binding), and he product eluted by boiling the washed column in 2% SDS (Elution). **(B)** A similar total protein stain reveals the proteins present in the total Alternaria extract (Alternaria), the active DEAE sepharose fraction (DEAE), the non-binding supernatant from the streptavidin column, a wash fraction in 2 M urea, and the product eluted (elution). The visible bands in the molecular weight range of the allergen trypsin-like enzymes that were excised for sequencing are indicated by red arrows.

4.4 Discussion

Having quantified and characterized the trypsin-like enzymes in both the crude cockroach and Alternaria allergen extracts in Chapter 3, the allergen-derived enzymes were isolated and purified as described in this Chapter. The challenge in purifying the enzymes contained in the cockroach extract was to separate the three closely-related enzymes visualized by the ABP labeling. An affinity chromatography-like approach using an immobilized trypsin inhibitor was considered. However this approach was not able to separate the three bands into distinct fractions, and the yield of proteinase activity eluted off the column was too low for use in subsequent experiments. Another group had successfully applied such an approach with a cockroach frass extract[84]. However, using different columns and conditions, we developed an ion-exchange chromatography protocol that successfully isolated each of the three cockroach enzymes in individual high-activity fractions, and subsequently purified each to a high degree. There was only one trypsin-like enzyme in the Alternaria allergen, so an initial isolation step was not necessary prior to partially purifying the enzyme.

The three approaches that we developed to isolate and purify the allergen-derived enzymes yielded fractions of varying enzyme concentration and purity, which were each best suited for the different assays to be described in subsequent chapters. For the cockroach extract, the High S column yielded fractions containing only one of each of the three enzymes with relatively high concentrations, which made these fractions suitable to assess the activation of the PARs as described in Chapter 6. However there were a number of contaminating proteins visible with a total protein stain, and greater enzyme purity is desirable for the determination of the biochemical properties with

enzyme kinetics since non-specific proteins could theoretically interact with the enzymes or inhibitors in those assays. Therefore, a second purification step was performed. The three High S fractions containing each individual cockroach enzyme were applied to a High Q column for this further purification, and the fractions collected contained fewer contaminating proteins with sufficient enzyme activity to perform the enzyme kinetics assays presented in Chapter 5. Furthermore, that the cockroach enzymes differentially bind to the different ion-exchange columns (E1 binds to the cation-exchange column while E2 and E3 do not) indicated that they carry different relative charges in solution. This result is the first piece of evidence that the enzymes are biochemically distinct from one another, which I will discuss in detail in Chapter 5.

For the Alternaria allergen, only one trypsin-like enzyme was present in both preparations so a High Q chromatographic purification of the enzyme was the first step. This step yielded a fraction of a high degree of purity, with few contaminating proteins visible in the total protein stain. The trypsin-like enzyme had also been separated from the non-trypsin-like enzyme(s) in the crude extract, so this fraction was used for both the enzyme kinetics (Chapter 5) and the PAR signaling (Chapter 6) assays. However, the continued presence of contaminating proteins in all of the fractions containing isolated enzymes from both allergens collected from the High Q columns precluded these fractions from being used for mass spectral analysis. Therefore, a final purification step was required.

For this third purification step, we developed a novel method to purify serine proteinases to a high degree using activity-based probe-mediated affinity chromatography. This method consists of an initial crude isolation of the enzymes using

a DEAE sepharose column from which the fractions collected were biotinylated with the activity-based probe and purified further using a streptavidin column in an affinity purification step. This method was highly effective in purifying large amounts of each enzyme with minimal protein contamination, and so would be applicable to identify the proteinases in other allergens, and in other crude proteinase-containing mixtures. The enzymes purified in this manner were not active since the serine residues in the enzymes' active sites are irreversibly alkylated by the probe. However, the proteins eluted from this column were ideal for mass spectral sequencing when resolved by SDS/PAGE. I will discuss the mass spectral analysis of the enzymes in Chapter 5.

In summary, the cockroach allergen contains three trypsin-like enzymes and the Alternaria allergen contains one trypsin-like enzyme. We developed three approaches to isolate and purify the enzymes, so each enzyme was collected in three separate fractions with varying enzyme concentration and purity. These fractions were then used to assess the biochemical properties of the enzymes, as well as their abilities to regulate signaling via the PARs, which I will discuss in the following chapters.

CHAPTER 5 BIOCHEMICAL CHARACTERIZATION OF THE ISOLATED ALLERGEN-DERIVED PROTEINASES

5.1 Introduction

In the previous Chapter, three approaches were used to isolate and purify the trypsin-like enzymes contained in the cockroach and Alternaria allergens. To this point, only one of the four trypsin-like proteinases that I have identified in the previous chapters had been characterized or sequenced; Ock et al[80] identified a trypsin-encoding sequence through analysis of an expressed-sequence tag (EST) library generated from a German cockroach cDNA library, which they then cloned and expressed. Since I identified three potential trypsin-like enzymes in the cockroach allergen, it was important to analyze the sequences of each to determine whether they represent distinct enzymes or variant forms of the cloned trypsin-like enzyme from the EST library. Likewise, the Alternaria-derived enzyme had not been identified and sequenced, and it was not known whether different preparations of Alternaria allergen (total mould extracts vs. culture media filtrates) contained different trypsin-like proteinases. It was thus an important distinction to make to put into context experimental results using the different Alternaria preparations.

To achieve this aim, I characterized the biochemical properties of each allergen-derived enzyme with enzyme kinetics assays, and identified the primary sequences of each enzyme with mass spectral analysis. Each purification approach in the previous Chapter yielded fractions containing each enzyme with varying levels of activity and purity, so the fractions collected in each were appropriate for use in different assays; the High Q fractions contained the enzymes at the highest level of purity, and so were used

to characterize the biochemical properties of the enzymes. However, the High Q fractions contained some contaminating proteins and thus were not appropriate for mass spectral sequencing. The inactive, ABP-bound enzymes collected from the affinity chromatography step contained sufficient amounts of the enzymes to cut an isolated gel band to send for sequencing. Finally the High S fractions had sufficient enzyme activity to assess the regulation of PAR signaling, which I will discuss in Chapter 6.

5.2 Assessment of the biochemical properties of each enzyme with enzyme kinetics

5.2.1 Determination of Km values for each isolated enzyme

Having separated and partially purified each allergen-derived enzyme, enzyme kinetics assays were performed to characterize each enzyme biochemically. The aims were: (1) to assess the biochemical similarity between trypsin-like enzymes from the diverse allergen sources, which could be important in targeting allergen-derived trypsins therapeutically, and (2) to assess biochemical differences among the three enzymes in the cockroach allergen, which would be evidence that the three ABP-labeled bands in that allergen represent three distinct enzymes. First, the affinities of each enzyme to different fluorogenic peptide substrates were measured as the Michaelis constant (Km) value, representing the substrate concentration at which enzyme activity is half-maximal. The substrates chosen all contained arginine (R) residues in the cleavage site (P1 position), but two different amino acids in the two immediate residues N-terminal to the P1 position (P2 and P3 positions). The High Q fractions (collection described in Sections 4.2.2 and 4.2.4) containing each individual enzyme were used with equivalent

concentrations of trypsin-like activity determined with the QAR-AMC substrate (0.2 U/ml; 10 mU total activity/50 µl reaction volume). Each of the three cockroach enzymes had similar Km values for QAR-AMC, within 1 µM of substrate concentration (E1 = 3 µM; E2 = 2 µM; E3 = 3 µM), whereas Alt had a lower affinity for this substrate (16 µM). By contrast, there was substantial variation of Km values between the four enzymes for the FVR-AMC substrate (E1 = 81 µM, E2 = 19 µM, E3 = 11 µM, Alt =366 µM), and the GGR-AMC substrate (E1 = 60 µM, E2 = 99 µM, E3 = 28 µM, Alt = 98 µM). For reference, each enzyme also demonstrated different kinetic profiles from porcine trypsin (Km: QAR-AMC = 5 µM; FVR-AMC = 24 µM; GGR-AMC = 1.0 mM) (summarized in Table 5.2.1; kinetic curves presented in Appendix A).

	Substrate Km (µM)			Inhibitor Ki	
	QAR	FVR	GGR	SBTI	TLCK
	Trypsin	Thrombin	Urokinase	(pM)	(nM)
E1	3 ± 0.8	81 ± 15	60 ± 12	4 ± 0.2	277 ± 28
E2	2 ± 0.3	19 ± 3	99 ± 7	8 ± 1.4	3 ± 0.2
E3	3 ± 0.3	11 ± 2	29 ± 4	3 ± 0.7	8 ± 1.7
Alt	16 ± 2	366 ± 57	98 ± 31	18 ± 4	12879 ± 842
Trypsin	5 ± 0.6	24 ± 5	1054 ± 211	4 ± 0.4	2 ± 0.2

Table 5.2.1 – Allergen enzymes have distinct biochemical identities
Substrates: Glutamine-alanine-arginine (QAR)-aminomethylcoumarin (AMC), phenylalanine-valine-arginine (FVR)-AMC, and glycine-glycine-arginine (GGR)-AMC; 25°C, pH 8 : Inhibitors: Soybean trypsin inhibitor (SBTI) and Nα-tosyl L-lysine chloromethylketone (TLCK); 25°C, pH 8. Km and Ki values are presented ± the standard error values calculated by the Graphpad Prism software (n=3). Kinetic curves for each value are presented in Appendix A.

5.2.2 Determination of Ki values for two trypsin inhibitors

In addition to determining the relative affinities of each enzyme for different peptide substrates, the relative sensitivities of each enzyme to different trypsin inhibitors were explored. Curves representing fractional activity over inhibitor concentration were generated and the inhibition constant (Ki) for each enzyme calculated by relating the observed inhibitor concentration at which enzyme activity is half of the uninhibited condition (IC50) to the Km and substrate concentration in the experimental setup (described in the methods Chapter). Beginning with the soybean trypsin inhibitor (SBTI), similar sensitivities were observed for the cockroach enzymes, within a four-fold range of inhibitor concentration, whereas the Alternaria enzyme demonstrated less sensitivity (E1 = 3 pM, E2 = 3 pM; E3 = 8 pM. Alt = 18 pM). Sensitivities to the other inhibitor that was used, N-α-tosyl-L-lysine chloromethylketone (TLCK) were observed to vary more substantially. While both E2 (3 nM) and E3 (8 nM) were within a similar range, E1 (278 nM) was substantially less sensitive to this inhibitor, and Alt the least sensitive (1.5 µM). Again, for reference trypsin was highly sensitive to both of the inhibitors used (Ki: SBTI = 4 pM; TLCK = 2 nM) (summarized in table 4.2.1; kinetic curves presented in Appendix A).

5.3 Mass spectral sequencing of the allergen-derived enzymes

5.3.1 Mass spectral sequence analysis

The affinity chromatography procedure described in Chapter 4 yielded purified enzymes in high abundance. The enzymes were resolved with SDS/PAGE and stained with Coomassie Blue total protein stain (procedure presented in Figure 4.3.1, p. 106).

Bands visible to the naked eye in the molecular weight range of the ABP-labeled enzymes (20-27 kDa) were excised and sent for mass spectral analysis. Since the genomes of neither the German cockroach nor the Alternaria mould have been sequenced, EST libraries derived from species-specific cDNA libraries generated in-house by the University of Arizona (Alternaria*)* and provided to us by a Korean group led by Dr. Mee-Sun Ock (cockroach) were used. Comparisons to the EST sequences were used to identify the mass spectra of peptides generated from the excised gel bands to establish the sequences of each enzyme.

The Alternaria enzymes isolated from both the commercially available total mould extract and the University of Arizona mould culture media filtrate were found to match a common EST sequence with homology to trypsin-like enzymes with 50% (filtrate) -71% (extract) coverage (Figure 5.3.1). In both cases, the mass spectra generated matched with 100% probability to this EST sequence, indicating that the EST represents the sequence of the enzyme isolated form both of the Alternaria preparations. These results confirm that there is one common trypsin-like enzyme in both the total mould extract and the filtered culture media.

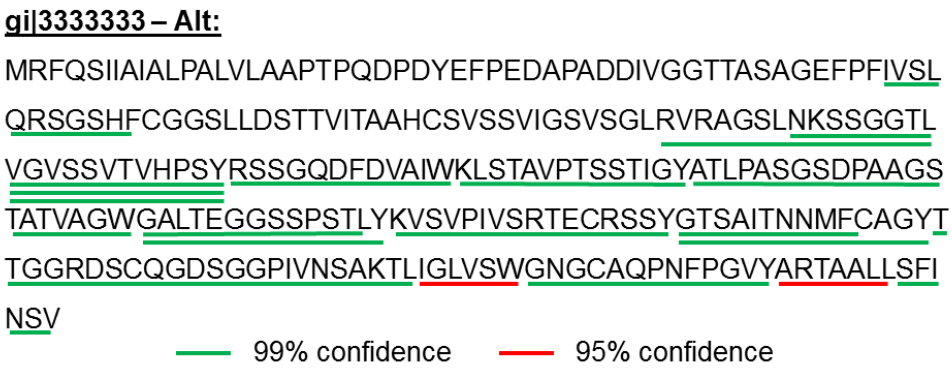


Figure 5.3.1 – Mass spectral sequencing of the Alternaria enzyme.
Mass spectra generated from the purified Alternaria enzyme were compared to an EST library generated from Alternaria cDNA, matching to a sequence with 100% probability. The unique peptides identified are presented as lines under the translated EST sequence. Green lines indicate a >99% confidence interval and red lines a >95% confidence interval.

For the cockroach allergen, the EST library was annotated so that each sequence, when theoretically transcribed, was characterized by homology to known proteins. This annotated EST library was searched for sequences that were described to have homology to trypsin-like enzymes or to serine proteinases generally. The search yielded three distinct sequences, identified as (1) Cluster (CL)8, Contig1, (2) CL1Contig9, and (3) CL53Contig1, with variable homologies to the cloned TRYP1 sequence cloned by Ock et al[80] (sequence alignment in Figure 5.3.2).

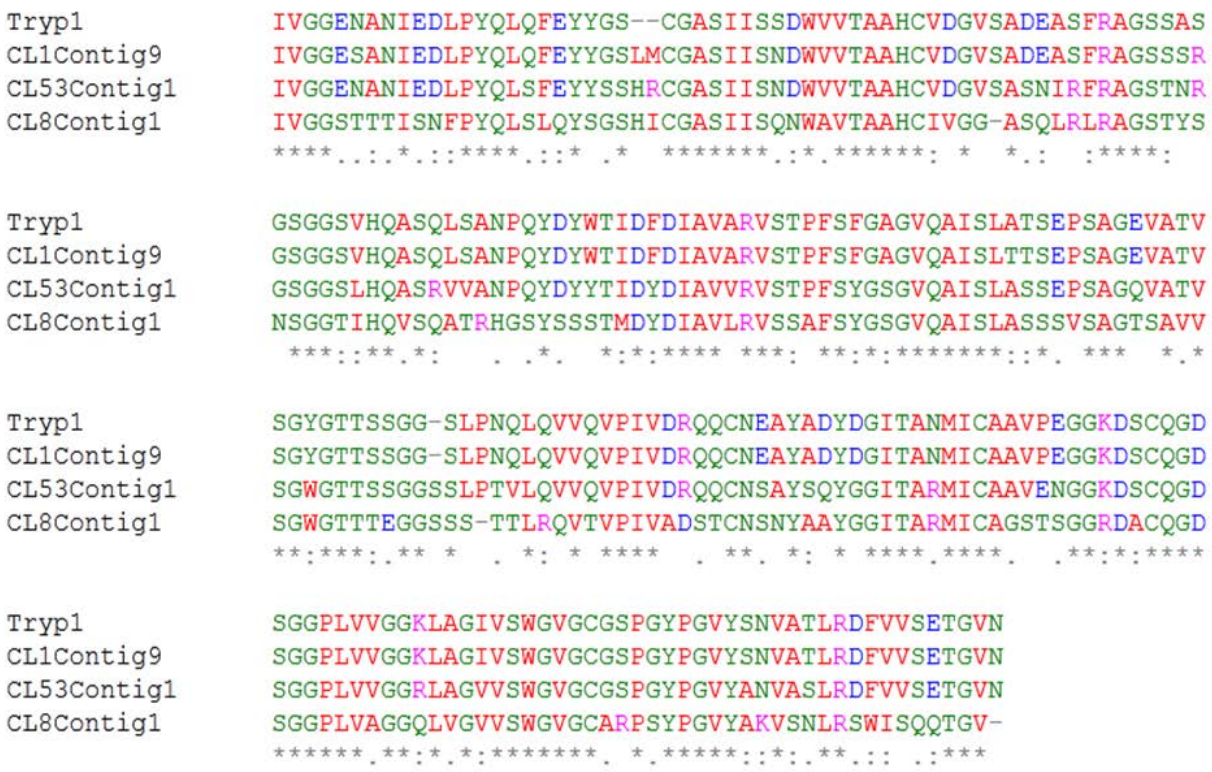


Figure 5.3.2 – Sequence alignment of three trypsin-homologous cockroach EST sequences and the cloned cockroach trypsin Tryp1.

Residues common to the four sequences are indicated by stars below the sequences. Amino acids are colour-coded based on the characteristics of their side chains as follows: red = non-polar; blue = negatively charged; pink = positively charged; green = polar

Comparing the mass spectra generated from the chymotryptic digest-generated peptide fragments to the three EST sequences identified in the library demonstrated that each enzyme best matched to distinct sequences (E1 = CL8Contig1; E2 = CL53Contig1; and E3 = CL1Contig9; Figure 5.3.3). The E3-containing sample matched only one EST sequence, as well as the TRYP1 sequence, indicating that E3 is the enzyme that was previously cloned. However, there was overlap among the samples

containing E1 and E2; unique peptides matching each of the three EST sequences were identified in both samples (summarized in Table 5.3.1). This overlap suggests possible contamination of each enzyme in the gel bands, likely due to the crude initial purification step, as can be observed in the ABP-labeled blot in Chapter 4 (Figure 4.2.3 A), or to contamination during the SDS/PAGE procedure as E1 and E2 were excised from the same gel, and E3 from a separate gel. For the E2-containing sample in particular (DEAE 2), this effect was expected due to the co-elution of both E2 and E3 from the DEAE sepharose column. Nevertheless, a quantitative analysis performed using the Scaffold 4 software, which can estimate the relative abundance of unique peptide fragments specific to each EST sequence detected for each sample, suggested that the greatest number of mass spectra in each excised gel band matched distinct EST sequences (Figure 5.3.4), further suggesting that the three ABP-labeled bands in the cockroach allergen represent unique enzymes encoded by distinct genes.

CL1Contig8 – E1:

MFLLLALCALVASGSALPPIRSLKPQLDGRIVGGSTTTISNFPYQLSLQYSGSHI
CGASIISQNWAVTAAHCIVGGASQLRLRAGSTYSNSGGTIHQVSQATRHGSYS
SSTMDYDIAVLRVSSAFSYGSGVQAISLASSSVSAGTSAVVSGWGTTTEGGS
SSTTLRQVTVPIVADSTCNSNYAAYGGITARMICAGSTSGGRDACQGDSGGPL
VAGGQLVGVVSWGVGCARPSYPGVYAKVSNLRSWISQQTGV

CL53Contig1 – E2:

KNLKMLPLVLASLLIVGCLAGTRLVRPRPRLDGRIVGGENANIEDLPYQLSFEY
YSSHRCGASIISNDWVVTAAHCVDGVSASNIRFRAGSTNRGSGGSLHQASRV
VANPQYDYYTIDYDIAVVRVSTPFSYGSGVQAISLASSEPSAGQVATVSGWGT
TSSGGSSLPTVLQVVQVPIVDRQQCNSAYSQYGGITARMICAAVENGGKDSC
QGDSGGPLVVGGRLAGVVSWGVGCGSPGYPGVYANVASLRDFVVSETGVN

CL9Contig1 (Cockroach TRYP1) – E3:

MFRLVVIATLLVASCLGAAPRGRPRPRMHGRIVGGESANIEDLPYQLQFEYYG
SLMCGASIISNDWVVTAAHCVDGVSADEASFRAGSSSRGSGGSVHQASQLS
ANPQYDYWTIDFDIAVARVSTPFSFGAGVQAISLTTSEPSAGEVATVSGYGTTS
SGGSLPNQLQVVQVPIVDRQQCNEAYADYDGITANMICAAVPEGGKDSCQGD
SGGPLVVGGKLAGIVSWGVGCGSPGYPGVYSNVATLRDFVVSETGVN

99% confidence 95% confidence

Figure 5.3.3 – Cockroach enzyme mass spectral analysis.

Mass spectra generated from each purified cockroach enzyme were compared to the trypsin-homologous EST sequences identified above. Each enzyme best matched to distinct EST sequences; the unique peptides identified from each enzyme are presented as lines under the EST sequence. Green lines indicate a >99% confidence interval and red lines a >95% confidence interval.

Bio Sample	Accession	Prob	%Spec	#Pep	#Unique	#Spec	%Cov	mw (kDa)
E1	*CL8Contig1|*	*100%*	*0.037745*	*18*	*32*	*229*	*0.725191*	*27*
	CL53Contig1|	100%	0.007417	12	17	45	0.616858	27
	gi|73544890|gb|AAZ78212.1|	100%	0.002143	1	1	13	0.284047	26
	CL1Contig9|	100%	0.002143	1	1	13	0.284047	26
E2	CL8Contig1|	100%	0.012861	14	22	109	0.656489	27
	CL53Contig1|	*100%*	*0.023599*	*18*	*29*	*200*	*0.816092*	*27*
	gi|73544890|gb|AAZ78212.1|	100%	0.015221	2	3	129	0.766537	26
	CL1Contig9|	100%	0.014041	2	3	119	0.766537	26
E3	*gi|73544890|gb|AAZ78212.1|*	*100%*	*0.007815*	*1*	*2*	*21*	*0.2607*	*26*
	CL1Contig9|	89%	0.00521	0	0	14	0.18677	26

Table 5.3.1 – Mass spectral analysis of cockroach enzymes.

The results from the mass spectral analysis of the cockroach enzymes are summarized. Of note, each biological sample was found to match with 100% probability to the sequences identified in the EST library. The multiple matches found in the E1- and E2-containing samples may be due to contamination. The percentage of spectra identified for each sample matching to each EST sequence (%Spec) differs. The highest percentage of unique E1-generated spectra matches to the CL8Contig1 sequence, whereas the highest percentage of E2-generated spectra matches to the CL53Contig1 sequence.

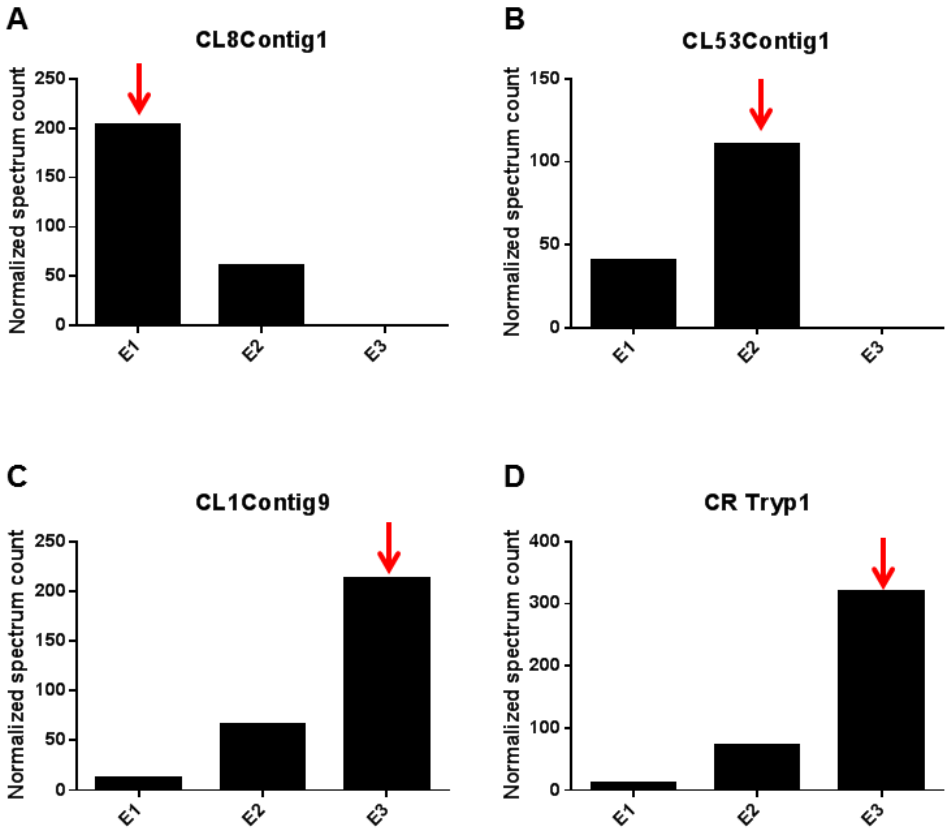


Figure 5.3.4 – Quantification of the relative abundance of each EST sequence using a normalized spectrum count.

A normalized spectrum count generated by the Scaffold 4 software, quantifies the number of unique peptides identified for each purified enzyme that match each EST sequence relative to all of the peptides identified. The excised cockroach enzyme that had the highest proportion of unique peptides matching to each EST sequence are marked by a red arrow. These results indicate that the E1 is encoded by CL8Contig1, E2 by CL53Contig1, and E3 by CL1Contig9. E3 matches with the cockroach trypsin (Tryp1) cloned by Ock et al[80].

5.4 Biochemical assessment of the proteolytic content of a cockroach frass extract

It is thought that the major source of environmental cockroach allergens is the frass (feces), which is excreted as small, light particles easily incorporated into airborne house dust[38]. Thus, the trypsin-like enzymes contributing to asthma pathogenesis are thought to be largely derived from digestive enzymes excreted in the frass. As such, it was important to compare the proteolytic content of the total body cockroach extract to an extract of cockroach frass to determine whether the enzymes that we identified were also plausibly present in the main source of environmental cockroach-derived proteinases. Unfortunately, we were unable to procure frass from the German cockroach, but a collaborator provided the frass collected from speckled cockroaches (*Nauphoeta cinerea*) to us. Though direct comparisons to the total body extract are thus not able to be made from this material, we establish the principle by which German cockroach frass could be similarly studied.

A biochemical approach was used to determine the molecular weight of the frass trypsin-like content (ABP labeling), the relative charge of the enzyme(s) (ion-exchange chromatography), and the sensitivity of the frass enzyme(s) to trypsin inhibitors. ABP labeling of the frass extract revealed a single band (Figure 5.4.1 A). When aligned with an ABP label of the total cockroach extract, the single frass band aligns at the same molecular weight as E1, the lowest molecular weight band in the total body extract. A DEAE sepharose column was used to fractionate the frass extract, and enzyme activity was monitored in the fractions collected from the column with a NaCl step gradient elution (Figure 5.4.1 B, dashed black line), similar to the protocol described in Chapter 4

(Section 4.2.3). A single peak of trypsin-like activity was observed in the non-binding fractions (Figure 5.4.1 B, black line), and labeling of the collected fractions with ABP reveals the same single band labeled in the active fractions (Figure 5.4.1 A). Thus, since the frass enzyme did not bind the positively-charged anion exchange column indicating a relative positive charge in the enzyme, both the molecular weight and the relative charge of the frass enzyme resemble E1 from the total cockroach extract. Finally, examining the biochemical properties of the frass enzyme revealed distinct inhibitor kinetics from E1. The frass enzyme demonstrated less sensitivity to both TLCK (frass IC50 = 186 µM; E1 IC50 = 42 µM) and SBTI (frass IC50 = 1.6 µM; E1 IC50 = 0.17 µM) than E1. Though similar in molecular weight and relative charge to E1, the two enzymes are biochemically distinct.

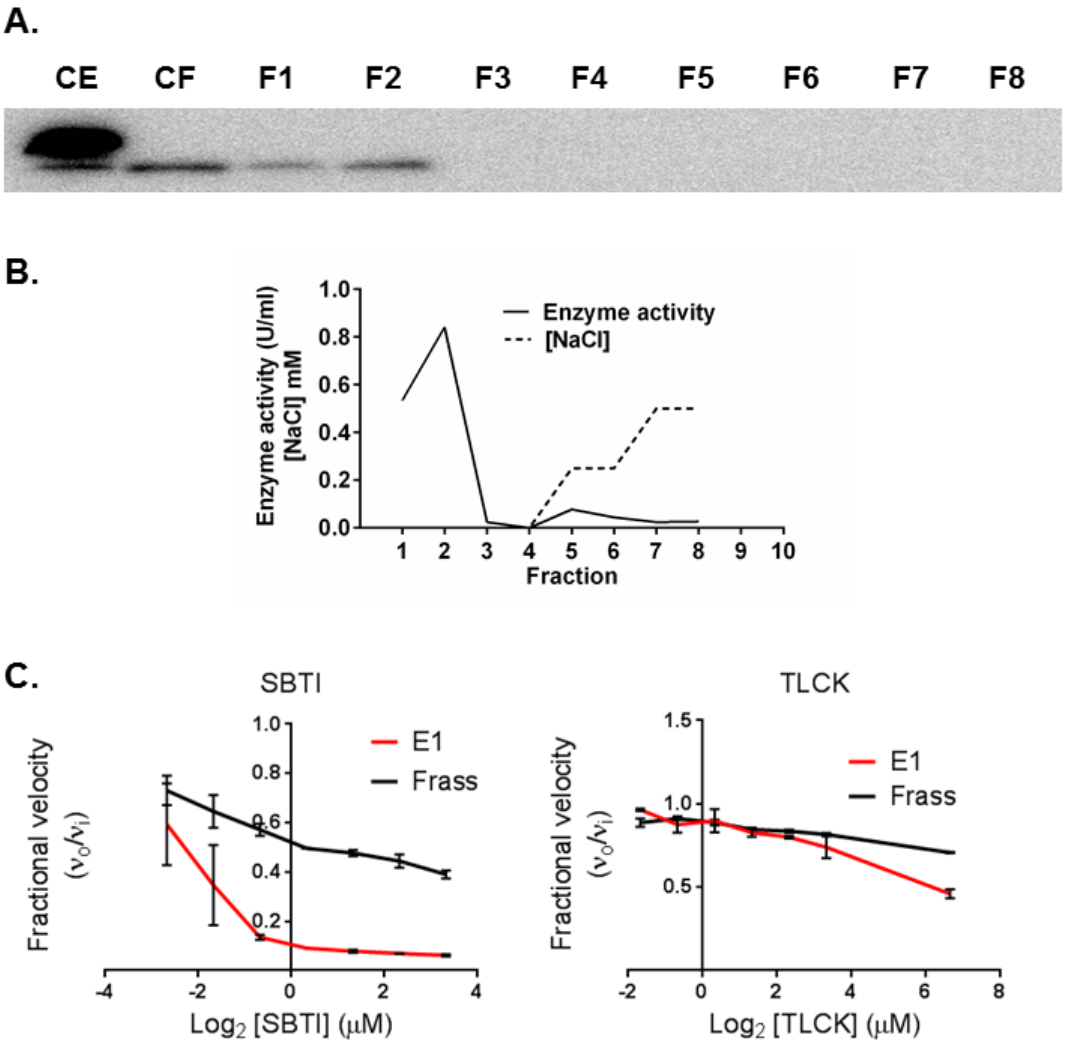


Figure 5.4.1 – A cockroach frass extract contains a single enzyme similar to E1. **(A)** Labeling the cockroach frass extract (CF) with the ABP reveals a single band that resolves in line with E1 from the total body extract (CE). **(B)** A High-Q anion-exchange column was used to purify the enzyme and assess its relative charge. A single peak of trypsin-like activity (black line) was present in the non-binding flow-through fractions, indicating a positively charged protein similar to E1. The fractions collected (F1-F8) were labeled with the ABP as shown in **(A)**. **(C)** Inhibitor kinetics assays reveal distinct biochemical properties between E1 and the frass enzyme. Though the inhibition curves for TLCK are similar, they diverge at higher inhibitor concentrations. The curves for SBTI also diverge with the frass enzyme displaying lower sensitivity to both inhibitors.

5.5 Discussion

Having isolated each of the allergen enzymes, I characterized each biochemically by measuring their enzyme kinetics values, and identified the amino acid sequences of each by mass spectral analysis. Though all four allergen-derived enzymes can be classified as trypsin-like due to (1) their cleavage of substrates with a positively charged residue (arginine) in the P1 position, and (2) their sensitivities to the trypsin-specific inhibitors SBTI and TLCK, the relative kinetic values indicate that the four enzymes have distinct biochemical properties and thus are functionally distinct enzymes. The affinities of proteinases to the different substrates depend on the favourability of the interaction between the enzymes' active pockets and the R-groups projecting from the residues adjacent to the cleavage site[194]. Different affinities to substrates with different P2 and P3 residues, as were used in these assays, indicate that the structural geometry of the active pocket is distinct between the four enzymes. Similarly, differences in observed Ki values between the enzymes indicate structural differences in the inhibitor binding sites that further support distinct biochemical properties for each enzyme. These distinct properties thus reveal three distinct potential pro-inflammatory trypsin-like enzymes in the cockroach allergen, and a unique trypsin-like enzyme in the Alternaria allergen. So, it is not possible to assume that since each enzyme can be described as 'trypsin-like' that its biochemical properties will be identical to that of each other 'trypsin-like' enzymes or to mammalian trypsins commonly used experimentally. It is thus necessary to consider each allergen-derived enzyme as a distinct proteinase with the potential to elicit distinct physiological responses in the airway. Likewise, if the goal of therapeutic intervention involves targeting allergen-

derived proteinases with inhaled inhibitors, it cannot be assumed that all trypsin-like enzymes will be inhibited equally by the equivalent doses of inhibitors.

To definitively identify each allergen-derived enzyme, and to confirm that the three cockroach enzymes represent distinct gene products, mass spectral analysis was performed for each purified enzyme. Using EST libraries generated for each allergen species, we identified the sequences of the Alternaria enzyme and the three cockroach enzymes. Having thus identified the cDNA sequences that likely encode each enzyme, the possibility now exists to clone and recombinantly express each enzyme, which could provide abundant, highly pure preparations of each enzyme for use in future experiments. One cockroach-derived trypsin-like enzyme, which I have identified as E3, has been cloned and expressed[80], so a repetition of this procedure for each of the other three allergen-derived enzymes may be possible. A main drawback of my experimental approach of ion-exchange chromatographic purification of the allergen-derived enzymes was that enzyme abundance in the allergen material was often limited, so I was in turn limited in this way in the experiments that I could perform. Generating recombinant enzymes could potentially solve that problem since enzyme generation would not be constrained to the extraction process from the allergen species. A common sequence for the Alternaria trypsin was identified in both a total-mould extract and a filtered culture media preparation, indicating that the enzyme is likely a secreted factor to which exposure may not require exposure to the mould itself. This is significant in that the same trypsin-like enzyme in the Alternaria allergen can be studied using different preparations.

Finally, analysis of the trypsin-like activity in a frass extract generated from the speckled cockroach revealed one ABP-labeled enzyme with similar molecular weight and relative charge to E1. Inhibitor kinetics revealed that the frass enzyme and E1 are biochemically distinct. Though these enzymes may represent homologous trypsin-like proteinases, they originate in different species of cockroach and thus are likely to diverge in primary sequence. However, these results highlight the potential value in this line of comparison between the total body extract and German cockroach frass. Since the frass is thought to be the major source of cockroach allergens in the environment[38], this would be important to determine which enzyme(s) present in the total body extract are likely to be encountered in the environment, and thus capable of contributing to airway inflammation and allergic sensitization.

In summary, the cockroach and Alternaria allergens collectively contain four distinct trypsin-like enzymes, with unique primary amino acid sequences and differing biochemical properties. Differences in substrate specificities among the four enzymes raise the possibility that biological targets in the airway, and thus the host response induced, may also differ between the four enzymes. To explore this possibility, the ability of each enzyme to regulate signaling via PARs 1 and 2 is assessed in the following Chapter.

CHAPTER 6 REGULATION OF SIGNALING VIA PAR1 AND PAR2 BY THE ALLERGEN-DERIVED PROTEINASES

6.1 Introduction

In the previous Chapter, all of the four allergen-derived trypsin-like enzymes that I have identified and isolated were characterized for their biochemical properties and for their primary amino acid sequences. Each of the three cockroach enzymes were found to be distinct from one another, both in their enzyme kinetics values and in their sequences, indicating that each represents a distinct gene product with distinct substrate specificities and inhibitor sensitivities. The Alternaria enzyme also demonstrated distinct biochemical properties from each of the cockroach enzymes, and the sequence was also identified. These distinct biochemical properties of the four enzymes indicate that they differentially target and cleave different substrates, which could affect the physiological responses evoked by each in the context of the airway.

In particular, we were interested in possible differences in how the enzymes regulate signaling via PARs 1 and 2, so the aims explored in this Chapter were to assess the ability of each isolated enzyme (1) to target and cleave the tethered ligand sequences of PARs 1 and 2, and (2) to regulate the intracellular signaling pathways known to be activated by the PARs. For both receptors we focused on the activation of two distinct PAR-regulated signaling pathways: Ca^{2+} signaling and ERK 1/2 phosphorylation. Differences in the intracellular responses evoked by each enzyme were taken as evidence for potential downstream physiological effects, which I will discuss in the following Chapter.

6.2 Signaling via PAR2 by the allergen-derived enzymes

6.2.1 Cleavage of a synthetic peptide representing the tethered-ligand sequence of PAR2

Having previously shown that the crude cockroach and Alternaria allergen extracts can both activate Ca^{2+} signaling via PAR2, the next step was to assess the ability of each individual allergen-derived enzyme to activate PAR2-mediated Ca^{2+} signaling. For all of the experiments in this Chapter, the fractions containing each isolated enzyme obtained by passing the crude cockroach extract through the High S column (Section 4.2.1) and the fraction obtained by passing the crude commercial Alternaria extract through a High Q column (Section 4.2.4) were used. To demonstrate the theoretical ability of each isolated enzyme to target PAR2, and to establish possible cleavage sites, cleavage of a synthetic peptide representing the tethered ligand sequence of the rat PAR2 protein was monitored. Incubation of the peptide with all of E1, E2, E3, and Alt, as well as a porcine trypsin control, yielded a common peak in the HPLC readout corresponding to the cleavage of the peptide at the arginine residue representing the canonical trypsin cleavage site (R36 in the intact receptor) and revealing the tethered ligand sequence of SLIGRL (representative traces in Figure 6.2.1; data summarized in Table 6.2.1). Thus, each allergen enzyme demonstrated the ability to cleave PAR2 in a way that could activate signaling similarly to porcine trypsin.

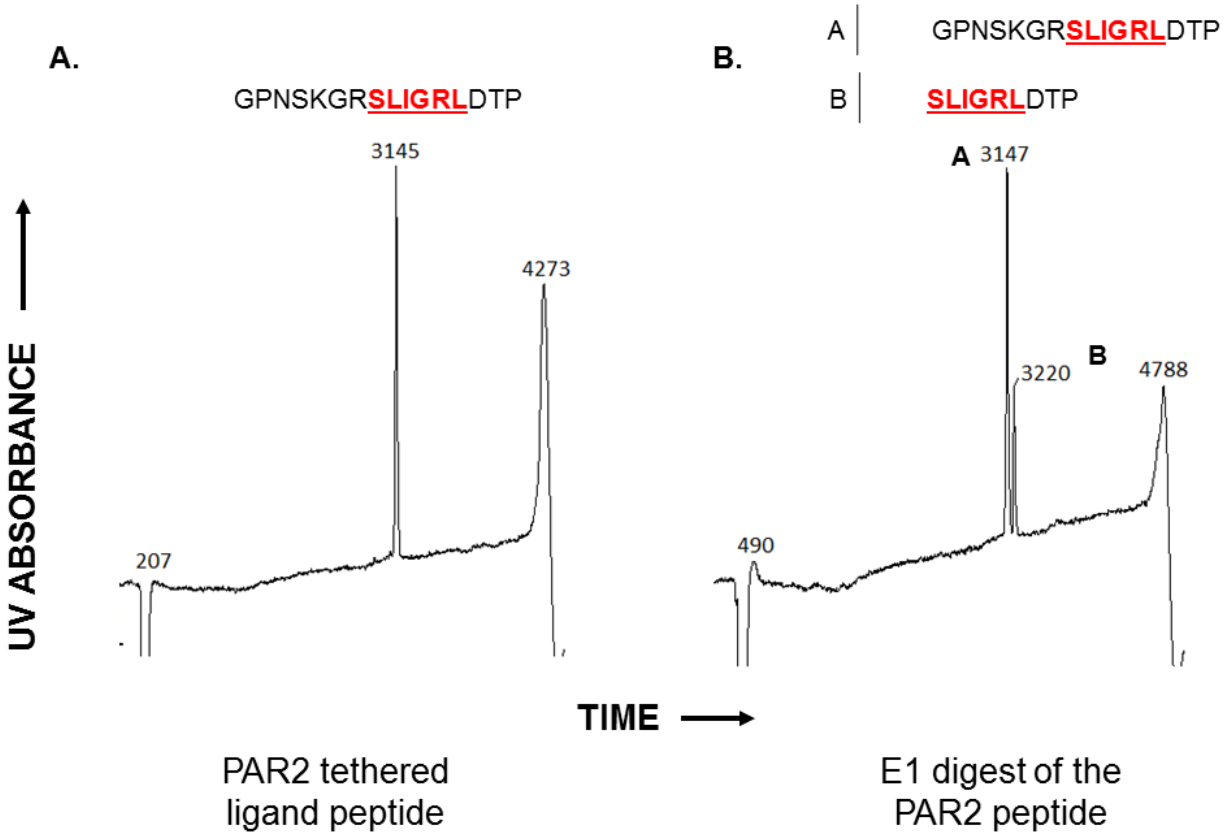


Figure 6.2.1 – E1-mediated cleavage of a synthetic PAR2 tethered ligand peptide. **(A)** The single peak in a HPLC readout represents a synthetic peptide representing the tethered ligand sequence of rat PAR2. **(B)** Incubating the PAR2 peptide with 1 U/ml E1 reveals cleavage of the receptor. The two peptides identified represent the total peptide sequence (peak A) and the sequence that has been cleaved at the residue representing R36 in the receptor, revealing the activating tethered ligand sequence SLIGRL. Similar results were obtained by incubating the PAR2 peptide with each of the allergen-derived enzymes.

Peptide	E1	E2	E3	Alt
PAR1	1.NATLDPR	1.NATLDPR	1.NATLDPR	1.NATLDPR
NATLDPRSFLLRNPNDKYE	2.SFLLRNPNDKYE	2.SFLLRNPNDKYE	2.SFLLRNPNDKYE	2.SFLLRNPNDKYE
PAR2	1.GPNDKGR	1.GPNDKGR	1.GPNDKGR	1.GPNDKGR
GPNSKGRSLIGRLDTP	2.SLIGRLDTP	2.SLIGRLDTP	2.SLIGRLDTP	2.SLIGRLDTP

Table 6.2.1 – Each allergen enzyme cleaves synthetic peptides representing the tethered ligand sequences of PARs 1 and 2.

Each of the allergen-derived enzymes cleaved synthetic peptides to unmask the tethered ligand sequences (underlined in red) of both PAR1 and PAR2. Representative traces presented in Figures 6.2.1 (PAR2) and 6.3.1 (PAR1).

6.2.2 Cleavage of a PAR2-N luciferase construct expressed in an intact cell

Having demonstrated the ability of each enzyme to cleave a synthetic PAR2 tethered ligand peptide, cleavage of a construct consisting of rat PAR2 linked to N-luciferase in the N-terminal region of the receptor was monitored to assess the ability of each enzyme to cleave the intact receptor in a cell membrane environment. The Nluc contained in the construct is N-terminal to the trypsin cleavage site, so it is expressed in the peptide sequence that is released following cleavage and unmasking of the tethered ligand sequence. Thus, the release of a luciferase signal into the supernatant was taken to represent the cleavage of the receptor by each enzyme (construct illustrated in Figure 6.2.2 A). Treatment of the cells by each of the allergen enzymes at a concentration of 1 U/ml of trypsin-like activity resulted in significant Nluc signals in the supernatant, indicating that each enzyme cleaved the PAR2 construct at that

concentration (Figure 6.2.2 B). The magnitude of the luciferase signal was approximately similar for each enzyme, and to a trypsin control. A slightly higher, signal was generated by both E2 and Alt compared to E1, E3 and trypsin. These findings support the conclusion that each allergen-derived enzyme can cleave PAR2, and so individually all are potential PAR2 agonists.

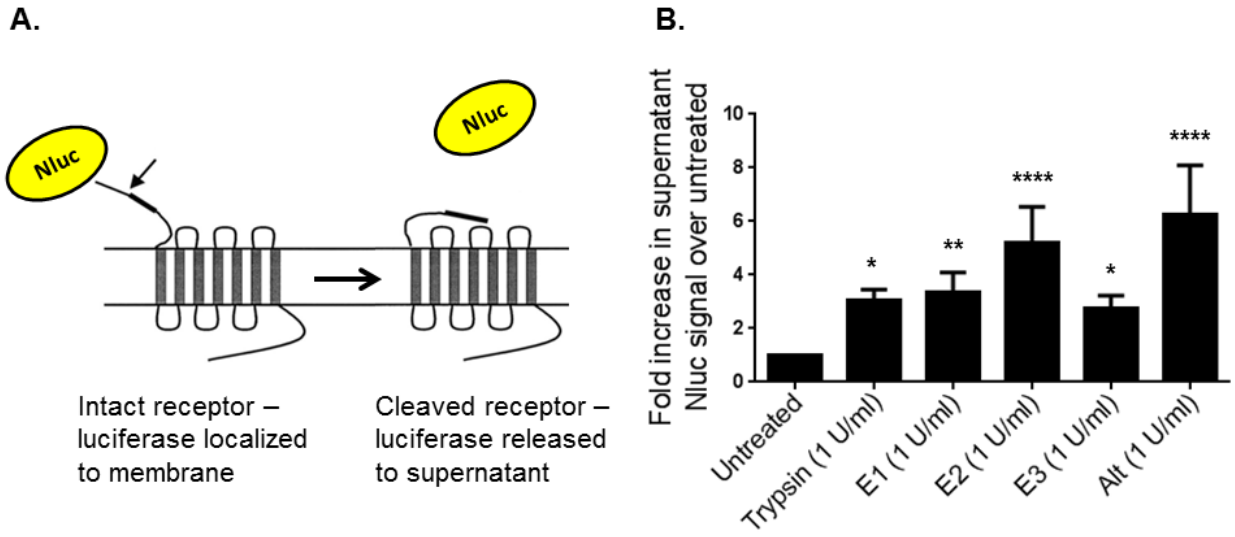


Figure 6.2.2 – Allergen enzymes cleave a PAR2-Nluc construct.
(A) The Nluc expressed in the N-terminal region of the PAR2 receptor is released into the supernatant when the receptor is cleaved by an agonist enzyme. **(B)** Each of the allergen-derived enzymes cleaved the PAR2 construct at a concentration of 1 U/ml (*p<0.05, **p<0.01, ****p<0.0001; n=4).

6.2.3 PAR2-mediated Ca^{2+} signaling

With the ability of each enzyme to cleave PAR2 established, the intracellular signaling events induced following receptor cleavage were assessed. First, PAR2-expressing KNRK cells were used to assess the ability of each allergen-derived enzyme to activate Ca^{2+} signaling via PAR2. Monolayers of the cells in 96-well microtiter plates were loaded with the cell permeant, Ca^{2+}-sensitive Fluo-4 dye and each enzyme was added to the cells individually. Each of the cockroach enzymes induced a positive Ca^{2+} response at a concentration of 1 U/ml comparable to the response induced by trypsin. The responses induced by each enzyme were reduced or blocked both by prior desensitization of the PAR2 in the cells by pre-treating with 2fLI, and by inhibition of the enzyme with soybean trypsin inhibitor (SBTI) prior to administering to the cells (representative traces in Figure 6.2.3 A-C). The Alternaria enzyme also induced a positive Ca^{2+} response at a concentration of 1 U/ml that was sensitive to desensitization of the cells to PAR2 (representative traces in 6.2.3 D). However, there was not sufficient Alternaria material to test the sensitivity to SBTI inhibition. The enzyme-activated Ca^{2+} responses were quantified by normalizing to the response induced following treatment of the cells by 2 µM of the Ca^{2+} ionophore A23187 (Figure 6.2.3 E).

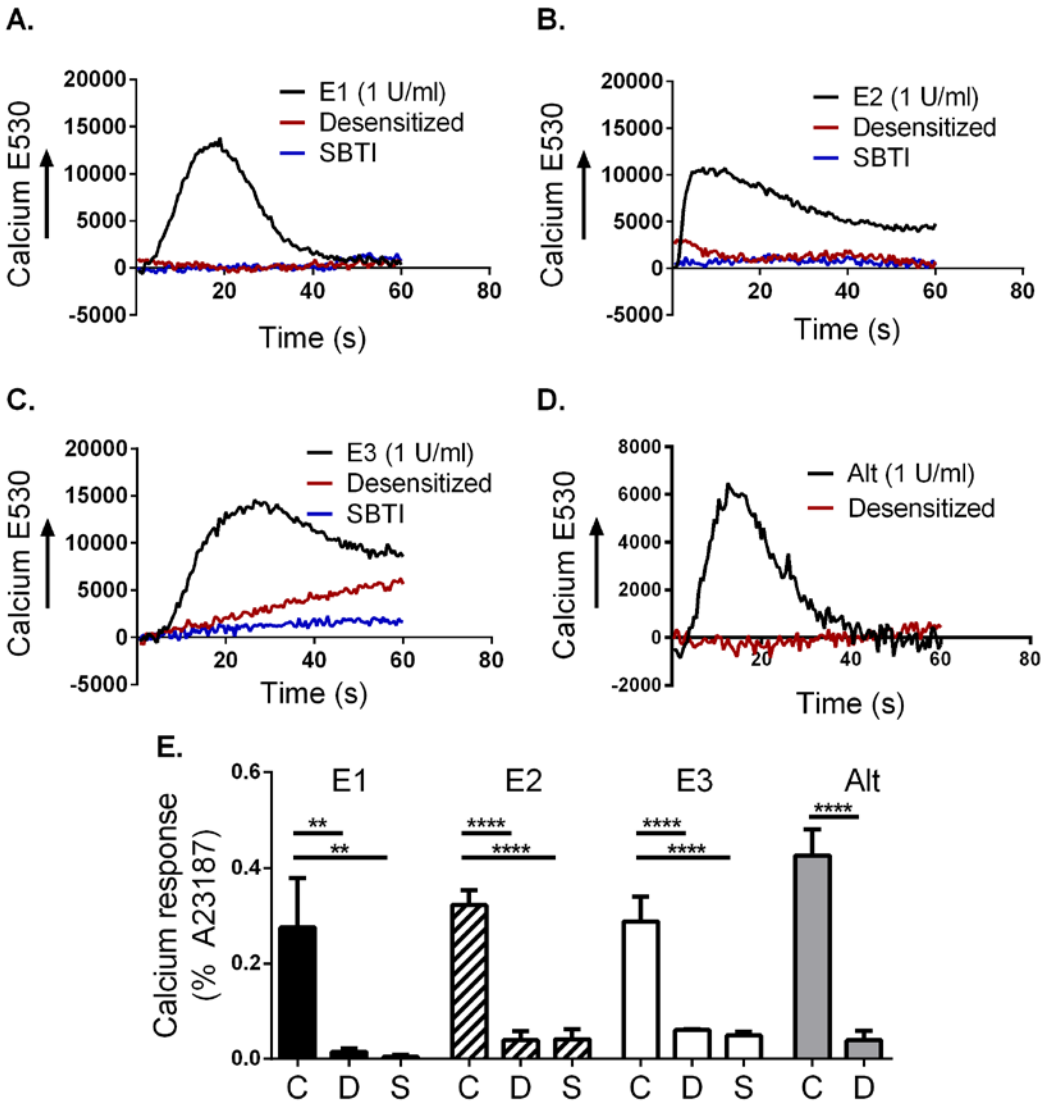


Figure 6.2.3 – Allergen enzyme-driven PAR2-mediated Ca^{2+} signaling.
PAR2-expressing KNRK cell monolayers were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4. 1 U/ml each of E1 **(A)**, E2 **(B)**, E3 **(C)** and Alt **(D**; representative traces) activate Ca^{2+} transients (black traces), which are blocked by either pre-treating the enzymes with 50 µM SBTI (blue traces) or desensitizing the cells with the PAR2 agonist peptide 2fLI (red traces). **(E)** Quantification of the Ca^{2+} transients as a percentage of the response induced by the Ca^{2+} ionophore A23187 (2 µM) in the control (C), desensitized (D) and SBTI-inhibited (S) conditions. (**p<0.01, ****p<0.0001; n=4)

6.2.4 PAR2-mediated ERK 1/2 phosphorylation

In addition to Ca^{2+} signaling, activation of MAPK signaling, in this case ERK 1/2, the other main signaling arm activated downstream of PAR2 activation, was assessed using the PAR2-expressing KNRK cells. Following a 10 min incubation, each enzyme was able to activate ERK 1/2 signaling via PAR2 at a concentration of 1 U/ml (Figure 6.2.4 A). In the case of the three cockroach enzymes, the signal was blocked by pre-treating the fractions with 50 µM SBTI prior to administering to the cells (Figure 6.2.4 A, left blot). However, the Alternaria signal was not sensitive to SBTI inhibition (Figure 6.2.4 A, right blot). This result could reflect the low sensitivity of this enzyme to that inhibitor, as indicated by the relatively high Ki value determined in Chapter 4. Densitometric analyses of the blots were performed to quantify the MAPK activation responses, and the band intensities normalized to the GAPDH loading control (Figure 6.2.4 B). The responses induced by each enzyme were also found to be PAR2-dependent since treatment with equivalent concentrations of each enzyme of PAR non-expressing KNRK cells transfected with the pcDNA vector alone did not result in an increased p-ERK 1/2 signal relative to the untreated control Figure 6.3.9).

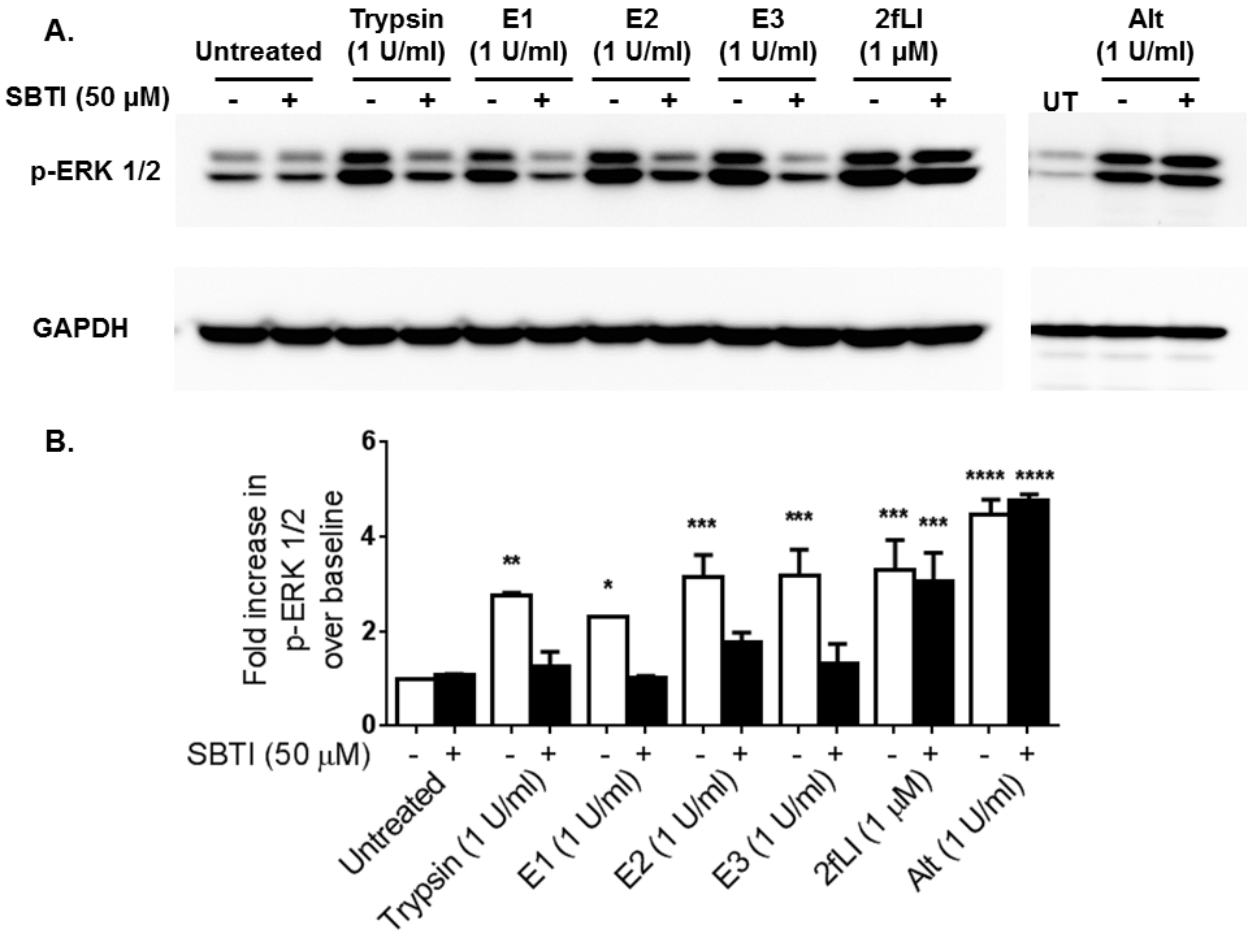


Figure 6.2.4 – PAR2-mediated ERK 1/2 phosphorylation.

PAR2-expressing KNRK cells were treated with each enzyme or agonist for 15 min in the presence or not of 50 µM SBTI. **(A)** Respresentative blots (UT=untreated). **(B)** Densitometric analysis reveals that each allergen-derived enzyme activates ERK 1/2 phosphorylation similarly to porcine trypsin, and that pre-treatment with SBTI blocks that response, with the exception of the Alternaria enzyme which was resistant to SBTI inhibition. (P values are relative to the untreated control; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; n=3)

6.2.5 BRET-based detection of the recruitment of β-arrestin to PAR2 following receptor activation

Having established each enzyme as a non-biased agonist for PAR2, activating both of the major downstream signaling pathways, the final step was to assess the ability of each enzyme to recruit β-arrestin 1 and/or 2 to PAR2. This recruitment plays a role both in β-arrestin scaffold signaling and the initiation of the receptor internalization mechanism. Each enzyme was incubated with the HEK293 cells expressing both PAR2-YFP and β-arrestin 1-R-luciferase or β-arrestin 2-R-luciferase. The cells were then incubated with the coelenterazine luciferase substrate, which results the activation of the luciferase bioluminescence and emission of light with a wavelength of 480 nm. When recruited to PAR2, and thus in close proximity to the YFP tag on the C-terminus of the PAR2 construct, the luciferase-emitted photons are absorbed by and activate the YFP. This process results in the emission of light with a wavelength of 530 nm. Thus, the presence of the YFP emission signal indicates close proximity to Rluc, and by extension, recruitment and binding of β-arrestin to PAR2 (illustrated in Figure 6.2.5 A). Each enzyme was initially administered to the cells at a concentration of 1 U/ml for 15 min, following which light emission at both 395 nm and 530 nm was monitored and compared to light emission from untreated cells. Treatment with E1 resulted in significant recruitment of both β-arrestin 1 and 2, whereas treatment with E2 resulted in significant recruitment of β-arrestin 1 but not 2, and E3 recruited β-arrestin 2 but not β-arrestin 1 (Figure 6.2.5 B). In each of the latter case the signals observed were higher than the untreated control, though not significantly, so the lack of significance may be

due to sample size. Regardless, activation of PAR2 by each cockroach enzyme was found to result in the recruitment of β-arrestin 1 and/or β-arrestin 2.

By contrast, and unexpectedly, treatment of the cells with the Alternaria enzyme at 1 U/ml did not result in significant recruitment of either β-arrestin 1 or 2 (Figure 6.2.5 B). Treatment of the PAR2-expressing KNRK cells with the same aliquot of the Alt-containing fraction activated both Ca^{2+} signaling and ERK 1/2 activation in a PAR2-dependent manner. This result suggests that the Alternaria enzyme activates PAR2 in such a way as to initiate the major intracellular signals without the recruitment of β-arrestin to the receptor. If so, it may represent a unique mechanism of signal regulation and receptor internalization for a trypsin-like enzyme, although further experiments will be necessary.

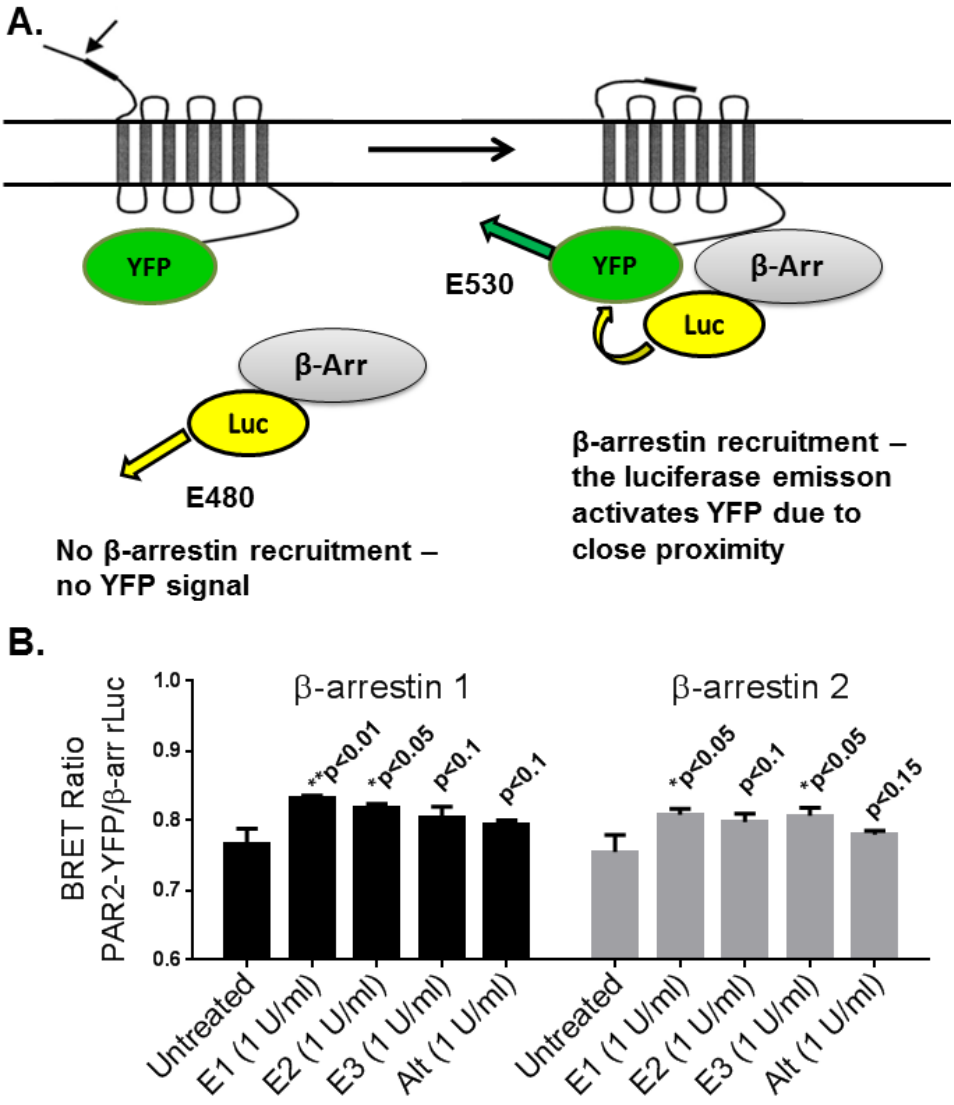


Figure 6.2.5 – BRET-based β-Arrestin recruitment to PAR2.

HEK293 cells expressing YFP-linked PAR2 and rLuc-linked β-arrestin 1 or 2 were used to measure recruitment of β-arrestin to PAR2 following treatment with the allergen-derived enzymes. **(A)** Illustration of the BRET mechanism. **(B)** E1 induced significant ($p<0.05$) recruitment of both β-arrestin 1 and 2 at a concentration of 1 U/ml, whereas E2 induced significant recruitment of only β-arrestin 1, E3 of only β-arrestin 2 and Alt did not induce a significantly increased BRET signal. (P values are relative to the untreated control; n=3.)

6.2.6 Regulation of PAR2 signaling by thrombin

A response to an allergen that contains proteolytic activity can in principle result in activation of the extrinsic clotting cascade to generate thrombin activity. Therefore, to explore an additional avenue through which allergen-derived enzymes could indirectly regulate PAR2 signaling via thrombin, we were interested in exploring the ability of thrombin to cleave and activate PAR2. In the following sections, we assessed the ability of the trypsin-like allergen-derived enzymes to activate biased signaling via PAR1. In a similar vein, we hypothesized that thrombin may induce a similar biased signaling mechanism via PAR2. Though we don't demonstrate the activation of thrombin via regulation of the clotting cascade by the allergen-derived enzymes, these preliminary experiments served as a proof of concept for future research.

To assess the ability of thrombin to target PAR2, we used the Nluc-tagged PAR2 construct as discussed above, expressed in a CHO cell background. Treating CHO cells expressing the Nluc construct with 2 U/ml thrombin induced the significant release of the Nluc signal to the supernatant (Figure 6.2.6 A).

Since thrombin has been reported not to activate PAR2 Ca^{2+} signalling[102], we aimed to assess the ability of thrombin to activate MAPK signaling via PAR2. Treating PAR2-expressing KNRK cells with both low (2 U/ml) and relatively high (20 U/ml) concentrations of thrombin induced ERK 1/2 phosphorylation (Figure 6.2.6 B, C). The response induced by treating PAR1-expressing KNRK cells with 1 U/ml thrombin is included as a reference (Figure 6.2.6 B, C – right images)

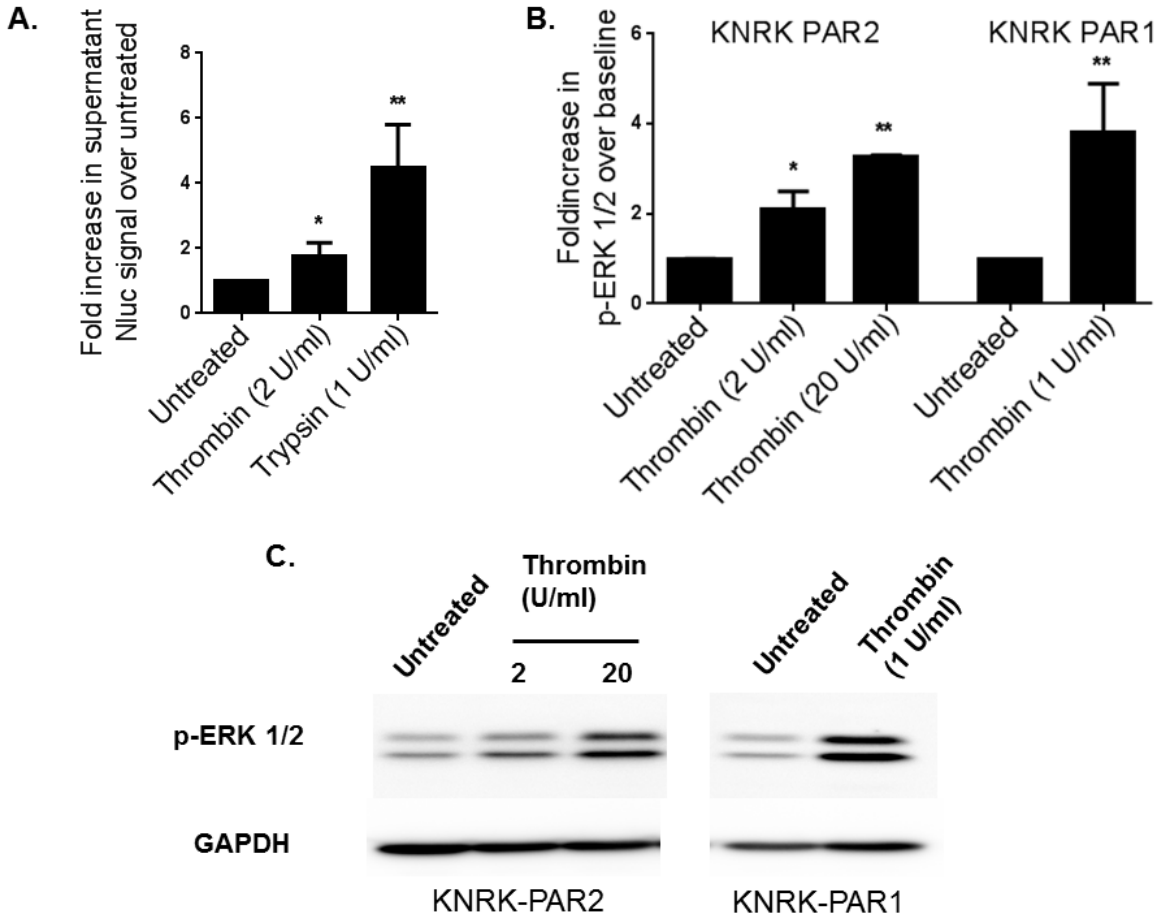


Figure 6.2.6 – Thrombin cleaves PAR2 and activates ERK 1/2 signaling via PAR2. **(A)** Treatment of CHO cells expressing the Nluc-PAR2 construct with 2 U/ml thrombin resulted in the significant release of the luciferase signal to the supernatant. **(B)** Treating PAR2-expressing KNRK cells with thrombin (2 and 20 U/ml) induces the phosphorylation of ERK 1/2 .The response evoked by 1 U/ml thrombin in PAR1-expressing KNRK cells is shown for comparison. **(C)** Representative blots. (P values are relative to untreated controls; *p<0.05, **p<0.01; n=3)

6.3 Signaling via PAR1 by the allergen-derived enzymes

6.3.1 Cleavage of a synthetic peptide representing the tethered-ligand sequence of PAR1

The ability of allergen-derived trypsin-like enzymes to target PAR1 has not been studied previously. Trypsin is not considered a conventional activator of PAR1, and its action on PAR1 has been described as inactivating the receptor by cleaving and disarming the tethered ligand sequence, preventing thrombin-induced Ca^{2+} signaling[186]. However, the role of trypsin in regulating the activation of the MAPK arm of PAR1 signaling has not been evaluated. To determine whether the cockroach and Alternaria enzymes are capable of unmasking the PAR1 tethered ligand, cleavage of a synthetic peptide representing the tethered ligand-containing N-terminal tail sequence of PAR1 by each allergen enzyme was monitored with HPLC and mass spectral analysis, as was described in Section 6.2.1 for the PAR2 peptide. Each allergen-derived enzyme, as well as porcine trypsin, was observed to cleave the PAR1 peptide at the arginine residue representing the thrombin cleavage site (R41) (representative tracing in Figure 6.3.1; summarized in Table 6.2.1, page 130).

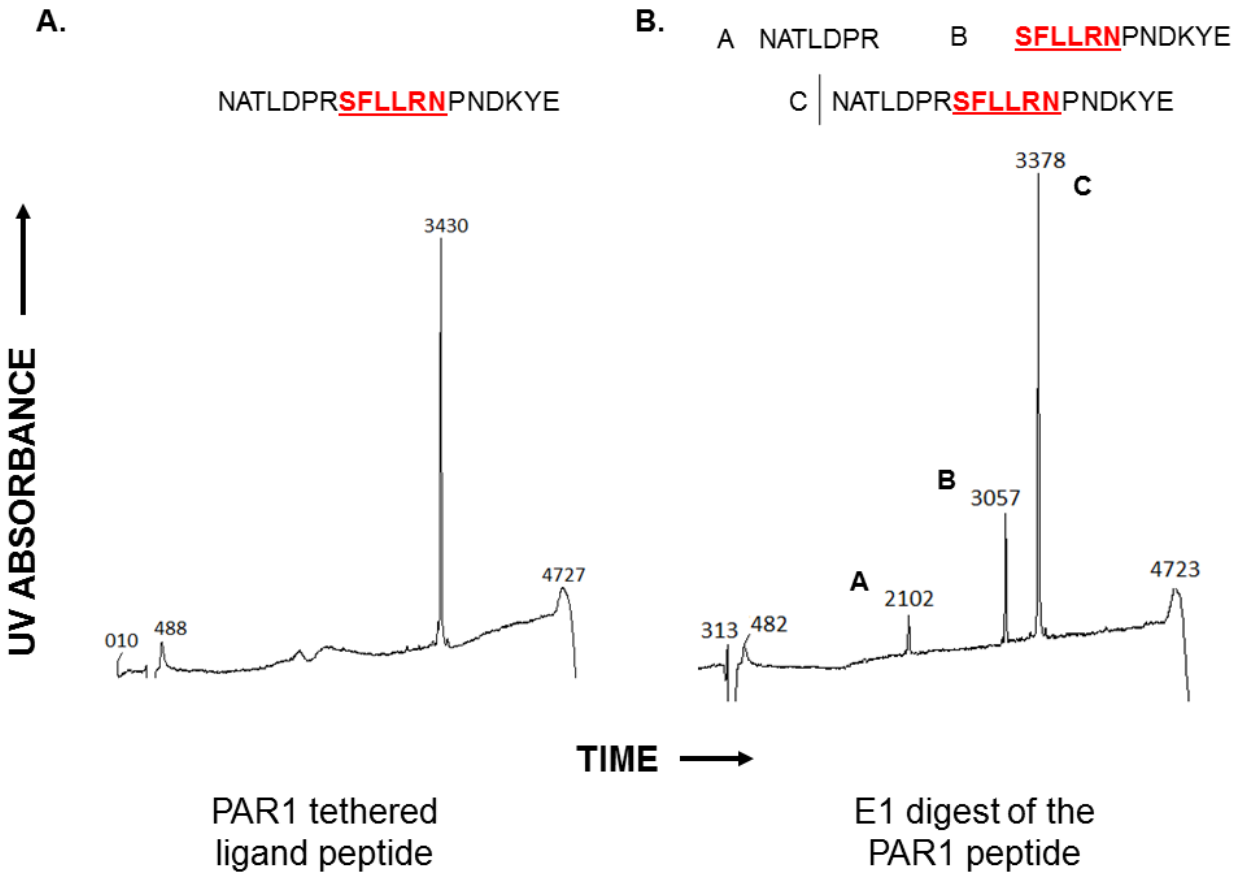


Figure 6.3.1 – E1-mediated cleavage of a synthetic PAR1 tethered ligand peptide. **(A)** The single peak in a HPLC readout represents a synthetic peptide representing the tethered ligand sequence of human PAR1. **(B)** Incubating the PAR1 peptide with 1 U/ml E1 reveals cleavage of the peptide at the residue representing the thrombin cleavage site, R41. The three peptides identified represent the total peptide sequence (peak C) and the cleavage products representing the peptide sequence that is removed from the receptor following cleavage (peak A) and the activating tethered ligand sequence SFLLRN (peak B). Similar results were obtained by incubating the PAR1 peptide with each of the allergen-derived enzymes.

6.3.2 Cleavage of a PAR1-N luciferase construct expressed in an intact cell

Having established that each allergen-derived trypsin-like enzyme is capable of cleaving the PAR1 peptide at the conventional thrombin activation site, an Nluc-tagged PAR1 construct expressed in an intact cell setting to determine if the allergen proteinases are able to cleave the receptor when in the cell surface membrane environment.. This assay is important since the ability of proteinases to target and cleave a substrate can depend on the accessibility of the substrate as well as the substrate specificity of the enzyme. Each of thrombin, E2 and Alt caused a significant increase in the release of the luciferase-containing peptide into the supernatant at a concentration of 1 U/ml with varying efficiencies. However, at this level of enzyme activity porcine trypsin, E1 and E3 did not significantly cleave the receptor construct (Figure 6.3.2 A). At higher concentrations (2 U/ml), each of trypsin, E1 and E3 were able to induce the significant release of the Nluc signal (Figure 6.3.2 B). E2 and Alt induced stronger supernatant luciferase signals than E1, E3 and trypsin at equivalent concentrations, indicating that the former are more efficient at cleaving the receptor than the latter, with E3 appearing to be the least efficient at cleaving the receptor. These results suggest that each allergen enzyme can cleave PAR1, but the efficiency with which they do so varies in descending order such that Alt>E2>E1>trypsin>E3.

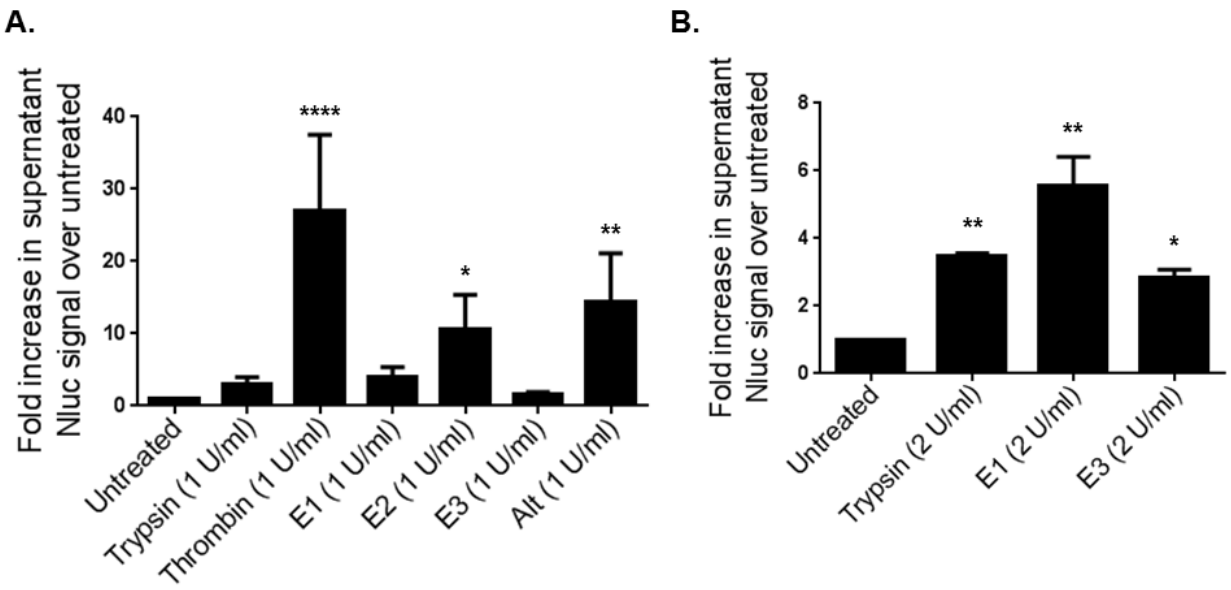


Figure 6.3.2– Cleavage of a PAR1-Nluc construct.

(A) Thrombin, E2 and Alt significantly cleaved the PAR1-Nluc construct at a concentration of 1 U/ml. **(B)** Trypsin, E1 and E3 significantly cleaved the construct at a concentration of 2 U/ml (P values are relative to the untreated controls; *p<0.05, **p<0.01, ****p<0.001; n=3-4).

6.3.3 PAR1-mediated Ca^{2+} signaling

Having established that the allergen enzymes can target and cleave PAR1 with varying efficiencies, the next aim became to establish which, if any, intracellular signaling pathways were activated following this receptor cleavage. As previously mentioned, trypsin has been described as a disarming agent of PAR1 that cleaves the receptor in a way that prevents subsequent activation by conventional agonist enzymes like thrombin. Therefore, it was expected that the allergen enzymes would display the same disarming mechanism. Similar to the PAR2 signaling assay described in Section

6.2.3, KNRK cells expressing human PAR1 were loaded with the Ca^{2+}-sensitive dye to monitor Ca^{2+} signaling. The allergen-derived enzymes, as well as trypsin and thrombin as negative and positive controls respectively, were applied to the cells and fluorescence was monitored. As expected due to the results of the Nluc-construct cleavage assay, at concentrations of 1 U/ml none of trypsin, E1 or E3 induced Ca^{2+} transients. However, both E2 and Alt induced positive Ca^{2+} fluorescence signals in the cells (Figure 6.3.3 A). As previously mentioned, none of the allergen-derived or control enzymes used induced a positive response in PAR non-expressing cells transfected with the pcDNA vector alone, indicating that the responses induced by E2 and Alt were dependent on the presence of PAR1 (Figure 6.3.4).

Since it has been shown that high concentrations of trypsin can activate Ca^{2+} signaling via PAR1 while also disarming $PAR1^{186}$, E1 and E3 were applied to the cells at increasing concentrations to determine if those enzymes were capable of activating PAR1-mediated Ca^{2+} signaling at high concentrations. E1 induced positive Ca^{2+} signals at and above concentrations of 8 U/ml, but not at 4 U/ml indicating that the threshold of PAR1 activation for this enzyme lies between 4-8 U/ml. E3 was able to induce a positive Ca^{2+} response at a concentration of 16 U/ml but not at 8 U/ml, and porcine trypsin, induced a positive Ca^{2+} response at 64 U/ml but not at 32 U/ml (Figure 6.3.3 B). These results suggest that E1 has a greater ability to activate PAR1-mediated Ca^{2+} signaling than E3 and porcine trypsin. Taken together these results demonstrate that each of the cockroach enzymes and the Alternaria enzyme display differential abilities to activate Ca^{2+} signaling via PAR1 such that Alt>E2>E1>E3.

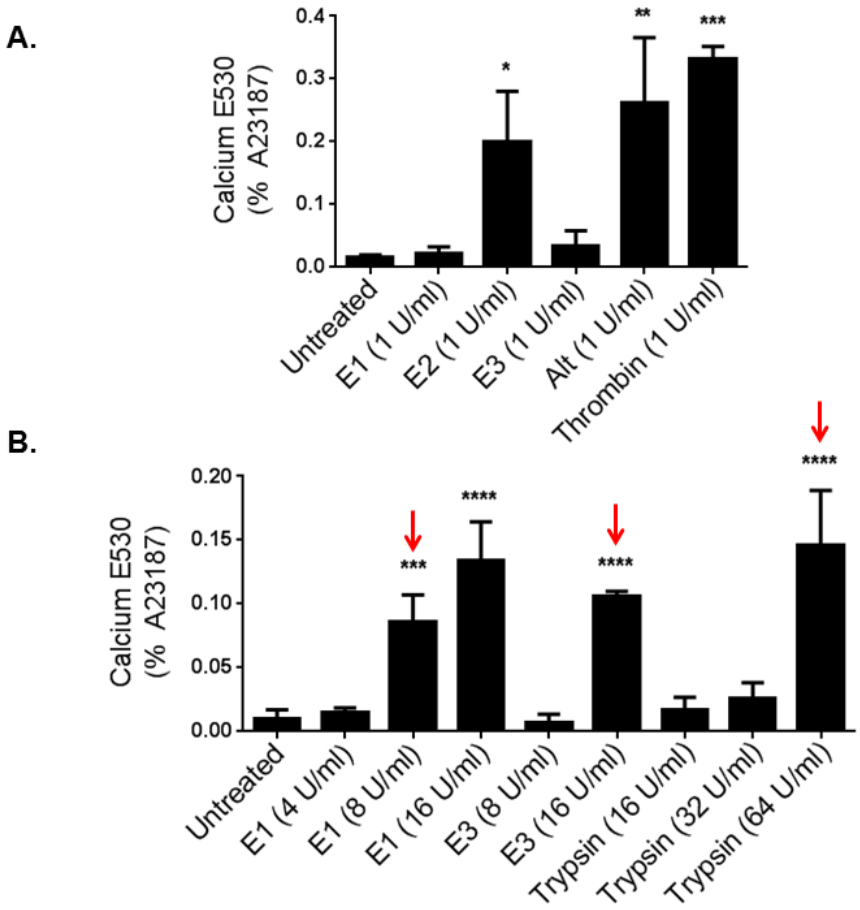


Figure 6.3.3 – Activation of PAR1-mediated Ca^{2+} signaling.

(A) The allergen-derived enzymes displayed differential activation of PAR1-mediated Ca^{2+} signaling at a concentration of 1 U/ml. E2 and Alt induced a positive response, whereas E1 and E3 did not. **(B)** E1 and E3 activate detectable PAR1-mediated Ca^{2+} signaling (the lowest concentrations observed to activate Ca^{2+} signaling for each enzyme are indicated by red arrows) at concentrations of 8 U/ml (E1) and 16 U/ml (E3). Trypsin also activated a Ca^{2+} response at 64 U/ml. (P values are relative to the untreated control; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; n=3-4)

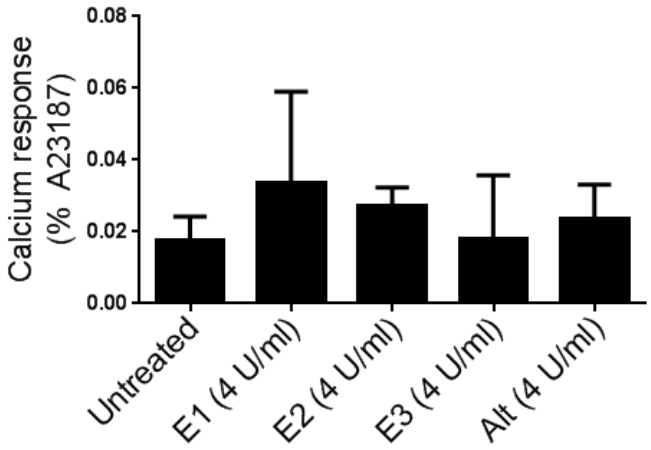


Figure 6.3.4 – Ca^{2+} signal in KNRK cells transfected with pcDNA vector alone. None of the allergen-derived proteinases activated a significant Ca^{2+} response in KNRK cells transfected with empty pcDNA vectors alone. The concentration of 4 U/ml is sufficient to induce strong Ca^{2+} transients in at least one of the PAR1- or PAR2-expressing KNRK cells, indicating that those responses were dependent on PAR expression (n=3).

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6.3.4 PAR1-mediated ERK 1/2 phosphorylation

In addition to Ca^{2+} signaling, the ability of each allergen-derived enzyme to activate ERK 1/2 signaling downstream of PAR1 was monitored. It has been shown that certain 'biased agonist' enzymes (activated protein C[135], proteinase-3 and neutrophil elastase[136]) can activate MAPK signaling via PAR1 in the absence of Ca^{2+} signaling. Since all of the trypsin-like enzymes were shown to cleave the PAR1 peptide and Nluc construct, we hypothesized that the trypsin-like enzymes that do not activate PAR1 Ca^{2+} signaling may function as biased agonists. Treatment of the PAR1-expressing KNRK cells with each of the allergen-derived enzymes at a concentration of 2 U/ml, with the

exception of E3, induced the phosphorylation of ERK 1/2 (Figure 6.3.5). For each of the cockroach enzymes, the PAR1-mediated ERK 1/2 activation was blocked by pre-treatment with SBTI, whereas the Alternaria enzyme was again resistant to inhibition by SBTI (Figure 6.3.5 A, right blot). E3 was found to activate PAR1-mediated ERK 1/2 phosphorylation at higher concentrations, with a noticeable increase at a concentration of 4 U/ml (Figure 6.3.6). All of E1, E3 and trypsin were found to activate MAPK signaling at concentrations below the threshold at which they activate Ca^{2+} signaling in the PAR1-expressing cells. These results confirm that the allergen-derived enzymes regulate PAR1 differentially. The data suggest that while both Alt and E2 are non-biased agonists for PAR1, E1, E3 and porcine trypsin are biased agonists for PAR1. Thus, at low concentrations, these enzymes are able to activate MAPKinase selectively without triggering Ca^{2+} signaling.

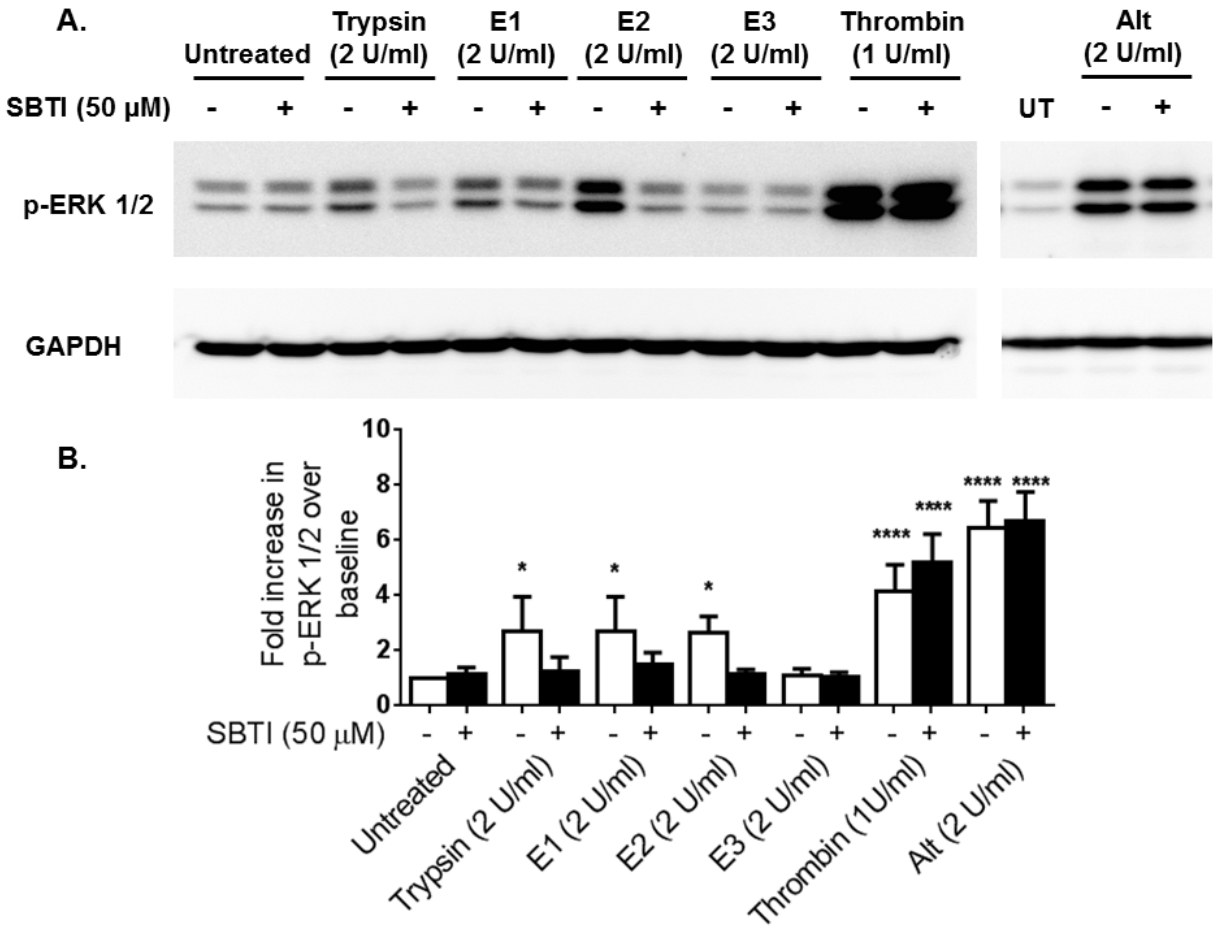

Figure 6.3.5 – ERK 1/2 phosphorylation via PAR1.

Each of trypsin, E1, E2 and Alt activate ERK 1/2 signaling in PAR1-expressing KNRK cells. Pre-treatment of the enzymes with 50 µM SBTI reduces the signal to baseline, with the exception of Alt which was not sensitive to SBTI inhibition in this assay. A thrombin control of 1 U/ml is also not sensitive to SBTI inhibition. **(A)** Representative blots. **(B)** Densitometric analysis of the blots. (P values are relative to the untreated control; *p<0.05, ****p<0.0001; n=3-4)

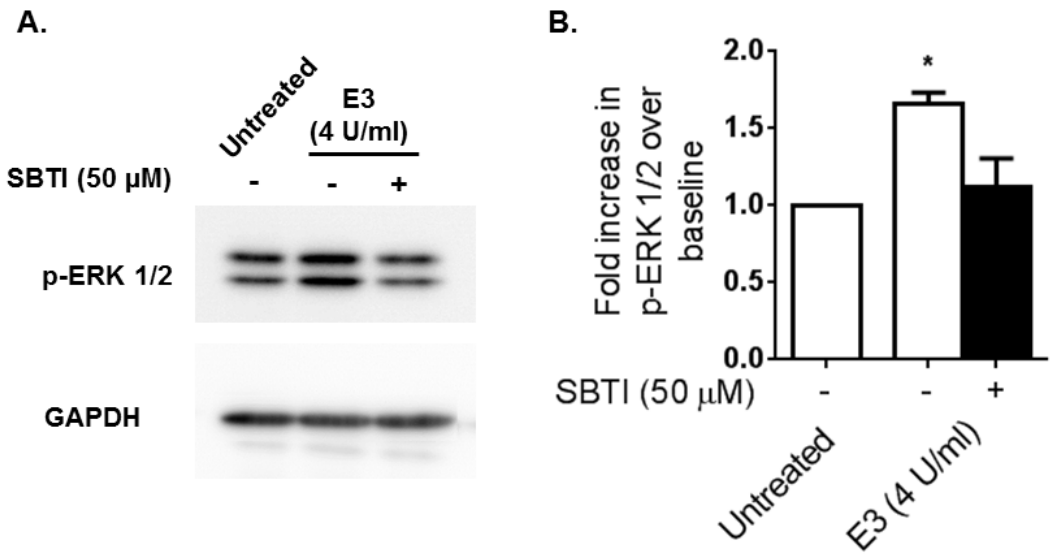

Figure 6.3.6 – Activation of PAR1-mediated ERK 1/2 signaling by E3.
E3 activates PAR1-mediated MAPK signaling at a concentration of 4 U/ml, which is reduced by pre-treating the enzyme with 50 μM SBTI. **(A)** Representative blot. **(B)** Densitometric analysis of the blots (P values are relative to the untreated control; *p<0.05; n=3).

Additionally, since the magnitudes of PAR1-mediated ERK 1/2 activation at a concentration of 2 U/ml varied among the allergen enzymes, concentration-effect curves for each enzyme were generated to illustrate the differential efficiencies with which each enzyme activates that pathway. For porcine trypsin, E1 and E2, curves from 0.5 – 4 U/ml were generated. E3 was a relatively weaker PAR1 agonist, so a curve ranging from 0.5 – 16 U/ml was generated, whereas Alt was a relatively stronger PAR1 agonist so a curve from 0.5 – 2 U/ml was used for that enzyme (Figure 6.3.7). Differences in the observed MAPK phosphorylation responses for each enzyme are visible at a concentration of 0.5U/ml, and the responses for Alt, E1, E2 and porcine trypsin converge from 1-4 U/ml indicating that the potency with which those enzymes activate

PAR1-mediated MAPK phosphorylation is similar. The E3 curve is situated to the right of all of the other enzymes, indicating that it is indicating that it is the PAR1 agonist with the lowest potency of all of the allergen-derived enzymes. However, even with a low potency, the E3 enzyme can generate a response with a magnitude equivalent to that of the other allergen-derived enzymes. Additionally, to illustrate the preferential activation of the MAPK response over the Ca^{2+} response via PAR1 by the biased agonist enzymes (E1, E3, trypsin), the responses that each enzyme induced are presented in comparison to concentration-effect curves for the Ca^{2+} responses that were presented in Figure 6.3.3 B. In all cases, the curve for the Ca^{2+} response is shifted to the right from the curve for the MAPK phosphorylation response, indicating the preferential activation of MAPK signaling over Ca^{2+} signaling at low concentrations (Figure 6.3.8). Due to insufficient enzyme concentrations in the ion-exchange chromatography fractions, I was not able to generate full concentration-effect curves to determine the absolute maximum responses induced by each enzyme, so absolute EC50 values were not able to be calculated. The curves presented in Figure 6.3.8 are relative to the maximum observed responses.

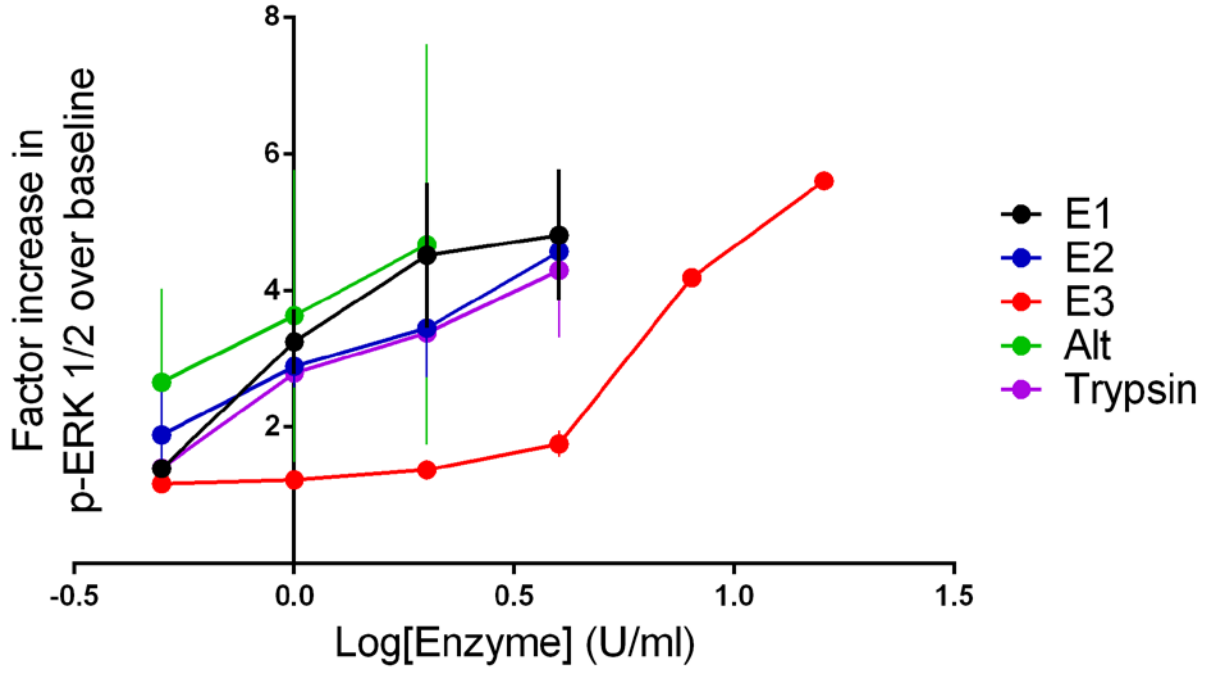

Figure 6.3.7 – Allergen enzyme-mediated ERK 1/2 phosphorylation concentration-effect curves.

Concentration-effect curves of each enzyme ranging from 0.5 - 2 U/ml (Alt), 0.5 - 4 U/ml (E1, E2, trypsin) and 0.5 - 16 U/ml (E3) were generated to visualize the potency with which each enzyme activates PAR1-mediated MAPK signaling. The curves for E1 (black), E2 (blue), Alt (green) and trypsin (purple) were similar, whereas E3 (red) activated MAPK signaling with less potency as its curve was situated to the right of the other enzymes.

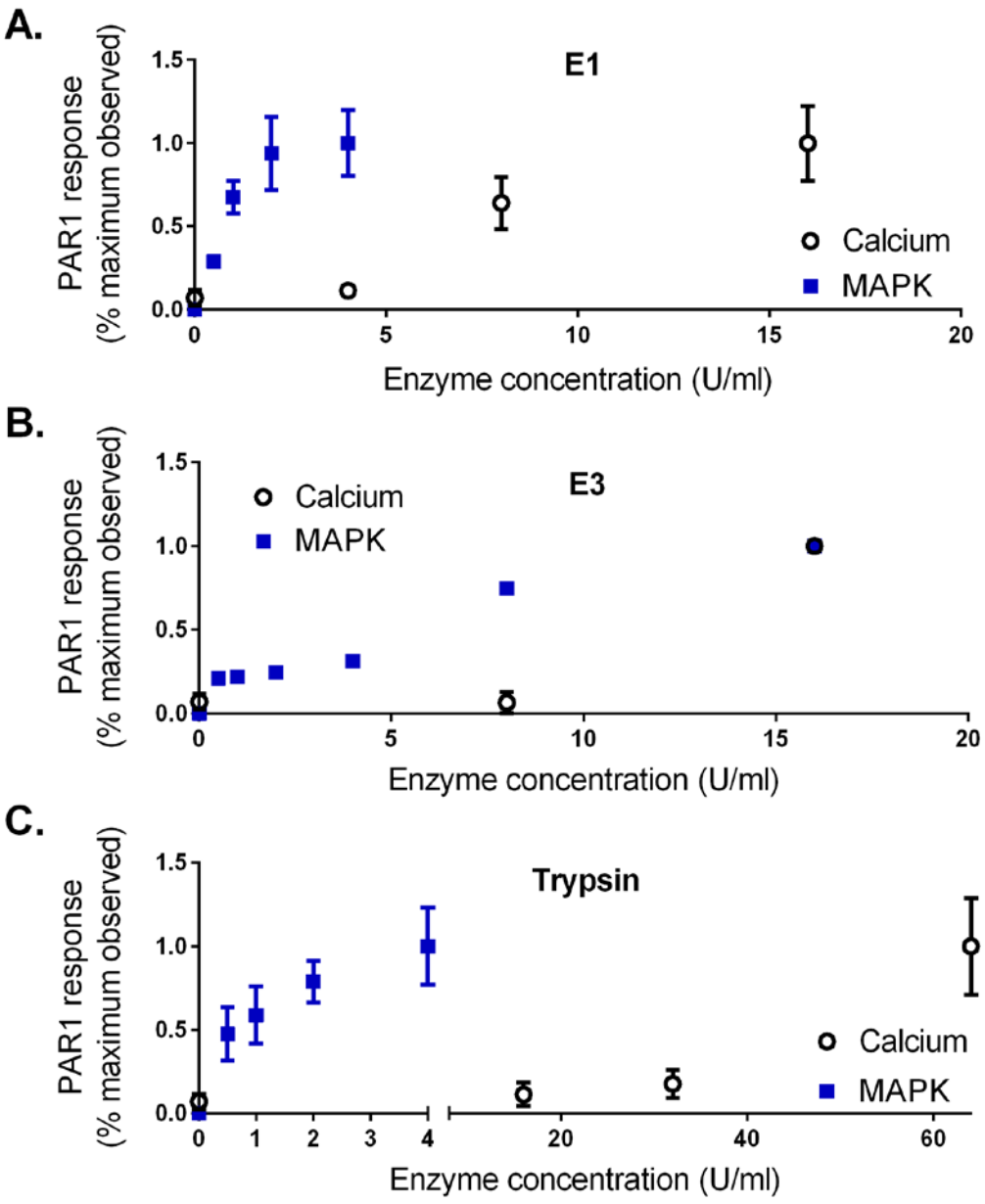

Figure 6.3.8 – Comparison of concentration-effect curves for the Ca^{2+} signaling and ERK 1/2 phosphorylation responses generated for the PAR1 biased agonists.

The curves generated from the Ca^{2+} responses (open circles) of the PAR1 biased agonist enzymes E1 **(A)**, E3 **(B)** and trypsin **(C)** were situated to the right of the curves generated from the ERK 1/2 phosphorylation responses (blue squares). These results illustrate the selective activation of PAR1-mediated MAPK signaling over Ca^{2+} signaling at low concentrations.

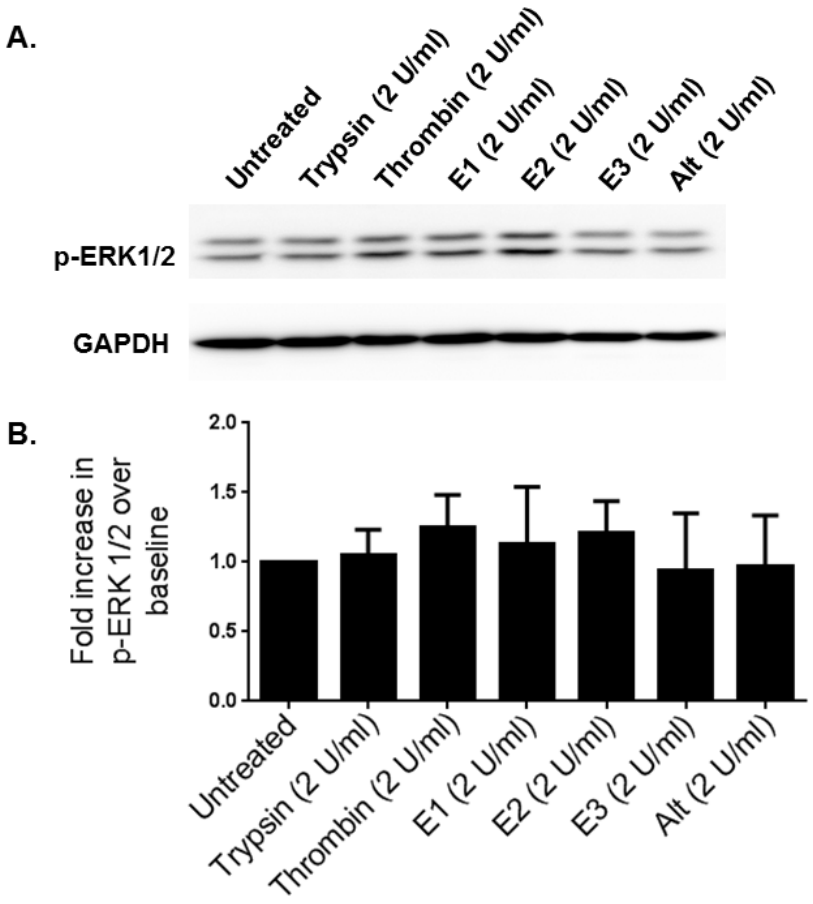

Figure 6.3.9 – ERK 1/2 phosphorylation in KNRK cells transfected with pcDNA vector alone.

There is no significant MAPK phosphorylation response activated by any of the allergen-derived or control proteinases in KNRK cells transfected with the pcDNA vector alone at a concentration of 2 U/ml. This concentration induces a strong MAPK signal in at least one of the PAR1- or PAR2- expressing cells for each enzyme, demonstrating the PAR-dependence of those responses. (n=3)

6.3.5 Cleavage and internalization of a dual-fluorescent PAR1 construct

With the ability of each allergen-derived enzyme to differentially activate signaling via PAR1 having been shown in the previous sections, the final aspect of signaling was

in receptor internalization kinetics. Conventional G protein coupled receptor signaling involves activation of the receptor, followed by termination of the signal and internalization of the receptor, followed by recycling to the membrane or, as with the PARs, degradation of the receptor. To monitor whether the distinct PAR1 activation profiles of each receptor translated into distinct receptor internalization, a dual-fluorescent PAR1 construct was used. A red mCherry tag was inserted in the receptor in the N-terminal tail upstream of the tethered ligand sequence, and a green YFP tag was inserted in the intracellular C-terminal domain of PAR1. In this system, an intact receptor is visualized by co-localized red and green signals (shown as a yellow signal). Receptor cleavage results in the release of the mCherry signal in the N-terminal pro-peptide, leaving only a green signal in the receptor. This green YFP signal can then be tracked to assess the internalization of the receptor; the green signal will either remain on the cell membrane, or be redistributed to foci in the cytoplasm (illustrated in Figure 6.3.10 A). Treating A549 cells expressing this dual-fluorescent PAR1 construct with a high concentration of thrombin (2 U/ml) reveals the impact of receptor activation by its 'canonical' enzyme-unmasked tethered ligand. Untreated cells express a yellow signal at the cell membrane, indicating the intact receptor (Figure 6.3.10 B); thrombin-treated cells fixed after 3 min express a green signal on the cell membrane, indicating a cleaved receptor (Figure 6.3.10C); thrombin-treated cells fixed after 30 min express green foci in the cytoplasm with minimal green signal localized to the cell membrane, indicating that the PAR1 of the cell has been internalized (Figure 6.3.10 D).

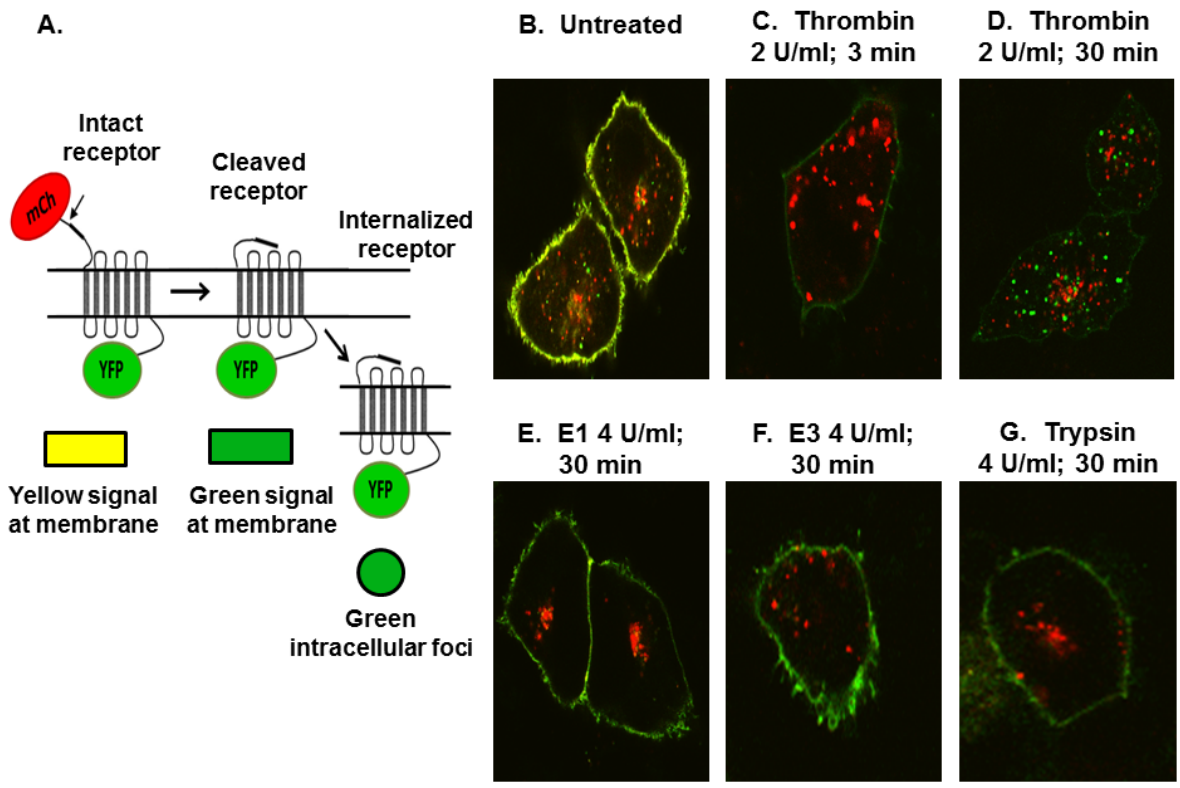

Figure 6.3.10 – Monitoring cleavage and trafficking of a dual-fluorescent PAR1 construct.

(A) A dual-fluorescent PAR1 construct, consisting of a C-terminal YFP tag and a N-terminal mCherry tag, was used to track receptor cleavage and trafficking. Compared to the membrane-localized yellow signal representing the intact receptor in untreated cells **(B)**, thrombin cleaved the receptor after 3 min, visualized by the disappearance of the red signal at the membrane **(C)**, and followed by receptor internalization to intracellular foci after 30 min **(D)**. By contrast, each of E1 **(E)**, E3 **(F)** and trypsin **(G)** cleaved the receptor but the green signal remained at the membrane after 30 min.

Treating the A549 cells expressing the dual-tagged PAR1 with E1, E3 or trypsin at 4 U/ml (Figure 6.3.10 E-G) reveals a distinct receptor activation process: cells fixed 30 min after treating with each of E1, E3 and trypsin express a green signal localized to the cell membrane, indicating that the receptor was cleaved in a way that did not induce internalization, as is caused by thrombin (Figure 6.3.10 D). Together these results indicate a novel signaling mechanism for trypsin-like enzymes via PAR1. They cleave the receptor in a way that activates the MAPK signaling arm, but not the Ca^{2+} signaling arm or the receptor internalization process.

6.4 Discussion

In Chapter 4, it was confirmed that the four trypsin-like enzymes contained in the cockroach and Alternaria allergens are distinct from one another, both biochemically as determined by enzyme kinetics, and in amino acid sequence as determined by mass spectral analysis. In this Chapter the regulation of signaling via both PAR1 and PAR2, with which potential physiological effects of each enzyme in the airway could be inferred, were explored. As expected, each of the allergen-derived trypsin-like enzymes were able to activate PAR2 in a similar manner to porcine trypsin; in addition to effectively cleaving the synthetic peptide at the canonical R36 cleavage site and the Nluc-tagged construct, each enzyme activated PAR2-dependent Ca^{2+} signaling and ERK 1/2 signaling at equivalent concentrations. The significance of these findings is that each of the three cockroach enzymes and the Alternaria enzyme are likely contributors to the PAR2-mediated enhancement of airway inflammation observed *in vivo* in allergy models. Therefore, all of the allergen enzymes may evoke similar effects in

environmental allergen exposure in asthma patients, and so all may represent therapeutic targets. As demonstrated in the previous Chapter, each of the enzymes has distinct substrate specificities and inhibitor sensitivities, so this consideration may be important in determining the most effective way to counteract this signaling mechanism in the clinical setting. The Alternaria enzyme in particular was not sensitive to SBTI inhibition in the PAR2 MAPK activation assay at a concentration of 1 U/ml, so it may not be valid to assume that using conventional trypsin inhibitors such as SBTI are as effective in neutralizing all allergen-derived trypsin-like enzymes as mammalian trypsin-like enzymes.

The ability of the allergen-derived enzymes to regulate PAR1 signaling differentially was an unexpected finding. Each enzyme cleaved the PAR1 tethered ligand peptide and the Nluc-tagged receptor. All of the enzymes except E3 activated MAPK signaling via PAR1 at relatively low concentrations. Cockroach E2 and the Alternaria enzyme also activated Ca^{2+} signaling via PAR1 at the same low concentrations, inducing a response akin to a non-biased PAR1 agonist such as thrombin. These findings for E2 and Alt are unusual for trypsin-like enzymes, which have conventionally been classified as disarmers of PAR1, cleaving the receptor at a different residue than the canonical activation site and thus preventing further PAR1 activation.

E1 and E3 are biased agonists for PAR1, preferentially activating PAR1-driven MAPK signaling over Ca^{2+} signaling. Treating cells expressing the dual-fluorescent PAR1 construct with these biased agonist enzymes reveals a mechanism in which the receptor is cleaved but remains at the cell membrane, which distinguishes PAR1

trafficking induced by these enzymes from thrombin, which induces the robust internalization of the receptor. This suggests a mechanism of receptor activation similar to that described for neutrophil elastase, which involves cleavage of the receptor at the non-canonical R46 cleavage site producing a truncated tethered ligand sequence which then induces the intracellular signal biased toward MAPK activation[136]. The specific cleavage site and mechanism of trypsin-driven biased signaling via PAR1 would be an interesting topic for future research.

This differential PAR1 activation is a novel aspect of the physiological effects driven by allergen-derived trypsin-like enzymes. Since, as described in the introduction of this thesis, cells throughout the airway have been found to express both PAR1 and PAR2, these findings suggest a scenario in which inhaled environmental allergen-derived proteinases activate both PAR1 and PAR2 concurrently. The specific contributions of each receptor to the physiological response in such a scenario have not been explored, but would be worthwhile avenue of future research. Specifically, any differences between the responses induced by the non-biased agonists E2 and Alt, in which the Ca^{2+} and MAPK signaling arms downstream of both PAR1 and PAR2 are activated, and the biased agonists E1 and E3, in which only the MAPK arm of the PAR1 signal is activated along with the non-biased PAR2 response. For example, it has been reported that the thrombin-driven differentiation of airway fibroblasts to myofibroblasts is dependent on the PAR1-mediated activation of the Ca^{2+}-dependent PKC signaling pathway[195]. A similar assay comparing the non-biased PAR1 agonists to the biased PAR1 agonists, which presumably differ in their ability to activate PKC downstream of

PAR1 cleavage, would be valuable in determining the specific effects of each enzyme and each receptor in the airway response to inhaled allergens.

Another interesting distinction between cellular responses induced by the allergen-derived proteinases was in the recruitment of β-arrestin following the activation of PAR2. Treatment with each of the cockroach enzymes at a concentration of 1 U/ml resulted in the significant recruitment of at least one β-arrestin isoform, whereas the treatment with the Alternaria enzyme did not. Equivalent concentrations of the same Alternaria fraction used in this assay robustly activated both Ca^{2+} signaling and MAPK activation in the PAR2-expressing KNRK cells, so the lack of response in the BRET assay was unexpected. A collaborator, Dr. Kathryn Defea, conducting similar experiments has demonstrated in unpublished data that the Alternaria enzyme can induce the recruitment of β-arrestin to PAR2, so a comparison of the experimental designs would be beneficial. β-arrestin signaling downstream of PAR2 activation, rather than G-protein signaling, has been proposed as crucial in the pro-inflammatory response seen *in vivo*[196,197]. Since Alternaria allergen has been shown to enhance airway inflammation dependent on trypsin-like activity and PAR2 signaling, the ability or inability of the Alternaria-derived trypsin-like enzyme to drive β-arrestin recruitment to PAR2 will be important to determine to elucidate its role in airway inflammation.

In summary, these data demonstrate that each of the four isolated allergen-derived enzymes are non-biased agonists of PAR2 and differentially regulate signaling via PAR1. Therefore, each enzyme could possibly induce different physiological responses when presented to the airway, which presents a more complex picture of the effects of allergen-derived trypsin-like enzymes in the airway. However, since the PAR

expression system used in this Chapter is an artificial overexpression system, our ability to infer similar PAR regulation in the airway is limited. Therefore, in the next Chapter we assess the ability of the allergen-derived enzymes to regulate PAR signaling in more physiologically relevant airway-derived cells and tissues.

CHAPTER 7 REGULATION OF PAR SIGNALING BY THE ALLERGEN-DERIVED PROTEINASES IN AIRWAY-DERIVED CELLS AND TISSUES

7.1 Introduction

In the previous chapters, the presence of trypsin-like proteinases in cockroach and Alternaria *alternata* allergens that can target and activate signaling via PARs 1 and 2 was established. Our final aim was to assess (1) the expression of active PAR1 and PAR2 in airway-derived cells and tissues, (2) the ability of the allergen-derived enzymes to activate PAR signaling and (3) the physiological effects of PAR activation in these cells and tissues. Each of the cockroach- and Alternaria-derived enzymes was shown to be non-biased agonists for PAR2, and to regulate PAR1 signaling differentially. However, the PAR-overexpressing KNRK cells used in the previous Chapter represent an artificial system used to define possible targets of the allergen-derived proteinases, and so the physiological effects of PAR activation by the allergen-derived enzymes may not be reliably inferred. For this reason, the expression of PARs and the ability of the allergen-derived enzymes to regulate PAR signaling in airway-derived cells and tissues were explored. Assessing this ability in a more tissue-relevant system could then allow the physiological effects of PAR signaling in the airway reported in the literature to be applied to each of the allergen enzymes.

The ability of the allergen-derived enzymes to activate PAR signaling in airway-derived cell lines was assessed. As in Chapter 6, both Ca^{2+} signaling and MAPK activation were used as indicators of PAR activation, and the expression of the pro-inflammatory cytokine IL-8 was monitored as a model physiological effect following exposure of the cells to the allergen-derived enzymes. Additionally, signaling via both

PAR1 and PAR2 has been found to regulate smooth muscle tone in vascular and bronchial tissues[106]. To assess the ability of the allergen-derived enzymes to regulate processes at the tissue-level via PAR1 and PAR2, smooth muscle relaxation in aortic and bronchial tissues harvested from PAR1- or PAR2-null mice was monitored. Much of the work in this Chapter is preliminary, establishing the principle for future experiments. Assessing the reproducibility of some of the data and performing statistical analyses will be the targets of future work. However, a detailed examination of the *in vitro* physiological effects of PAR signaling by allergen-derived enzymes was outside the scope of this thesis.

7.2 PAR signaling in airway-derived cultured cell lines

First, we aimed to assess the presence of PARs 1 and 2 in common airway-derived cell lines: type II alveolar carcinoma-derived A549 cells and transformed bronchial epithelial cell-derived BEAS-2B cells. Fluo-4 dye uptake in monolayers of both of the cell lines used was inconsistent, with variable responses both to PAR agonists and to the Ca^{2+} ionophore A23187, so Ca^{2+} signaling for these cells was performed in suspension. Due to the larger volumes required for the Ca^{2+} signaling assay in suspension, only the most abundant and easily isolated enzyme, cockroach E1, was applied to the A549 cells, and only PAR agonist peptides were used with the BEAS-2B cells.

7.2.1 PAR-mediated Ca^{2+} signaling in A549 cells

Treating dye-loaded A549 suspensions with both the PAR1-activating peptide TFLLR (PAR1; Figure 7.2.1 A, left traces) and the PAR2 activating peptide 2fLI (PAR2; Figure 7.2.1 A, right trace) resulted in positive Ca^{2+} transients, indicating the presence of both receptors in A549 cells. The signal elicited by TFLLR at a concentration of 10 µM was relatively low, suggesting a low expression level of active PAR1 in the A549 cells, so a second dose of 100 µM was also used, which induced a Ca^{2+} transient with a higher magnitude. Since the higher concentration of TFLLR can also activate PAR2[111], the cells were also desensitized for PAR2 by administering 2fLI prior to adding the PAR1 peptide (Figure 7.2.1 A, right trace). The TFLLR signal was not diminished following PAR2 desensitization, confirming the presence of active PAR1 in the A549 cells.

The expression of PAR1 appeared to be low, so the response induced by thrombin was also assessed. Treating the cells with a thrombin concentration of 2 U/ml, which is a saturating concentration in most PAR1-expressing cells such as the KNRK-PAR1 cells used previously, evoked a slight Ca^{2+} transient (Figure 7.2.1 B, left trace). Some cells have been found to secrete cysteine proteinases that can disarm PAR1, preventing activation by thrombin, so the cells were also incubated for 24 h with the cysteine proteinase inhibitor E-64 to assess this possibility. This treatment did not appreciably increase the Ca^{2+} response to a 2 U/ml dose of thrombin (Figure 7.2.1 B, middle trace). A 4 U/ml treatment did increase the response, and a second treatment did not induce a response indicating that this concentration fully desensitized the cells to PAR1 and induced a maximal response (Figure 7.2.1 B, right trace).

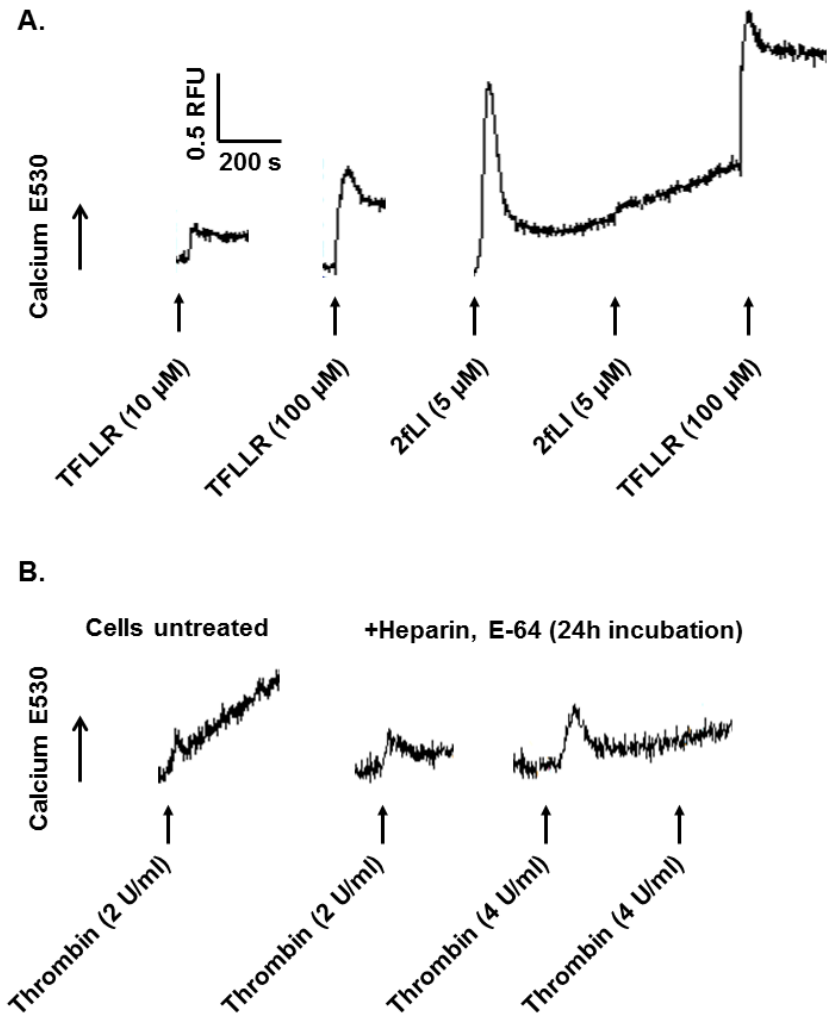

Figure 7.2.1 – Detection of functional PAR1 and PAR2 in A549 cells with a Ca^{2+} signaling assay.

(A) Application of both the PAR1 agonist peptide TFLLR and the PAR2 agonist peptide 2fLI evoked Ca^{2+} responses in the A549 cells. 10 µM TFLLR induced a weak response, so the concentration was raised to 100 µM. That concentration that can target PAR2, so to confirm that the response is due to PAR1 activation the TFLLR response was repeated following desensitization of the cells with 2fLI. **(B)** Similar to the TFLLR response, the PAR1 activating enzyme thrombin evoked a weak Ca^{2+} response at a concentration of 2 U/ml. Since many cells can release endogenous proteinases that can disarm PAR1, the cells were pre-treated with the cysteine proteinase inhibitor E-64 and heparin, which did not appreciably increase the thrombin response. A concentration of 4 U/ml was found to be saturating, as a second application did not evoke a response.

Having confirmed the expression of PAR1 and PAR2 in the A549 cells, the ability of cockroach E1 to activate Ca^{2+} signaling via the PARs in these cells was also assessed. Administering E1 to the cells resulted in a positive Ca^{2+} transient (Figure 7.2.2, left trace). Desensitizing the cells for PAR2 with 2fLIGRL prior to administering E1 completely blocked the signal induced by E1, indicating that the Ca^{2+} transient evoked by E1 was entirely PAR2-dependent (Figure 7.2.2, right trace).

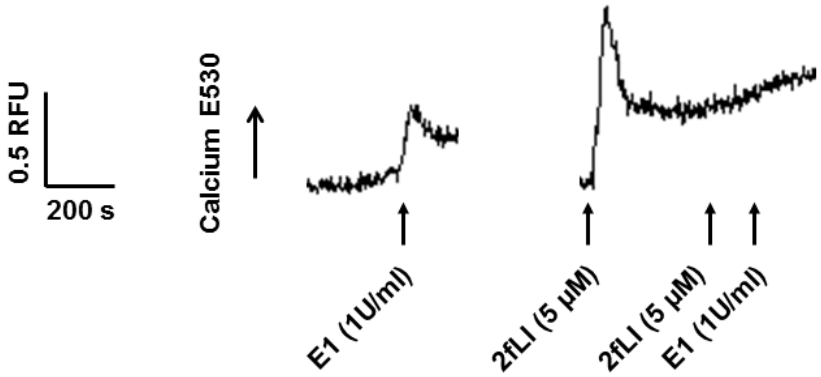

Figure 7.2.2 – Cockroach E1 activates PAR2-mediated Ca^{2+} signaling in A549 cells.

A 1 U/ml concentration of E1 induced a positive Ca^{2+} response in the A549 cells (left trace). Desensitizing the cells for PAR2 with 2fLI eliminated the E1-induced Ca^{2+} signal, indicating that the E1-mediated response is via activation of PAR2 and not PAR1 (right trace).

7.2.2 PAR-mediated MAPK signaling in A549 cells

In addition to the Ca^{2+} signaling assays, the ability of the allergen-derived enzymes to activate PAR-mediated MAPK signaling was assessed. First, the ability of conventional PAR1 and PAR2 agonists to activate MAPK signaling in these cells was confirmed using trypsin and 2fLI (PAR2), as well as thrombin and TFLLR (PAR1). All of

the control agonists were found to induce the phosphorylation of ERK 1/2 in the A549 cells (Figure 7.2.3).

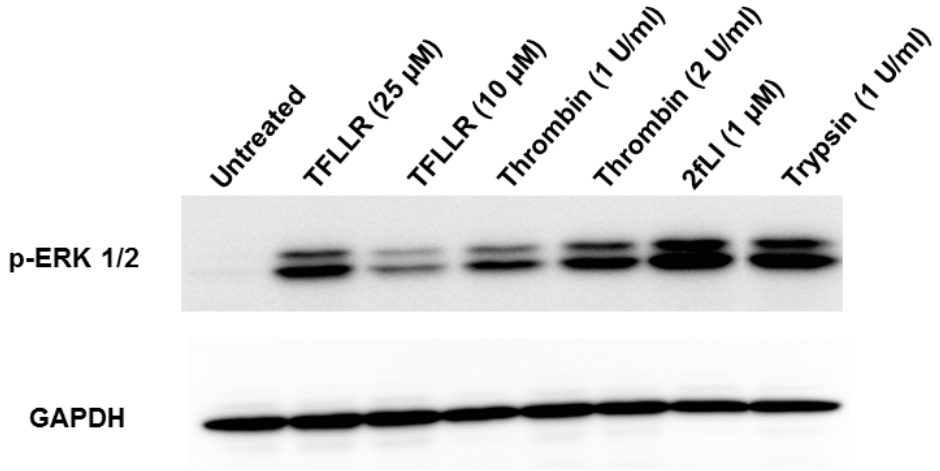

Figure 7.2.3 – PAR-mediated ERK 1/2 phosphorylation in A549 cells.
The PAR1 agonists TFLLR and thrombin and the PAR2 agonists 2fLI and trypsin all induced the phosphorylation of ERK 1/2 in A549 cell monolayers.

Additionally, the ability of the allergen-derived enzymes to activate MAPK signaling in the A549 cells was assessed. Each of E1, E2, E3 and Alt induced the phosphorylation of ERK 1/2 at a concentration of 1 U/ml, and these responses were blocked or reduced by pre-treating the enzymes with 50 μM SBTI (Figure 7.2.4). In this assay, E2 demonstrated a resistance to SBTI inhibition, whereas the Alt-induced response was reduced by pre-incubation with the inhibitor. These results contrast with the results shown in Chapter 6 (Figures 6.2.4 and 6.3.5). Aliquots of the same fractions containing each enzyme were used in this assay and in the MAPK activation assays in Chapter 6, so the nature of this observed difference in SBTI sensitivities is unknown.

Further experiments to explore this difference, as well as to assess the PAR-dependence of the responses induced, may be necessary.

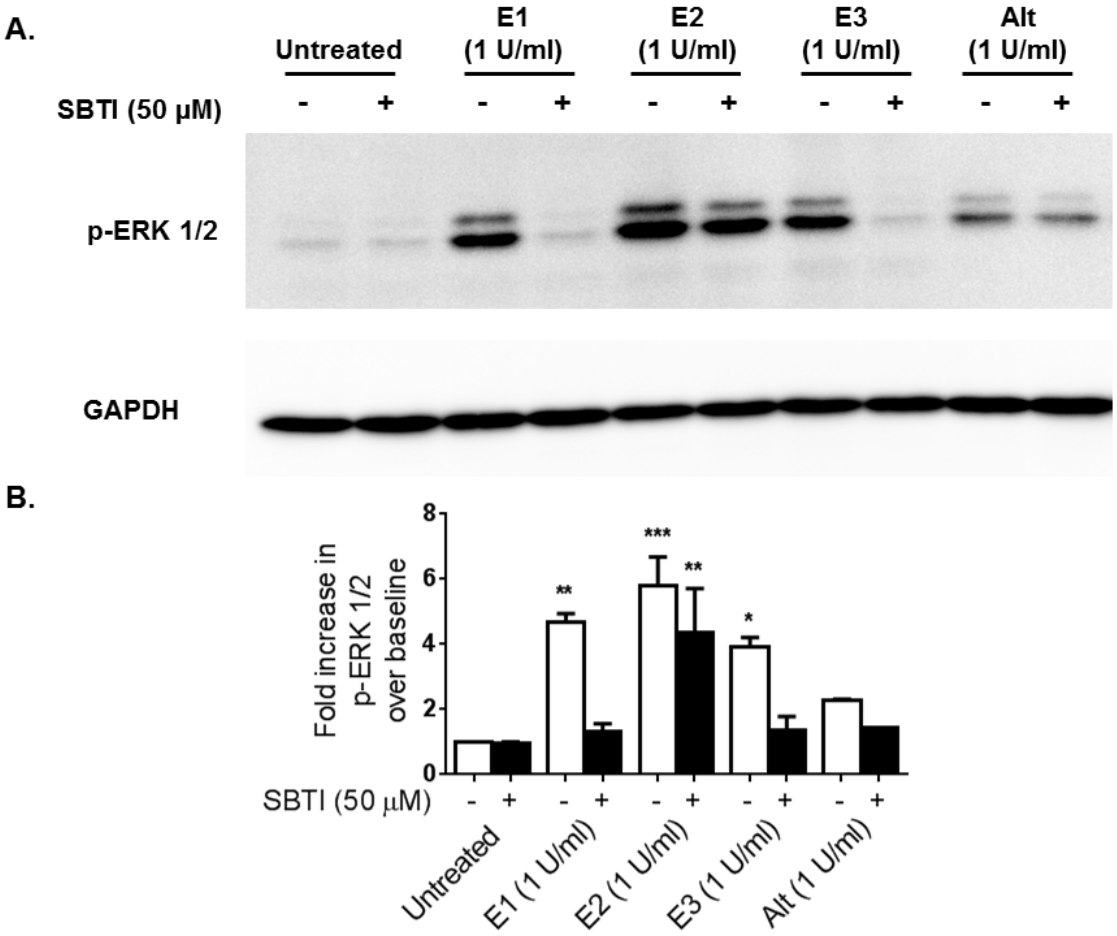

Figure 7.2.4 – Allergen-derived enzyme-mediated ERK 1/2 phosphorylation in A549 cells.

Treatment of A549 cell monolayers with 1 U/ml concentrations of each of cockroach E1, E2, E3 resulted in the phosphorylation of ERK 1/2. Pre-treatment of each enzyme with SBTI prior to administering to the cells resulted in the elimination or reduction of the ERK 1/2 activation response. (Data are presented as the mean with range, P values are relative to the untreated control; *p<0.05, **p<0.01, ***p<0.001; n=2)

7.2.3 Allergen proteinase-driven IL-8 expression in A549 cells

Finally, the ability of the allergen-derived enzymes to induce the expression of IL-8 at the mRNA level in the A549 cells was assessed as a marker of the pro-inflammatory physiological effects of allergen-mediated PAR2 signaling. Both of E1 and Alt induced a significant increase in Il-8 mRNA (Figure 7.2.5). Trypsin and thrombin controls also induced a significant expression of IL-8.

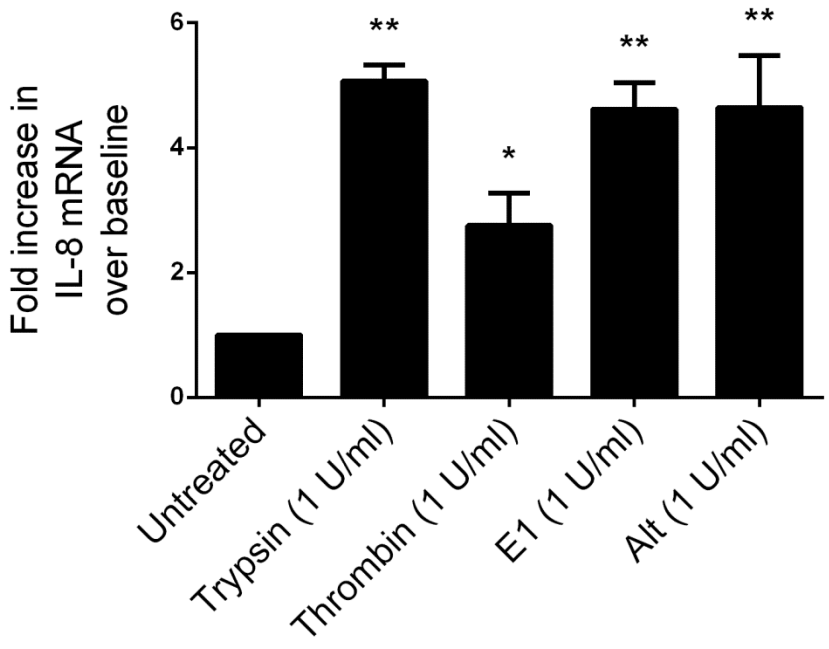

Figure 7.2.5 – Allergen-derived enzymes induce the expression of Il-8 in A549 cells.

1 U/ml concentrations of each of porcine trypsin, thrombin, cockroach E1 and the Alternaria enzyme (Alt) induced the expression of IL-8 mRNA in A549 cells. (p values are relative to the untreated control. **p<0.01, *p<0.05; n=3)

7.2.4 PAR-mediated Ca^{2+} signaling in BEAS-2B cells

The expression of PAR1 and PAR2 in BEAS-2B cells was also assessed by monitoring Ca^{2+} signaling as above. This cell line was used to compare the relative functional PAR expression levels between the BEAS-2B cells and the A549 cells. Application of 25 µM TFLLR and 5 µM 2fLI both elicited robust Ca^{2+} signals (Figure 7.2.6), indicating a high expression level of both PAR1 and PAR2.

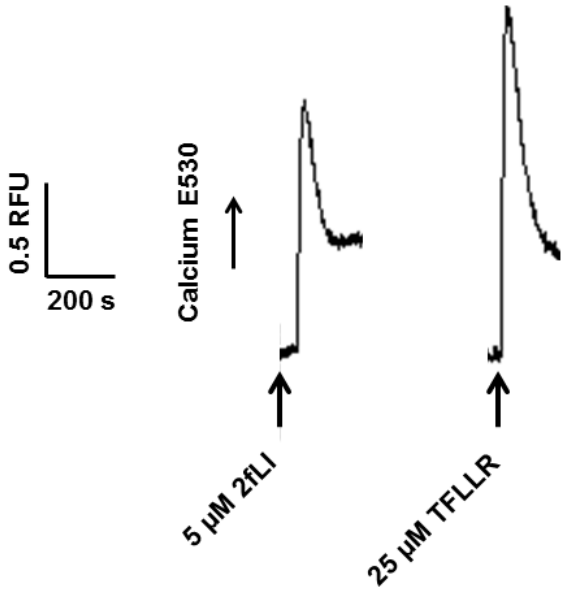

Figure 7.2.6 – Detection of functional PAR1 and PAR2 in BEAS-2B cells with a Ca^{2+} signaling assay.

Application of both 2fLI (left tracing) and TFLLR (right tracing) induced strong Ca^{2+} signals in the BEAS-2B cells, indicating that both PAR1 and PAR2 are highly expressed.

7.3 Allergen proteinase-mediated vascular relaxation in mouse aortic tissue

7.3.1 Vascular relaxation via PAR2 in PAR1-null mouse aortic tissue

The data in the previous sections of this Chapter have demonstrated both the presence of PARs 1 and 2 in airway-derived cells, and that the allergen-derived proteinases can signal via the PARs in these airway-derived cells. These findings thus bring the PAR signaling results in the artificial PAR expression system from Chapter 6 into physiological relevance, and the finding that both E1 and Alt induce the expression of IL-8 in the A549 cells suggests that each can promote a potential pro-inflammatory response in the airway. Our final aim was to demonstrate the ability of the allergen enzymes E1 and Alt to regulate PAR signaling at the tissue level by monitoring smooth muscle relaxation in whole tissue mounts. First, mouse aortic tissue was used as a model system due to determine whether activation of the PARs by the allergen-derived enzymes could be detected at the tissue level. To obtain the data presented in the following sections, the experiments were designed, set up and performed with the crucial assistance Dr. Mahmoud Saifeddine.

Aortic tissues harvested from PAR1-null mice were used to assess the enzymes' abilities to induce vascular smooth muscle relaxation via PAR2. Each of the preparations were pre-treated with 2.5 μM of the α1-adrenergic receptor agonist phenylephrine to contract the smooth muscle, and a control treatment with 1 μM acetylcholine verified the ability of the aortic rings to relax via endothelial GPCR signaling (Figure 7.3.1 A, B left tracings). Phenylephrine-treated tissues were subsequently treated with the Alternaria enzyme at a concentration of 0.6 U/ml, which induced a robust relaxation (Figure 7.3.1 A, right tracing). Pre-treating the tissues with

the nitric oxide synthase (NOS) inhibitor N^5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride (L-NAME) prior to the addition of the Alternaria enzyme eliminated the Alt-induced relaxation (Figure 7.3.1 B). This indicates that the Alternaria response is mediated by the synthesis of nitric oxide.

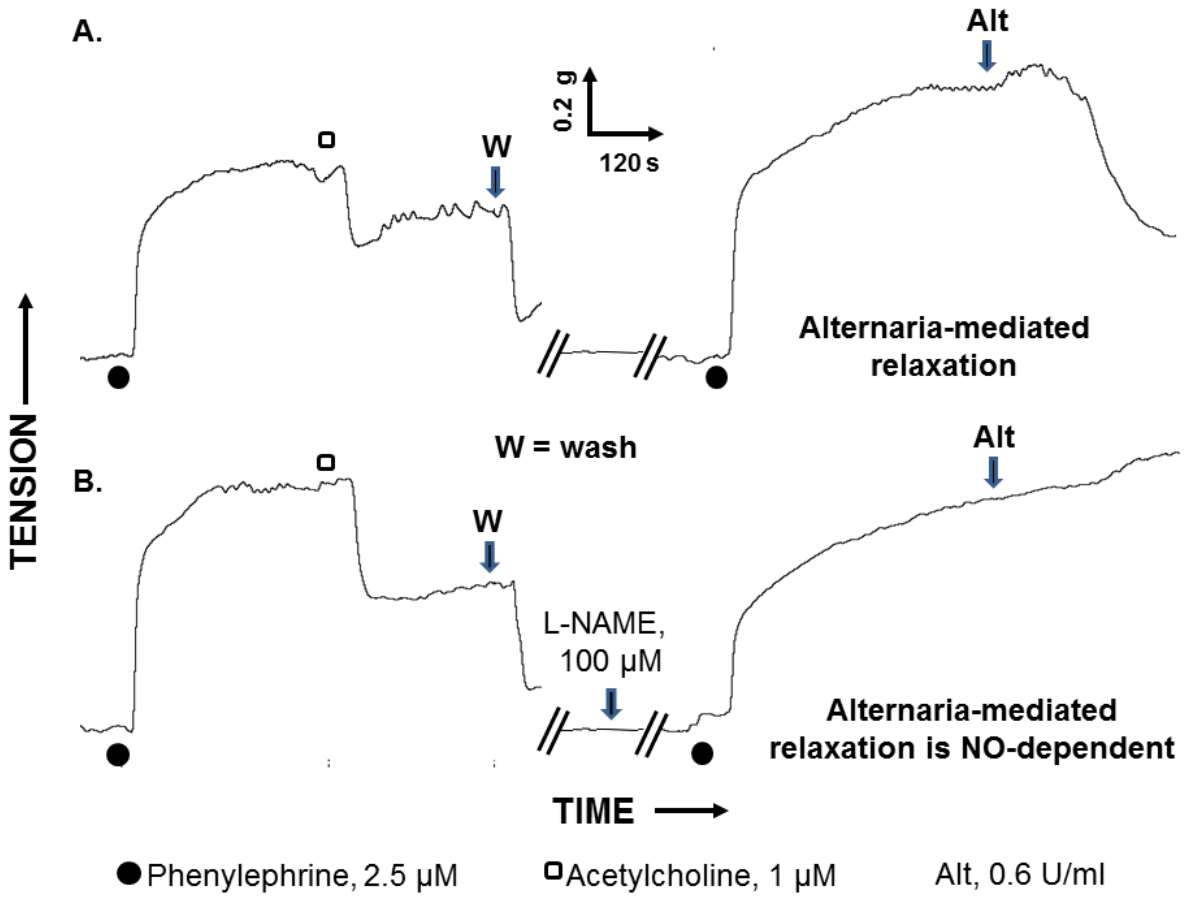

Figure 7.3.1 – Alt-induced aortic relaxation in PAR1-null tissue.

(A) Application of 0.6 U/ml Alt to the PAR1-null aortic tissue resulted in a strong relaxation response (right tracing). **(B)** Pre-treatment of the tissue with the nitric oxide synthase inhibitor L-NAME eliminated the Alt-induced relaxation. This result indicates that the Alt-induced response is dependent on the generation of nitric oxide. (The left tracings in both (A) and (B) confirm the viability of the endothelium in the tissues by demonstrating contractility to acetylcholine.)

The ability of cockroach E1 to regulate vascular tone was assessed in a similar experiment (Figure 7.3.2). As above, the viability of the tissues was assessed by first inducing a contraction response with 2.5 µM phenylephrine, followed by a relaxation response with 1 µM acetylcholine (left tracings). Following a wash step, application of 1 U/ml E1 to the phenylephrine-treated tissues induced a strong relaxation response (Figure 7.3.2 A, right tracing). As above, pre-treatment of the tissue with L-NAME eliminated the E1-induced relaxation response, indicating that this response is also NO-dependent (Figure 7.3.2 B, right tracing).

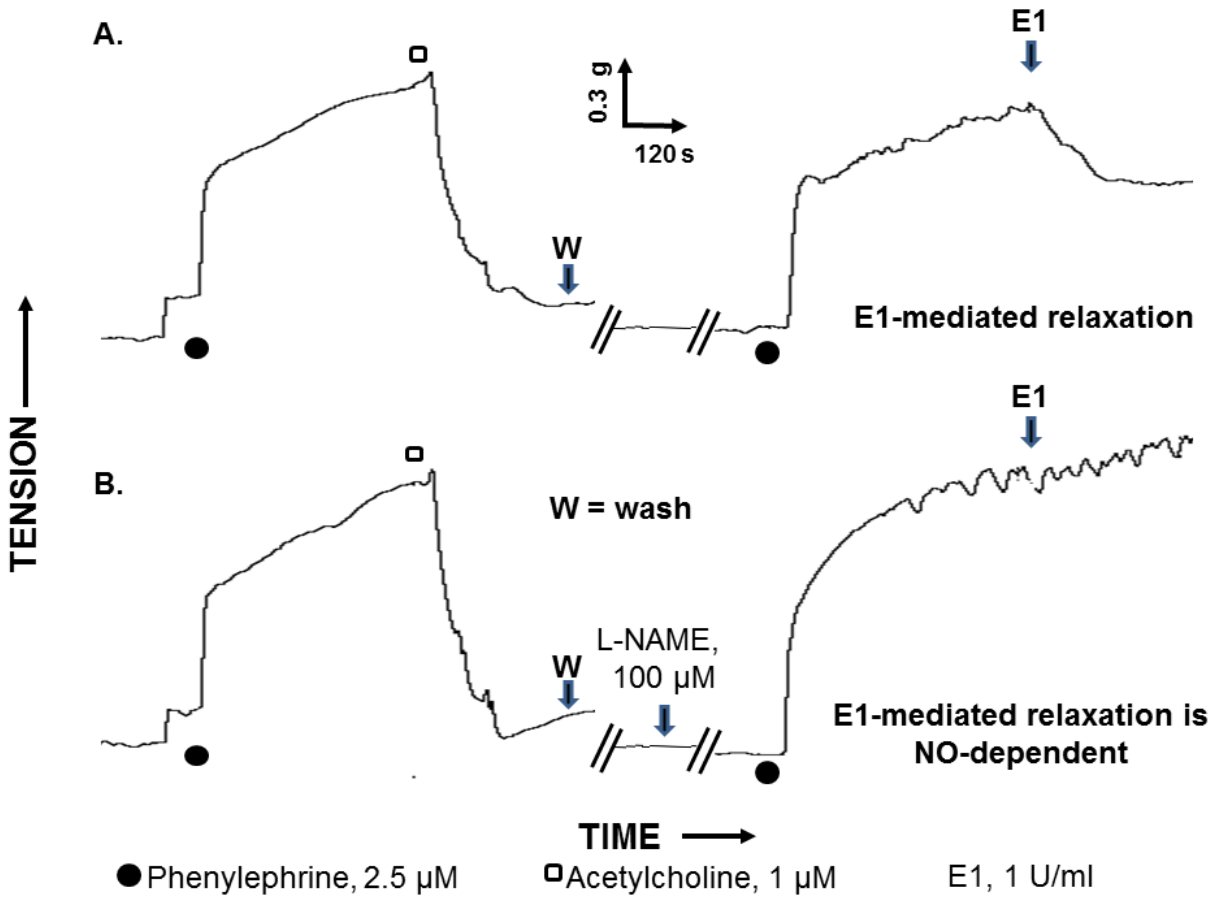

Figure 7.3.2 – E1-induced aortic relaxation in PAR1-null tissue.

(A) Treatment of PAR1-null aortic tissue with 1 U/ml E1 induced a relaxation response. **(B)** Pre-treatment of the tissue with L-NAME eliminated the E1-induced relaxation, indicating that this effect is dependent on nitric oxide synthesis.

7.3.2 Vascular relaxation via PAR1 in PAR2-null mouse aortic tissue

Similarly, aortic tissues harvested from PAR2-null mice were used to assess the enzymes ability to signal via PAR1. As above, phenylephrine and acetylcholine were applied to the tissues to verify their viabilities. Phenylephrine-contracted tissue was treated with 1 µM of 2fLI to confirm the absence of PAR2 in the tissue, followed by application of 0.6 U/ml of the Alternaria enzyme, which induced a robust relaxation in the tissue (Figure 7.3.3 A). To verify that the Alt-induced relaxation was due to the activation of PAR1, the phenylephrine-contracted tissues were desensitized for PAR1with saturating 25 µM concentrations of TFLLR, following which treatment with 0.6 U/ml Alt failed to induce a relaxation response (Figure 7.3.3 B), confirming the PAR1-dependence of the initial response. Treating phenylephrine-contracted tissues with 1 U/ml E1 did not result in tissue relaxation, indicating that this enzyme does not signal via PAR1 in the aortic tissue (Figure 7.3.4). The tissues were responsive to acetylcholine following the application of E1, demonstrating the viability of the endothelium in the preparation.

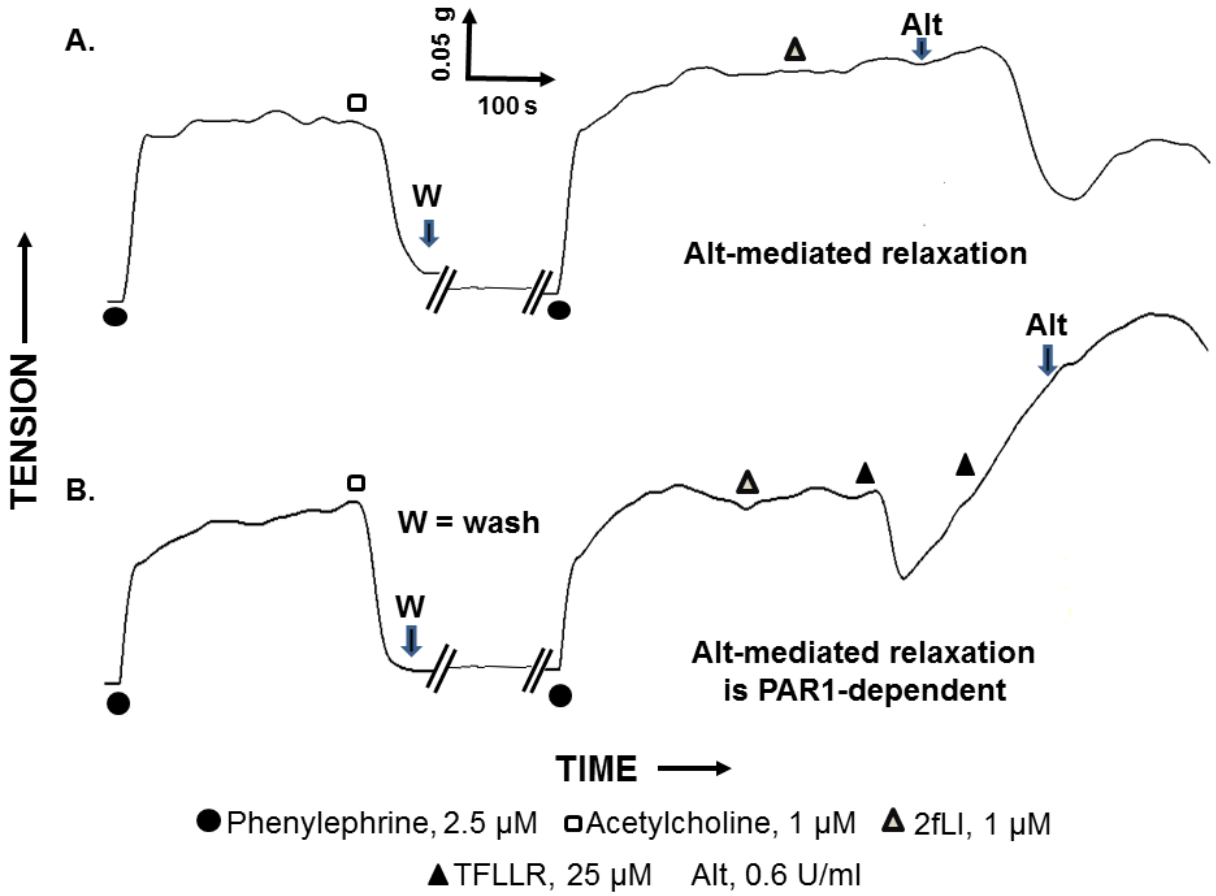

Figure 7.3.3 – Alt induces PAR1-dependent aortic relaxation in PAR2-null tissue. **(A)** The application of 0.6 U/ml of the Alternaria enzyme (Alt) to the PAR1-null aortic tissue results in a strong relaxation response. Prior administration of 2fLI (open triangle) did not induce a response, confirming the absence of PAR2. **(B)** Pre-desensitizing the tissue for PAR1 with 25 µM TFLLR (black triangle) eliminated the Alt-induced response, which indicates that the Alt response is PAR1-dependent.

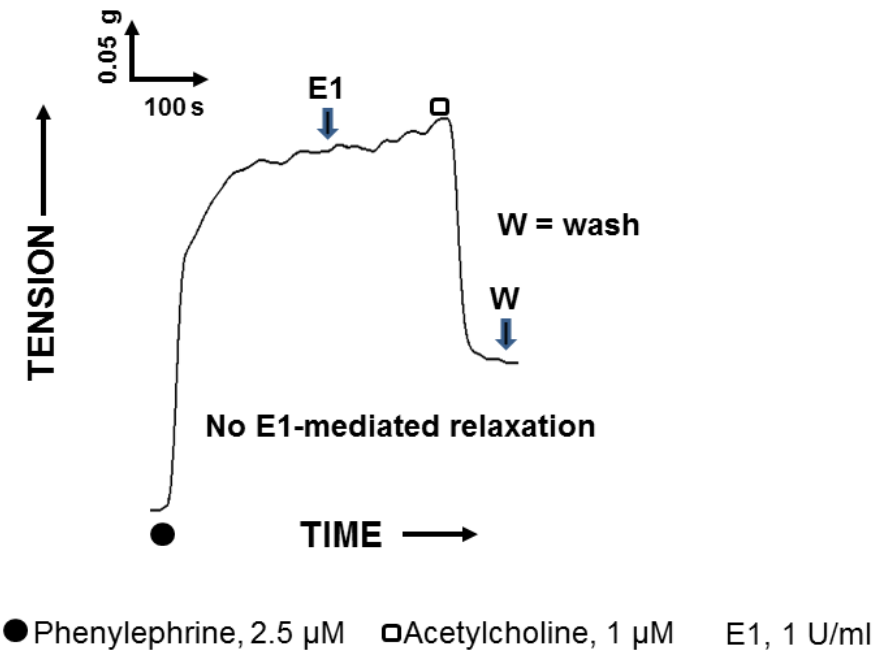

Figure 7.3.4 – E1 does not induce relaxation in PAR2-null aortic tissue.
Treatment of phenylephrine-contracted PAR2-null bronchial tissue with 1 U/ml E1 does not result in a relaxation response. This result indicates that this enzyme cannot induce a relaxation response via PAR1, and that the relaxation response observed in the PAR1-null tissue is PAR2 dependent. The subsequent application of 1 µM acetylcholine verified the viability of the tissue.

7.4 Alt-mediated relaxation in mouse bronchial tissue

7.4.1 Alt-induced bronchial relaxation via PAR2

In the previous Section, the allergen-derived enzymes were shown to differentially regulate the PARs in a manner reflective of the data presented in Chapter 6; E1 was found to activate smooth muscle contraction via PAR2 but not PAR1, whereas Alt signaled via both receptors. However, since the aorta does not represent a tissue relevant for the pro-allergenic action of allergen-derived proteinases, we

performed similar experiments assessing the ability of Alt to regulate both PAR1 and PAR2 signaling in mouse bronchial tissues. Bronchial rings harvested from PAR1-null mice were contracted with 1 µM of the cholinergic receptor agonist carbachol, following which they were relaxed by treating with 20 nM of the neuropeptide substance P to assess the viability of the epithelium in the preparation (Figure 7.4.1, left tracing). The tissues were then washed and allowed to recover for over 10 min, following which they were again contracted with carbachol treatment and exposed to 0.6 U/ml of the Alternaria enzyme, which induced a robust relaxation of the tissue (Figure 7.4.1, right tracing).

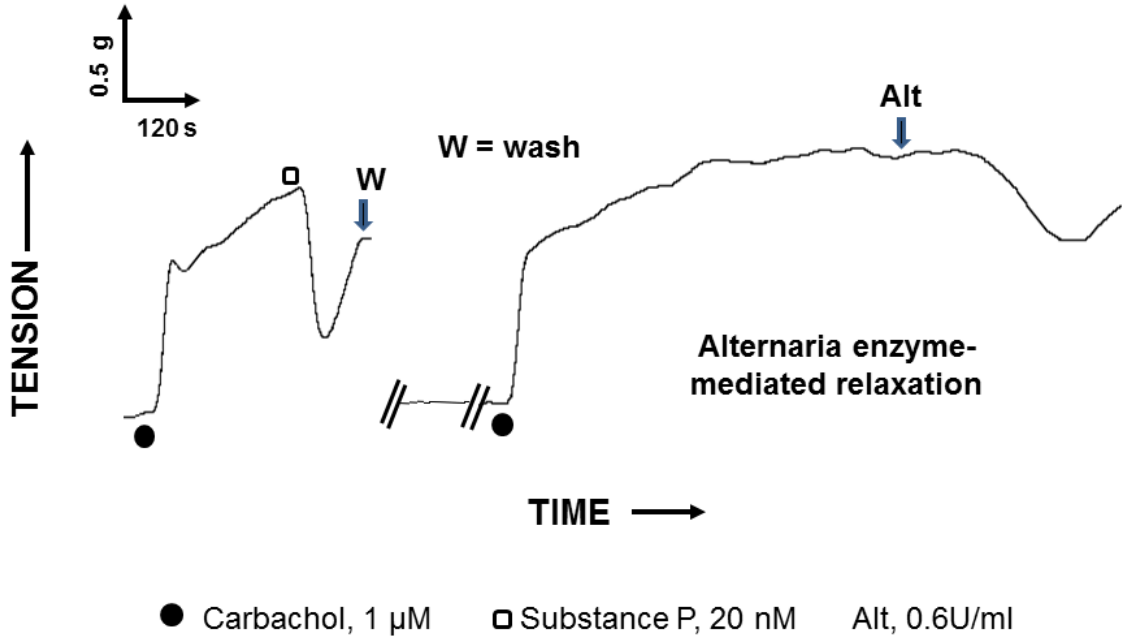

Figure 7.4.1 – Alt-induced relaxation of PAR1-null bronchial tissue.
Applying 0.6 U/ml of the Alternaria enzyme (Alt) to PAR1-null bronchial tissue evokes a robust relaxation response. The left trace of substance P-mediated relaxation demonstrates an intact epithelium in the preparation.

7.4.2 Alt-induced bronchial relaxation via PAR1

As above, bronchial tissues harvested from PAR2-null mice were used to assess the regulation of bronchial relaxation by the Alternaria enzyme via PAR1. The viability of the tissue preparations were tested in the same way, by contracting with carbachol and relaxing with substance P (Figure 7.4.2 A, B; left tracings). After a wash and recovery period, the tissues were contracted with carbachol treatment and 0.6 U/ml of the Alternaria enzyme was applied, which again induced a strong relaxation response (Figure 7.4.2 A, right tracing). It has been reported that PAR-mediated bronchial relaxation is due to epithelial production of cyclooxygenase (COX) products[198], so to assess whether this Alt-induced relaxation response was mediated by a similar mechanism the tissue was pre-treated with 3 μM of the COX inhibitor indomethacin (Figure 7.4.2 B). The relaxation response induced by 0.6 U/ml Alt was eliminated in the indomethacin-treated tissue (Figure 7.4.2 B, right tracing), indicating that the enzyme's relaxation response is dependent on COX activity.

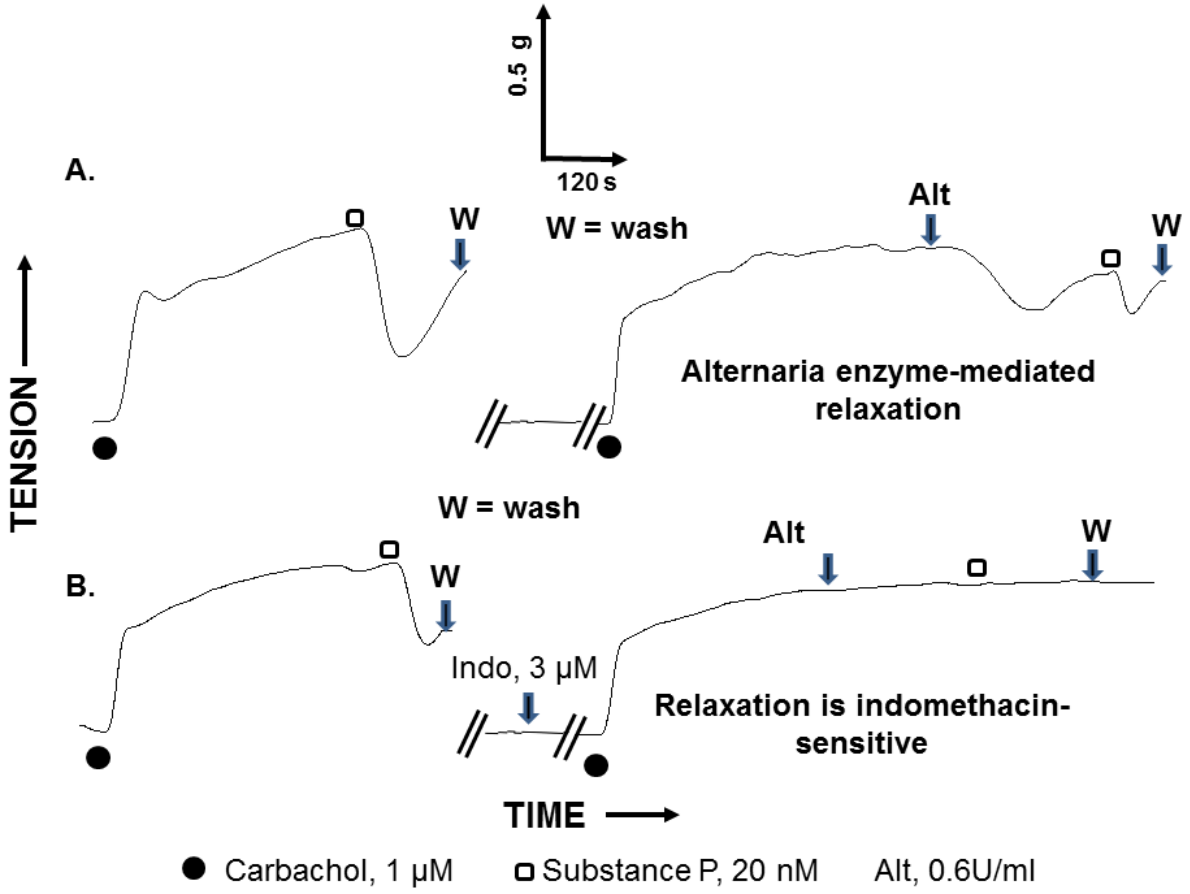

Figure 7.4.2 – Alt-induced relaxation of PAR2-null bronchial tissue.

(A) Applying 0.6 U/ml Alt to the carbachol-contracted bronchial tissue results in a robust relaxation response. **(B)** Pre-treatment of the tissue with the COX inhibitor indomethacin eliminates the Alt relaxation response, indicating that this response is due to the release of COX products.

7.5 Discussion

In this Chapter, preliminary data suggested that the allergen-derived enzymes regulate PAR signaling in airway-derived cells and tissues, extending the PAR regulation observed in Chapter 6 in the PAR-expressing KNRK cells to systems more relevant for defining the physiological effects of the enzymes. Ca^{2+} signaling and MAPK phosphorylation assays, similar to those presented in Chapter 6, were used in airway epithelial-derived cell lines. These assays were used to assess both the presence and apparent abundance of active PARs in these cell lines, as well as the ability of the allergen-derived enzymes to activate similar signaling pathways downstream of PAR activation in the airway cells as in the artificial PAR2-expressing KNRK cell lines. However since both cell lines were found to express both PAR1 and PAR2, further experiments would be necessary to assess the ability of the enzymes to regulate each receptor individually. However, given the apparently low expression of PAR1 in the A549 cells, it is likely that the majority of the responses induced by the allergen-derived enzymes in these cells are due to PAR2 activation. Both of E1 and Alt were also shown to induce the expression of IL-8, though the PAR-dependence of this effect will need to be assessed in future experiments. However, this result could provide a pathophysiological role of these isolated enzymes in the airway.

An interesting finding in comparing the Ca^{2+} responses evoked in both cell lines was the difference in magnitude of the PAR1 responses; the A549 cells required high concentrations of PAR1 agonists for a visible response to be induced, whereas the BEAS2-B cells responded robustly to both thrombin and TFLLR. This finding indicates that the two cell lines express different levels of functional PAR1, which could have

implications in the choice of a cell line for a model assessing the physiological role of epithelial PAR1 in the airway.

Finally, the ability of the allergen-derived enzymes to regulate PAR-mediated responses at the tissue level was assessed in aortic and bronchial tissues. Of particular interest was determining whether the differential regulation of PAR1 observed with the allergen enzymes in Chapter 6 could be replicated at the tissue level; due to a lack of enzyme abundance my main focus was the comparison of cockroach E1, a biased agonist for PAR1, to the Alternaria enzyme, a non-biased PAR1 agonist. As expected, both enzymes induced relaxation in the PAR1-null tissues. These responses were sensitive to pre-treatment of the tissues with the nitric oxide synthase inhibitor L-NAME, which is consistent with the previously reported mechanism of endothelial PAR-mediated vasorelaxation[199]. Again as expected, only Alt induced relaxation in the PAR2-null aortic tissue, and this relaxation was blocked by desensitizing the PAR1 in the tissue with the PAR1-activating peptide TFLLR. These results support the conclusion from Chapter 6 that these two enzymes regulate PAR1 signaling differentially, and extend these signaling properties beyond the artificial KNRK expression system used in that Chapter.

Similar relaxation responses in bronchial rings harvested from both PAR1- and PAR2-null mice by the Alternaria enzyme suggest that the ability of that enzyme to activate both PAR1 and PAR2 extends to the airway. We could not directly assess the PAR-dependence of the responses in the bronchial tissues, since these tissues do not recover well from the prior desensitization protocol used in the aortic tissue. However, the elimination of the Alt-induced relaxation response following pre-treatment of the

tissue with indomethacin the COX inhibitor indomethacin suggests a relaxation mechanism that is consistent with mechanisms previously reported for the PARs. These results support the thrombin-like action of the Alternaria trypsin-like enzyme in regulating PAR1 signaling in airway tissue, which provides a novel pathophysiological mechanism by which certain allergen-derived enzymes may contribute to airway disease.

CHAPTER 8 DISCUSSION

8.1 General rationale

Proteolytic activity has been widely reported in diverse airborne allergens, and though the enzymes themselves have not necessarily been identified as immunogenic antigens against which a specific adaptive immune response is raised, proteolytic activity in general has been found to promote airway inflammation by signaling via PAR2 in airway allergic sensitization models. These findings have raised the possibility of targeting this signaling system therapeutically, either by inhibiting the environmental proteinases or by blocking PAR2 signaling in the airway. Two major allergen species to which sensitization is a significant asthma risk factor, German cockroach and *Alternaria alternata* mould, are among the allergen species that have been found to contain active proteinases, however the individual trypsin-like enzymes had not been identified or characterized. Such a characterization is important in the eventual development of therapeutic enzyme inhibitors for two main reasons: (1) the substrate specificities and inhibitor sensitivities may differ among enzymes derived from diverse allergen species, so a single therapeutic strategy to target all allergen-derived enzymes may not be the most effective strategy, and (2) identifying the sequences of the enzymes by matching to EST library sequences would provide the ability to express recombinant forms of the enzymes, allowing a more in-depth analysis on the specific physiological effects of these enzymes that is not practical using crude allergen extracts.

Furthermore, it was important to characterize each isolated allergen enzyme for its ability to signal via the PARs and to identify the intracellular mechanisms through which the signal is transmitted. The ability of the crude allergens to signal via PAR2 has

been established, and our work, among others, has implicated PAR2 signaling as a potent enhancer of airway inflammation *in vivo*[170]. In assessing the ability of each enzyme to activate PAR2, the hypothetical physiological effects evoked by each can therefore be inferred based on previous findings in the literature.

In addition, PAR1 has been an overlooked potential contributor to the mechanism of the allergen-derived enzymes in driving asthma pathogenesis. PAR1 has been found to be expressed in a number of airway-derived cells, but trypsin-like enzymes have conventionally been described as driving 'dis-arming,' or inhibition, of PAR1 signaling. Recent studies using enzymes such as activated protein C (APC) and neutrophil elastase demonstrated that enzymes previously described as dis-armers of PAR1 are in fact biased agonists[135,136], cleaving the receptor in a way that drives activation of the G12/13-mediated MAPK pathway and not the Gq-mediated Ca^{2+} signaling pathway. The mechanism of biased signaling reveals a new avenue to define proteinase activators of the PARs, so I aimed to explore the possible role of trypsin-like enzymes as regulators of PAR1 signaling.

8.2 Proteinases in the allergen extracts

8.2.1 Proteinases in the cockroach allergen

Using the activity-based probe to label active proteinases in the allergens, we have identified three distinct trypsin-like enzymes in the German cockroach extract and one trypsin-like enzyme common between the two different preparations of the *Alternaria alternata* allergen. The enzymes were all found to differ from one another in a number of ways. Differences observed specifically among the cockroach-derived

enzymes included: (1) molecular weights as determined by ABP labeling and resolution by SDS/PAGE; (2) distinct relative pI values as determined by their interactions with different ion-exchange columns; (3) distinct substrate and inhibitor affinities determined by enzyme kinetics; (4) primary amino acid sequences determined by mass spectral analysis; and (5) differential regulation of PAR1 signaling in PAR1-expressing KNRK cells. Together these results confirm that the total body cockroach extract contains three distinct trypsin-like enzymes that activate distinct cellular signaling pathways.

Analysis of the frass from the speckled cockroach revealed a single enzyme with similar, though not identical, biochemical properties to cockroach E1. As mentioned, the frass is assumed to represent the primary source of inhaled environmental cockroach allergens[38], so a similar analysis of a German cockroach frass extract will be an important target of future investigation. In particular, determining which enzyme(s) are present in the frass, and therefore to which enzymes patients are likely exposed, will be important in inferring which signaling pathways are activated in the airway. In a similar vein, analysis of the proteinases present in house dust collected from cockroach-infested homes would be a valuable area to explore. E1 and E3 are biased agonists for PAR1 at lower concentrations, but can activate both signaling arms at higher concentrations, so determining the concentration of cockroach-derived enzymes present in the environment could also aid speculation on the relevant signaling mechanisms involved in airway sensitization and asthma.

8.2.2 Proteinases in the Alternaria allergen

The Alternaria enzyme was also distinct from the cockroach enzymes and from porcine trypsin in its substrate specificities, inhibitor sensitivities, and ability to regulate

signaling via PAR1. In particular, the trypsin-specific inhibitor sensitivities observed for this enzyme were substantially lower than those observed for the cockroach enzymes and especially porcine trypsin. At lower concentrations, as in the substrate cleavage assays, the enzyme displayed sensitivity to inhibition by SBTI. However, at the higher concentrations used in the MAPK signaling assay, the enzyme was resistant to inhibition by SBTI. This reduced sensitivity to SBTI inhibition is reflected in the Ki value calculated for the enzyme, which was 6-8X higher than the values calculated for the cockroach enzymes. SBTI resistance in a trypsin-like enzyme has been reported with the mouse trypsin 4 isoform, so an exploration into similarities between that enzyme and the Alternaria enzyme could be interesting[200]. The ability of the enzyme to activate non-biased signaling via PAR1 likewise distinguishes it from the conventionally defined actions of trypsin-like enzymes. Though the Alternaria enzyme can be classified as trypsin-like due to its substrate specificities and to its sensitivity to SBTI inhibition, these distinctions reveal an unusual trypsin-like enzyme.

A common trypsin-like enzyme was found to be present in both the commercial total mould extract and in the in-house generated mould culture media filtrate, so environmental exposure to factors secreted by the mould, as well as the mould material itself, could result in exposure to this enzyme. However, in Chapter 3 we detected evidence of (a) non-trypsin like enzyme(s) that label with the chymotrypsin specific activity-based probe in the total mould extract but not in the filtrate. This could indicate a difference in the enzymatic content of the environmental Alternaria allergen depending on the source of the airborne material. Non trypsin-like serine proteinase activity in the *Alternaria* mould has not been previously reported, so whether these enzymes can

contribute to pathophysiological processes is unknown, but may provide an avenue of future research. Determining whether clinical symptoms differ depending on the source of Alternaria allergens would be an interesting area to explore in assessing the role of these non-trypsin-like enzymes.

8.3 Regulation of PAR signaling by the allergen-derived enzymes

8.3.1 PAR2 signaling by allergen-derived enzymes

The ability of the trypsin-like enzymes in the crude cockroach and Alternaria extracts to signal via PAR2 had been established in the literature in several *in vitro* and *in vivo* models, so the ability of each of the four allergen-derived enzymes that we identified to activate non-biased signaling via PAR2 was not surprising. The responses evoked in the artificial PAR2 expression systems in (1) cleaving the Nluc-tagged PAR2 construct, (2) activating Ca^{2+} signaling and (3) driving phosphorylation of ERK 1/2 were of similar magnitudes among all four enzymes, indicating that each target and activate PAR2 with similar efficiencies. Likewise in the airway-derived epithelial cells, E1 activated both PAR2-dependent Ca^{2+} signaling and, along with the other allergen-derived proteinases, SBTI-sensitive ERK 1/2 activation. The activation of MAPK signaling may be due to the combined cleavage of both PAR1 and PAR2 in the A549 cells, which is a distinction that could be made with further experiments. Finally, E1 and Alt both induced aortic tissue relaxation in tissues harvested from PAR1-null mice. E1 did not induce a similar relaxation in PAR2-null tissue, which indicates that this response was PAR2-dependent. Alt also induced the relaxation of PAR1-null bronchial tissues, although we were unable to assess the PAR2-dependence of this response.

These findings, along with the previously reported pro-inflammatory effects of PAR2 activation in the airway discussed in the introduction, support our proposal that all four enzymes present in these allergens are potential therapeutic targets in the clinical setting.

8.3.2 PAR1 signaling contributes to airway fibrosis

A role of PAR1 signaling by the allergen-derived trypsin-like enzymes had not previously been considered. In Chapter 6 all of the allergen enzymes were found to cleave and activate PAR1 signaling with different efficiencies at similar concentrations. PAR1 signaling in the airway has been shown to contribute to subepithelial fibrosis, and so it may have pathophysiological significance. It has been shown that activation of PAR1 by thrombin induces the expression of the chemokine CCL2[201] and the pro-fibrotic signaling molecule connective-tissue growth factor (CTGF/CCN-2) in airway-derived fibroblasts[202], and CCL2[203] as well as platelet-derived growth factor in airway epithelial cells[184]. PAR1 signaling also induces the differentiation of airway fibroblasts to a myofibroblast phenotype[195], which is a known contributor to subepithelial fibrosis.

Additionally, the direct inhibition of thrombin reduced fibroblast proliferation, procollagen production, and connective tissue growth factor (CTGF) mRNA levels in cultured fibroblasts and reduced lung collagen accumulation and inhibited α1(I) procollagen and CTGF mRNA levels, but had no effect on the recruitment of immune cells to the airway, in a bleomycin-induced lung injury model in mice[204]. In a similar bleomycin injury model using PAR1-null mice, it was found that PAR1 deficiency reduced both the deposition of collagen and the expression of the pro-inflammatory and

pro-fibrotic factors monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-beta-1 (TGF-β1), and CTGF in the airways of the treated mice[205]. In the same study, the authors found an increase in PAR1 expression in lung tissues taken from patients with fibrotic lung diseases. Furthermore, a similar fibrosis model in mice has shown that PAR1 signaling results in a potent increase of the chemokine CCL2, which contributes to the fibrotic response[203].

These results suggest that PAR1 signaling can contribute to airway fibrosis, and its role as a potential target in fibroproliferative lung disease has been considered[206]. A recent study by Zhu et al[207] has provided evidence that thrombin signaling via PAR1 contributes to airway remodeling induced by allergic sensitization in an ovalbumin allergy model in rats. It was found that the expression of PAR1 and TGF-β, as well as indicators of airway remodeling including Goblet cell hyperplasia, mucus secretion, collagen deposition and smooth muscle thickness, were increased in OVA-sensitized rats co-treated with thrombin, and these effects were reduced when either thrombin or PAR1 were pharmacologically inhibited. Finally, as mentioned in the introduction, the airway smooth muscle can respond directly to the inhaled environment, and it has been found that thrombin signaling via PAR1 can stimulate the contraction of bronchial smooth muscle[129], so since we observed that the Alternaria enzyme can activate a PAR1-mediated smooth muscle response in bronchial tissue, it could possibly induce a similar response and contribute to the airway constriction in asthma in that way. The relaxation response that we observed in the mouse bronchial tissues was dependent on epithelial COX activity, but the enzyme may induce different responses in the context of the asthmatic airway. Asthma has been described as a persistent, unresolved injury-

repair scenario[26]. We therefore propose that signaling via PAR1 by allergen-derived trypsin-like enzymes may contribute to airway remodeling.

The role of biased signaling via PAR1 to contribute to airway pathology is an area that will require future investigation. As mentioned previously, at least one pro-fibrotic effect of PAR1 signaling, the thrombin-driven differentiation of fibroblasts to myofibroblasts, has been shown to be dependent on PAR1-mediated Ca^{2+} signaling[195]. If that mechanism is common for the PAR1-mediated pro-fibrotic responses, it could be expected that E1 and E3 may not contribute to fibrosis in that way. As we demonstrated in Chapter 5, the dual-fluorescent PAR1 construct remains on the membrane after cleavage by E1 and E3. This phenomenon has also been shown with the PAR1 biased agonist APC, following cleavage by which PAR1 is found to remain on the membrane in the presence of thrombin[134]. These findings could suggest a potential protective role of the biased PAR1 agonist allergen-derived enzymes in lung fibrosis, cleaving the receptor and preventing the pro-fibrotic response induced by thrombin-like signaling. On the other hand, if the pro-fibrotic PAR1 responses are due to MAPK activation, then all of the allergen enzymes could be potential contributors to airway fibrosis via PAR1.

A further confounding principle in inferring the physiological role of PAR signaling by the allergen-derived enzymes is in recently observed interactions between PAR1 and PAR2. In the artificial PAR-expressing KNRK system that I have used to assess the ability of the allergen enzymes to signal via PARs, only one of PAR1 or PAR2 were expressed in each cell line. This allowed a detailed examination of the regulation of each receptor individually, however as outlined in earlier sections both PAR1 and PAR2 are expressed in a number of airway cells. PAR1 and PAR2 have been shown to form

receptor heterodimers, which can potentially allow for receptor transactivation and can affect the signaling pathways activated downstream of the dimer activation[208]. This signaling mechanism has been shown to influence the effect of thrombin signaling in breast cancer; knocking out PAR2 from breast cancer cells attenuated the responses induced by thrombin, as well as growth of a xenograft in a mouse model, and PAR1 and PAR2 were found to interact in an immunoprecipitation assay[209], and indicating that the receptors directly interact and are both necessary in this system. Likewise, activation of a PAR1-PAR2 heterodimer demonstrated distinct receptor trafficking following thrombin treatment than PAR1 monomers, involving the recruitment of β-arrestin and endosomal localization[210]. Whether similar PAR heterodimerization may influence signaling by allergen-derived proteinases in airway tissues is not known, but it may provide further distinctions in the responses induced by the PAR1 biased agonists E1 and E3 compared to the non-biased agonists E2 and Alt.

8.4 PAR signaling in airway cells and tissues

Our Ca^{2+} signaling and MAPK activation results demonstrated that the A549 cells, in our culture conditions, have a low apparent expression of functional PAR1. By contrast, the BEAS-2B cells in similar culture conditions displayed a robust activation of PAR1-mediated Ca^{2+} signaling. Furthermore, the mouse bronchial tissues displayed strong relaxation responses to PAR1 agonists, indicating a high expression level of the receptor in the tissues. This relaxation was sensitive to the inhibition of COX by indomethacin; COX-mediated prostaglandin synthesis has been reported as a mechanism contributing to epithelial PAR-driven regulation of bronchial tone[198].

Together these results, along with the ability of the allergen-derived enzymes to target and activate PAR1 signaling discussed earlier, highlight that PAR1 is likely present and targeted by allergen-derived trypsin-like enzymes in the airway. Considering the PAR1-PAR2 interactions discussed above, PAR1 should therefore not be overlooked as a contributor to the physiological effects of inhaled allergen-derived proteinase activity in the airway.

8.5 Conclusions

In summary, we have identified and characterized three distinct trypsin-like enzymes in the German cockroach allergen and one trypsin-like enzyme in the *Alternaria alternata* mould allergen. We propose that all four of these enzymes contribute to airway inflammation and allergic sensitization in individuals exposed to these allergens by regulating signaling via PAR1 and PAR2. The allergen-derived trypsin-like enzymes that we have described are all potent agonists for PAR2, activation of which has been identified as a pro-inflammatory signaling mechanism in the context of allergic airway inflammation. These findings identify each of the four enzymes as potential therapeutic targets. Additionally, to my knowledge this is the first time that the activation of PAR1 signaling by trypsin-like enzymes has been described. The allergen enzymes regulate signaling via PAR1 differentially, with Alt and E2 activating thrombin-like, non-biased signaling via PAR1 while E1 and E3 activate biased signaling, preferentially activating MAPK signaling over Ca^{2+} signaling. The contribution of PAR1 signaling to allergic airway inflammation may thus warrant further investigation. Overall, a better understanding of the biochemical and signaling properties of proteinases

derived from diverse airborne allergens may be valuable in the development of therapeutic interventions for allergies and asthma.

CHAPTER 9 FUTURE STUDIES

The main outcome of the work described in this thesis is the complete biochemical characterization of the trypsin-like enzymes in cockroach and Alternaria allergens, including their catalytic/inhibitor properties and their complete amino acid sequences. Further, the ability of the four isolated enzymes to regulate tissue function by cleaving and activating PARs has been documented for the first time. These data are now included in three manuscripts in preparation. The manuscripts will describe (1) the characterization of the allergen enzymes, (2) the differential signalling generated via PARs 1 and 2 by the cockroach vs the Alternaria enzymes and (3) the role of β-arrestin signaling for the impact of the Alternaria enzyme on a murine asthma model. The experiments to be presented in these manuscripts have been completed, and the data included were presented in this thesis as described in Appendix B. The working titles and authors are as follows:

1. Cockroach allergen serine proteinases: Isolation, sequencing and signaling via proteinase-activated receptors (PARs) (*working title*). Danny Polley, Koichiro Mihara, Rithwik Ramachandran, Harissios Vliagoftis, Bernard Renaux, Mahmoud Saifeddine, Michael Daines, Scott Boitano and Morley D. Hollenberg.

2. Allergen-derived serine proteinases regulate signaling via PAR1 differentially (*working title*). Danny Polley, Koichiro Mihara, Rithwik Ramachandran, Harissios Vliagoftis, Bernard Renaux, Mahmoud Saifeddine, Michael Daines, Scott Boitano and Morley D. Hollenberg.

3. Proteinase-activated-Receptor-2-Induced Signaling through β-Arrestin-2 Mediates Alternaria Serine Proteinase-induced Airway Inflammation. Heddie L. Nichols, Michael

Yee, Kasturi Pal, Kyu Lee, Danny Polley, Emma H. Wilson, Michael Daines, Morley D. Hollenberg, Scott Boitano and Kathryn A. DeFea.

In addition, data stemming from the thesis have already been included in a number of publications as follows:

1. Biased signalling and proteinase-activated receptors (PARs): targeting inflammatory disease.

Hollenberg MD, Mihara K, Polley D, Suen JY, Han A, Fairlie DP, Ramachandran R.

Br J Pharmacol. 2014 Mar;171(5):1180-94.

PMID: 24354792

2. Proteinase-activated receptor-2 activation participates in allergic sensitization to house dust mite allergens in a murine model.

Davidson CE, Asaduzzaman M, Arizmendi NG, Polley D, Wu Y, Gordon JR, Hollenberg MD, Cameron L, Vliagoftis H.

Clin Exp Allergy. 2013 Nov;43(11):1274-85.

PMID: 24152160

3. β-Arrestin-2 mediates the proinflammatory effects of proteinase-activated receptor-2 in the airway.

Nichols HL, Saffeddine M, Theriot BS, Hegde A, Polley D, El-Mays T, Vliagoftis H, Hollenberg MD, Wilson EH, Walker JK, DeFea KA.

Proc Natl Acad Sci U S A. 2012 Oct 9;109(41):16660-5.

PMID: 23012429

4. Mucosal allergic sensitization to cockroach allergens is dependent on proteinase activity and proteinase-activated receptor-2 activation.

Arizmendi NG, Abel M, Mihara K, Davidson C, Polley D, Nadeem A, El Mays T, Gilmore BF, Walker B, Gordon JR, Hollenberg MD, Vliagoftis H.

J Immunol. 2011 Mar 1;186(5):3164-72.

PMID: 21270400

Following is a synopsis of future work that will come from the data described in the thesis.

9.1 Chapter 5

In Chapters 3, 4, and 5 the allergen-derived enzymes were labeled, isolated from the allergen extracts and characterized. Having identified the sequences each of the enzymes with mass spectral analysis, the main focus of future work will be on cloning the cDNA sequences encoding enzyme and generating recombinantly expressed enzymes. The main drawback in my approach of isolating the enzymes from the crude extracts was that enzyme abundance in the extracts was a limiting factor. Recombinant expression of each enzyme could remove that limitation and allow more detailed explorations into the physiological effects of each of the enzymes.

Additionally, a future goal will be to characterize the trypsin-like enzyme(s) in a frass extract of the German cockroach. Since the frass extract is not commercially available, a source will have to be found to pursue this goal.

9.2 Chapter 6

In Chapter 6, the ability of each allergen-derived enzyme to regulate signaling via PAR1 and PAR2 was assessed. My focus was to demonstrate the general feature of receptor regulation, such as receptor cleavage and the activation of downstream signaling pathways. Future work will entail a more detailed analysis of the molecular basis of signal transduction. The PAR tethered ligand peptide cleavage assay revealed that each allergen-derived enzyme can target both the PAR1 and PAR2 receptors at the canonical cleavage site. However, the differential signaling observed following PAR1 activation could suggest that the enzymes cleave the intact receptors at different sites. An assay using the PAR1-Nluc construct with site-directed mutations of known PAR1 cleavage sites would identify the specific sites that the allergen enzymes are targeting.

Additionally, identifying which G proteins are mediating the intracellular signaling pathways induced by receptor activation would be important in fully assessing the differential regulation of PAR1 by the allergen-derived enzymes. This could be accomplished by knocking out individual Gα proteins in the PAR-expressing KNRK cell lines and subsequently monitoring Ca^{2+} signaling and ERK 1/2 activation.

The β-arrestin recruitment assay will also need revisiting. Concentration effect curves for each of the enzymes would be beneficial to determine the abilities and efficiencies with which the allergen-derived enzymes drive β-arrestin recruitment. In particular, the lack of significance observed for the recruitment response induced by the Alternaria enzyme will require further investigation.

9.3 Chapter 7

In chapter 7, preliminary experiments assessing the ability of the allergen-derived enzymes to regulate PAR signaling in airway-derived cells and tissues were presented. Future work with the cultured airway epithelial cells will involve assessing the ability of each allergen enzyme to regulate the PARs in these cells, with the possibility of pharmacologically blocking or knocking down one of PAR1 or PAR2 to explore each receptor individually. Additionally, the A549 cell line is not generally considered representative of the airway epithelium. A more appropriate cell, ideally primary bronchial epithelial cells, could be selected for these experiments. A thorough examination of the physiological effects of each allergen-derived enzyme, such as cytokine production, would also be valuable. Real-time PCR, gene array and ELISA assays could be used to detect the expression of cytokines and chemokines at the mRNA and protein levels.

Finally, the smooth muscle relaxation bioassays will be repeated and the responses analyzed statistically. Cockroach E2 and E3 were not used in these assays due to insufficient enzyme abundance in the ion-exchange chromatography fractions. With recombinant expression of those enzymes, they could possibly be assessed for their ability to mediate bronchial relaxation.

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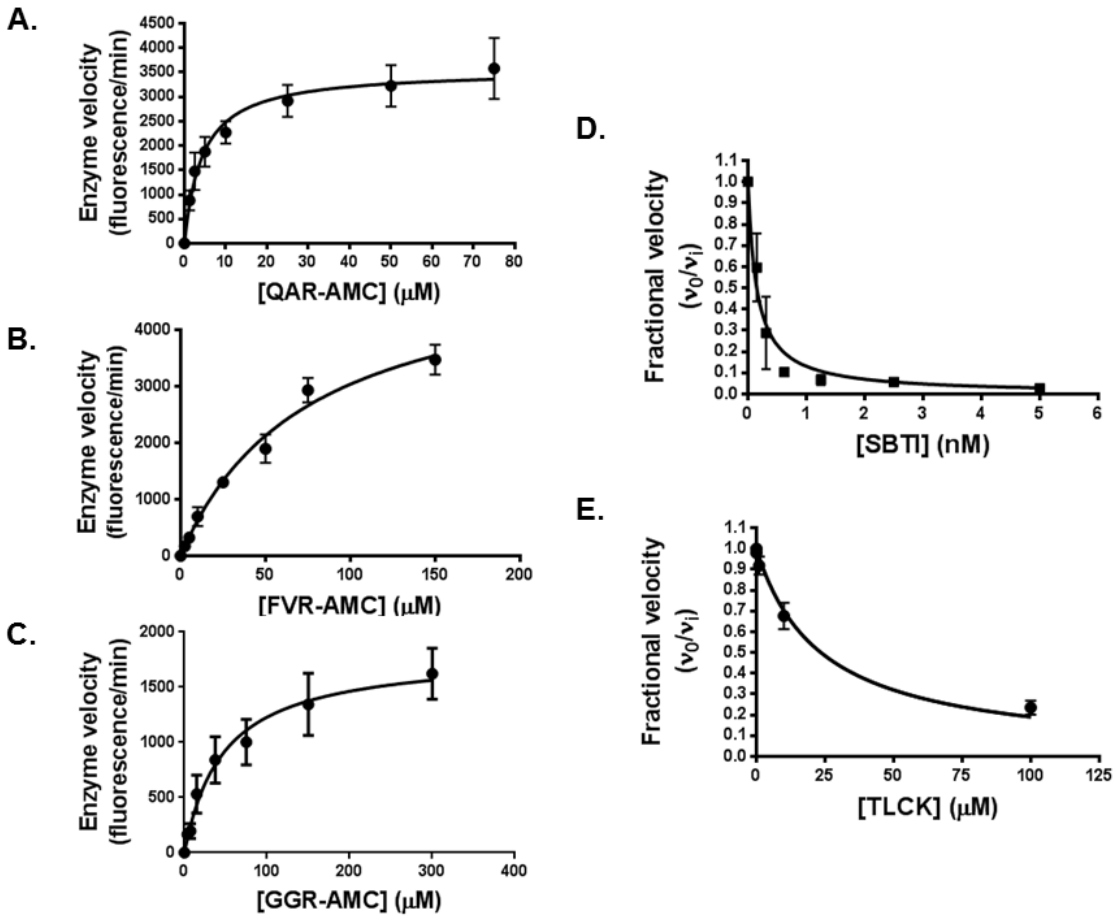
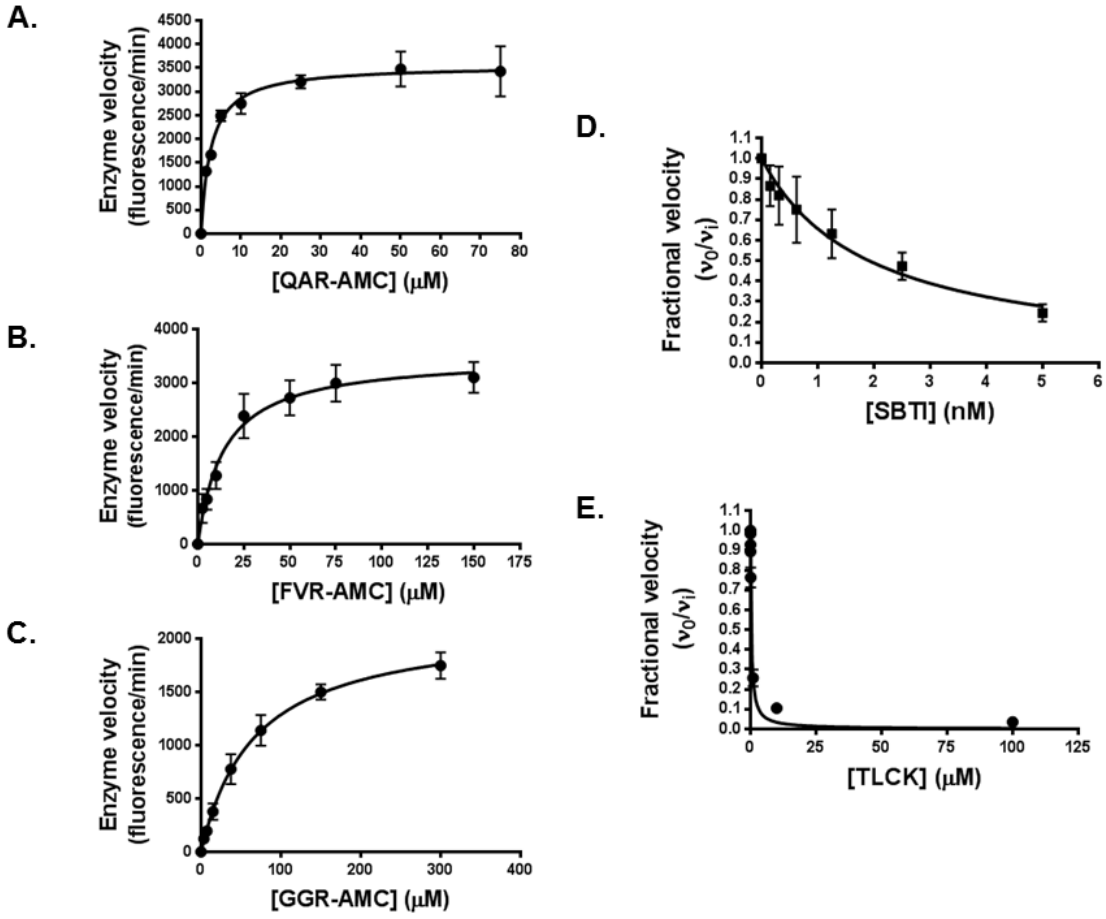
APPENDIX A: ADDITIONAL FIGURES

Cockroach E1 enzyme kinetics



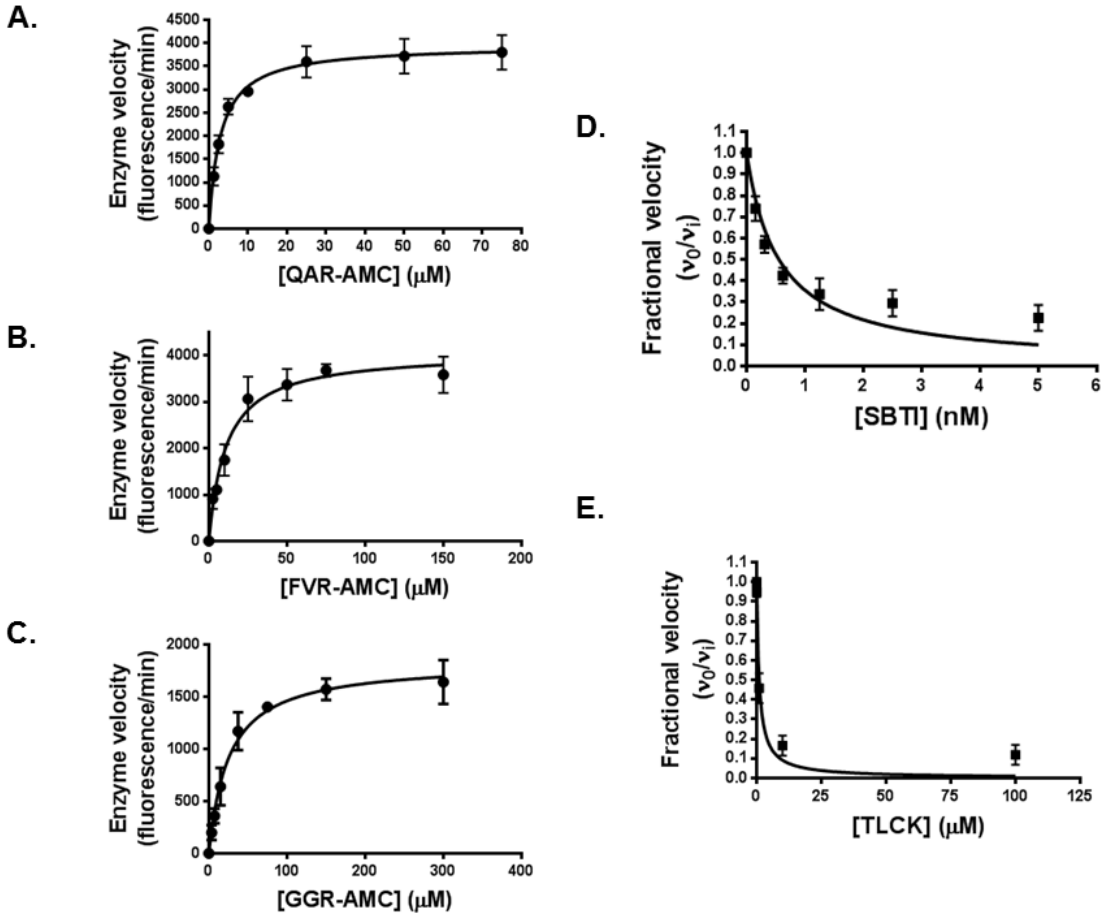
Kinetic curves for cockroach E1. Michaelis-Menten (Km) curves for QAR-AMC (**A**), FVR-AMC (**B**) and GGR-AMC (**C**). Inhibition (Ki) curves for SBTI (**D**) and TLCK (**E**). (n=3)

Cockroach E2 enzyme kinetics



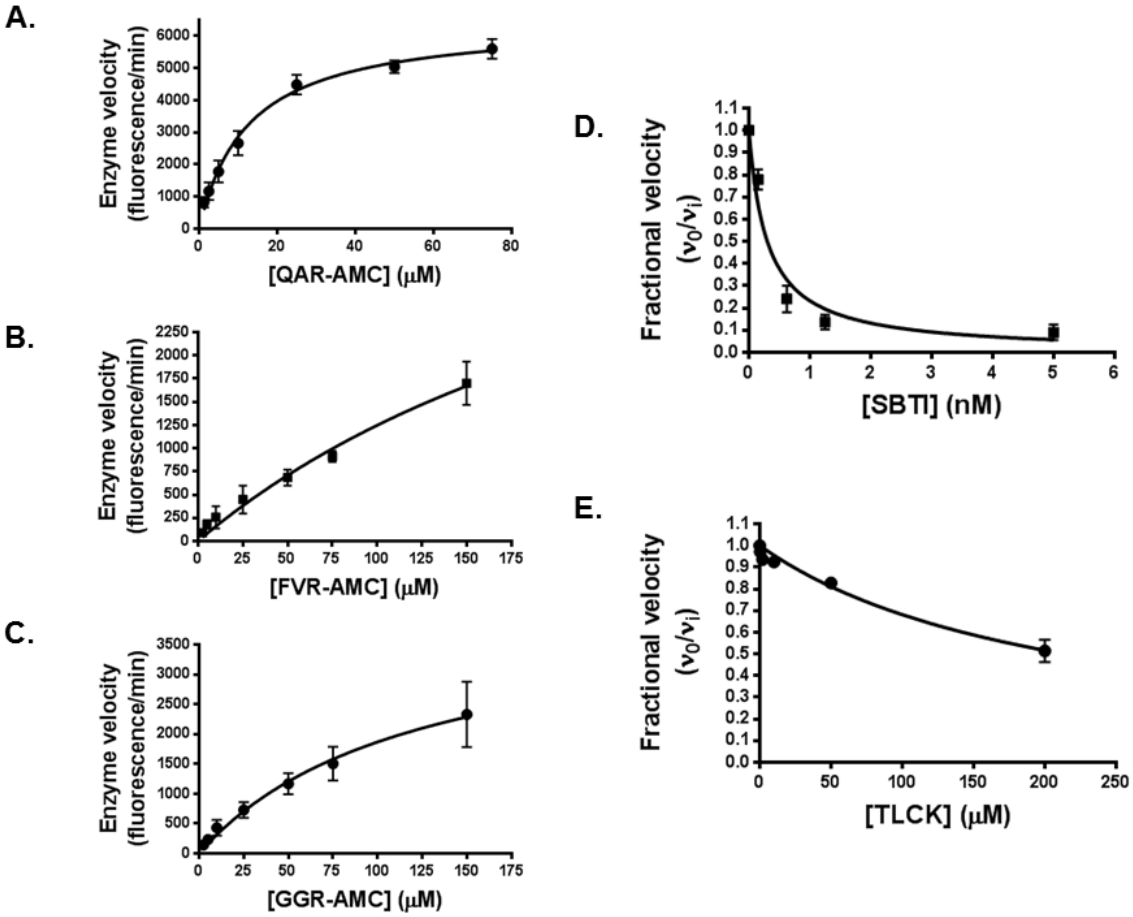
Kinetic curves for cockroach E2. Michaelis-Menten (Km) curves for QAR-AMC (**A**), FVR-AMC (**B**) and GGR-AMC (**C**). Inhibition (Ki) curves for SBTI (**D**) and TLCK (**E**). (n=3)

Cockroach E3 enzyme kinetics



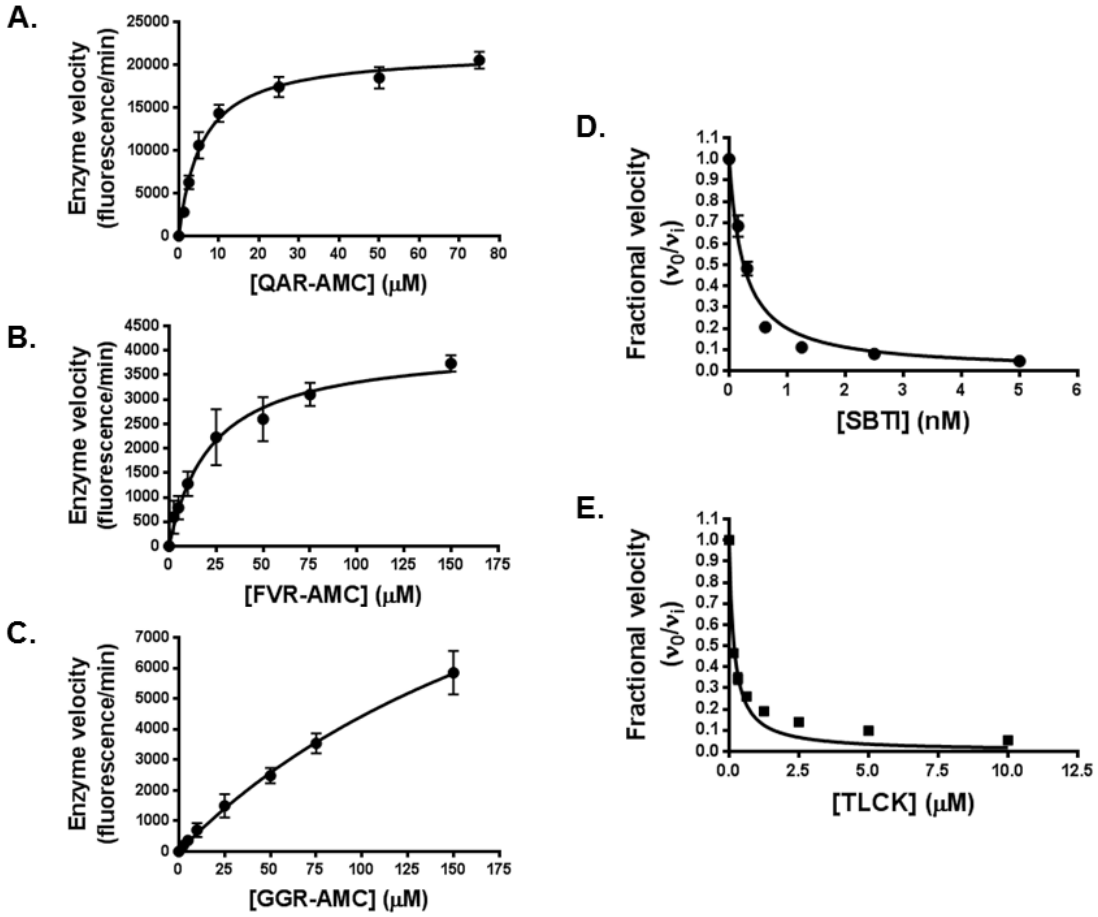
Kinetic curves for cockroach E3. Michaelis-Menten (Km) curves for QAR-AMC (**A**), FVR-AMC (**B**) and GGR-AMC (**C**). Inhibition (Ki) curves for SBTI (**D**) and TLCK (**E**). (n=3)

Alternaria enzyme kinetics



Kinetic curves for the Alternaria enzyme. Michaelis-Menten (Km) curves for QAR-AMC (**A**), FVR-AMC (**B**) and GGR-AMC (**C**). Inhibition (Ki) curves for SBTI (**D**) and TLCK (**E**). (n=3)

Porcine trypsin enzyme kinetics



Kinetic curves for porcine trypsin. Michaelis-Menten (Km) curves for QAR-AMC (**A**), FVR-AMC (**B**) and GGR-AMC (**C**). Inhibition (Ki) curves for SBTI (**D**) and TLCK (**E**). (n=3)

APPENDIX B: PUBLICATION LIST AND PUBLICATIONS IN PROGRESS

First author publications in progress:

1. Cockroach allergen serine proteinases: Isolation, sequencing and signaling via proteinase-activated receptors (PARs) (*working title*). Danny Polley, Koichiro Mihara, Rithwik Ramachandran, Harissios Vliagoftis, Bernard Renaux, Mahmoud Saifeddine, Michael Daines, Scott Boitano and Morley D. Hollenberg.

This manuscript presents the isolation and characterization of the cockroach proteinases, as well as the cockroach-mediated PAR2 signaling data. It contains: the enzyme isolation data in **Figures 4.2.1** (p. 94), **4.2.2** (p.97). **4.2.3** (p.99) and **4.3.1** (p.106); the enzyme kinetics data in **Table 5.2.1** (p. 112); the mass spectral sequencing data in **Figure 5.3.3** (p. 118) and **Table 5.3.1** (p. 119); the cockroach frass characterization data in **Figure 5.4.1** (p. 123); the PAR2 peptide cleavage data in **Figure 6.2.1** (p. 129) and summarized in Table **6.2.1** (p. 130); the PAR2-Nluc construct cleavage data in **Figure 6.2.2** (p. 131); the PAR2 Ca^{2+} signaling data in **Figure 6.2.3** (p. 133); the PAR2 MAPK activation data presented in **Figure 6.2.4** (p. 135); and the β-Arrestin recruitment data presented in **Figure 6.2.5** (p. 138).

2. Allergen-derived serine proteinases regulate signaling via PAR1 differentially (*working title*). Danny Polley, Koichiro Mihara, Rithwik Ramachandran, Harissios Vliagoftis, Bernard Renaux, Mahmoud Saifeddine, Michael Daines, Scott Boitano and Morley D. Hollenberg.

This manuscript presents the differential regulation of PAR1 by the allergen-derived enzymes. It contains: the PAR1 peptide cleavage data in **Figure 6.3.1** (p. 142) and **Table 6.2.1** (p. 130); the PAR1-Nluc cleavage data in **Figure 6.3.2** (p. 144); the PAR1-mediated Ca^{2+} signaling data in **Figure 6.3.3** (p. 146), the PAR1-mediated MAPK activation data in **Figures 6.3.5** (p. 149) and **6.3.6** (p.150); the dual-fluorescent construct cleavage and trafficking data in **Figure 6.3.10** (p. 156); the aortic tissue

relaxation data in **Figures 7.3.1** (p. 172), **7.3.2** (p.174), **7.3.3** (p.176), and **7.3.4** (p. 177); and the bronchial tissue relaxation data in Figures **7.4.1** (p. 178) and **7.4.2** (p. 180).

Contributing author publication in progress:

1. Proteinase-activated-Receptor-2-Induced Signaling through β-Arrestin-2 Mediates Alternaria Serine Proteinase-induced Airway Inflammation. Heddie L. Nichols, Michael Yee, Kasturi Pal, Kyu Lee, Danny Polley, Emma H. Wilson, Michael Daines, Morley D. Hollenberg, Scott Boitano and Kathryn A. DeFea.

As a co-author, I provided figures presenting the characterization of the Alternaria enzyme as well as its ability to activate PAR2-mediated Ca^{2+} signaling. This manuscript contains: the Alternaria activity-based probe labeling in **Figure 3.2.3** (p. 76); the Alternaria enzyme isolation data in **Figure 4.2.4** (p. 101); the PAR2 Ca^{2+} signaling data in **Figures 3.3.2** (p. 88) and **6.2.3** (p. 133); the enzyme kinetics data in **Table 5.2.1** (p. 112); and the mass spectral sequencing data in **Figure 5.3.1** (p. 115).

Contributing author publications:

1. Arizmendi NG, Abel M, Mihara K, Davidson C, Polley D, Nadeem A, El Mays T, Gilmore BF, Walker B, Gordon JR, Hollenberg MD, Vliagoftis H. Mucosal allergic sensitization to cockroach allergens is dependent on proteinase activity and proteinase-activated receptor-2 activation. *J Immunol*. *2011 Mar 1;186(5):3164-72*
As a co-author, I contributed the ABP-labeling and SBTI inhibition data for the cockroach extract, as well as performing the mucosal cockroach sensitization protocol, the whole-body plethysmography methacholine challenge and the BALF and blood collection in the wildtype and PAR2-null C57/BL6 mice.

2. Nichols HL, Saffeddine M, Theriot BS, Hegde A, Polley D, El-Mays T, Vliagoftis H, Hollenberg MD, Wilson EH, Walker JK, DeFea KA.β-Arrestin-2 mediates the

proinflammatory effects of proteinase-activated receptor-2 in the airway. *Proc Natl Acad Sci U S A. 2012 Oct 9;109(41):16660-5*
As a co-author, I performed the ovalbumin sensitization protocol in wildtype and β-arrestin-null C57/BL6 mice.

3. Davidson CE, Asaduzzaman M, Arizmendi NG, Polley D, Wu Y, Gordon JR, Hollenberg MD, Cameron L, Vliagoftis H. Proteinase-activated receptor-2 activation participates in allergic sensitization to house dust mite allergens in a murine model. Clin Exp Allergy. 2013 Nov;43(11):1274-85
As a co-author, I performed the mucosal house dust mite sensitization protocol, the whole-body plethysmography methacholine challenge and the BALF and blood collection in the wildtype and PAR2-null C57/BL6 mice.

4. Hollenberg MD, Mihara K, Polley D, Suen JY, Han A, Fairlie DP, Ramachandran R. Biased Signalling and Proteinase-Activated Receptors (PARs): Targeting Inflammatory Disease. Br J Pharmacol. 2013 Dec 19
As a co-author, I provided the figures presenting the cockroach-mediated Ca^{2+} signaling and MAPK activation via PAR1.