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Cockroach allergen serine proteinases: Isolation, sequencing and signalling via proteinase-activated receptor-2 (PAR2)

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SUMMARY

BACKGROUND—Allergy to the German cockroach (*Blattella germanica)* is a significant asthma risk factor for inner-city communities. Cockroach, like other allergens, contains trypsin-like enzyme activity that contributes to allergenicity and airway inflammation by activating proteinase-activated receptors (PARs). To date, the enzymes responsible for the proteolytic activity of German cockroach allergen have not been characterized.

OBJECTIVES—We aimed to identify, isolate and characterize the trypsin-like proteinases in German cockroach allergen extracts used for clinical skin tests. For each enzyme, we sought to determine (1) its substrate and inhibitor enzyme kinetics (Km and IC50); (2) its amino acid sequence and (3) its ability to activate calcium signaling and/or ERK1/2 phosphorylation via PAR2.

METHODS—Using a trypsin-specific activity-based probe, we detected three distinct enzymes that were isolated using ion-exchange chromatography. Each enzyme was sequenced by mass spectometery (deconvoluted with an expressed sequence tag library), evaluated kinetically for its substrate/inhibitor profile and assessed for its ability to activate PAR2 signaling.

FINDINGS—Each of the three serine proteinase-activity-based probe-labelled enzymes isolated were biochemically distinct, with different enzyme kinetic profiles and primary amino acid sequences. The three enzymes showed a 57 to 71% sequence identity with a proteinase previously

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Amino acids are designated by their one-letter codes e.g. A, alanine; F, phenylalanine

cloned from the American cockroach (Per a 10). Each enzyme was found to activate both Ca^{++} and MAPK signaling via PAR2.

CONCLUSIONS AND RELEVANCE—We have identified three different serine proteinases from the German cockroach that may, via PAR2 activation, play different roles for allergen-sensitization *in vivo* and may represent attractive therapeutic targets for asthma.

Keywords

Asthma; calcium signaling; cockroach; protease; Proteinase-activated receptor-2 (PAR2)

INTRODUCTION

Asthma is a substantial world-wide public health and financial burden. Thus, it is essential to understand the underlying pathogenic mechanisms of allergic sensitization to enable the development of more effective therapy. The majority of asthma cases that persist into adulthood are associated with early life exposure and sensitization to one or more airborne allergens [1]. This exposure is of particular concern for children living in low-income, inner city communities, where cockroach exposure represents one of the main risk factors associated with asthma morbidity [2]. There is growing evidence that proteolytic enzymes present in the inhaled allergens can play an important role in the development of allergic sensitization. An important role for specific allergen proteinases was first appreciated when potent immunogenic antigens from the house dust mite (*Dermatophagoides pteronyssinus*), a major asthma risk-factor allergen, were found to have structural homology to cysteine (Der p 1) [3] and serine proteinases (Der p 3: tryptic Der p 6: chymotryptic and Der p 9: collagenolytic) [4]. A number of diverse environmental allergens have since been found to contain proteolytic activity, including several species of moulds [5], pollens [6], the German cockroach (*Blattella germanica*) [7], and the American cockroach (*Periplaneta americana*) [8, 9], among others. Previous work suggests that, similar to the house dust mite allergen, the proteinases in these other allergen sources may contribute to their allergenicity [10].

In previous work with Der p-allergen-derived proteinases, we found that the two principle proteinases can stimulate asthma-associated cytokine production in target cells by mechanisms that are either PAR2-dependent (Der p 3, serine proteinase) or PAR2-independent (Der p 1, cysteine proteinase) [11]. We therefore hypothesized that for the cockroach allergens, proteolytic activity in the preparation used for clinical testing might play a role in its ability to synergize with other allergens to cause sensitization. Indeed, in keeping with the cloning of a serine proteinase from the German cockroach by Ock and colleagues [12], we found that the cockroach extract used for clinical testing contains three proteinases that are selectively labeled with a biotinylated serine proteinase activity-based probe (ABP: figure 4 in ref. 13); and we determined, using a murine sensitization model, that blocking PAR2 activation with an antagonist antibody can mitigate the cockroach extract-induced allergic response, including a reduction in specific IgG1 production [13]. Further, we established firstly that the mixture of cockroach allergen enzymes can activate signaling via proteinase-activated receptor 2 (PAR2) and secondly that blocking PAR2 activation with an antagonist antibody can mitigate the allergen-induced allergic response including a reduction in immunoglobulin IgG production [13]. However, we did not know at

the time which of the three serine proteinases (if any) present in the cockroach allergen was able to regulate PAR2 activity and thus to contribute to the sensitization process. Our aim was therefore to isolate each of the three enzymes present in a German cockroach extract allergen used for clinical testing, characterize them biochemically, determine their amino acid sequences and evaluate their ability to signal via PAR2.

MATERIALS AND METHODS

Cockroach allergen extract

German cockroach extract (CE), used for clinical testing, was purchased from Greer labs (Lenoire, NC; catalog #XPB46D3A4). Each vial of the extract was reconstituted in 5 ml 10mM Tris-HCl pH 7.2. The reconstituted extracts were dialyzed against 10 mM Tris-HCl pH 7.2 at 4°C for 2h Novagen D-Tube Maxi dialyzers (EMD Millipore, catalog # 71509) with a molecular weight cut off of 6–8 kDa to remove small molecule contaminants in the extract. This dialyzed allergen extract was used for all subsequent biochemical procedures as described in the following paragraphs.

Cockroach frass

Frass harvested from the speckled cockroach (*Nauphoeta cinerea*) was provided by Dr. Samantha Ross of the Defense Science and Technology Organisation, Melbourne, Australia. 10 g aliquots of the frass were shaken in 50 ml of distilled water for 4 h at 4°C to extract the soluble fraction containing proteolytic activity.

Cleavage of fluorogenic substrates for enzyme kinetic measurements

To assess the presence of different types of proteinases in the cockroach allergen extract, multiple flourogenic peptide substrates with different enzyme specificities were used. The four substrates used were: Boc-Glutamine-Alanine-Arginine (QAR)- aminomethylcoumarin (AMC; Bachem, Bubendorf, Switzerland; I1550) (trypsin substrate), Phenylalanine-Arginine (FR)-AMC (serine and cysteine cathepsin substrate; Bachem, I-1160), Alanine-Alanine-Proline-Phenylalanine-AMC [(AAPF)-AMC (chymotrypsin substrate; Calbiochem, San Diego, CA; 672159] and Glycine-Glycine-Arginine (GGR)-AMC (Urokinase substrate; Calbiochem, 230914). Each substrate was diluted to 1.5 mM in Tris-NP40-calcium buffer, pH 8 (50 mM Tris HCl pH 8, 0.2% NP40, 1.5 mM $CaCl_2$) (Proteinase Assay Buffer). Initially, serial dilutions of the extract were made from dilution factors of 10X – 10 000X in proteinase assay buffer. 50 μl of each cockroach extract dilution were loaded in triplicate in a black 96-well plate (Greiner Bio-One; Kremsmünster, Austria). Prior to the addition of the substrate, cockroach extract was treated or not with 50 μM of soybean trypsin inhibitor (SBTI, Sigma, St. Louis, MO) for 5 min at room temperature. 50 μl of each substrate was then added to the extract-containing wells for a final concentration of 750 μM in 100 μl. Immediately following addition of the substrate, cleavage was monitored as an increase in fluorescence in the Perkin-Elmer Victor X4 2030 Multilabel plate reader (excitation 480 nm; emission 530 nm) over a kinetic course of 15 minutes, with fluorescence measurements acquired every 2 min for 10 min and a final recording at 15 min. Enzyme activity was taken as the slope of the increase in fluorescence over time in the linear phase of the substrate cleavage curve (R^2 value ≥ 0.99).

Quantification of trypsin-like activity in cockroach allergen extract

The trypsin-like activity of the dialyzed crude extract was determined with the QAR-AMC substrate. To quantify the activity in the extract, substrate cleavage was compared to that of a trypsin standard curve using known unit (U)/ml concentrations of porcine trypsin (Type IX-S; Sigma, catalog # T0303, 16 000 BAEE U/mg; 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). The trypsin standard curve consisted of five concentrations ranging from 20 mU/ml to 300 mU/ml. 50 μl of each concentration of the trypsin standard solutions, as well as cockroach extract diluted 200X in Proteinase Assay Buffer, was loaded in triplicate to the 96-well black plate and 50 μM of the substrate was added for a final concentration of 750 μM as above. Enzyme activity in the cockroach extract was interpolated from the fluorescence (Y-axis) versus U/ml concentration (X-axis) curve generated from the trypsin standard curve.

Determination of protein concentration

Protein levels of each of the extracts were measured in a 96-well microtiter plate assay 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)-generated standard curve.

Activity-based Probe (ABP) labeling

A trypsin-specific, biotin-tagged biotin-linker-Pro-Lys-diphenyl phosphonate activity-based probe (ABP) was used to label the active serine proteinases in the crude extract specifically and covalently, essentially as described previously [13] with minor modifications. The total extract was diluted to 10 U/ml trypsin-like activity in 50 mM Tris pH 8, and a reaction mixture of 5 μl of diluted extract, 1 μl of 10X proteinase assay buffer (500 mM Tris-HCl pH 8, 2% NP40, 15 mM $CaCl_2$), 1 μl of 1 mM ABP and 2 μl H_20 for a total of 50 mU of trypsin-like activity in the 10 μl reaction volume. After a 2h incubation at room temperature, the reaction was terminated with the addition of 10 μl of 2X SDS sample buffer containing β-mercaptoethanol, and the reaction products were boiled for 3 minutes to denature the proteins. 15 μl of each 20 μl reaction volume was loaded into a Novex 4–20% gradient tris-glycine gel (Life Technologies, Burlington ON Canada) and resolved with SDS/PAGE. The gel contents were transferred to a Hybond-P PVDF membrane (GE Healthcare Canada, Mississauga, ON) and blocked overnight in 1% ECL prime blocking agent (GE Healthcare Canada, Mississauga, ON) in phosphate-buffered saline (PBS) with 0.1 % Tween-20 (PBST). Following blocking, the membranes were treated with the Extravidin-Peroxidase horseradish peroxidase-conjugated avidin buffered aqueous solution (Sigma; St. Louis, MO, cat. No. E2886) in a 1:10000 dilution in 25 mM Tris-fortified isotonic phosphate buffer pH 7.4 for 30 minutes and washed 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.

Gel electrophoretic analysis of protein and SYPRO Ruby protein detection

The crude cockroach extract was diluted to 5–6 mg protein/ml. Diluted extract (10 μl) was combined with an equal volume of 2X SDS sample buffer containing β-mercaptoethanol and boiled as above for a total of 50 μg of protein in the sample volume. An aliquot (15 μl) of this sample was then loaded onto a gel for SDS/PAGE as above. At the end of the gel run, the gel was fixed (15% acetic acid, 10% methanol for 1h), and was then incubated in SYPRO Ruby stain (Invitrogen, Life Technologies; Carlsbad, CA) overnight in the dark on a shaker. The following day the gel was washed in fixing solution for 2 h in the dark and protein was visualized as UV light-activated fluorescence in the Kodak Image Station gel doc.

Ion exchange chromatography

Aliquots of the dialyzed crude cockroach extract (250 μl) were diluted to 1 ml in 20 mM acetic acid-acetate buffer, pH 5. Aliquots (1 ml) were loaded onto a 5 ml High S cation exchange column (40 mm X 12.6 mm) (Bio-Scale Mini Macro-Prep High S Cartridge, catalog # 732-4132; Bio-Rad, Hercules, CA) with a fast-phase liquid chromatography (FPLC) system (Pharmacia, Stockholm, Sweden) and 1 ml fractions were collected at a flow rate of 1 ml/min. The first 10 low-salt flow-through fractions were collected, followed by elution with a linear NaCl gradient from 0 to 1 M over 25 ml between fractions 10 and 35. Each collected fraction was assessed for its trypsin-like activity with the QAR-AMC fluorogenic substrate. Each active fraction was labeled with the biotinylated activity-based probe to identify the enzymes. This procedure was repeated with multiple 250 μl aliquots of the crude extract, and fractions containing each isolated trypsin-like enzyme were pooled. Pooled fractions of 15–20 ml containing each individual enzyme were concentrated using 15 ml Amicon Ultra centrifugal filter devices with a molecular weight cut off of 10 kDa (EMD Millipore, Darmstadt, Germany). For enzyme kinetics assays, the concentrated fractions containing individual enzymes were further purified with a High Q anion-exchange column (40 mm X 12.6 mm) (Bio-Scale Mini Macro-Prep High Q Cartridge catalog # 732-4122; Bio-Rad Canada Mississauga, ON). The fractions were each dialyzed-equilibrated against 20 mM Tris-HCl, pH 7.8, and the High Q column was equilibrated in the same buffer. The elution protocol and enzyme identification procedures, as outlined above for the High S fractionation protocol, were repeated for the High Q column.

Enzyme kinetics for cleavage of fluorogenic substrates

The 96 well micro-titer plate fluorogenic peptide cleavage method was used to determine enzyme kinetic profiles for each allergen enzyme. To determine substrate Km, 10 mU trypsin-equivalents of each fraction containing the isolated enzymes was added per well in 50 μl of Proteinase-assay buffer (200 mU/ml), and increasing concentrations of the substrates QAR-AMC (trypsin), phenylalanine-valine-arginine (FVR)-AMC (thrombin substrate), and glycine-glycine-arginine (GGR)-AMC (urokinase substrate) were added to each enzyme-containing well in 50 μl volumes in triplicate for final reaction volumes of 100 μl. Michaelis-Menten curves were generated for each of the enzymes and each of the substrates, and the Km was determined using the Michaelis-Menten analysis tool in GraphPad Prism 6 software. To determine an inhibitor IC50, 10 mU of each enzyme was

added to 50 μl of Proteinase Assay Buffer containing increasing concentrations of the trypsin inhibitors, Soybean Trypsin Inhibitor (SBTI) and N-α-tosyl L-lysine chloromethyl ketone (TLCK: Sigma, St. Louis, MO). The enzymes were pre-incubated for 15 min (SBTI) or 1h (TLCK). Following incubation with the inhibitor, 50 μl of a 750 μM solution of the QAR-AMC substrate was added for a final reaction volume of 100 μl and fluorescence tracked as described above. The half-maximal inhibitory concentration of each inhibitor (IC50) was determined using GraphPad Prism 6 software.

Avidin affinity chromatographic purification of activity-based probe-biotinylated enzymes

Each enzyme was purified for mass spectral sequencing after reaction with the biotinylated activity-based probe. 1 ml of the crude extract (~200 U total trypsin-like activity) was first passed through a DEAE sepharose anion exchange column (DEAE Sephacel, Pharmacia; Stockholm, Sweden) and a crude isolation of each enzyme was achieved with a batch elution method. 5 ml of DEAE sepharose slurry was poured into a column (1 X 2.5 cm) and allowed to settle. 20 ml of 50 mM Tris-HCl, pH 7.2, with 50 mM NaCl was passed through the column to equilibrate, following which the extract was applied. A low-salt wash was collected by applying 1 ml volumes of the 50 mM NaCl buffer until 15 x 1 ml fractions had been collected. This step was repeated for intermediate and high salt elutions by applying 15 x 1 ml volumes of the Tris buffer containing 250 mM NaCl and 500 mM 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 cutoff of 10 kDa (EMD Millipore, Darmstadt, Germany). Aliquots of each fraction obtained from the DEAE column containing a total of 16 U of trypsin-like activity (from 40 to 350 μl; estimated to represent 1 μg of enzyme protein) were reacted with 100 μM of the activity-based probe to biotinylate each enzyme. Biotinylated enzymes were then applied to streptavidin-conjugated magnetic sepharose beads (Streptavidin MagSepharose; GE Healthcare catalog # 28-98372-99) and allowed to react for 1 h. The enzyme-bound beads were washed with 1 M urea to remove contaminants, and then the avidin bead-bound proteins were eluted by boiling for 5 min in 10 μl of 2% SDS. An equal volume of 2X SDS sample buffer containing β-mercaptoethanol was added to the eluted product and the mixture was denatured by boiling. The samples were then resolved by SDS/Polyacrylamide gel electrophoresis in a 4–20% tris-glycine gradient gel (Life Sciences) and the gel stained for total protein with Coomassie blue (Bio-Rad). Bands visible by eye in the 20–26 kDa range were excised from the gel and sent for mass spectral sequencing. All experiments were performed in a fume hood and all reagents used were passed through a 0.22 μm filter to reduce keratin contamination of the samples.

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

The previously reported annotated cockroach EST library [14] was provided by Dr. Mee Sun Ock (Kosin University College of Medicine, South Korea). Putative serine proteinases were identified by searching the annotated library 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 the ExPASy online translation program (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 (bgtryp-1) identified by Ock et al [12], were then used to deconvolute the sequences, based on the mass spectral analysis of the excised gel bands. Sequence alignment for the three *germanica* enzymes that we characterized, E1, E2 and E3, in comparison with each other and with the previously published cockroach sequences (bgtryp-1 and Per a 10 [8, 9, 12]) was done using the EMBL-EBI Clustal Omega online multiple sequence alignment tool that also generates the percent identity matrices (http://www.ebi.ac.uk/Tools/msa/clustalo/) [15]. The three *B. germanica* enzyme sequences were also compared in the same way for sequence alignment with the other *Blattella* antigen sequences in the database (Bla g 1 and Bla g 2) as well as the sequences of the dust mite proteinases, Der p 1, Der p 3 and Der p 6.

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 using previously published approaches [16–18]. Excised coomassie-stained protein gel bands following 1D SDS-PAGE were digested with chymotrypsin (10 μg/mL) at 37°C overnight. LC-MS/MS analysis of in-gel chymotrypsin digested-proteins was done 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 minutes, followed by a 35–45% gradient of solvent B over 9 minutes, and finally a 95% increase and hold over 0.1 and 5 minutes, 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 [17] 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 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 [18] as determined by a reversed database search.

Calcium signalling by PAR2-expressing KNRK cell monolayers

KNRK cells were transfected with a rat PAR2 construct C-terminally tagged with YFP (PAR2-YFP) inserted into a pcDNA vector [19] and grown in G418-containing Dulbecco's modified eagle medium (DMEM; Gibco, Life Technologies, Carlsbad, CA) with sodium pyruvate and Plasmocin treatment (Invitrogen) as described previously [19]. To monitor calcium signalling, the no-wash calcium-sensing dye Fluo-4 NW (Invitrogen, F36206) was dissolved in 10 ml of calcium assay buffer (1X HEPES-fortified Hanks buffered saline, pH 7.4 (10 mM HEPES, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$) at a final concentration of 5 μg/ml, along with 2.5 mM probenecid (Invitrogen). The PAR2-expressing KNRK cells were grown to confluence in a 96-well black cell culture plate (BD Falcon; Franklin Lakes, NJ) and loaded with the dye by incubating in 50 μl of the dye solution for 30 min at 37°C. Agonists were diluted to the desired concentrations in calcium assay buffer and 50 μl volumes were applied to the cells with the Perkin-Elmer plate reader's injector system. Kinetic traces of fluorescence measurements representing increased cytoplasmic calcium levels were recorded with an excitation wavelength of 480 nm and an emission wavelength of 530 nm with recordings every 0.5 s for 1 min. Fluorescence levels were normalized to the signal generated by 2 μM calcium ionophore A23187 (Sigma, St. Louis MO).

Monitoring activation of MAPKinase

The PAR2-expressing KNRK cells were grown to confluence in a 24-well plate (Thermo Scientific; 2 cm^2 area) and serum-starved by incubation overnight in serum-free Dulbecco's Minimal Essential Medium (DMEM) culture medium. Fresh serum-free medium was added the following day and the cells incubated another 4 h. PAR agonists and enzymes were diluted in serum-free DMEM and applied to the cells at room temperature for 10 min. The culture media were then aspirated and the cells were lysed in 100 μl cold lysis buffer containing Protease inhibitor cocktail (Calbiochem, San Diego, CA; set III) for 10 min on ice. The protein-containing lysis buffer (100 μl) was removed from the cells and clarified in a 1.5 ml microfuge tube by centrifugation at 4°C for 5 min at 1500 rpm. Supernatants were collected and combined with an equal volume of 2X SDS sample buffer containing β-mercaptoethanol followed by denaturation 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 anti-phospho-ERK 1/2 (T202/Y204) antibody (Cell Signaling Technologies, Danvers, MA) 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 anti-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 hours 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, 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.

Mapping cleavage of the PAR2-derived N-terminal tethered ligand-containing sequence using HPLC-mass spectroscopy

A synthetic peptide representing the N-terminal extracellular sequence of rat PAR2 was synthesized (Table 2) and evaluated for hydrolysis by the purified cockroach serine proteinases, as described previously [20, 21]. Peptides derived from the N-terminus of PAR2 (100 μM in a total volume of 150 μL) were incubated with enzymes (2 U/ml trypsin-like activity) for up to 30 min at 37 °C. Reactions were stopped by adding 150 μl of ice-cold 0.1% trifluoroacetic acid (TFA) in water. 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.

BRET-based detection of β-arrestin recruitment to PAR2

HEK293 cells were transfected with human PAR2-YFP and β-arrestin-1 or β-arrestin-2-rLuc and measurements of PAR2 agonist-stimulated receptor-β-arrestin interactions were monitored by bioluminescence resonance energy transfer measurements (BRET), as previously described [19]. PAR2 was activated by each of the cockroach enzyme-containing fractions at concentrations of 1 U/ml of trypsin-like activity and the interaction of PAR2 and β-arrestin-1 or β-arrestin-2 was monitored by the BRET signal.

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. Significance was assumed with a p value ≤ 0.05.

RESULTS

Cleavage of different fluorogenic substrates by crude cockroach allergen extract

In preliminary experiments we found that the crude dialysed cockroach allergen extract (0.5 U/ml trypsin-like activity, using the QAR-AMC substrate) cleaved all four of the AMC-tagged substrates to varying degrees, with the highest cleavage found for the trypsin substrate, QAR-AMC. For each of the substrates, treatment with soybean trypsin inhibitor (1 μg /ml) completely blocked cleavage, indicating that all of the proteolytic activity responsible for the cleavage of the substrates was trypsin-like (not shown). We thus used the QAR-AMC substrate to quantify the trypsin-like activity of the crude allergen and to monitor the enzyme activity eluted from the ion-exchange columns (below). On average, the crude extract, prepared in 5 ml aliquots (about 12 to 13 mg/ml protein), had a specific

activity of approximately 16 U/mg trypsin-like activity. This crude dialysed solution was used for further characterization. Our strategy was 1. to use conventional ion exchange chromatography to separate the enzymes from each other in sufficient purity to assess their individual enzymatic properties and 2. to use streptavidin affinity chromatography of the ABP-biotinylated enzymes to isolate sufficient amounts of each of the enzymes for mass spectral sequence analysis.

Activity based probe labeling of the cockroach allergen serine proteinases

For our work, it was essential to verify that the new batch of allergen provided to us by Greer contained the same proteinases we detected in previous preparations. Labeling of the proteins in the crude allergen extract with the serine proteinase-specific activity-based probe revealed only three distinct bands upon gel electrophoretic analysis (Figure 1, Right panel). The molecular masses of the three labeled enzymes were the same as those observed by us previously [13]. These three ABP-labelled proteins, singled out amongst the multitude of proteins detected by the SYPRO Ruby stain (Figure 1, Left panel), were denoted enzymes 1 to 3 (E1, E2, E3) in descending order of electrophoretic mobility (fastest to slowest; smallest to largest molecular size) in the SDS/PAGE gel. It was our goal to separate the three enzymes chromatographically and to characterize each one biochemically.

Ion-exchange chromatographic isolation of cockroach allergen trypsin-like enzymes

Each of the three enzymes was eluted from a High S cation-exchange column in distinct fractions (Figure 2A). In the ‘low-salt pass-through fractions’, isolated Enzyme E3 emerged first (fraction 4), followed by E2 (fractions 6–7). Enzyme E1 was then eluted with the salt gradient (fractions 19–21). Thus at pH 5, enzymes E2 and E3 had a net negative charge, and E1 had a net positive charge, enabling its binding to the negatively charged cation-exchange resin. The separation of the three enzymes was verified by the detection of only a single ABP-labeled protein in each pooled fraction (Figure 2B, left-hand panel). Each enzyme fraction was then purified further by fractionation using the High-Q anion exchange column from which only a single peak of enzyme activity was recovered for each enzyme (Figure 3A–3C), as indicated by the detection of only a single ABP-labeled constituent (Figure 3D–3F:). A comparison of the SYPRO Ruby-stained gels from the fractions eluted from the two ion-exchange columns showed that the majority of the proteins present in the initial crude allergen had been eliminated from the purified fractions obtained from the High-Q column (Compare the SYPRO Ruby-stained gels in Figures 2 and 3 for the fractions containing enzymes E1, E2 and E3) with the stained gel shown in Figure 1. Further fractionation on a size-separation column resulted in a substantial loss of enzyme activity and did not yield fractions with purities much greater than those recovered from the High-Q column (not shown).

Since only a single active ABP-labeled enzyme was recovered in each of the E1, E2 and E3-containing fractions from the High-S column, these fractions were deemed of sufficient purity for evaluating the ability of each enzyme to signal via PAR2. The higher purity fractions obtained from the High Q column had lower concentrations of active enzymes, and so were not appropriate for use in the PAR signaling assays. However, the high Q-purified fractions had sufficient enzyme activity to be used to assess the kinetic properties of the

three enzymes using different substrates (Km) and chemically distinct inhibitors (IC50), as summarized in the following section.

Biochemical characterization of enzymes E1, E2 and E3

The characteristics of each enzyme were determined by measuring the Km's for three distinct serine proteinase substrates (QAR-AMC, FVR-AMC and GGR-AMC), which are routinely used to measure the activity of trypsin, thrombin and urokinase, respectively. Each of the three cockroach enzymes was found to have comparable Km's for the trypsin-preferred substrate, QAR-QMC, but differential Km values with the substrates FVR-AMC and GGR-AMC (Table 1). The three enzymes also were inhibited with distinct IC50's by the inhibitors, SBTI and TLCK (Table 1). Thus, the three cockroach allergen serine proteinases were quite different in terms of 1. Chromatographic elution profiles, 2. substrate Km's and 3. inhibitor IC50's. Our next aim was to compare the three enzymes isolated from whole cockroach with those detected in frass and ultimately to determine their amino acid sequences.

Characterization of serine proteinases in cockroach frass

Because the aerosolized environmental cockroach-derived allergens to which individuals become sensitized may be more due to the frass deposited in a locale rather than from the aerosolized residue from intact dead insects, we were interested to compare the enzymes present in the cockroach extract used for clinical testing with the enzyme present in cockroach frass. For practical reasons, it was unfortunately necessary to use frass obtained from a cockroach species different from the one used to prepare the cockroach extract. Notwithstanding, the ABP labeling procedure revealed only one biotinlyated enzyme in the frass (Figure 4). The frass enzyme aligned in the SDS/PAGE gel in the 24–26 kDa region of the gel, along with enzyme E1 from the total body extract obtained from our Greer supplier (Figure 4A). Proteolytic activity from the frass extract, like E1 from the total body extract, was eluted in the low-salt non-binding fractions from a High Q anion-exchange column (compare Figure 4B with Figure 3A). The properties of the frass-derived enzyme were closest to those of enzyme E1; but frass enzyme F1 was distinct from E1 in terms of its sensitivity to SBTI, although the sensitivity of the two enzymes for inhibition by TLCK was comparable (Figure 4C).

The cockroach enzymes mimic trypsin cleavage of the synthetic PAR2 tethered ligand sequence

As shown in Table 2, all of the cockroach enzymes cleaved the synthetic PAR2 peptide representing the cleavage-activation sequence of rat PAR2 at arginine 36, the conventional activation site of PAR2 unmasked by trypsin in PAR2 found in all species to date, including humans. This cleavage reveals the PAR2 receptor-activating tethered ligand sequence, SLIGRL. Thus, all of the three enzymes E1, E2 and E3 would be expected to generate PAR2 signals like those caused by trypsin.

Amino acid sequences of enzymes E1, E2 and E3: mass spectral analysis of chymotryptic cleavage fragments deconvoluted with sequences obtained from a cockroach expressed sequence tag library

For mass spectral sequencing of enzymes E1, E2 and E3, we found it necessary to improve the yield of enzyme from a cation exchange column prior to ABP-biotinylating the three enzymes in preparation for avidin-sepharose affinity purification. Preliminary separation of the three enzymes was thus achieved using a DEAE-sepharose ion exchange column. The three DEAE-sepharose fractions (DEAE1, DEAE2, and DEAE3) which contained the three enzymes, E1, E2 and E3 respectively (Figure 5A, 5B) were then biotinylated with the activity-based probe (Figure 5A) for further isolation using streptavidin-sepharose. Each biotinylated enzyme was applied to the streptavidin column and the unadsorbed and eluted protein for each preparation was analysed by SDS/PAGE. This approach yielded in the streptavidin-column-eluted fractions, a SYPRO Ruby-stained band for each of fractions DEAE 1, DEAE 2 and DEAE 3 that corresponded to the mobilities of each of the ABP-labeled enzymes (Figure 5C). These bands were excised individually from the gel for mass spectral sequencing as described in Methods.

The mass spectral data for the chymotryptic cleavage products of the proteins recovered from the streptavidin affinity column were matched with three trypsin-like sequences found in the cockroach EST library: CL9Contig1, CL1Contig8 and CL53Contig1. Analysis of the mass spectra generated from multiple chymotryptic peptides generated from the three individual gel bands revealed that each EST sequence was found with 100% probability in the three samples: E1 was found to match to CL1Contig8, E2 matched to CL53Contig1, and E3 matched to CL9Contig1 (Figure 6). The sequences of the three *Blattella germanica* enzymes were highly homologous with about an 80% identity found between enzymes E2 and E3 (Table 3). Enzyme E1 had about a 55 to 60% sequence identity with enzymes E2 and E3. Enzymes E2 and E3 also showed a 71% sequence homology with the *Periplaneta americana* cockroach proteinase previously cloned (Per a 10: Table 3) [9], whereas enzyme E1 showed a 58% sequence homology with the *P. americana* enzyme. In contrast, the three isolated serine proteinases (E1, E2, E3) showed only a 20% sequence homology with the *Blattella. germanica* aspartic proteinase allergen, Bla g 2 and a 14 % homology with the Blattella antigen Bla g 1, which itself has only an 18% sequence homology with Bla g 2 (Table 3). Similarly, the proteinases we sequenced had only a 20% homology with the dust mite allergen cysteine proteinase, Der p 1; but there was a much higher degree of homology (40%) with the dust mite serine proteinase Der p 3, which is able to activate PAR2 [24,11] and Der p 6 (30%), which like Der p 3 is also a serine proteinase (Table 3, bottom two rows). Of note, the sequence in the vicinity of the key amino acids in the tryptic catalytic triad, histidine, aspartic acid and serine were conserved in all of the insect trypsin-related enzymes (Table 3, Figure 10 and sequences not shown).

Activation of calcium signaling via PAR2

Each isolated enzyme was found to elicit a Ca^{++} transient signal in the PAR2-expressing KNRK cells at a concentration of 1 U/ml (representative tracings shown in Figures 7A–7C) but not in non-transfected KNRK cells (not shown). The calcium signalling responses, normalized to the signal generated by calcium ionophore, were comparable for each of

enzymes E1, E2 and E3 at 1 U/ml. Further, these calcium concentration transients were blocked either by pre-treating the enzymes with soybean trypsin inhibitor (SBTI: blue tracings, Figs 7A to 7C) or by prior desensitization of the KNRK cell PAR2 receptor by exposure to an excess of the PAR2-selective agonist, 2fLI (red tracings, Figures 7A to 7C; summarized by histograms in Figure 7D).

Each cockroach enzyme activates MAPKinase-ERK 1/2 via PAR2

As shown by the representative blots in Figure 8A, each isolated enzyme induced an increased phosphorylation of MAPKinase-ERK 1/2 at a concentration of 1 U/ml trypsin-like activity. The degree of MAPKinase-ERK 1/2 activation over baseline by the cockroach enzymes was comparable to the signal generated by 1 U/ml trypsin or by 1 μM of the PAR2-activating peptide, 2fLI. Pre-treatment of the enzymes with SBTI diminished the activation of MAPKinase-ERK 1/2 by all of the enzymes, but had no effect on activation by the PAR2-activating peptide. Thus, in addition to activating PAR2 calcium signaling, each of the cockroach enzymes activated PAR2-mediated MAPKinase-ERK signaling.

Each cockroach enzyme induces β-arrestin recruitment to PAR2

Treatment of HEK293 cells expressing both a YFP-tagged PAR2 and rLuc-tagged β-arrestin (either β-arrestin 1 or 2) with the isolated enzymes obtained from the High S column revealed that each enzyme induces the recruitment of β-arrestin to PAR2 following receptor activation (Figure 9). There appeared to be small differences in the abilities of the three enzymes to recruit arrestin-1 vs arrestin-2 to the receptor.

DISCUSSION

Our main finding was that the crude *Blattella germanica* cockroach allergen extract used to monitor patient sensitivity contains three serine proteinases that are distinct according to their substrate and inhibitor characteristics (different Km’s and IC50’s) and their amino acid sequences. Further, all three enzymes are capable of signalling via PAR2 to drive both calcium and MAPKinase-ERK1/2 signaling along with promoting the interaction of PAR2 with beta arrestins-1 and -2. Thus, all three enzymes in principle can contribute to ability of PAR2 to enhance the allergic response [13].

The purification method that we developed using the activity-based probe to biotinylate the trypsin-like enzymes specifically in the crude extract and to purify the tagged enzymes using streptavidin-linked beads in amounts sufficient for mass spectral sequencing may be applicable in the identification of proteolytic enzymes in other allergens for comparison with the enzymes we describe here. Enzymes E2 and E3 are closely related to the proteinase (bgtryp-1) previously cloned by Ock et al. [12] with a sequence identity of 80 and 97% respectively. Very possibly enzyme E3, which differs slightly in sequence from bgtryp-1 is the same as bgtryp-1, but may represent a genetic polymorphism that can exist within the species. Our finding of three trypsin-like proteinases in the cockroach extract complements the identification of a single enzyme (Per a 10) from *Periplaneta americana* extracts isolated using a benzamadine affinity column [8, 9]. The *Blattella germanica* enzymes E2 and E3 exhibit 71% sequence identity with Per a 10, whereas the E1 enzyme has a 58% sequence

identity (Figure 10 and Table 3). However, all four cockroach enzymes have an identical zymogen cleavage//activation site sequence (GR//IVGG: see boxed in sequences in Figure 10) which is the same as the one found in *Drosophila* trypsin-alpha (see MEROPS database). Further, all enzymes show high sequence homology in the vicinity of the catalytic triad amino acids, histidine (H), aspartic acid (D) and serine (S) (Boxed amino acids in Figure 10) and all cockroach proteinases have 6 conserved cysteines, which are the same as those found in all trypsins ranging from human trypsin-1 to *Drosophila* trypsin-alpha (Figure 10; not shown for trypsin-alpha). Clearly the three *Blattella* serine proteinases we have isolated differ considerably from the *Blattella* aspartic acid proteinase antigen, Bla g 2 [25] and the other antigen, Bla g 1. Of note, the insect trypsins, which retain the conserved catalytic triad amino acids, lack two conserved cysteines found in all mammalian trypsins, one of which is just 7 residues C-terminal to the zymogen activation sequence; and the insect trypsins also lack two additional cysteines found in human trypsin-1 and mesotrypsin. Notwithstanding, overall, the disulphide-maintained conformation of all of the enzymes due to the 6 conserved cysteines would be expected to expose both common and distinct antigenic sites. Given the demonstrated reactivity of Per a 10-sensitized human subjects in terms of their intradermal response, IgE immunoreactivity and Per a 10-stimulated histamine release by peripheral blood leukocytes [8, 9], it is possible that there may be cross-sensitization to enzymes from all cockroach species and also possibly to mammalian and fish trypsins to which agricultural and fish plant workers are exposed [22, 23]. Whether or not the insect serine proteinases represent significant allergens themselves, the ones we have studied so far all appear to be able to signal via PAR2. Thus, all of the enzymes E1, E2 and E3 in isolation would be able to synergize with other allergens in the cockroach extract to trigger the allergic process.

Interestingly, the single enzyme we found in the frass of the speckled cockroach (*Nauphoeta cinerea)* aligns with enzyme E1, both in migration in a SDS/PAGE gel (thus, suggesting similar molecular weights), and in passage through an anion-exchange column, suggesting common charge properties. Although the frass enzyme inhibitor kinetics are distinct from those of the E1 enzyme, the frass enzyme appears to be more homologous with the German cockroach E1, and not with the previously cloned German and American cockroach trypsins (E3 and Per a 10). One possibility is that the frass contains an E1-like enzyme that has been partially processed by intestinal digestion prior to excretion. However, more likely, the species differences in the enzyme sequences between the German cockroach (*Blattella germanica)* and the frass source (*Nauphoeta cinerea)* may account for these differences in biochemical properties. Unfortunately we were unable to obtain German cockroach frass for a direct comparison; and we were not able to obtain sufficient quantities of the enzyme from *Nauphoeta* frass for sequencing. Further work with the frass-derived enzymes is warranted, since the frass may represent the major source of environmental cockroach allergens.

Ideally, we were aiming to isolate amounts of the three German cockroach enzymes that would enable us to evaluate their impact individually on sensitization *in vivo*, using our intranasal allergen challenge model [13]. Unfortunately, the abundance and enzyme activities of the purified E1, E2 and E3 fractions were not sufficient for the *in-vivo* assays. Nonetheless, by identifying the EST sequences in the cockroach library that correspond to each serine proteinase, it may now be possible to express each cockroach enzymes recombinantly to evaluate their effects *in vivo.* This approach would help to distinguish the

importance of the immunogenic properties of the enzymes themselves versus the ability of the enzymes to trigger epithelial PAR2 signalling events that amplify the allergen-induced response. Based on the data obtained with Per a 10, which is antigenic in humans [8, 9], and given the considerable sequence identity of Per a 10 with enzymes E1, E2, and E3, we expect that sensitization to Per a 10 will also confer sensitivity to all three of the enzymes we have isolated from the German cockroach (and vice-versa).

Our data show that each of the cockroach serine proteinases cleave and activate PAR2 in a manner similar to that of porcine trypsin, the conventional ‘agonist’ enzyme used for activating PAR2. Thus together, the enzymes in the cockroach extract activate both the calcium (Gq) and MAPKinase-ERK1/2 arms of the PAR2 signaling cascade in concert with triggering PAR2-beta-arrestin interactions. We thus propose that common signal transduction pathways triggered by PAR2 can be involved in the driving the allergic process by allergen-containing trypsins. Thus, both the enzyme activities themselves and their common signal pathways represent potential therapeutic targets to block the induction of lung epithelium-derived inflammatory mediators in the allergen-exposed airway.

In summary, our work has identified the three key PAR2-regulating trypsin-like serine proteinases present in the German cockroach extract that can, as a combined stimulus, amplify the allergenic response. Whether these enzymes themselves trigger the same kind of immune response as the allergens Bla g 1 and Bla g 2 remains to be determined. Nonetheless, by blocking the activity of these three enzymes along with targeting their PAR2 signal transduction pathways, it should in principle be possible to attenuate the allergenic asthma-generating actions of cockroaches.

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ABBREVIATIONS USED

ABP	serine proteinase-targeted activity-based probe
AMC	aminomethlylcoumarin; Amino acids are designated by their one-letter codes e.g. A = alanine; F = phenylalanine
BRET	bioluminescence resonance energy transfer
CE	defatted aqueous cockroach extract of intact *Blattella germanica* provided by Greer

CF	*Nauphoeta* cockroach frass
KNRK	Kirsten virus transformed rat kidney-derived epithelial cell line
PBS	phosphate-buffered isotonic saline, pH 7.4
PBST	PBS supplemented with 0.1 % Tween-20
PAR	proteinase-activated receptor (PAR1, PAR2)
SBTI	soybean trypsin inhibitor
TFA	trifluoro-acetic acid
TLCK	N-α-tosyl L-lysine chloromethyl ketone

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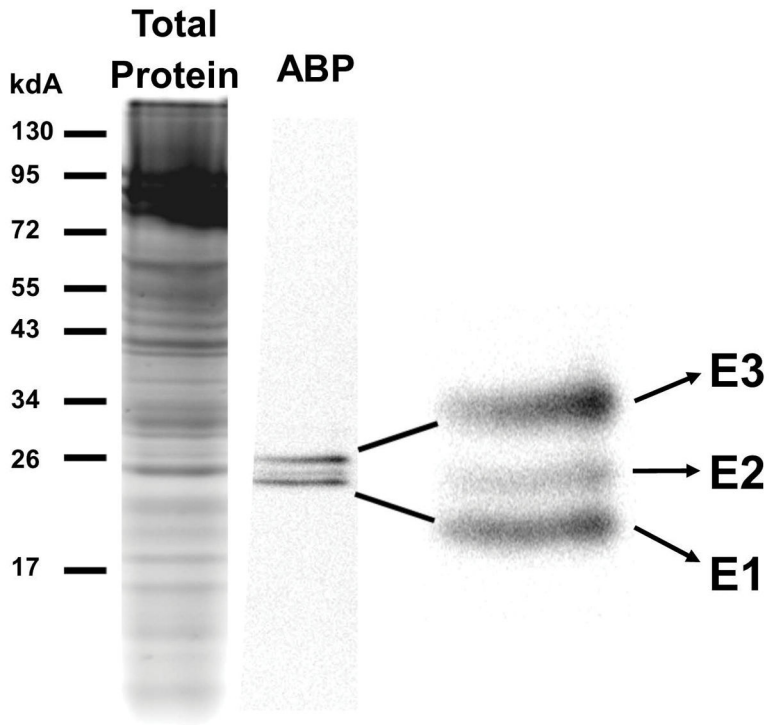


Figure 1. Activity-based probe labeling of serine proteinases E1, E2 and E3

Left Lane:Total cockroach extract was resolved with SDS/PAGE and stained for total protein with SYPRO Ruby fluorescent protein stain. Right lanes: Activity-based probe labeling of the total extract revealed three biotinylated enzymes (middle lane; expanded on the right).

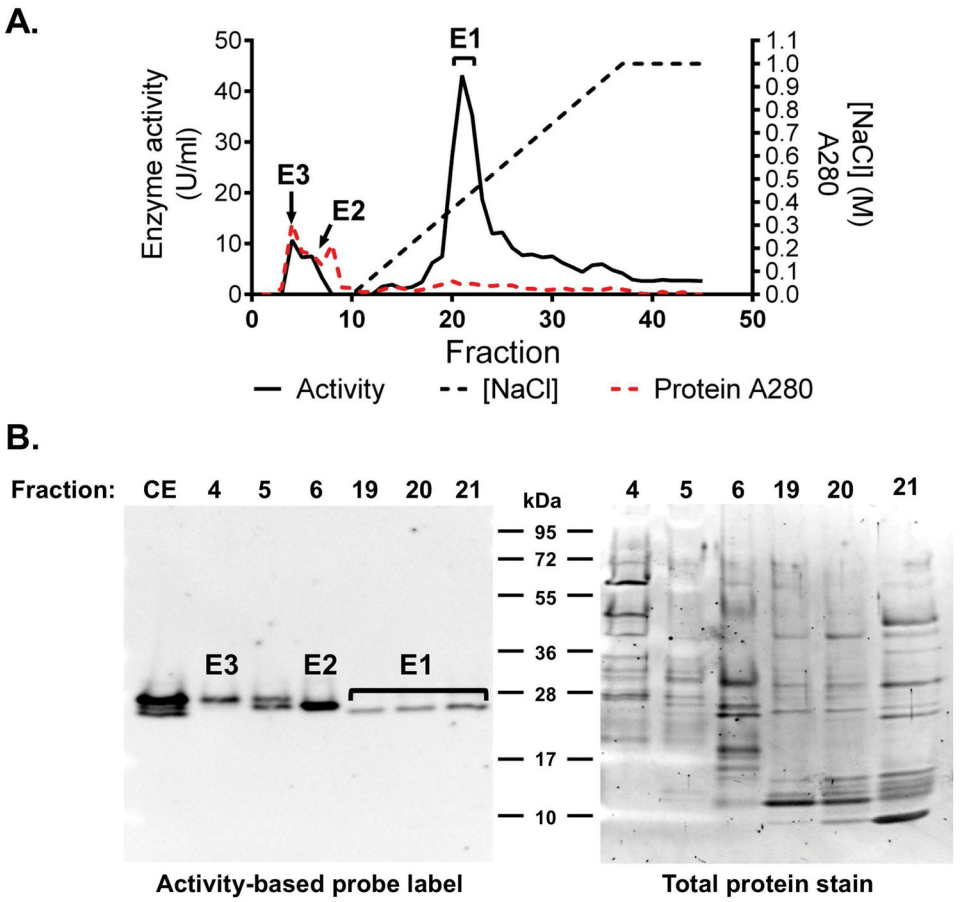


Figure 2. Cation-exchange fractionation of total cockroach extract

(A) The crude allergen extract was passed through a High S cation exchange column using a salt gradient (dashed lines, right-hand Y-axis) and collected in 1 ml fractions (X axis). Enzyme activity was tracked with the QAR-AMC substrate (black line, left Y axis), and protein was estimated by absorbance at 280 nm (red line, right Y axis). **(B)** Active fractions were labeled with ABP (left blot), revealing isolated E3 (fraction 4), E2 (fraction 6), and E1 (fractions 19–21). The total protein of each fraction resolved by SDS/PAGE was stained with SYPRO Ruby (right image).

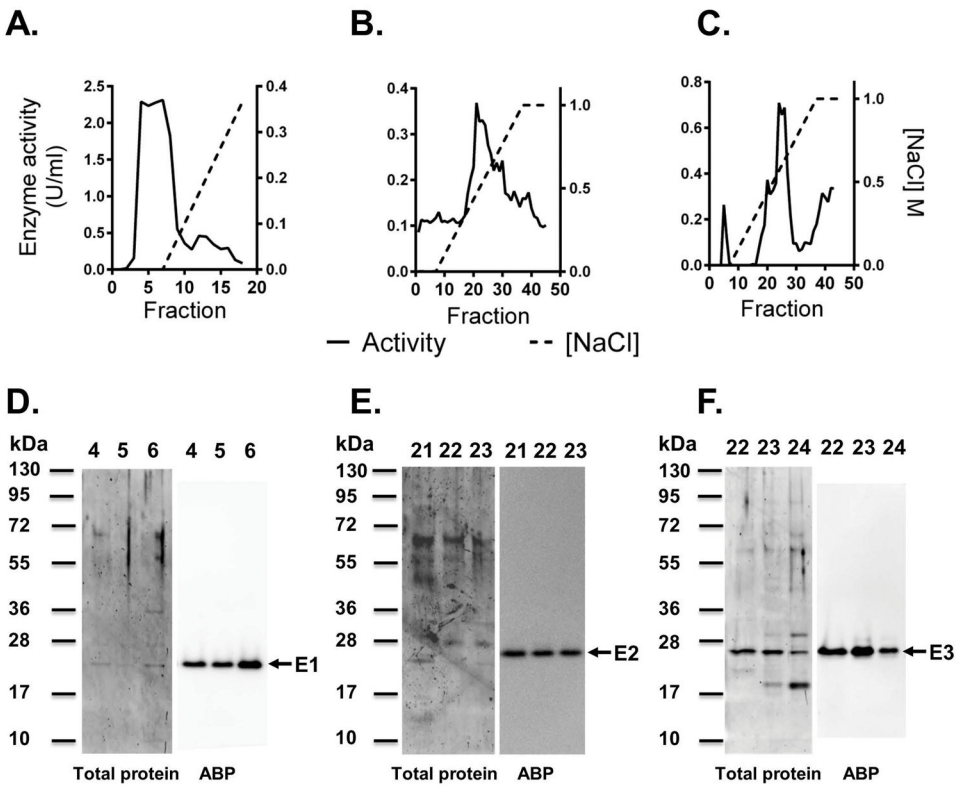


Figure 3. Anion-exchange chromatography of isolated cockroach enzymes

(A) Concentrated High S fractions containing each isolated enzyme were applied separately to a High Q anion-exchange column (Panels A, B and C) and eluted with a salt gradient (dashed lined) as outlined in Experimental Procedures. Enzyme activity (U/ml: solid lines) in the eluted fractions (numbers at bottom of chromatograms) was monitored by measuring the cleavage of QAR-AMC. Peak fractions containing enzyme activity (numbers at top of gels: D, E and F) were analysed by SDS/PAGE to visualize total protein (SYPRO Ruby total protein stain shown on the left side of each image pair) and by activity-based probe (ABP) biotinylation followed by SDS/PAGE-avidin blot detection to detect each serine proteinase (E1, E2 and E3: panels D, E and F respectively). This staining reveals a high degree of purity for each enzyme, labeled with the ABP (right side of each image pair). ABP labeling showed that each of the three enzymes E1, E2 an E3 was separated from the others by the chromatographic procedure.

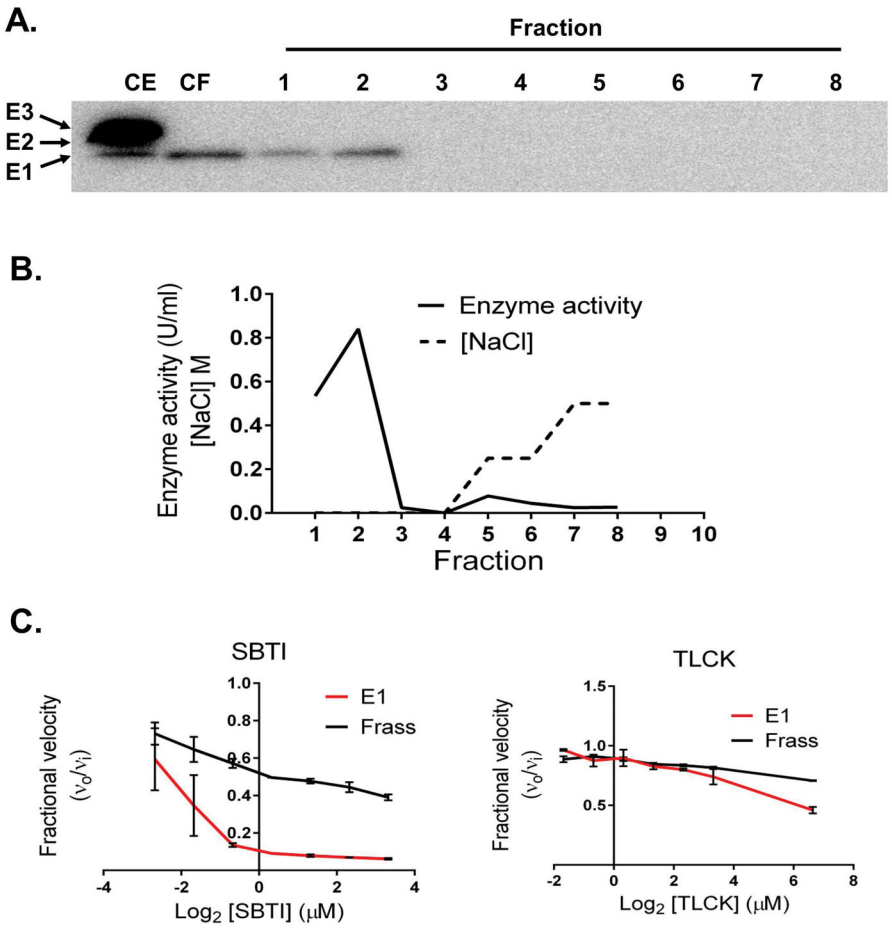
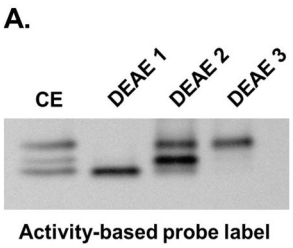


Figure 4. Cockroach frass contains one trypsin-like proteinase

(A) Activity-based probe labeling of cockroach extract and Frass extract. The single ABP-labeled enzyme in the cockroach Frass extract (CF) is compared with the enzymes labeled in the German cockroach allergen extract (CE: E1, E2, E3). **(B) Chromatography of Frass extract.** The non-binding fractions obtained using anion-exchange chromatography (low salt elution, before salt gradient: dashed lines) contained trypsin-like activity (black line). There is a single band in the Frass extract fractions 1 & 2 that is labeled with the activity-based probe, which aligns with the lowest molecular weight band (E1) in the total body extract (CE): Top panel, A. **(C) Inhibitor profile of Frass enzyme.** Inhibition of the Frass enzyme fraction by Soya Bean Typsin inhibitor and TPCK (Fractional velocity: Vo/Vi black tracing) is compared with the inhibition of enzyme E1 (red tracing). Values represent the mean ± SEM, for n = 3.



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

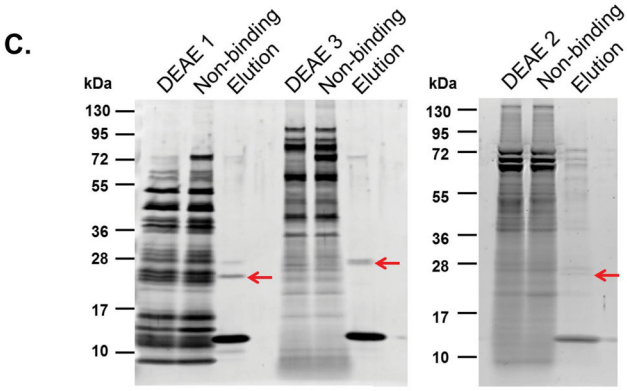


Figure 5. Affinity chromatography isolation of biotinylated cockroach proteinases with avidin-linked sepharose

(A) The crude extract was passed through a DEAE sepharose column and proteins collected in a batch elution method (DEAE 1 – 50 mM NaCl; DEAE 2 – 250 mM NaCl; DEAE 3 – 500 mM NaCl). **(B)** The specific activity of each fraction is summarized in the table. **(C)** Concentrated fractions from the DEAE sepharose column were biotinylated with ABP and applied to avidin-conjugated sepharose beads followed by SDS/PAGE analysis of the unbound and avidin bead-bound proteins. SYPRO staining of the unbound vs bead-bound proteins is compared with the starting DEAE fractions Bands from the eluted avidin beads, visible in the range of the enzymes (~20 – 28 kDa), were excised and sent for mass spectral sequence analysis (indicated by red arrows). Free avidin released from the affinity beads by the elution procedure shows as a biotin-binding protein in the 15 kDa region.

Figure 6. Amino acid sequences of enzymes E1, E2 and E3

The three sequences identified in the EST library (CL1Contig 8, CL53Contig1, CL9Contig1) matched with 100% probability to the mass spectrally-determined sequences obtained for the purified cockroach enzymes (E1, E2 and E3 respectively). The green lines underneath the sequences represent peptides that matched, with green representing 99% probability and red representing 95% probability based on an analysis of Sequest XCorr scores.

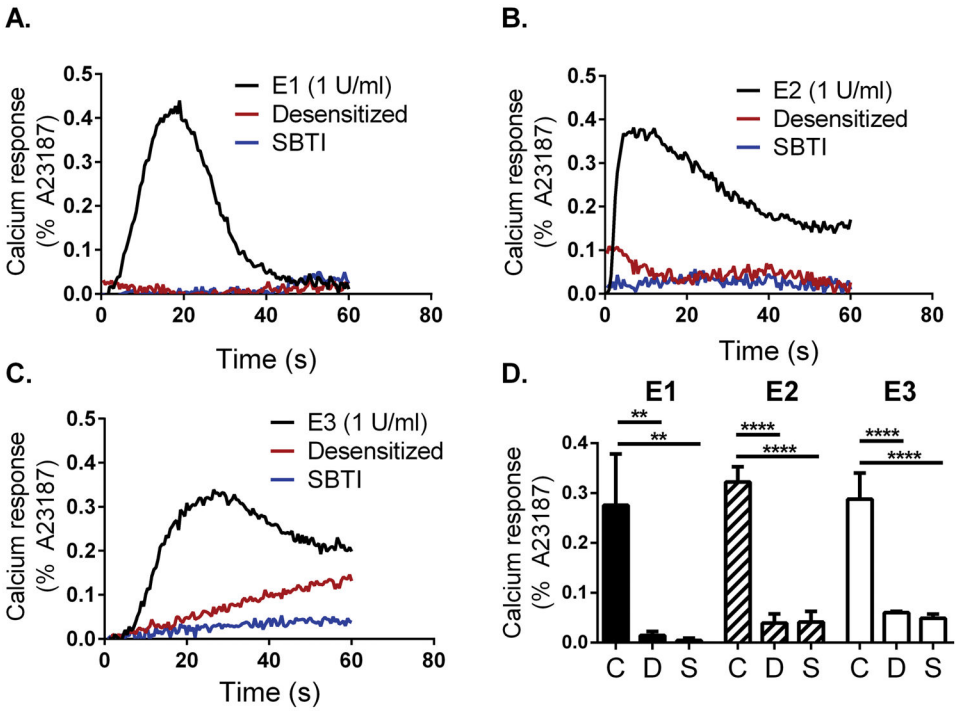


Figure 7. PAR2 calcium signalling stimulated by enzymes E1, E2 and E3

PAR2-expressing KNRK cell monolayers were loaded with the calcium-sensitive fluorescent dye Fluo-4. 1 U/ml each of E1 **(A),** E2 **(B)** and E3 **(C)** (representative traces) activate calcium concentration transients (black traces), which are blocked either by pre-treating the enzymes with 50 μM SBTI (blue traces) or by prior desensitization of the cell PAR2 by treatment with an excess of the PAR2-selective agonist peptide 2fLI (red traces). **(D)** Quantification of the calcium transients as a percentage of the response induced by the calcium ionophore A23187 (2 μM) in the control (**C**), desensitized (**D**) and SBTI-inhibited (**S**) conditions. Histogram values represent the mean responses ± SEM for n = 4 (**p<0.01, ****p<0.0001)

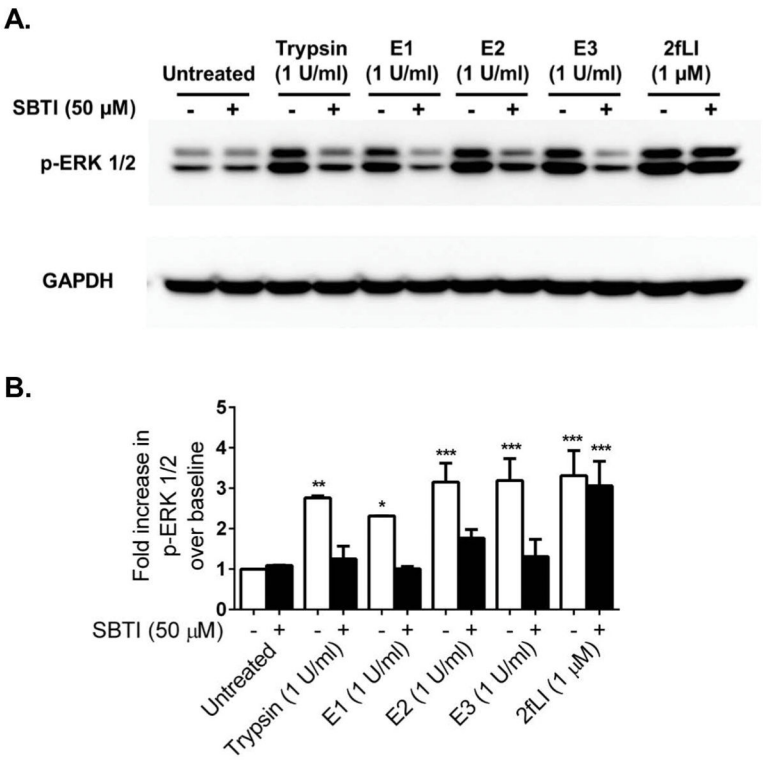


Figure 8. ERK 1/2 activation stimulated by enzymes E1, E2 and E3 via PAR2

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

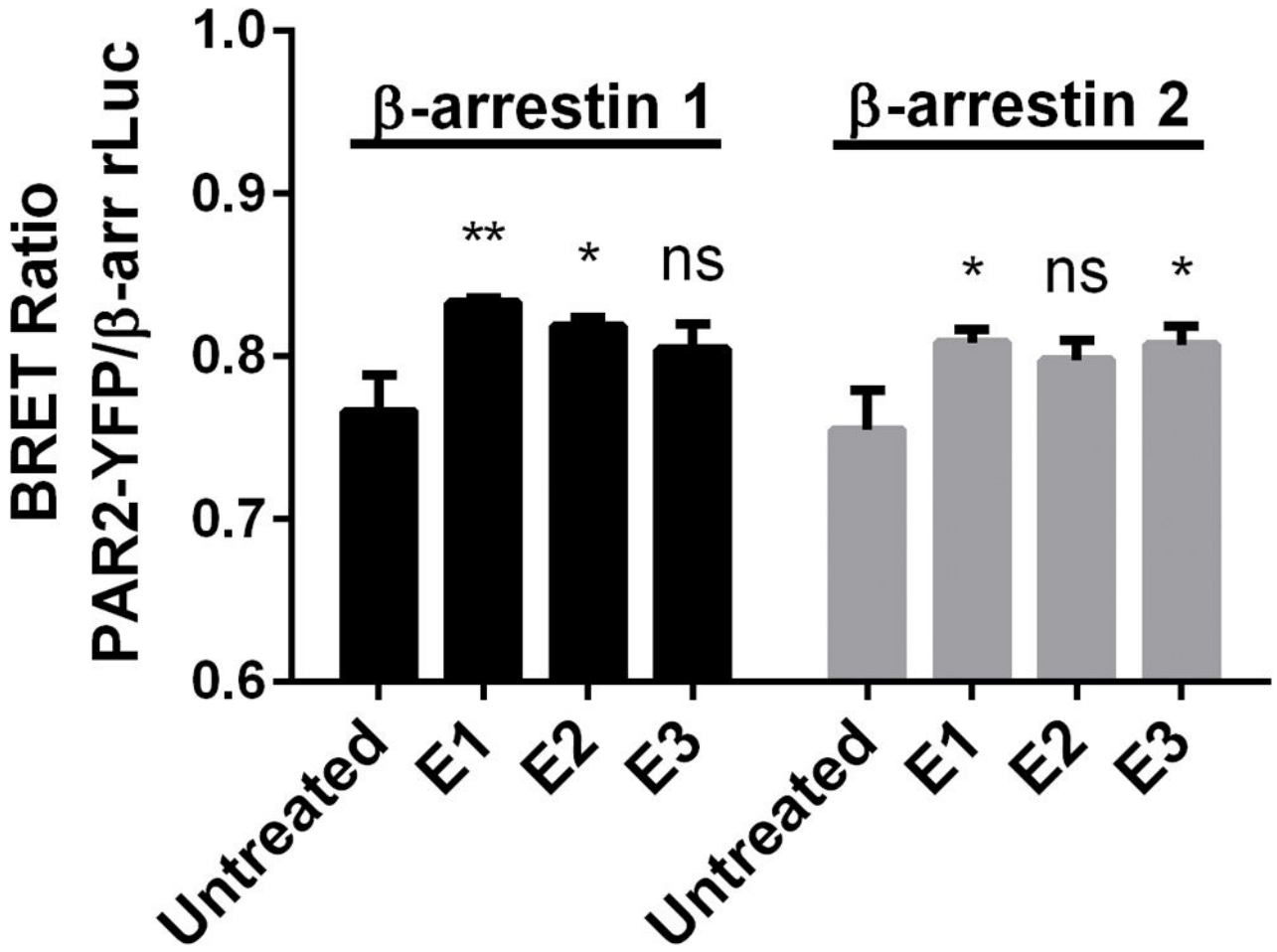


Figure 9. Recruitment of PAR2 to β-arrestin upon activation of PAR2 by enzymes E1, E2, and E3 measured by a BRET-based assay

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 cockroach enzymes. Histograms represent the mean BRET ratio ± SEM for n = 3. P values are relative to the untreated control.

	Substrate Km (μM)			Inhibitor Ki	
Enzyme	QAR (Trypsin)	FVR (Thrombin)	GGR (Urokinase)	SBTI (pM)	TLCK (nM)
E1	3	81	60	4	277
E2	2	19	99	8	3
E3	3	11	29	3	8
Trypsin	5	24	1054	4	2

Enzyme	Fragments identified
E1	1. GPNSKGR 2. SLIGRLDTP
E2	1. GPNSKGR 2. SLIGRLDTP
E3	1. GPNSKGR 2. SLIGRLDTP

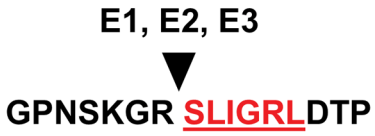


Figure 10. Sequence alignment of German cockroach trypsin-like enzymes E1, E2, E3 and *Periplaneta americana* serine proteinase, Per a 10

Using the Clustal W multiple sequence alignment procedure, the three sequences of enzymes E1, E2 and E3 described in the text are aligned along with the deduced sequence of Per a 10 (labeled: Pera10) described previously. The boxes indicate for all of the enzymes, the common proenzyme cleavage-activation site (R/IVGG: first row of sequences) along with the regions of identical sequences in the vicinity of the key histidine, aspartic acid and serine residues (H: second row, D: third row, S: fifth row) that comprise the serine proteinase catalytic triad as found in mammalian trypsin. The symbol * below the sequences denotes amino acid identity for all enzyme sequences.

Table 1

Cockroach extract enzymes E1, E2, and E3 have distinct substrate Km's and inhibitor sensitivities (IC50's)

The Km's for three different substrates (Glutamine-alanine-arginine (QAR)-aminomethylcoumarin (AMC), phenylalanine-valine-arginine (FVR)-AMC, and glycine-glycine-arginine (GGR)-AMC) and the IC50's for two distinct inhibitors (Soybean trypsin inhibitor (SBTI) and Nα-tosyl L-lysine chloromethylketone (TLCK)) were determined as described in Experimental Procedures. Values represent the mean ± SEM; n=3.

	Substrate Km (μM)			Inhibitor IC50	
	QAR	FVR	GGR	SBTI	TLCK
Enzyme	Trypsin	Thrombin	Urokinase	(nM)	(μM)
E1	3 ± 0.8	81 ± 15	60 ± 12	0.5 ± 0.03	32 ± 3
E2	2 ± 0.3	19 ± 3	99 ± 7	1.2 ± 0.2	0.5 ± 0.04
E3	3 ± 0.3	11 ± 2	29 ± 4	0.3 ± 0.07	0.8 ± 0.2
Trypsin	5 ± 0.6	24 ± 5	1054 ± 5	0.2 ± 0.05	0.2 ± 0.01

Table 2

Cockroach enzymes cleave a synthetic peptide representing the tethered ligand sequence of PAR2 to unmask the tethered ligand

Cleavage of the synthetic peptide representing the N-terminal rat PAR2 sequence by enzymes E1, E2 and E3 (bottom) generated the peptide representing the PAR2 tethered ligand sequence (underlined in red), as identified by the procedures outlined in Experimental Procedures.

Enzyme	Fragments identified
E1	1. GPNSKGR
	2. **SLIGRL**DTP
E2	1. GPNSKGR
	2. **SLIGRL**DTP
E3	1. GPNSKGR
	2. **SLIGRL**DTP

E1, E2, E3

▼

GPNSKGR **SLIGRL**DTP

Table 3

Sequence homology between cockroach serine proteinases E1, E2, E3 and Per a 10 and *Blattella* (Bla g 1, Bla g 2) and dust mite (Der p 1, Der p 3 and Der p 6) antigens

The percent identities (% identity matrix) between the three enzymes isolated from the German cockroach (E1, E2 and E3) and that cloned from *Periplanata americana* (Per a 10) are compared with each other and with the sequences of the other insect antigens from Blattella (Bla g 1, Bla g 2) and dust mite (Der p 1, Der p 3 and Der p 6) found in the database. The % identity was calculated from their sequence alignments e.g. Figure 6 and Figure 10, as outlined in Experimental Procedures. ND = not determined.

ENZYME	E1	E2	E3	Bla g 1
E1	100	60	55	13
E2	60	100	79	14
E3	55	79	100	15
Per a 10	58	71	71	ND
Bla g 1	13	14	15	100
Bla g 2	20	20	22	19
Der p 1	22	20	21	ND
Der p 3	40	40	39	ND
Der p 6	29	31	31	ND