



Chapter 14

Novel Method for the Purification of House Dust Mite Allergen Der p 1 and Its Use in Structure-Based Chemical Design of Novel Inhibitors

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Abstract

House dust mites are globally significant triggers of allergic disease. Notable among their extensive repertoire of allergens are the Group 1 cysteine peptidase allergens which function as digestive enzymes in house dust mites. Compelling evidence suggests that the proteolytic activity of these molecules plays a key role in the development and maintenance of allergic diseases through the activation of innate immune mechanisms which exploit genetic predispositions to allergy. Growing interest in this area creates a requirement for high-quality purified protein, whether natural or recombinantly expressed. It has also identified these allergens as therapeutic targets for a novel approach to allergy treatment through modulation of innate immune responses. The purpose of this chapter is to describe a new method for the purification of Der p 1 and use of the protein produced in a screening assay designed for the discovery of novel inhibitors of Group 1 house dust mite allergens.

Key words House dust mite, Der p 1 purification, Allergen delivery inhibitors, Innate immunity, Structure-based drug design, Protein purification, Protease assay

1 Introduction

In 1921 the American physician Richard Kern postulated that house dust was a cause of allergic disease because many patients with asthma or rhinitis had positive skin responses to extracts of dust from their dwellings. This concept was developed further by Willem Storm van Leeuwen whose work led him to believe that house dust mites (HDM) were key components of this house dust allergy. However, formal proof of this idea was elusive and it was not until the work of Voorhorst and Spieksma in 1967 that the involvement of HDM was eventually acknowledged by most allergists. Under environmental conditions which enable HDM to thrive, they elicit the production of allergen-specific IgE and form a notable allergen source strongly associated with asthma. Many species of mite are found in house dust, with the Pyroglyphid

family (e.g., *Dermatophagoides pteronyssinus*, *D. farinae*, and *Euroglyphus maynei*) dominating in most parts of the world. The unusually extensive allergen repertoire of HDM includes proteins of several classes [1]. Of these, the Group 1 allergens have attracted particular scrutiny because of their strong association with sensitization and disease. In studying Group 1 HDM allergens, Der p 1 has frequently been used as a meaningful archetype for Group 1 allergens from other mite species. This approach is reasonable because Group 1 allergens from different HDM species show a high degree of sequence identity, giving encouragement that their immunological, and other, behavior should be similar if not the same.

In HDM, Group 1 allergens function as digestive enzymes belonging to the cysteine protease family. In fact, they represent a novel subfamily of 25 kDa cysteine proteases based on the length, structure, and behavior of the propiece which regulates latency of these enzymes [2]. The recognition that Group 1 allergens were cysteine proteases prompted interest into the possible relevance of this bioactivity to the mechanisms of allergy, in particular fundamental questions surrounding the molecular basis of allergenicity [1]. It is clear from exploration of this topic that proteolytic activity of Group 1 HDM allergens is significant for the development of sensitization to themselves, other mite allergens, and allergens from unrelated sources. Proteolytic activity acts as a means of facilitating allergen delivery to antigen-presenting cells, as an adjuvant to stimulate a vigorous immune response which exhibits Th2 polarization, and contributes to mechanisms which have chronic pathophysiological consequences [3]. These biophysical and innate immune mechanisms are summarized in Table 1. A recent, and unexpected, finding was the discovery that Der p 1 is a prothrombinase capable of triggering interstitial thrombin formation in the absence of coagulation pathway activation [4]. This triggering of thrombin formation initiates a complex signaling cycle in which the intracellular generation of ROS eventuates, and whose operation forms a nexus with inflammatory signaling from Toll-like receptors 3 and 7 which act as sensors for viral RNA [4, 5].

The realization that these events are relevant to allergic sensitization and, significantly, exert a collateral priming effect for the sensitization to other allergens has prompted interest in Group 1 HDM allergens as novel therapeutic targets for asthma and other allergic conditions. Conveniently, amino acid sequence similarities between Group 1 allergens from different species of HDM mean that they represent a single target for drug design purposes. This is pleasingly helpful compared to their epitopes which, although showing many similarities, are sufficiently different to be immunologically distinguishable.

Provisioning a drug discovery campaign requires a supply of purified target protein for screening, and generally recombinantly

Table 1**Actions of Der p 1 mediated through pathways central to innate immunity**

Biosignature	Notes
Cleavage of tight-junction adhesion molecules/allergen delivery	Demonstrated for Der p 1. Molecular targets are occludin and claudins. Reversible increase in epithelial permeability eventuates. Prevented by ADI drugs
Prothrombinase activity	Intrinsic property of Der p 1. Prevented by ADIs
Activation of protease activated receptor (PAR) 1 and 4	Due to formation of thrombin by prothrombinase action. Prevented by ADIs
Activation of protease-activated receptor 2	Direct cleavage of receptor by Der p 1
Breakdown of immune tolerance—development of allergic sensitization	Der p 1 promotes sensitization to itself and unrelated allergens. Prevented by ADIs
Cleavage of low-affinity IgE receptor (CD23)	Regulates IgE biosynthesis in B cells
Cleavage of DC-SIGN/R	Regulate recruitment of dendritic antigen-presenting cells
Cleavage of IL-2R (CD25)	Promotes Th2 polarization of immune responses
Inactivation of surfactant proteins A and D	Demonstrated for Der p 1 and Der f 1. Predicted to promote allergy development
Inactivation of airway antiproteases, e.g., α_1 -antitrypsin	Der p 1 impairs natural defense against other proteases
IgE-independent mast cell stimulation	Demonstrated for Der p 1
Cytokine and chemokine release (e.g., IL-6, IL-8, GM-CSF, CCL-5, CCL-11 from epithelial cells)	Demonstrated for Der p 1. Possibly mediated through PAR activation
Activation of epidermal growth factor receptor (EGFR) signaling	Dependent on Der p 1. Thought to be cross talk from PAR activation. Requires activation of a disintegrin and metalloprotease (ADAM) 17. Prevented by ADIs
Pannexon gating in airway epithelial cells	ATP release and signaling through P_2X_7 receptors eventuate. Dependent on Der p 1 and thought to be downstream of EGFR signaling. Prevented by ADIs
Activation of ADAM 10	Downstream signaling consequence of ATP release by Der p 1. Exerts significant control over IgE biosynthesis (as a CD23 sheddase) and involved in stem cell factor-dependent recruitment of mast cells. Prevented by ADIs
Thrombin formation	Endogenously formed from prothrombin by ADAM 10-dependent mechanism activated as above. Prevented by ADIs
Intracellular reactive oxidant formation in airway epithelial cells	Downstream consequence of Der p 1. Mechanism involves PAR, EGFR signaling, ATP, and ADAM 10 components as described above. Provides fundamental redox-dependent transcriptional control of cytokine expression. Prevented by ADIs
Inflammatory cell recruitment in vivo	Innate mechanisms activated by Der p 1 and prevented by ADIs

expressed protein is used for this purpose. However, in the case of Der p 1 inhibitor design this was not an immediately available option. Although recombinant Der p 1 had been expressed prior to drug discovery work, this was produced by prokaryotic expression and lacked catalytic competence due to the absence of correct protein folding. Purified protein is also the basis of structure-based drug discovery approaches because the availability of high-resolution target structures facilitates rational decision-making in chemical design. In the initial absence of definitive structural data for Der p 1 itself, we generated its likely structure by homology modeling using high-quality crystallographic data for the cysteine peptidases cathepsin K, papain, and actinidin. Subsequently, high-resolution crystal structures became available for Der p 1 and Der f 1 from *D. farinae* and, with only few notable differences, these are generally consistent with our homology models [6, 7]. In its monomeric form, Der p 1 comprises a pair of globular domains between which is located a substrate-binding groove whose composition, as predicted by empirical examinations of substrate and inhibitor kinetics, is highly conserved at both the isoallergen and homologous species levels. This substrate-binding groove contains the Cys, His, and Asn catalytic triad typical of the C1 family of CA clan cysteine peptidases. The availability of high-quality structural data, together with information gathered during screening using highly purified Der p 1, corrects an erroneous report that Der p 1 allergens (and by implication other Group 1 HDM allergens) are mechanistically bifunctional enzymes with both cysteine and serine protease activity [8]. No credible evidence exists to support this suggestion, which most likely originates from inadequacies in protein purification based on immunoaffinity chromatography which has been a commonly employed method of obtaining Der p 1 for research studies. Der p 1 prepared as described herein will not degrade *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, which is an effective substrate for chymotrypsin, and while trypsin and Der p 1 are both able to utilize *N*-Bz-Phe-Val-Arg-*p*-nitroanilide as substrate, the latter enzyme's behavior is not affected by the broad-spectrum serine peptidase inhibitor 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF), whereas it is blocked by the thiol-reactive Der p 1 inhibitor ADZ 50,000 [3]. Indeed, structural inspection of Der p 1 reveals that none of the serine residues it contains are located in a proximity to His or Asp residues for the creation of a catalytic triad.

Examination of protease substrate specificity provides an efficient means of identifying target motifs which may be useful as scaffolds in structure-based drug design. Screening a library of fluorescence resonance energy transfer (FRET) substrates of general formula Abz-B-C-D-E-Tyr(NO₂)-Asp-NH₂ revealed preferences as follows: B: Val > Ala, Gln, Leu, Phe; C: Ala >> Gln or Lys; D: Leu, Nle or Ala > Ser; E: Ser. This information was employed in

the elaboration of substrates into inhibitors such as acyloxymethyl ketones based around the scaffold Val-Ala-Nle-Ser (Fig. 1).

An unfortunate corollary of this otherwise valuable approach is that while initial inhibitor “hits” may exhibit exquisite potency, they lack significant drug-likeness [3, 9]. Thus, progress toward developable candidate drugs requires careful optimization of necessary credentials (potency, selectivity, pharmacokinetic properties, physical form, etc.). An additional concern in the case of the acyloxymethyl ketone derivatives is that their mechanism of Der p 1 inhibition is irreversible, creating the potential for hapten generation. This negates the optimization of this particular series for the intended therapeutic indications where the intended patient population has a genetic predisposition to allergy. However, considerable success was achieved in optimization of a lead series based around a pyruvamide motif [9]. These inhibitors have a reversible mechanism on the target enzyme and through a structure-based design campaign around this series we obtained a range of develop-

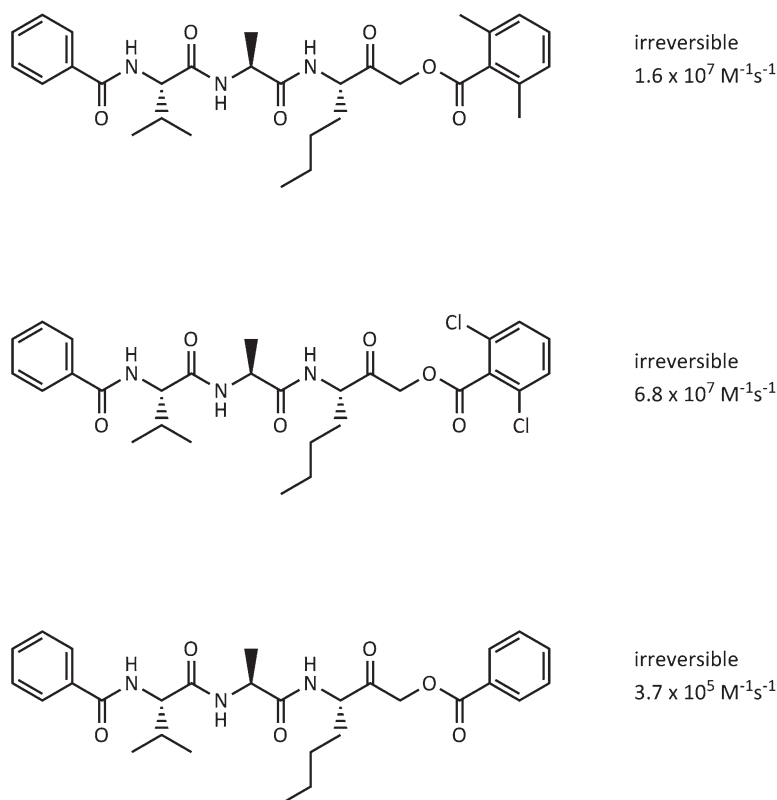


Fig. 1 Exemplification of acyloxymethyl ketone inhibitors of the proteolytic activity of Group 1 HDM allergens. Inhibitory activity is expressed as second-order inhibitor rate constant for each compound when evaluated against Der p 1. These compounds were identified by making systematic variations to a peptide sequence identified from peptide library screening

Table 2

Structure-activity relationships for simple modifications to a pyruvamide scaffold and their effects on the ability to inhibit the proteolytic activity of Der p 1 and cathepsin B

P_1	P_2	P_3	P'	Der p 1 IC_{50} (nM)	Cathepsin B IC_{50} (nM)
<i>n</i> -Bu	Me	Benzyl	Cyclohexyl	8 ± 1	17 ± 2
<i>i</i> -Pr	Me	Benzyl	Cyclohexyl	18 ± 2	52 ± 5
<i>i</i> -Pr	Me	Benzyl	Benzyl	12 ± 2	50 ± 5
<i>t</i> -Bu	Me	Benzyl	Cyclohexyl	9167 ± 880	<i>Not Determined</i>
<i>i</i> -Pr	Me	<i>t</i> -Bu	Cyclohexyl	14 ± 3	378 ± 27
<i>i</i> -Pr	Me	C(Me) ₂ Ph	Benzyl	42 ± 6	446 ± 11
<i>i</i> -Pr	<i>n</i> -Pr	benzyl	Cyclohexyl	164 ± 24	67 ± 1

Cathepsin B inhibition was measured using enzyme from human liver preactivated by 2.5 mM 1,4 dithioerythritol at 37 °C for 10 min. Substrate was ABz-Gly-Ile-Val-Arg-Ala-Lys-DNP-OH. Progress curves were measured at 30 °C by detection of fluorescence (excitation/emission 320/420 nm)

able compounds with the required combination of properties for delivery by inhalation in dry powder form [9]. Arrival at this position required iterative rounds of screening and counter-screening of compounds in which structure variations at key positions in the basic pyruvamide scaffold were introduced systematically. An illustration of one small aspect of this, made using the screening method described herein for Der p 1 purified as described, is shown in Table 2. Comprehensive details of structure-activity relationships for the Der p 1 target are available elsewhere [9]. We call this new class of drugs “allergen delivery inhibitors” (ADIs) because of their ability to inhibit the transepithelial delivery of allergens to subepithelial dendritic antigen-presenting cells. However, their efficacy profile extends considerably beyond this action given the wide-ranging effects of the target’s proteolytic activity on innate immune responses.

2 Materials

2.1 Reagents for Der p 1 Extraction and Purification

1. House dust mites (*Dermatophagoides pteronyssinus*) are maintained in continuous solid-phase culture with controlled temperature and relative humidity (25 °C, 75%) in a custom-built containment system. Mite growth medium comprises 50%

(w/w) of Brewer's Yeast (*Saccharomyces cerevisiae*) and 50% desiccated liver that has been ground in a Waring blender and hot air sterilized at 80 °C for 3–4 h in a Stericell oven. Cultures grown for 1.5–3 months provide a suitable feedstock for Der p 1 purification.

2. Dulbecco's phosphate-buffered saline, without calcium chloride and magnesium chloride (D-PBS).
3. Ethylenediaminetetraacetic acid disodium salt (EDTA) 100 mM in H₂O: Dissolve 18.6 g EDTA into 500 mL deionized water to make up 100 mM EDTA solution.
4. Elution buffer for Sephacryl S200 column (EB):
 - (a) Add 98 mL 0.2 M Na₂HPO₄, 10 mL 100 mM EDTA, and 890 mL double-deionized water into a 1000 mL beaker.
 - (b) Weigh out 29.22 g NaCl and dissolve in the above solution.
 - (c) Carefully adjust the pH to 7.4 with 0.2 M NaH₂PO₄.
 - (d) Add double-deionized water to a total volume 1000 mL.
 - (e) Vacuum filter through 0.2 µm porosity membrane. The filter device can be of any type having a suitable pore size. For routine batch preparations a filter device of 500 mL volume is adequate.
5. Buffer A (20 mM Tris–HCl buffer, pH 8.0):
 - (a) Dissolve 60.5 g Trizma base in 900 mL deionized water, and adjust to pH 8.0 using 50% (v/v) HCl.
 - (b) Add deionized water to a total volume of 1000 mL to produce 0.5 M Tris–HCl buffer, pH 8.0.
 - (c) Add 20 mL 0.5 M Tris–HCl buffer, pH 8.0, to 480 mL deionized water to yield buffer A.
6. Buffer B (20 mM Tris–HCl buffer, pH 8.0, containing 2 M NaCl): Dissolve 58.4 g NaCl into 500 mL buffer A.
7. 0.1 M Glycine-HCl buffer, pH 2.8 (GB):
 - (a) Dissolve 7.5 g glycine in 995 mL deionized water, and adjust the pH to 2.8 using 50% (v/v) HCl.
 - (b) Adjust volume to 1000 mL using deionized water.

2.2 Reagents for SBTI (Trypsin Inhibitor from Soybean) Affinity Column

1. SBTI (from Sigma-Aldrich, UK).
2. CNBr-activated Sepharose 4 Fast Flow (from Sigma-Aldrich, UK).
3. 0.1 M NaHCO₃ pH 8.3 containing 0.5 M NaCl.
4. 1 mM HCl.

5. 0.5 M Tris-HCl, pH 8.0 buffer: Dissolve 60.5 g Trizma base in 900 mL deionized water, and adjust to pH 8.0 with 50% (v/v) HCl. Then adjust to a final volume of 1000 mL with deionized water to produce 0.5 M Tris-HCl, pH 8.0. Add 100 mL of this solution to 400 mL deionized water to produce 0.1 M Tris-HCl buffer, pH 8.0
6. 0.1 M acetate buffer pH 3–4 containing 0.5 M NaCl: Dissolve 8.2 g sodium acetate in 500 mL deionized water to produce a 0.2 M solution. Mix 10 mL glacial acetic acid with 865 mL deionized to produce a 0.2 M solution. Take 410 mL of the acetic acid solution and adjust to pH 4.0 using 0.2 M sodium acetate (requires about 90 mL). Dilute twofold with deionized water to produce 0.1 M acetate buffer and dissolve 14.6 g NaCl into 500 mL of this.
7. 0.1 M Tris-HCl buffer pH 8–9 containing 0.5 M NaCl.
8. Binding buffer: EB.

**2.3 Reagents for Der
p1 Identification
by Matrix-Assisted
Laser Desorption/
Ionization Time-of-
Flight (MALDI-TOF)
Mass Spectrometry**

1. 50 mM Ammonium bicarbonate solution.
2. Ammonium bicarbonate, 40 mM, pH 8.9, containing 9% v:v acetonitrile.
3. 50% v:v Acetonitrile.
4. 1 mM Hydrochloric acid (HCl): Add 1 mL concentrated HCl to 11 mL deionized water yielding 1 M HCl which should then be diluted 1000-fold with deionized water to provide, finally, 1 mM HCl.
5. Trypsin solution: 20 µg Trypsin (Sigma Aldrich, UK) is supplied lyophilized in vials. Reconstitute the lyophilized trypsin in 100 µL 1 mM HCl and mix the vial briefly until completely dissolved. Add 900 µL ammonium bicarbonate, 40 mM, pH 8.9, containing 9% v:v acetonitrile to the vial and mix. The final concentration of trypsin is 20 µg/mL. Aliquot and store at –20 °C (*see Note 1*).
6. Matrix: α -Cyano-4-hydroxycinnamic acid (CHCA) for peptides with molecular weight <10 kDa. Weigh out 10 mg CHCA and add 700 µL acetonitrile and 300 µL 0.1% v:v trifluoroacetic acid (TFA) in deionized water. Vortex mix to dissolve completely.

**2.4 Reagents for Der
p 1 Activity Test
with or Without ADZ
50,000**

1. 50 mM Potassium phosphate buffer containing 1 mM EDTA, pH 8.29 (PPE) (*see Note 2*):
 - (a) Mix 97.4 mL, 0.5 M K_2HPO_4 with 10 mL, 100 mM EDTA, and 890 mL deionized water.
 - (b) Adjust pH to 8.29 with 0.5 M KH_2PO_4 (about 2.6 mL).
 - (c) Adjust to 1000 mL using deionized water.

2. Substrate solution (125 μ M substrate in buffer PPE, reagent I): Weigh out 10 mg Der p 1 substrate ((3S,6S,9S,12S,15S,18S)-1-(2-aminophenyl)-9-butyl-18- carbamoyl-1 5-(4-hydroxy-3-nitrobenzyl)-12-(hydroxymethyl)-3-isopropyl- 6-methyl-1,4,7,10,13,16-hexaoxo-2,5,8,11,14,17-hexaazaicosan-20-oic acid), and dissolve in dry dimethyl sulfoxide (DMSO) to make a 10 mM stock solution. Aliquot as 50 μ L/vial and store frozen at 4 °C or lower. Before use, add 987.5 μ L buffer PPE into 12.5 μ L 10 mM substrate solution. Vortex mix immediately and thoroughly.
3. 10 mM DL-dithiothreitol (DTT) (reagent II):
 - (a) 1 M DTT stock solution: Dissolve 1.55 g DTT in 8 mL water and adjust the final volume to 10 mL. Aliquot as 50 μ L/vial and store frozen at -20 °C.
 - (b) Make up 10 mM DTT from the stock solution by adding 10 μ L 1 M DTT stock solution into 990 μ L buffer PPE.
4. 9 mM DTT (reagent II*): Make up 9 mM DTT by adding 9 μ L 1 M DTT stock solution into 991 μ L buffer PPE.
5. Reagent III (2.5 μ g/mL Der p 1 in PPE): Add 7.5 μ L 1 mg/mL Der p 1 into 2992.5 μ L PPE. Mix well. For pre-activation: Add Der p 1 (7.5 μ L $\times$ 1 mg/mL) to 2989.5 μ L PPE and 3 μ L 1 M DTT. Mix well and leave at room temperature for 5 min (*see Note 3*).
6. Inhibitor preparation: Dissolve inhibitors in dry DMSO to make 100 mM stock solutions if they can be dissolved; otherwise the stock solutions could be 50 mM or 10 mM (the default inhibitor stock concentration). Prior to use, dilute the stock solution to 10 mM with DMSO.

2.5 Apparatus and Columns

1. Amicon Stirred Cell 50 mL (Merck Millipore, UK).
2. Ultracel membrane with 10 kDa cutoff (Merck Millipore, UK).
3. Cylinder of oxygen-free, compressed nitrogen (or nitrogen line from an infrastructure “boil off” supply).
4. Magnetic stirrer.
5. Balances, analytical and general laboratory.
6. pH meter.
7. pH paper.
8. Sephacryl S-200 column, Hiprep, 16/60 (GE Healthcare, UK).
9. Resource Q column, 1 mL (GE Healthcare, UK).
10. Refrigerated centrifuges with fixed-angle rotors: The g force needs to be $23,500 \times g$ or above for 30 mL tubes and $10,000 \times g$ for 15 mL microcentrifuge tubes.

11. Microplate reader equipped with fluorescence reading modes and heated plate carrier.
12. ÄKTA Purifier or similar equipment for protein chromatography.
13. MALDI-TOF mass spectrometer.

3 Methods

All Der p 1 purification steps are carried out at 4 °C. This may be achieved by working in a cold room or, more comfortably, in a suitably equipped laboratory cold cabinet.

3.1 Preparation of the SBTI Affinity Column

1. Dissolve 400 mg SBTI in 40 mL coupling buffer (0.1 M NaHCO₃, pH 8.3, containing 0.5 M NaCl) to make up 10 mg/mL SBTI as coupling solution.
2. Add 20 mL of cold 1 mM HCl to 2 g dried CNBr-activated Sepharose 4 Fast Flow. Wait until all the powder is wet, then pack the matrix into a 25 mL syringe, and wash with 10–15 column volumes of cold 1 mM HCl.
3. Cycle load 40 mL of 10 mg/mL SBTI in coupling buffer and allow to react for 4 h (or overnight) at 4 °C (*see Note 4*).
4. Wash away excess ligand with at least five column volumes of coupling buffer.
5. After coupling, non-reacted groups on the matrix should be blocked. To do this, run 100 mL 0.1 M Tris–HCl pH 8.0 buffer through the column and allow to stand for at least 2 h (*see Note 5*).
6. Wash the coupled matrix using alternate low- and high-pH buffers. Recommended buffers are 0.1 M acetate buffer pH 3–4 containing 0.5 M NaCl and 0.1 M Tris–HCl buffer pH 8–9 containing 0.5 M NaCl. A suitable procedure is three column volumes of Tris–HCl buffer followed by three column volumes of acetate buffer. Repeat this cycle 3–6 times.
7. The coupled matrix is now ready for use. To prevent microbial growth, store in 20% v:v ethanol.
8. Equilibrate the column with binding buffer before applying samples.

3.2 Preparation of PBS Extracts and 50% Ammonium Sulfate Fraction

1. Der p 1 PBS extracts from house dust mite medium.
 - (a) Add 200 mL D-PBS to 50 g spent medium from house dust mite cultures in a 500 mL beaker.
 - (b) Cover with parafilm and stir at 4 °C for 4 h to overnight. Ensure that the stirrer is operating satisfactorily in order to achieve efficient extraction.

- (c) Transfer the mixture into suitable centrifuge tubes (e.g., 30 mL for a Sorvall RC-5B centrifuge). Weigh the tubes to make sure that each pair of tubes has the same weight so that the rotor can be balanced.
 - (d) Put a pre-cooled fixed-angle rotor in the centrifuge, and ensure that it is correctly located on the spindle.
 - (e) Put the tubes into the rotor in a balanced (symmetrical) position.
 - (f) Centrifuge at $23,500 \times g$ and $4\text{ }^{\circ}\text{C}$ for 30 min.
 - (g) Remove the tubes gently to avoid disturbing the pellets and carefully transfer the supernatants to a 250 mL measuring cylinder to measure the volume. Then transfer the combined supernatants to a 500 mL beaker. This combined supernatant is called the PBS extract (PBSE).
 - (h) Clean the rotor with water and wipe dry with tissues. Return the rotor to cool storage.
2. 50% Ammonium sulfate precipitates.
- (a) Add 100 mM EDTA (stock solution in H_2O) to PBSE so the final concentration for EDTA is 1 mM.
 - (b) Calculate the amount of solid ammonium sulfate (ammonium sulfate (grams) = (supernatant volume/100) $\times$ 30) required for the supernatant to make up 50% saturation.
 - (c) Add the required solid ammonium sulfate to PBSE gradually, and stir at $4\text{ }^{\circ}\text{C}$ for 4 h or overnight.
 - (d) Centrifuge at $23,500 \times g$ and $4\text{ }^{\circ}\text{C}$ for 30 min.
 - (e) Discard the supernatants. Gently raise the pellets with D-PBS to remove excess ammonium sulfate (*see Note 6*).
 - (f) Add 3 mL D-PBS to each tube, and redissolve the precipitates.
 - (g) Centrifuge again, as above, and collect the supernatants.
 - (h) Transfer the supernatants carefully to an Amicon filtration cell equipped with a 10 kDa cutoff membrane for concentration with stirring at $4\text{ }^{\circ}\text{C}$ under compressed nitrogen (*see Note 7*).
 - (i) After concentration, wash the 50% ammonium sulfate fraction into 1–3 mL Sephacryl S200 EB buffer (**Note 7**).
 - (j) Centrifuge at $23,500 \times g$ and $4\text{ }^{\circ}\text{C}$ for 10 min and transfer the supernatant into a clean vial for chromatography. This supernatant is called the concentrated 50% ammonium sulfate fraction.

3.3 Isolation of Der p 1 by Chromatography

1. Equilibrate a Sephacryl S-200 column (16/60, Hiprep) on an ÄKTA Purifier system using three column volumes (CV) of buffer EB.
2. Apply 2–3 mL (*see Note 8*) of the concentrated 50% ammonium sulfate fraction to the column and elute with EB. Collect the fraction with a retention volume of 90–120 mL (RT90) (Fig. 2).
3. Equilibrate the SBTI affinity column with EB before applying samples.
4. Load the sample RT90 on the SBTI affinity column by cycle loading for 4 h to overnight using a flow rate of 0.1 mL/min.
5. Collect the flow-through solution and concentrate it under pressurized nitrogen in an Amicon cell equipped with 10 kDa cutoff ultrafiltration membrane.

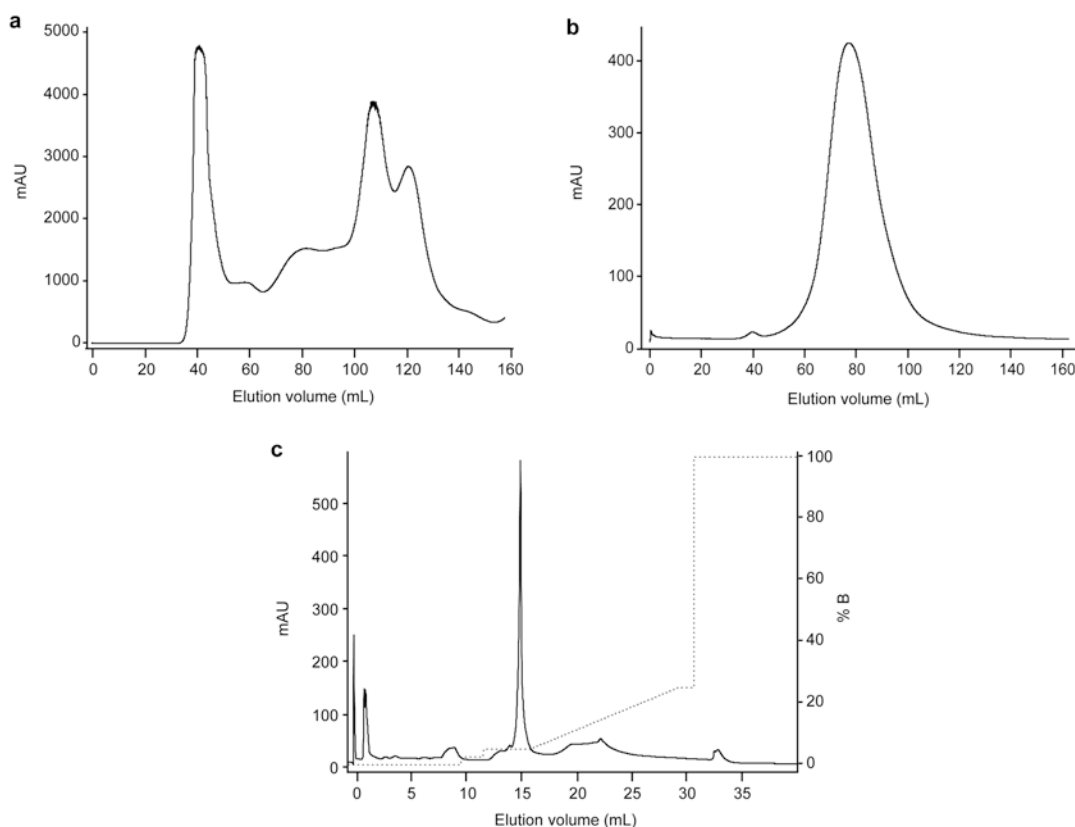


Fig. 2 Purification of Der p 1. (a) Initial chromatography of HDM allergen extract on Sephacryl S200 with monitoring of absorbance at 280 nm. (b) Rechromatography of HDM allergen extract on Sephacryl S200. (c) Resource Q chromatography of Der p 1 monitored by absorbance at 280 nm. The peak pattern is dependent on sample purity, and indicates various forms of Der p 1 caused by different degrees of glycosylation, autolysis, or cleavage of the propeptide from pro-Der p 1 at different cleavage sites. More than one peak containing Der p 1 is not unusual at this stage. The dotted line shows the gradient profile

6. After each run, regenerate the SBTI affinity column by washing it with 5 CV 0.1 M glycine-HCl buffer, pH 2.8, and re-equilibrate it with 5 CV of EB for the next run.
7. Add elution buffer for Sephacryl S200 chromatograph, transfer the concentrated sample into 1.5 mL vials, and then centrifuge at $13,400 \times g$, for 10 min at 4 °C, to get rid of any insoluble precipitates. Then repeat **steps 2–5** (Fig. 2).
8. Concentrate and desalt the samples obtained from **step 7** (*see Note 9*) and then redissolve them in 20 mM Tris-HCl, pH 8.0 buffer, followed by centrifugation at $23,400 \times g$, for 10 min at 4 °C with fixed-angle-head rotor. Collect the supernatant for Resource Q chromatography.

3.4 Chromatography on Resource Q

1. Equilibrate a Resource Q column with 5 CV of pH 8.0, 20 mM Tris-HCl buffer before use. Then load the samples.
2. Gradient elution from 0.05–0.5 M NaCl is done with buffer A and buffer B (Fig. 2c) (*see Note 10*). It is possible to modify the gradient to improve the resolution if samples show complex behavior. Table 3 illustrates one possible alternative.
3. Collect each peak and concentrate and desalt them using an Amicon cell as above. Then transfer into D-PBS for quality assurance.
4. Determine the concentration of the fractions from the Resource Q column by measuring A_{280} in a quartz cell or microplate. The concentration of Der p 1 is calculated using the equation (Der p 1 concentration (mg/mL) = absorption $A_{280\text{nm}}/1.8$).
5. Run the Der p 1 activity assay with or without inhibitor ADZ 50,000 to confirm the purity from a proteolytic class activity perspective.
6. Determine the purity of purified Der p 1 by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (ref. Molecular Cloning: A Laboratory Manual, by Sambrook, Fritsch, and Maniatis) (*see Note 11*).
7. Identify Der p 1 in each eluted fraction by MALDI-TOF mass spectrometry.
8. Combine the Der p 1 fractions which combine high purity on SDS-PAGE with good proteolytic activity (*see Note 12*).
9. Redetermine the concentration of the combined Der p 1 sample and its activity.
10. Aliquot and store at –20 °C or lower.

In a typical run, about 0.4 mg (measured by $A_{280\text{nm}}$) purified Der p 1 is obtained from 50 g spent house dust mite culture medium (*see Note 13*). The purity of Der p 1 is $\geq 90\%$ on SDS-PAGE by Coomassie blue staining (Fig. 3). Fractions eluted from Resource Q column were characterized by mass spectrometry, and

Table 3
Fine gradient elution program for Resource Q chromatography using an ÄKTA Purifier system with Buffer A and Buffer B containing 1 M NaCl

Start_Conc_B	Start_ConcB {%B}	0
Column_Equilibration	Equilibrate_with {CV}	1
Sample_Injection	Empty_loop_with {ml}	5
Wash_Out_Unbound_Sample	Wash_column_with {CV}	2
Gradient_Segment_1	Target_ConcB_1 {%B}	5
	Length_of_Gradient_1 {CV}	0
Gradient_Segment_2	Target_ConcB_2 {%B}	5
	Length_of_Gradient_2 {CV}	5
Gradient_Segment_3	Target_ConcB_3 {%B}	10
	Length_of_Gradient_3 {CV}	5
Gradient_Segment_4	Target_ConcB_4 {%B}	10
	Length_of_Gradient_4 {CV}	5
Gradient_Segment_5	Target_ConcB_5 {%B}	17.5
	Length_of_Gradient_5 {CV}	20
Gradient_Segment_6	Target_ConcB_6 {%B}	20
	Length_of_Gradient_6 {CV}	10
Gradient_Segment_7	Target_ConcB_7 {%B}	50
	Length_of_Gradient_7 {CV}	5
Gradient_Segment_8	Target_ConcB_8 {%B}	50
	Length_of_Gradient_8 {CV}	5
Gradient_Segment_9	Target_ConcB_9 {%B}	100
	Length_of_Gradient_9 {CV}	5
Gradient_Delay	Gradient_Delay {ml}	5
Clean_after_Elution	Clean_with {CV}	10
Length_of_Reequilibration	Reequilibrate_with {CV}	10

combined for activity testing. The enzymatic activity of purified Der p 1 was confirmed by assay with the specific substrate which incorporates a FRET fluorescor-quencher pair.

**3.5 Der p 1
Confirmation
by MALDI-TOF Mass
Spectrometry**

1. Carefully excise the band of interest from the SDS-PAGE gel and transfer to a 1.5 mL microcentrifuge vial.
2. Cut the excised band into smaller pieces and wash with 50 mM ammonium bicarbonate followed by 50% acetonitrile in water (500 µL). Then dehydrate the gel with 100% acetonitrile.

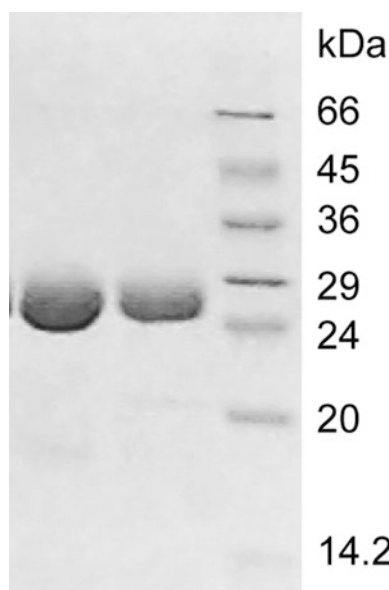


Fig. 3 SDS-PAGE of purified Der p 1

3. Remove acetonitrile and cover the vial with pierced parafilm. Vacuum dry the gel for 20 min in a freeze dryer.
4. Add 20 μL (0.4 μg of trypsin) of the trypsin solution prepared for in-gel digests to the sample and rehydrate for 15 min.
5. Add sufficient 9% v:v acetonitrile in 40 mM ammonium bicarbonate to cover the gel and incubate for 4 h to overnight at room temperature.
6. After incubation, pipette 1 μL solution onto a MALDI-TOF target and mix with 1 μL matrix α -cyano-4-hydroxycinnamic acid. Air-dry the samples before running.
 - (a) MALDI-TOF mass spectrometry can be performed using any suitable instrument. Details are given here for work performed using a Kratos Axima instrument operated in positive reflectron mode at an accelerating voltage of 100 kV. The calibrant contained peptides with m/z^+ from 700 to 2000.
 - (b) The ions obtained by MALDI-TOF mass spectrometry of trypsin-digested Der p 1 (m/z^+ : 622.49, 647.51, 741.66, 1830.83, 1734, 1759, 2056.57, 2585.88, 3214.87) can be observed on the sample mass spectrum (Fig. 4) and match to the calculated data of the Der p 1 sequence (*see Note 14*). Among them, m/z^+ 1830.83 is the variant of fragment $^{209}\text{FGISNYCQIYPPN}\underline{\text{V}}\text{NK}$, in which V^{222} was changed to A^{222} FGISNYCQIYPPNNANK, as reported in UniProtKB/Swiss-Prot.

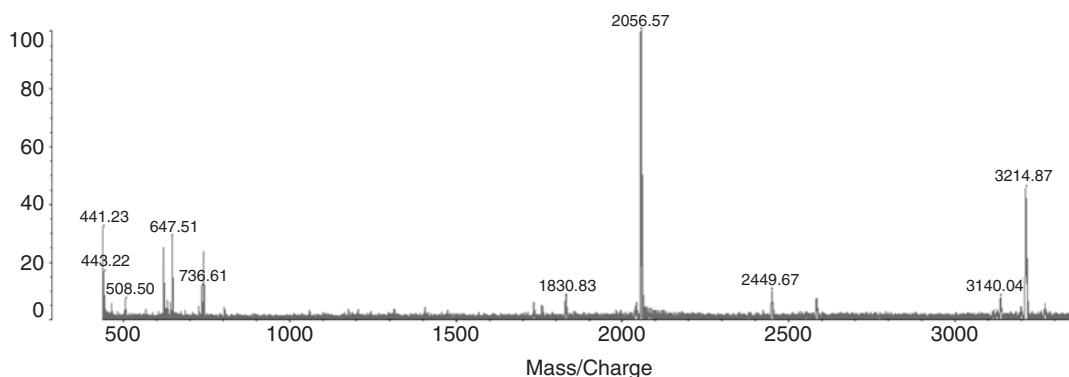


Fig. 4 MALDI-TOF mass spectrum of Der p 1 following trypsin digestion as described in these methods

3.6 Der p 1 Proteolytic Activity Measurement with or Without ADZ 50,000

The purpose of this assay is to measure the enzymatic activity of Der p 1 and to provide assurance that the measured activity is free from serine peptidase contamination. This is achieved using a potent and selective inhibitor, ADZ 50,000. The proteolytic activity assay is performed on a black 96-well plate (Optiplat, Perkin Elmer, Buckinghamshire, UK) by adding reagent I (10 μ L), PPE (70 μ L), and reagent II (10 μ L) into each well, and then adding 10 μ L reagent III to initiate the reaction. The plate is read in a kinetic assay mode for 33 min at 30 $^{\circ}$ C using excitation/emission at 330/420 nm immediately after adding reagent III. Our preference has been to perform these assays using Perkin Elmer Fusion or Envision microplate readers, but recognized alternatives are available. The ADZ 50,000 inhibition component of the assay is performed as described above except for the addition of 10 μ L/well of ADZ 50,000 at a range of concentrations into wells before adding substrate. To accommodate this, the volume of PPE is reduced to 60 μ L in these wells. This modification is also used in the screening of inhibitors, e.g., in the evaluation of novel compounds synthesized in a drug discovery campaign directed against the enzymatic activity of Group 1 house dust mite allergens (*see* description below). Example progress curves for Der p 1 activity assays are shown in Fig. 5.

3.7 $K_{obs}/[I]$ Measurement of Irreversible Inhibitors

The assay is set up on a 96-well plate containing different concentrations of inhibitors according to the format shown in Table 4. Examples of progress curves for determining inhibitor constant $K_{obs}/[I]$ are presented in Fig. 6. $[I]$ is the inhibitor concentration. The reproducibility was measured by determining $K_{obs}/[I]$ of ADZ 50,000 on Der p 1. K_{obs} is calculated as shown below:

$$[P]_t = V_z / K_{obs} (1 - e^{-K_{obs} \cdot t}).$$

In this equation, $[P]_t$ is the product concentration at time t (RFU in this case).

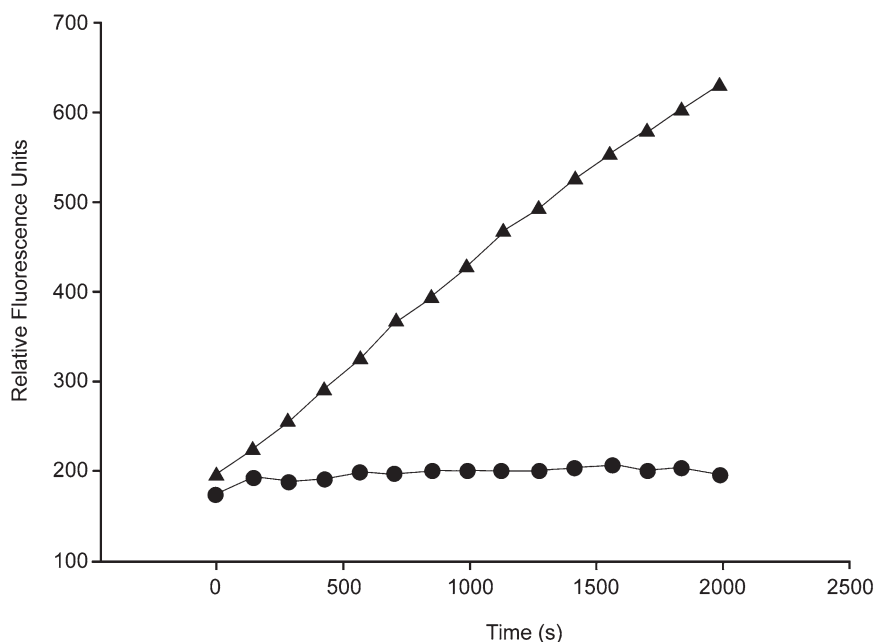


Fig. 5 Measurement of Der p 1 enzymatic activity by FRET assay performed as described herein using (3S,6S,9S,12S,15S,18S)-1-(2-aminophenyl)-9-butyl-18-carbamoyl-15-(4-hydroxy-3-nitrobenzyl)-12-(hydroxymethyl)-3-isopropyl-6-methyl-1,4,7,10,13,16-hexaoxo-2,5,8,11,14,17-hexaazaicosan-20-oic acid as substrate. Triangles show activity in the absence of any inhibitor. Circles depict reaction progress in the presence of 25 nM of the acyloxymethyl ketone derivative ADZ 50,000

Table 4
Map for screening Der p 1 inhibitors using a 96-well format

C1	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.0975	0.0488	NC
C1	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.0975	0.0488	NC
C2	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.0975	0.0488	NC
C2	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.0975	0.0488	NC
C3	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.0975	0.0488	PC
C3	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.0975	0.0488	PC
C4	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.0975	0.0488	PC
C4	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.0975	0.0488	PC

C1–C4: Compound identifier

The unit of concentration for the inhibitors is μM

PC: Positive control (Der p 1 without inhibitors)

NC: Blank without Der p 1

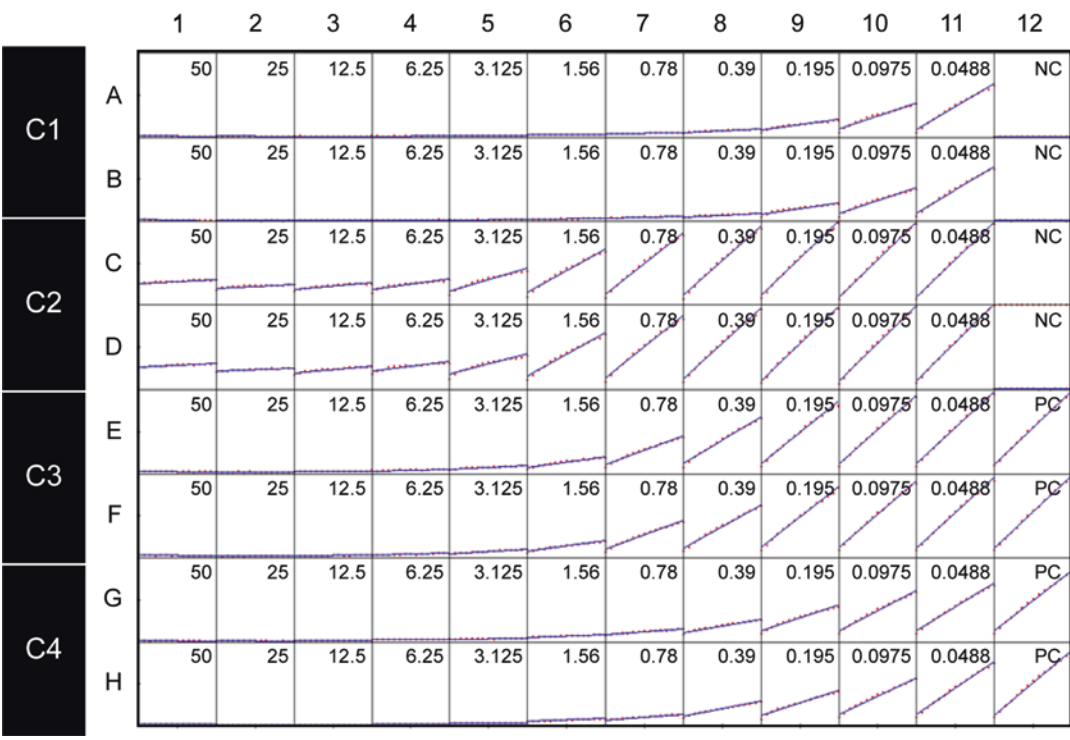


Fig. 6 Annotated screenshot of sample progress curves showing the activity of Der p 1 in the absence or presence of various experimental inhibitors from an experiment to determine inhibition constants

Table 5
Second-order rate determinations for acyloxymethyl ketone derivative ADZ 50,000 vs. Der p 1 showing example assay standard deviation (SD)

K_{obs}/I ($\times 10^8 \text{ M}^{-1} \text{ s}^{-1}$)	Assay 1	Assay 2	Assay 3	Assay 4	SD inter-daily
Plate 1a	1.42	1.74	1.48	1.33	0.1761
Plate 1b	1.52	1.33	1.15	1.35	0.1513
Plate 2a	0.97	1.0	0.93 ^a	0.83 ^a	0.0741
Plate 2b	1.18	1.29	0.95 ^a	0.99 ^a	0.1603
Within-day SD	0.2470	0.3045	0.2551	0.2568	/

K_{obs}/I was measured in five replicates except where indicated ^a when $n = 4$

V_z is the velocity of uninhibited reaction (mRFU/min).
 K_{obs} is a pseudo first-order rate constant.
 $K_{\text{obs}}/[I]$ unit is $\text{s}^{-1} \text{ M}^{-1}$ and calculated with SigmaPlot (*see Note 13*).

The inter-daily standard deviation and within-daily standard deviation of $K_{\text{obs}}/[I]$ are below 0.3 (Table 5), suggesting that the assays display good reproducibility and reliability.

3.8 Measurement of IC_{50} for Reversible Inhibitors

For reversible inhibitors, the screening assays are constructed similarly, except a 20-min preincubation is required prior to initiating the reaction by substrate addition. Inhibitory activity is analyzed from progress curves of reactions in the presence of a range of inhibitor concentrations. Initial reaction velocities are calculated by computational nonlinear regression and degree of inhibition produced by compounds determined, from which the concentration required to inhibit the reaction by 50% (IC_{50}) is calculated according to the scheme below:

Initial velocity in each well is converted to fractional activity by Eq. 1:

$$\text{Fractional activity} = (V_t / V_0) \times 100 \quad (1)$$

where

V_t : Initial rate at inhibitor concentration $[X]$.

V_0 : Initial rate at inhibitor concentration zero.

Then, IC_{50} is determined by fitting the data of fractional activity and inhibitor concentration to a four-parameter logistic curve, using Eq. 2:

$$V = V_{\min} + [V_{\max} - V_{\min}] / \left[1 + (X / IC_{50})^{\text{Hillslope}} \right] \quad (2)$$

where

V is the fractional activity of the enzyme in the presence of inhibitor at concentration $[X]$.

$[X]$ is the inhibitor concentration.

$V_{\min}$ is the minimum of V observed at high inhibitor concentration.

$V_{\max}$ is the maximum of V observed at zero inhibitor concentration.

Hill slope is the slope of the dose-response (inhibition) curve.

4 Notes

1. Trypsin solution can be stored at -20°C . Minimize the aliquot volumes so it can be used with little wastage because the activity will drop significantly if the enzyme is refrozen and thawed.
2. The pH will drop slightly after adding DTT, so the working pH would be very close to 8.25. If >2.6 mL NaH_2PO_4 is required to adjust the pH to 8.29, the total volume will be slightly over 1000 mL, but the buffer can still be used as normal.

3. Der p 1 is a cysteine peptidase and the active site contains reduced cysteine residues. When the catalytic cysteine is oxidized, the activity of Der p 1 drops. During purification, the cysteine residues in Der p 1 might have been oxidized, so pre-activation of Der p prior to use is very necessary. Therefore, reducing reagents such as DTT need to be added in the reaction to ensure maximal activation of the enzyme. Activation of Der p 1 by DTT is fast so this step should be done immediately prior to use. Der p 1 should not be stored in DTT to minimize the risk of autolysis.
4. The coupling process can be done as cycle loading to increase the coupling efficiency.
5. For good blocking efficiency, more blocking buffer can be used and the blocking time of non-reacted groups on the medium can be longer up to overnight.
6. When raising the pellets with D-PBS, be quick and gentle to avoid D-PBS dissolving the pellets.
7. Make sure that the membrane is intact. An Ultracel membrane with 10 kDa cutoff is used. Carefully pipette EB buffer into the cell, try to dissolve all materials in the cell, and divide the mixture into 2×1.5 mL Eppendorf vials for centrifugation. To avoid damaging the filtration membrane, do not touch it with pipette tips. The membrane can be reused after washing thoroughly with buffer and stored in water at 4 °C.
8. For the first chromatography on Sephacryl S200 the sample volume can be up to 5 mL.
9. For concentration of **step 7** samples using Amicon cell ultrafiltration, try to filter all liquid through the membrane to remove salt as much as possible. Residual salt may affect the binding efficiency of samples on Resource Q columns.
10. For the elution gradient on Resource Q, the gradient change from 0.05 to 0.5 M NaCl can be adjusted according to different purposes. For example, to polish samples the gradient could be set as a linear change from 0.05 to 0.5 M. For complex samples, the gradient can be finely tuned using a lower NaCl concentration in buffer B (e.g., 1 M can be used instead of 2 M to achieve a small gradient change). Table 3 is an example of this application.
11. On SDS-PAGE, there will be more than one fraction containing Der p 1 eluted by the NaCl gradient. This is due to different isoforms of Der p 1 in the mite medium. Although these contain Der p 1 they may also contain significant impurities and therefore should not be used for experimental purposes where purity is paramount. Der p 1 appearing in different frac-

tions of Resource Q column indicates the different forms of Der p 1.

12. In activity tests, Der p 1 in different eluted peaks displays different activities due to autolysis and/or Der p 1 isoforms. The fractions with significantly lower activity should not be combined in the purification workup.
13. The yield of purified Der p 1 is highly dependent on the initial production in the mite medium and this should be borne in mind when comparing different batches purely according to gross yield.
14. SigmaPlot is our preferred data-processing software, but suitable alternatives are available (e.g., Prism or WorkOut).

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