

Original article

Comparative enzymology of native and recombinant house dust mite allergen Der p 1

Background: The cysteine peptidase activity of group 1 house dust mite allergens is important for their allergenicity and may offer new therapeutic targets for allergy treatment. Hitherto, the design of specific inhibitors has been impeded because the availability of pure, fully active allergens has limited the implementation of drug screening campaigns. Similarly, investigation of the mechanisms by which peptidase allergens promote sensitization has also been restricted. Our aim was to compare the enzymology of recombinant and native forms of Der p 1 to establish if an easily expressed recombinant form of Der p 1 could be used as a drug discovery tool.

Methods: Enzymatic activity of natural and recombinant Der p 1 was compared fluorimetrically using a novel specific substrate (ADZ 50,059) and a novel specific active site titrant (ADZ 50,000). The effect of recombinant Der p 1 prodomain on the catalytic activity of both Der p 1 preparations was also examined.

Results: Although differing substantially in molecular weight, the enzymological properties of recombinant and native Der p 1 were indistinguishable. Our data show clearly by experiment that, in contrast to some suggestions, Der p 1 is not an enzyme of bifunctional mechanism.

Conclusion: The catalytic activity of Der p 1 is tolerant of glycosylation differences that occur at N150 when the protein is expressed in *Pichia pastoris*. This suggests that this recombinant protein may be suitable for drug design studies and in the elucidation of how peptidase activity promotes sensitization to peptidase and nonpeptidase bystander allergens.

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The peptidase activity of Group 1 mite allergens such as Der p 1, Der f 1 and Eur m 1 has cellular and molecular effects which potentially make important contributions to allergic sensitization (1–8). Furthermore, *in vivo* studies with dust mite or fungal peptidase allergens suggest that the proteolytic activity contributes to sensitization to both the peptidase allergen and nonpeptidase bystander allergens from the same or other sources (9–12). The availability of pure allergens in fully catalytic forms is vital to the further investigation of how peptidase activity contributes to allergy and to the design and high throughput screening of inhibitors that may have therapeutic application as novel allergy therapies.

Attempts to express active Der p 1 or Der f 1 in recombinant forms have met with varied success. Recombinant Group 1 mite allergens have been manufactured in both mature and proenzyme forms in bacterial, yeast and insect cell expression systems, but difficulties with insolubility, altered immunogenicity and little or no enzyme activity have been encountered (13–17). Expression of recombinant Der p 1 (rDer p 1) or rDer f 1 using the methylotropic yeast *Pichia pastoris* is

an alternative means of recombinant protein production (18–22).

It is common for proteins expressed in *P. pastoris* to exhibit abnormal glycosylation. Although the glycan composition of native Der p 1 (nDer p 1) has not been characterized, it is generally considered that only one glycosylation site exists in native mature Der p 1 at N150 (numbering from the N-terminus of unprocessed prepro Der p 1). The glycosylation in nDer p 1 is probably not extensive because only a small difference exists between the observed molecular weight of the native protein and that predicted from its amino acid sequence. In contrast, the abnormally glycosylated rproDer p 1 expressed in *P. pastoris* differs significantly from its native proDerp 1 counterpart because of abnormal glycosylation at N150, and also at N213 and N34 in the propiece (21, 23).

Prevention of rDer p 1 hyperglycosylation by producing an N150Q mutant have shown that rDer p 1-N150Q is enzymatically active but kinetic studies of the enzyme were incomplete due to the lack of a specific substrate and inhibitor (22, 24). Although the reasons are unclear, data

previously obtained suggest that the recombinant N150Q mutant has augmented enzymatic activity (24). This mutant may therefore be of uncertain value for applications where kinetic fidelity is paramount. Furthermore, expression of mutant Der p 1 is less efficient than the hyperglycosylated variant of the native protein (25), and this may have implications for the capacity to mount automated high throughput screening campaigns against this peptidase.

Der p 1, Der f 1 and Eur m 1 are members of the C1 family of cysteine peptidases in enzyme clan CA (8). These are initially synthesized as prepro forms from which latent proenzymes of ~36 kDa are produced on cleavage of the signal peptides. The prodomain prevents the activity of the enzyme by binding in retro orientation through the catalytic site (21) and, by analogy with other peptidase prodomains, it may orchestrate correct folding of the protein. The prodomain of Der p 1 sterically hinders conformational epitopes of this allergen, resulting in proDer p 1 being less immunogenic than mature Der p 1 (18, 21, 26). During maturation of the peptidase the 80 residue prodomains are removed, enabling the mature ~25 kDa proteins to gain proteolytic activity and to develop maximum immunogenicity. We have recently shown that the Der p 1 prodomain is rapidly inactivated, suggesting a novel mechanism that may contribute to the potency of this allergen (27).

Using a novel Der p 1 substrate and inhibitor, our objective in this study was to establish how the catalytic properties, including interaction with free prodomain, of abnormally glycosylated rDer p 1 compare with those of native enzyme purified from house dust mite cultures and thus to determine whether the recombinant enzyme can be used generally to study the peptidase properties of the allergen and serve as a drug design template.

Materials and methods

Expression and purification of rDer p 1 and prodomain

The proDer p 1.0105 isoform coding sequence was cloned into a *P. pastoris* expression vector and recombinant protein produced by fermentation as described elsewhere (22). Recombinant proDer p 1 was converted to its mature form by dialysis under acidic conditions and purified by cation exchange chromatography. The molecular weight of rDer p 1 was determined initially by SDS-PAGE and confirmed by gel filtration on Superdex G200 on an Äkta Explorer system.

Recombinant Der p 1 prodomain was expressed as a soluble protein (PP-02) in *Escherichia coli*. A flush end construct encoding the full length of the prodomain sequences (residues 19–98) was cloned, expressed and purified as described (27).

nDer p 1 purification

House dust mites of the species *Dermatophagoides pteronyssinus* were cultured as described (27). Der p 1 was purified chromatographically (27) and its concentration determined in a quartz

cuvette by absorbance at 280 nm using an extinction coefficient of 47 705/M/cm.

Synthesis of Der p 1 substrate and inhibitor

(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 (ADZ 50,059) is a novel substrate for Der p 1 and was synthesized in-house using solid phase chemistry.

Der p 1 inhibitor ADZ 50,000 ((S)-3-((S)-2-((S)-2-benzamido-3-methylbutanamido)propanamido)-2-oxoheptyl 2,6-bis(trifluoromethyl)benzoate) was synthesized as described (27).

Instrumental analyses

Chemical compounds were identified by LC-MS and ¹H-NMR. Proteins were analysed by MALDI-TOF using conventional methods.

Der p 1 enzyme activity assay

Assays were assembled in 96-well format using a Perkin Elmer MultiPROBE robot (27). Reactions were initiated by adding 10 µl of Der p 1 dissolved in reaction buffer at 2.5 µg/ml and progress at 30°C monitored by excitation/emission at 330/420 nm. Kinetic constants (*K_m* and *V_{max}*) were calculated from initial reaction velocities determined from the progress curves and fitted to the Michaelis–Menten equation by regression analysis.

Inhibitor kinetics for ADZ 50,000 were analysed from progress curves (28). Data were fitted by computational nonlinear regression and the apparent inactivation rate constant (*K_{obs}*) was calculated from the equation:

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

where *[P]_t* is the product concentration at time *t*, *V_z* is the velocity of uninhibited reaction and *K_{obs}* is a pseudo first order rate constant.

To investigate the class specificity of Der p 1, investigations were conducted using N-Bz-Phe-Val-Arg-*p*-nitroanilide hydrochloride or N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma-Aldrich, Dorset, UK) as substrates. TPCK-treated trypsin and chymotrypsin (Sigma-Aldrich) were used as reference enzymes and 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride (AEBSF, Sigma-Aldrich) as a generic serine peptidase inhibitor. Reactions were assembled in 96 well format in a final volume of 100 µl, consisting of 10 µl enzyme, 10 µl AEBSF (400 µM final), 10 µl DTT (1 mM final), and 65–70 µl reaction buffer (75–80 µl for inhibitor-free controls). Reactions were performed at 30°C and started by addition of 5–10 µl substrate to give a final concentration of 200–500 µM. Progress was monitored by absorbance at 405 nm in a Fusion platereader. Reaction concentrations of trypsin and chymotrypsin were 2 µg/ml, whilst for the Der p 1 preparations it was 20 µg/ml.

Results

Physicochemistry

Automaturation of proDer p 1 expressed in *P. pastoris* resulted in a protein with a significantly greater molecular

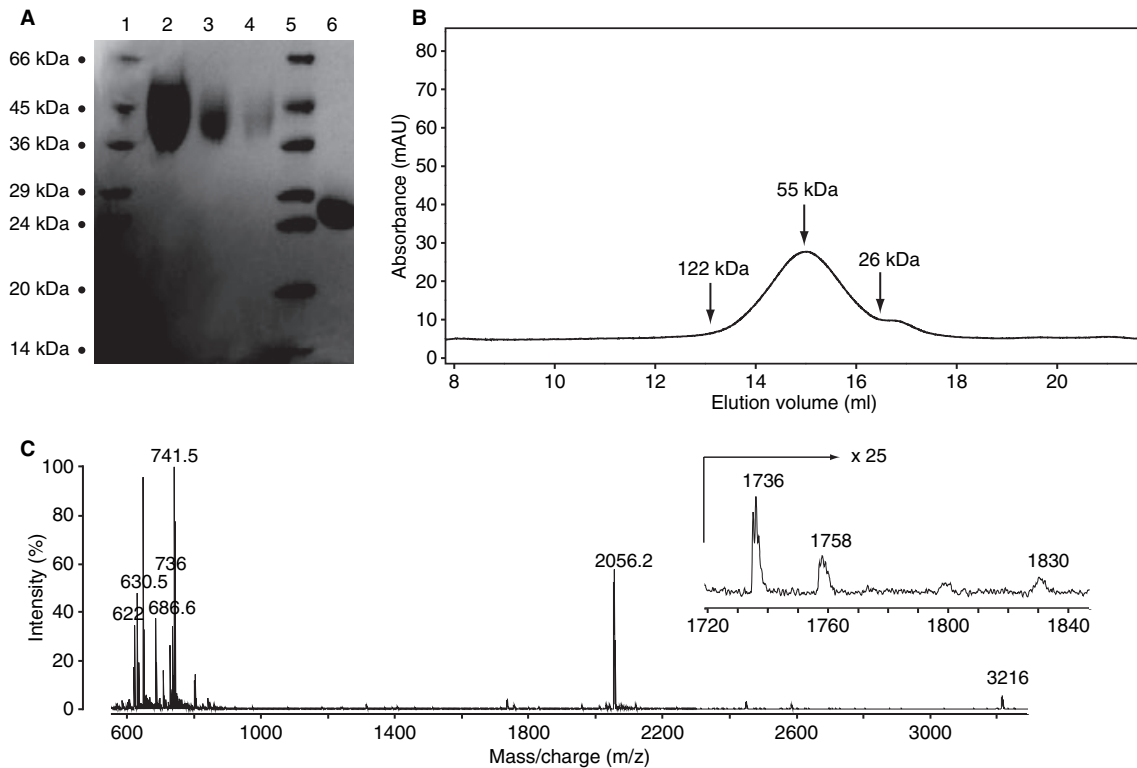


Figure 1. Panel A depicts SDS-PAGE of nDer p 1 and rDer p 1. Key: Lanes 1,5 molecular weight markers; Lanes 2–4 contain 40, 20, 10 μ g rDer p 1 respectively; Lane 6 nDer p 1. Determination of the molecular weight of rDer p 1 by gel filtration on Superdex G200 is shown in B. Panel C shows a MALDI-TOF mass spectrum of a tryptic digest of rDer p 1, essentially similar to that obtained for nDer p 1. The m/z range 1720–1840 is shown on an expanded scale (inset) to illustrate the MH^+ ion at 1758 which confirms the presence of an authentic N-terminus.

weight than Der p 1 purified from faecally-enriched *D. pteronyssinus* culture medium (Fig. 1A). On SDS-PAGE, rDer p 1 ran as a smear from 36 to 60 kDa and by gel filtration it eluted with an average molecular weight of 55 kDa compared to $\sim$ 25 kDa for nDer p 1 (Fig. 1B).

A comparison of MALDI-TOF mass spectra of tryptic digests of nDer p 1 and rDer p 1 revealed an essentially similar profile of ions. No fragments were observed in either nDer p 1 or rDer p 1 which were obviously associated with the tryptic cleavage predicted to contain the glycosylation sequence. A representative mass spectrum for rDer p 1 is shown in Fig. 1C. The following fragments, representing 58% sequence coverage, were identified: m/z (MH^+) 3216 ^{260} DNGYQP NYH AVNIVGYSNAQGV DYWIVR ^{287} , m/z (MH^+) 2056 ^{177} GIEYIQHNGV VQESYYR ^{193} , m/z (MH^+) 1830 ^{209} FGISNYCQIYPPNANK ^{224} (V222 to A222 substitution in Der p 1.0105 isoform), m/z (MH^+) 1758 ^{99} TNACSINGNAPAEIDLR ^{115} , m/z (MH^+) 1736 ^{227} EALAQTHSAIAVIGIK ^{243} , m/z (MH^+) 742 ^{203} RPNAQR ^{208} , m/z (MH^+) 736 ^{244} DLDAFR ^{249} , m/z (MH^+) 687 ^{119} TVTPIR ^{124} , m/z (MH^+) 648 ^{250} HYDGR ^{254} , m/z (MH^+) 631 ^{255} TIIQR ^{259} and (MH^+) 622 ^{198} EQSCR ^{202} .

Enzyme kinetics

In the absence of reducing agent to activate the enzyme, rDer p 1 had low intrinsic activity but in the presence of 1 mM DTT its activity towards ADZ 50,059 was increased $\sim$ 10-fold (Fig. 2A). Both native and recombinant Der p 1 displayed high specific activities and were kinetically indistinguishable (Fig. 2B). For nDerp 1 and rDer p 1 the measured K_m values ($\pm$ SEM) from four experiments were 15.4 ± 2.1 and 14.4 ± 3.1 μ M respectively.

Next, we examined whether the Der p 1 preparations were comparable in their behaviour towards ADZ 50,000, a potent and selective inhibitor of Der p 1 that irreversibly inactivates the peptidase. Inactivation rate constants were calculated for both Der p 1 preparations when tested against ADZ 50,000 concentrations ranging from 0.78–800 nM. The calculated inactivation rate $K_{obs}/[I]$ for nDer p 1 was $(1.4 \pm 0.2 \times 10^8)/M/s$ and was $(1.3 \pm 0.3 \times 10^8)/M/s$ for rDer p 1 ($n = 4$ experiments).

Illustrative progress curves for both enzymes in the absence and presence of one equivalent of ADZ 50,000, which is devoid of activity towards serine peptidases, establishes that all of the activity towards ADZ 50,059

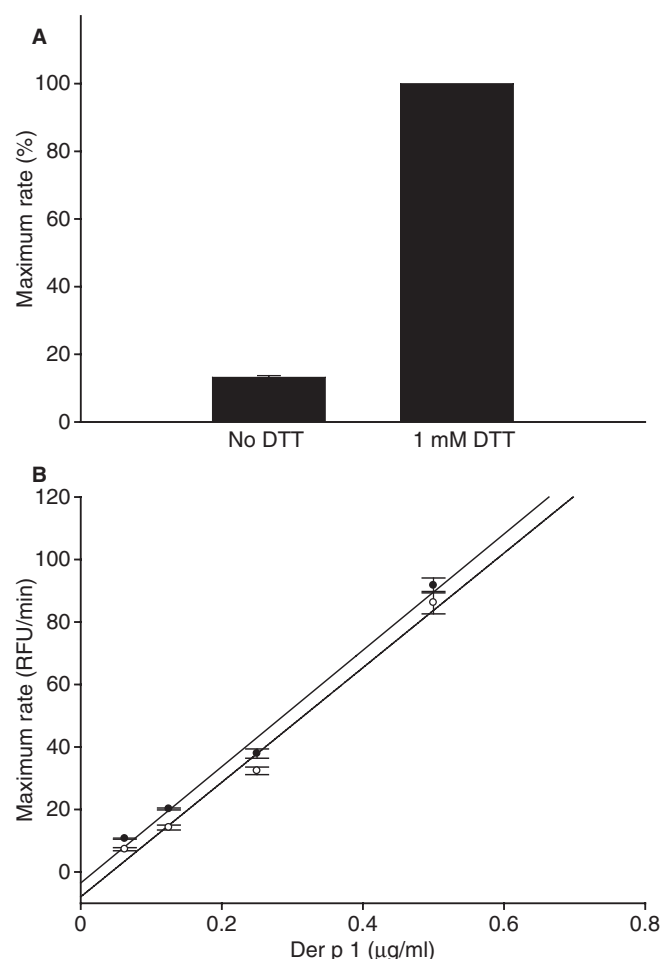


Figure 2. In the absence of reducing agent, rDer p 1 has minimal activity against ADZ 50,059 substrate, but this is significantly increased in the presence of 1 mM dithiothreitol, A. Panel B shows the relationship between Der p 1 concentration and maximum reaction velocity across a range of enzyme concentrations. Open symbols show nDer p 1 and filled symbols rDer p 1. Data are mean $\pm$ SEM from four observations at each concentration.

could be inhibited (Fig. 3A), enabling this to be used as a superior active site titrant as shown in Fig. 3B for nDer p 1 and rDer p 1 using the residual activity method.

Comparison of the activity of Der p 1 and trypsin on the substrate N-Bz-Phe-Val-Arg-*p*-nitroanilide, a preferred substrate of serine peptidases but which is degraded by Der p 1 at high enzyme concentrations, showed that it was rapidly cleaved by trypsin and the reaction was inhibited effectively by AEBSF (Fig. 4A). However, ADZ 50,000 failed to inhibit the reaction (Fig. 4A). At similar enzyme concentrations (2 μ g/ml), Der p 1 was poorly active on this substrate, so to test whether cleavage could be inhibited by ADZ 50,000 or AEBSF, the concentration of Der p 1 was increased ten-fold in order to obtain an acceptable rate of proteolysis. As shown in Fig. 4B,C, neither nDer p 1 nor rDer p 1 was

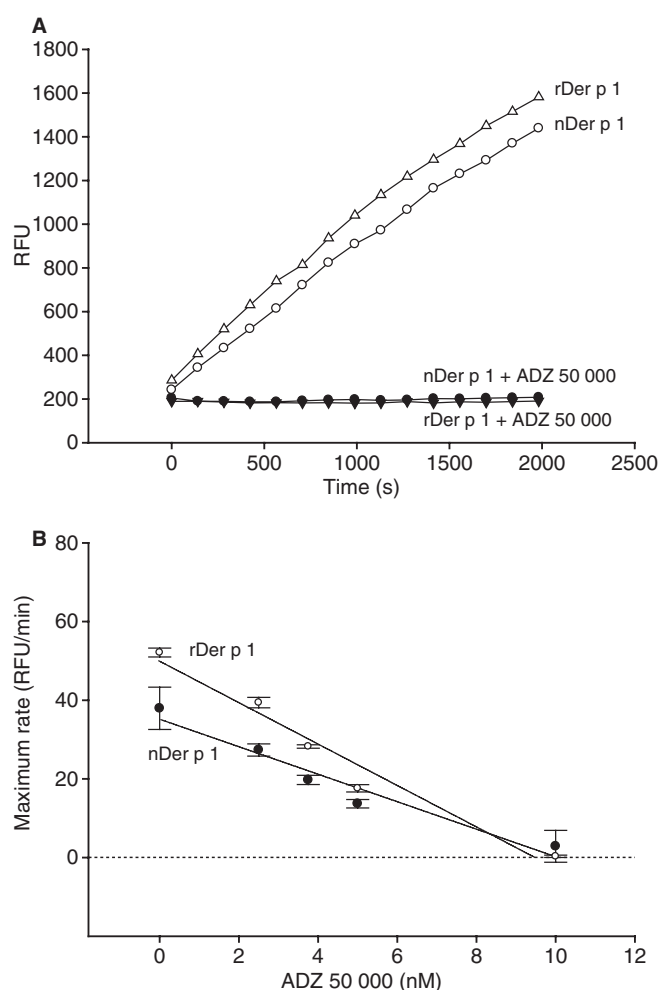


Figure 3. Panel A shows progress curves for the degradation of ADZ 50,059 by rDer p 1 in the absence (unfilled triangles) and presence (filled triangles) of one equivalent of ADZ 50,000. Corresponding curves for nDer p 1 are depicted by unfilled and filled circles, respectively. Panel B depicts active site titration of rDer p 1 (unfilled circles) and nDer p 1 (filled circles) using ADZ 50,000 as titrant and ADZ 50,059 as substrate. Data are shown as mean $\pm$ SEM from four observations at each concentration of ADZ 50,000. Enzyme concentration was 0.5 μ g/ml. Lines were fitted by unweighted least squares linear regression to determine the active site concentrations at the intercepts on the abscissa.

inhibited by AEBSF, whereas substrate cleavage was blocked by ADZ 50,000.

Figure 4D shows that chymotrypsin effectively degraded a specific substrate and that this was blocked by AEBSF. However, Der p 1 was ineffective on this substrate, even at a high enzyme concentration. Collectively, these findings show that Der p 1 has no serine peptidase activity.

The next objective was to examine the effect of the Der p 1 prodomain on the activity of the peptidase. To do this, the full-length Der p 1 prodomain was expressed as a

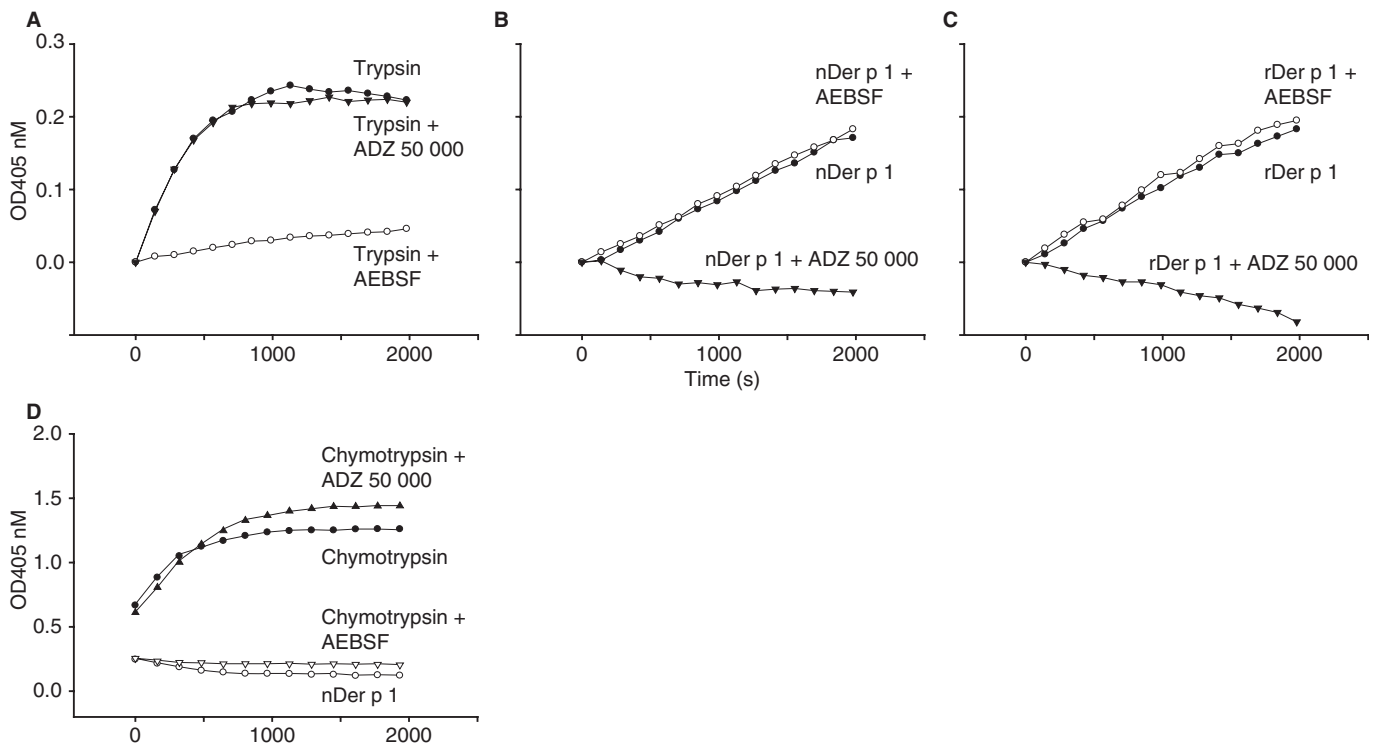


Figure 4. Representative progress curves showing the cleavage of N-Bz-Phe-Val-Arg-*p*-nitroanilide (A–C) or N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (D). (A) Trypsin (2 μ g/ml) in the absence (filled circles) of inhibitors and in the presence of 80 μ M AEBSF (unfilled circles) or 8 μ M ADZ 50,000 (triangles). (B,C) nDer p 1 and rDer p 1 (both at 20 μ g/ml) respectively and the effects of 80 μ M AEBSF or 8 μ M ADZ 50,000. (D) Chymotrypsin (2 μ g/ml, filled circles) and in the presence of ADZ 50,000 (40 μ M, filled triangles), AEBSF (400 μ M, open triangles). nDer p 1 (20 μ g/ml, open circles).

soluble protein (PP-02) and purified to homogeneity. The prodomain retarded the degradation of ADZ 50,059 by both nDer p and rDer p 1, as exemplified by the progress curves shown in Fig. 5A for rDer p 1 in the presence of 50 nM PP-02. PP-02 was a potent inhibitor with IC_{50} values of 56.9 ± 6.1 nM and 51 ± 3.5 nM against nDer p 1 and rDer p 1, respectively (mean $\pm$ SEM from four experiments) (Fig. 5B). The action of PP-02 was apparently competitive because it significantly ($P < 0.05$) increased K_m to 54.8 ± 9.6 and 48.5 ± 10.4 μ M for nDer p 1 and rDer p 1, respectively, without reduction in V_{max} (Fig. 5C).

At room temperature there was a progressive loss of enzymatic activity for both nDer p 1 and rDer p 1. This is illustrated by rDer p 1 where the residual peptidase activity assayed after 30 or 120 min preincubation is shown in Fig. 6. The effect was significantly greater for enzyme activated by DTT during the preincubation period, suggestive of autolysis.

Discussion

This study provides the first detailed kinetic comparison of a recombinant Der p 1 with its native counterpart.

Using a substrate, ADZ 50,059, created by positional scanning of the Der p 1 catalytic site and by means of an active site titrant of Der p 1, ADZ 50,000, which incorporates the substrate targeting sequence, we have shown that rDer p 1 produced in a yeast expression system is catalytically indistinguishable from native Der p 1. This suggests that the active site of the recombinant protein is fully preserved and that enzyme activity is unaffected by hyperglycosylation of the recombinant protein.

The rDer p 1 used in this study was prepared by automaturization of pro-rDer p 1 in acidic solution. The construct used for expression encoded 302 residues comprising the entire, unmodified proDer p 1 sequence which on cleavage of the prodomain at E98 should yield a mature protein of 25 kDa, considerably less than the average molecular weight of 55 kDa actually recorded.

It is well established that expression of proDer p 1 in *P. pastoris* results in hyperglycosylation and that this can be prevented in N150Q or N150E mutants. Despite these mutations, the matured recombinant proteins have similar immunogenicity to nDer p 1 and are enzymatically active (21, 22, 24, 29).

The glycosylation of native Der p 1 is uncharacterized and its influence on the properties of mature Der p 1 is

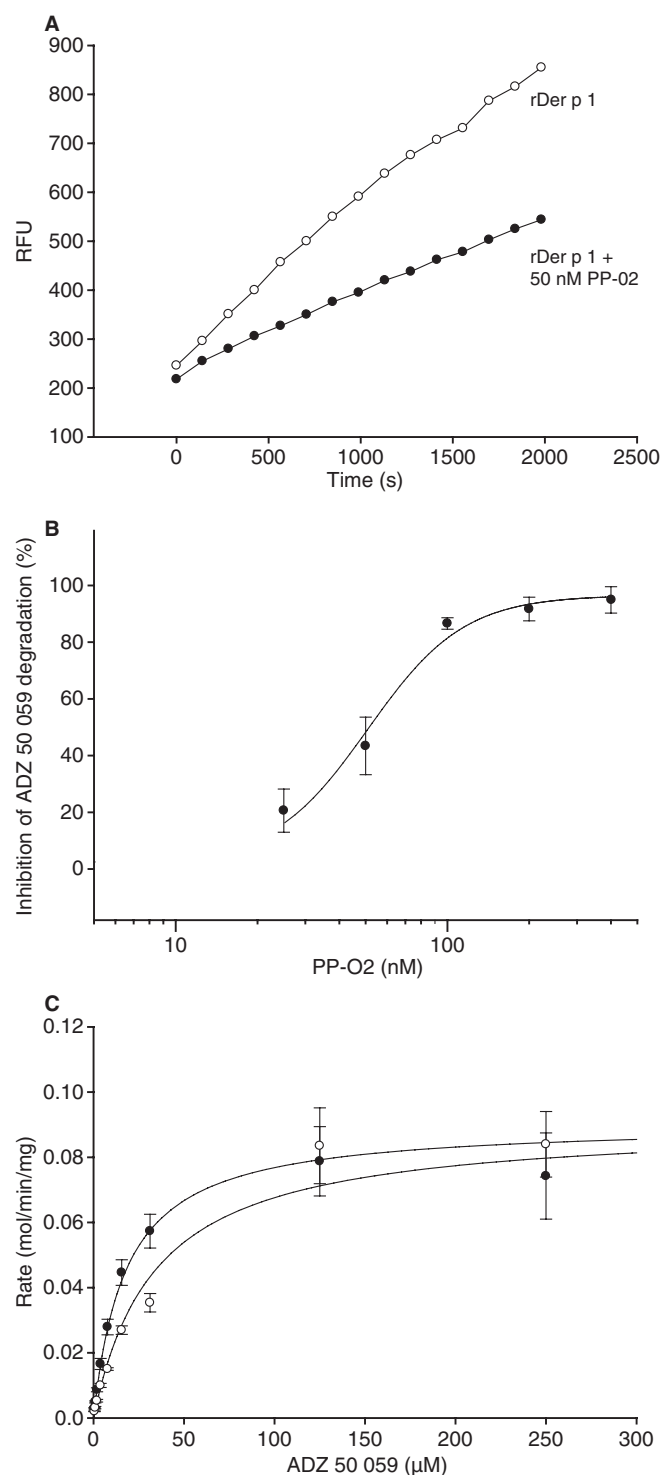


Figure 5. Panel A shows progress curves for the degradation of ADZ 50,059 by rDer p 1 (0.25 μg/ml) in the absence (unfilled circles) and presence (filled circles) of 50 nM PP-02. A concentration-inhibition curve for PP-02 at a single enzyme and substrate concentration is depicted in B, with data shown as mean ± SEM from four observations at each concentration. Panel C shows a Michaelis-Menten plot obtained in the absence (filled circles) or presence (unfilled circles) of 20 nM PP-02. Data are mean ± SEM from four observations at each concentration.

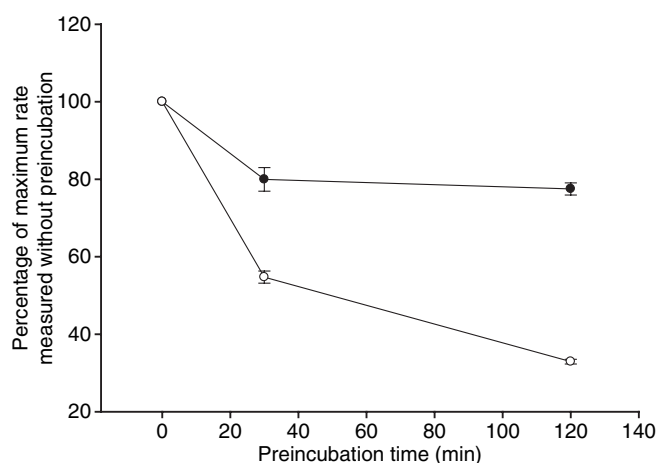


Figure 6. Decrease in the activity of rDer p 1 during prolonged incubation with DTT at room temperature. Reaction mixtures were assembled as described in Methods and ADZ 50, 059 added immediately (time zero control), after 30 min or 120 min. The progress curves were then followed at 30°C. Filled circles depict the maximum rates measured during preincubation in the absence of DTT (DTT was added with substrate to perform the assays in these cases). Unfilled circles depict maximum rates under conditions where DTT was present throughout the preincubation period and in the assay. The maximum rate measured in each case was expressed as a percentage of that in the relevant control that had not been preincubated. Data are mean ± SEM from four observations.

largely unknown. The potential N150 glycosylation site of Der p 1 is distal to the binding groove for substrate and so minimal effects on catalysis would be anticipated. However, previous characterization of the N150Q mutant unexpectedly suggested that its activity may be higher than that of the native Der p 1 comparator (24), indicating that it may not be a suitable surrogate of the native protein in all applications. However, the exploration of enzymatic properties in that case was based on nonselective substrate behaviour and contrasts with the selective, novel tools used in the present investigation. One possible effect of the glycosylation motif of Der p 1 may be to permit binding to the carbohydrate recognition domains of certain proteins, as demonstrated with lung surfactant proteins –A (SP-A) and –D (SP-D). Although Der p 1 has been shown to cleave SP-A and SP-D under *in vitro* conditions (30) it may be that the dominant immunological consequence of their interaction is attenuation of allergen function.

In electing to characterize the properties of the hyperglycosylated recombinant Der p 1 we have taken a different approach because we were interested in this providing a convenient source of protein for use in high-throughput studies of the catalytic site of Der p 1. The glycosylation of rDer p 1 used in this study is uncharacterized and it was not an aim of the present work to investigate this aspect. Despite the proximity of a

theoretical tryptic cleavage, no fragments bearing the glycosylation site were identified in mass spectra, possibly because heterogeneous glycosylation results in poor properties for MALDI-TOF. However, it is clear that despite being of abnormally high mass, the reactivity of the recombinant protein towards substrates or inhibitors was unaffected. Our results demonstrate that this form of rDer p 1 could be an acceptable alternative to nDer p 1 for applications where protein with mutated amino acid residues may be undesirable. It further benefits from expression at a generally higher level than N150Q/E mutants, and this may be advantageous for the provision of therapeutic discovery campaigns.

ADZ 50,059 is a preferred substrate of Der p 1 derived by screening of a positional scanning peptide library. It shows some reactivity towards human cathepsins but is less reactive with serine peptidases (e.g. K_m for chymotrypsin is $> 130 \mu\text{M}$). Reactivity of nDer p 1 and rDer p 1 towards ADZ 50,059 was identical. ADZ 50,000 is an inhibitor of Der p 1 which forms an irreversible complex with the enzyme. It has more than 500-fold greater reactivity with Der p 1 than does E-64. E-64 is a commercially available inhibitor which, with the exception of one study (31), has been the principal tool with which to study the contribution of Der p 1 peptidase activity to allergy. Unlike E-64, which can react with serine peptidases, ADZ 50,000 is devoid of such activity when tested at concentrations several thousand fold in excess of the enzyme (our unpublished observations). For nDer p 1 and rDer p 1, comparison of reaction rates showed no observable difference between the enzymes in their interaction with ADZ 50,000, suggesting that their binding is identical.

There is a growing understanding of the cleavage specificity of Group 1 mite allergens which is based on studies using model peptides, recombinant proteins and whole cells (2, 30, 32, 33). Taken together with data from our own drug discovery campaign, Der p 1 appears to have a limiting preference for small hydrophobic residues at the P2 position and broader tolerance at other positions. These preferences were exploited in the selection of ADZ 50,059 and the design of ADZ 50,000.

To avoid the possibility that a highly reactive titrant of the active site such as ADZ 50,000 might nevertheless fail to reveal subtle differences between the nDer p 1 and rDer p 1 we investigated the effects of PP-02, a recombinant form of the Der p 1 prodomain. The prodomains of Der p 1, and by

implication all related Group 1 HDM allergens, show unusual behaviour compared to other C1 family cysteine peptidases. Whereas the untethered prodomains of other C1 peptidases are stable and continue to function as potent inhibitors after maturation in order to prevent promiscuous proteolysis (34–38), the behaviour of the Der p 1 prodomain is entirely different. It is rapidly cleaved by nDer p 1 and its apparent inhibitory action is because it is a highly effective substrate (27). Comparison of nDer p 1 and rDer p 1 showed that PP-02 behaved identically with both, further supporting the view that they are kinetically indistinguishable.

Based on studies with commercially available inhibitors and insulin B chain as substrate, an earlier report suggested that Der p 1 might be an unusual enzyme with both serine and cysteine peptidase character (39). However, it has been argued that the trypsin-like serine peptidase activity reported in this surprising study was due to contamination arising from inadequacies in conventional purification strategies for Der p 1. The recent solution of the crystal structure of Der p 1 provides no theoretical support for the notion of Der p 1 being a bifunctional enzyme with serine and cysteine peptidase character (21, 22). The studies we report here, using novel and highly selective probes of Der p 1, now establish clearly that Der p 1 is a cysteine peptidase and that its sequence-based assignment to the C1 family of clan CA is appropriate. Any deviation from the well established archetypes of the C1 family is encoded in the prodomains of Der p 1 and other Group 1 mite allergens and has no bearing on the mechanistic class to which these peptidases belong (27).

In conclusion, this study has performed the first detailed comparison of nDer p 1 and rDer p 1 using a series of novel Der p 1-selective reagents. The data suggest that hyperglycosylation has little influence on the catalytic activity of this major allergen, and that heterogeneously glycosylated rDer p 1 is a useful molecular tool for the investigation of how peptidase activity contributes to allergy.

Acknowledgments

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