

High-level expression of recombinant house dust mite allergen Der p 1 in *Pichia pastoris*

A. Jacquet, M. Magi, H. Petry and A. Bollen

Department of Applied Genetics, Université Libre de Bruxelles, Belgium

Summary

Background The major house dust mite Der p 1 allergen is associated with allergic disease. Heterologous over-expression of biologically active Der p 1 was previously attempted but with limited success.

Objective The aim of this study was to establish an efficient system for the production of recombinant Der p 1.

Methods The proform of Der p 1 was expressed in *Pichia pastoris* as a fusion with the α mating factor signal sequence. The recombinant product was purified from culture medium and compared to Der p 1 isolated from mite culture, in terms of enzymatic activity as well as IgE binding capacity.

Results ProDer p 1 was efficiently secreted into culture medium as a hyperglycosylated protein of 40–60 kDa. Postpurification dialysis in acidic buffer was required for the autocatalytic processing of Der p 1. During this treatment, the prosequence was efficiently removed to give highly glycosylated recombinant mature Der p 1. Competition ELISA experiments as well as cysteine proteinase activity assays indicated that recombinant processed Der p 1 was similar to natural Der p 1 isolated from mite cultures in terms of IgE binding and enzymatic activities. However, the histamine releasing activity of recombinant Der p 1 was slightly weaker than that of natural Der p 1.

Conclusion This efficient system for recombinant Der p 1 expression leads the way for the design of new diagnostics for house dust mite allergy, epitope mapping, allergen engineering, structural and immunological studies and new immunotherapeutic treatments.

Keywords cysteine proteinase, Der p 1, IgE reactivity, processing, recombinant allergen

Submitted 15 October 2001; revised 14 January 2002; accepted 21 February 2002

Introduction

Recombinant allergens represent an attractive alternative to allergen extracts in diagnostics and allergen-specific immunotherapy [1]. Indeed, the reproductibility of well-characterized recombinant allergen productions can be guaranteed contrary to allergen extracts which are often heterogeneous from batches to batches. Moreover, the risk of anaphylactic reactions in immunotherapy, induced by repeated injections of allergen extracts, can be minimized by the use of recombinant 'hypoallergens', in which the IgE reactivity was altered by deletions or mutagenesis [2].

Allergens from the house dust mite *Dermatophagoides pteronyssinus* are commonly associated with allergic hypersensitivity reactions such as perennial rhinitis, atopic dermatitis or asthma [3]. Amongst the dozen mite proteins recognized by IgE from human allergic sera, the Der p 1 allergen is the strongest IgE-mediated immune response inducer [4, 5]. Although this 30 kDa protein is well characterized in terms of

its enzymatic activity, allergenicity and gene cloning, heterologous overexpression of Der p 1 was problematic, probably because this cysteine proteinase is synthesized as a PreProDer p 1 precursor. According to the cDNA sequence, Der p 1 is expressed as a precursor of 320 amino acid residues, including an 18-amino acid signal peptide, an 80-amino acid N-terminal prosequence and two potential N-glycosylation sites [6, 7]. This enzymatically inactive form is subsequently processed into a 222-amino acid mature and biologically active Der p 1.

The first attempts at recombinant Der p 1 productions were shown to be unsuccessful in *S. cerevisiae* and *E. coli* [8–10]. Recombinant Der p 1 was poorly expressed, mainly under an insoluble form, and displayed weaker IgE-binding reactivity than the native allergen. Recently, we reported the successful production of a recombinant secreted form of ProDer p 1 in insect and Chinese hamster ovary (CHO) cells [11, 12]. The potential value of this recombinant Der p 1 precursor in diagnostics as well as in allergen-specific immunotherapy was previously demonstrated as its IgE reactivity is modulated according to whether the recombinant allergen is coated or in solution.

The *Pichia pastoris* expression system offers many advantages compared to other systems since it leads to very high levels of secretion into an almost protein-free medium, is easy for fermentation to high cell density and can be scaled up without loss of yield. This methylotrophic yeast expression system

Correspondence: Dr Alain Jacquet, Department of Applied Genetics, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, Rue des Professeurs Jeener et Brachet, 12, B-6041 Gosselies, Belgium. E-mail: ajacquet@sga.ulb.ac.be

recently allowed the high-level production of the proform of Der f 1, the major *D. farinae* allergen having more than 80% homology with Der p 1 [13, 14].

The present study reports the efficient production of recombinant ProDer p 1 by *P. pastoris*. Our results showed that the recombinant allergen is secreted at very high-level in the culture medium. During expression, ProDer p 1 did not undergo an efficient processing to lead to mature Der p 1 as reported for ProDer f 1. However, acidic treatments of ProDer p 1 triggered autocatalytic processing of Der p 1. The allergen properties of recombinant processed Der p 1 such as IgE-binding, histamine release and proteolytic activities were comparable to those of natural Der p 1.

Materials and methods

Construction of ProDer p 1 expression vector

The ProDer p 1 coding cassette from pNIV4846 (full-length 1–302aa ProDer p 1 synthetic cDNA with optimized mammalian codon usage [12]) was amplified by PCR using the following primers: 5'ACTGACAGGCCTCGGCCGAGCTCCATTAA3' (*StuI* restriction site in bold, forward) and 5'CAGTCA CCTAGGTCTAGACTCGAGGGGAT3' (*AvrII* restriction site in bold, reverse). The amplified fragment was cloned into the pCR2.1 TOPO® cloning vector (Invitrogen, Groningen, the Netherlands). The correct ProDer p 1 cassette was verified by DNA sequencing. Recombinant TOPO vector was digested with *StuI*-*AvrII* to generate a 918-bp fragment. This DNA piece was subsequently introduced into the pPIC9K expression vector (Invitrogen) restricted with *SnaBI*-*AvrII*. The resulting plasmid, pNIV4878, contains the ProDer p 1 cassette downstream to the *S. cerevisiae* α factor

Transformation of *P. pastoris*

Plasmid pNIV4878 was introduced into *P. pastoris* using the spheroplast transformation method. Transformants were selected for histidinol deshydrogenase (His^+) prototrophy. The screening of His^+ transformants for geneticin (G418) resistance was performed by plating clones on agar containing increasing concentrations of G418.

Production of ProDer p 1 by recombinant yeast

G418 resistant clones were grown at 30 °C in Buffered Minimal Glucose (BMG) medium to an $\text{OD}_{600\text{nm}}$ of 2–6. Cells were collected by centrifugation and resuspended to an $\text{OD}_{600\text{nm}}$ of 1 in 100 mL of BMG medium. ProDer p 1 expression was induced by daily addition of methanol 0.5% for 6 days. The supernatant was collected by centrifugation and stored at –20 °C until purification.

Purification of ProDer p 1 from yeast culture supernatant

Supernatants were diluted 10 times with water and, after pH adjustment to 9, directly loaded onto a Q sepharose column equilibrated in 20 mM Tris-HCl pH 9. The column was washed with the starting buffer. Protein elution proceeded by step-wise increasing NaCl concentration in the buffer. The ProDer p 1-enriched fractions were pooled and concentrated by ultrafiltration onto a Filtron membrane (Omega series, cut-off: 10 kDa). The ProDer p 1 purification was achieved by a

gel filtration chromatography onto a superdex-75 column (1 × 30 cm, Amersham Pharmacia Biotech, Roosendaal, the Netherlands) equilibrated in phosphate buffered saline (PBS) pH 7.3. Purified ProDer p 1 was concentrated and stored at –20 °C.

SDS-PAGE and Western blot analysis

Proteins were analysed by SDS-PAGE on 12.5% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes using a semidry transblot system (Bio-Rad, Nazareth, Belgium). Membranes were saturated for 30 min with 0.5% Instagel (PB Gelatins) in TBS-T buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 0.1% Tween 80) and incubated with mouse polyclonal serum raised against denatured Der p 1 diluted in saturating solution (1 : 5000). Immunoreactive materials were detected using alkaline phosphatase-conjugated goat anti-mouse antibodies (Promega, Leiden, the Netherlands, 1 : 7500) and 5-bromo, 4-chloro,3-indolylphosphate (BCIP, Boehringer: Roche Molecular Biochemicals, Brussels, Belgium)/nitroblue tetrazolium (NBT, Sigma, Bornem, Belgium) as substrates.

Glycopeptidase F digestion

Recombinant ProDer p 1 (5 µg) was incubated with 1 unit glycopeptidase F (Boehringer) in PBS pH 7.2 containing 0.5% SDS, 1% CHAPS and 10 mM EDTA at 37 °C for 17 h. Reaction was stopped by addition of sample buffer and proteins were analysed by SDS-PAGE

Autocatalytic processing of Der p 1

Purified ProDer p 1 (100 µg) was dialysed against 100 mM sodium acetate buffer pH 4 at 4 °C for different periods of time. The maturation was monitored by SDS-PAGE.

Enzymatic assays

Enzymatic assays were performed in 50 mM Tris-HCl pH 7, containing 1 mM EDTA and 20 mM L-cysteine at 25 °C in a total volume of 1 mL. Hydrolysis of Boc-Gln-Ala-Arg7-amino-4-methylcoumarin (Boc-Gln-Ala-Arg-AMC) (Sigma) (final concentration of 100 µM) was monitored using a SLM 8000 spectrofluorimeter with $\lambda_{\text{ex}} = 380$ nm and $\lambda_{\text{em}} = 460$ nm. Assays were started by addition of cysteine-activated allergen to a final concentration of 100 nM. Before any assay, purified Der p 1 or ProDer p 1 was incubated with a mixture of aprotinin- and p-aminobenzamidine-agarose resins (Sigma) to remove any putative trace of serine protease activity. In some experiments, recombinant allergen was incubated with the irreversible cysteine proteinase inhibitor L-trans-epoxy-succinyl-leucylamido-(4-guanidino)-butane (E-64, final concentration 10 µM, Sigma) before any assay.

Protein determination

Total protein concentration was determined by the bicinchoninic acid procedure (MicroBCA, Pierce, Erembodegem, Belgium) with bovine serum albumin (BSA) as standard.

Der p 1 ELISA

Recombinant ProDer p 1 was detected with an ELISA kit using Der p 1 specific monoclonal antibodies 5H8 and 4C1 (Indoor Biotechnologies, Charlottesville, VA, USA). The

Der p 1 standard (UVA 93/03) used in the assay was at a concentration of 2.5 µg/mL.

IgE-binding activity

Immunoplates were coated overnight with natural or recombinant Der p 1 (500 ng/well) at 4 °C. Plates were then washed 5 times with 100 µL per well of TBS-T and saturated for 1 h at 37 °C with 150 µL of the same buffer supplemented with 1% BSA. Sera from allergic patients to *D. pteronyssinus* and diluted at 1/8 were then incubated for 1 h at 37 °C. The specific anti-*D. pteronyssinus* IgE values (RAST assays) of sera were above the upper cut-off value of 100 kU/L. Plates were washed 5 times with TBS-T buffer and the allergen-IgE complexes were detected after incubation with a mouse anti-human IgE antibody (dilution 1/2000 in TBS-T buffer; Southern Biotechnology Associates, Birmingham, AL, USA) and a goat anti-mouse IgG antibody coupled to alkaline phosphatase (dilution 1/7500 in TBS-T buffer, Promega). The enzymatic activity was measured using the p-nitrophenylphosphate substrate (Sigma) dissolved in diethanolamine buffer (pH 9.8). OD_{410nm} was measured in a Biorad Novapath ELISA reader.

For IgE inhibition assays, plates were coated with natural Der p 1 (500 ng/well). Three human sera from allergic patients (RAST value > 100 kU/L) were preincubated overnight at 4 °C with various concentrations (from 0.1 to 200 µg/mL) of natural or recombinant Der p 1 as inhibitors and added on ELISA plates. IgE-binding was detected as described above.

Histamine release

The histamine release was assayed using leucocytes from the peripheral heparinized blood of an allergic donor and by the Histamine-ELISA kit (Immunotech, Marseille, France). Basophils were incubated with serial dilutions of natural or recombinant Der p 1 for 30 min at 37 °C. The total amount of histamine in basophils was quantified after cell disruption with the detergent IGEPAL CA-630 (Sigma).

Results and Discussion

Two recent studies described the efficient production of recombinant Der f 1 in *Pichia pastoris*. Recombinant enzymatically active Der f 1 was obtained after ProDer f 1 expression and removal of the prosequence in acidic conditions [13]. Moreover, Best and coworkers surprisingly showed that expression of ProDer f 1, downstream to the α factor signal peptide, leads to direct secretion of mature Der f 1 from the methylotrophic yeast, without any acidic treatment [14]. Based on these results, we evaluated the expression of recombinant ProDer p 1 in this system. As optimization of codon usage did not appear essential for ProDer f 1 expression, a synthetic ProDer p 1 cDNA based on the mammalian prevalent codons was inserted into the *P. pastoris* expression vector pPIC9K downstream to the α factor signal peptide. *P. pastoris* cells were transformed with linearized recombinant plasmid pNIV4878 by the spheroplast method. Fifty-two clones, obtained after selection of transformants on histidine-deficient medium, were grown in the presence of G418 (0.25–4 mg/mL) to identify clones carrying multiple copies of ProDer p 1 coding cassette. Seven transformants resistant to 4 mg/mL G418 were tested for

ProDer p 1 expression. Supernatants of methanol-induced cultures were analysed on SDS-PAGE and expression products were detected by immunoblotting using mouse polyclonal antibodies directed to purified ProDer p 1 produced in CHO cells.

One secreted immunoreactive product with a molecular weight ranging from 35 to 65 kDa was detected in the culture supernatant of pNIV4878-transformed *P. pastoris*. As expected, no immunoreactive molecule could be found in supernatant of control pPIC9K-transformed yeast. The ProDer p 1 expression level in the culture supernatants was estimated by an ELISA assay, using two anti-Der p 1 monoclonal antibodies. The best producing clone expressed ProDer p 1 up to 150 mg/L (average: 70 mg/L), a result comparable with those previously obtained with ProDer f 1 production. Recombinant ProDer p 1 was then purified to homogeneity starting from spent culture medium of the highest secreting yeast transformant. The combination of anion-exchange (Q-sepharose) and gel filtration (superdex 75) chromatographies yielded about 100 mg of purified ProDer p 1 per litre of culture medium.

On SDS-PAGE, purified ProDer p 1 migrated as a diffuse band of 35–60 kDa suggesting the presence of large post-translational modifications on the recombinant allergen. Heterologous proteins secreted by *P. pastoris* are often hyperglycosylated with chains of mannose units of up to 50–150 residues carried by N-linked glycosylations sites [15]. The heterogeneity of recombinant ProDer p 1 could be attributed to different levels of glycosylations since ProDer p 1 carries two potential N-glycosylation sites at residues 16 and 132. The same heterogeneity at the level of glycosylations was observed for ProDer f 1 which contains only one N-glycosylation site. By treatment with N-glycopeptidase F, ProDer p 1 migrated as a discrete band of 32 kDa, demonstrating that the main ProDer p 1 post-translational modifications are N-linked glycosylations.

To confirm that ProDer p 1 processing did not occur during allergen expression, the ProDer p 1 band was submitted to an amino-terminal amino acid sequencing. The N-terminal sequence of ProDer p 1 started at residue Ala20 (ProDer p 1 numbering, the obtained sequence was Ala-Thr-Phe-Glu-Asp) showing that an internal cleavage of the prosequence occurred between Tyr19 and Ala20. A partially cleaved prosequence was also reported for ProDer f 1 secreted by *P. pastoris*. Consequently, no correctly processed mature Der p 1 was obtained. This result also showed that only one putative glycosylation site at residue Asn132 is present on secreted ProDer p 1.

Purified ProDer p 1 was incubated in acidic conditions for 24 or 48 h to trigger autocatalytic processing of Der p 1. To clearly visualize the putative ProDer p 1 maturation, acid-treated ProDer p 1 samples were deglycosylated prior to SDS-PAGE analysis. After a 24–48 h acid treatment period, the ProDer p 1 band shifted to a molecular weight close to that of natural Der p 1 (Fig. 1). N-terminal sequencing of the band clearly showed that ProDer p 1 incubation in acidic conditions led to two major products: a correctly processed mature Der p 1 (sequence Thr-Asn-Ala-Cys-Ser-Ile-Asn...) and a mature Der p 1 containing the last two residues of the prosequence (sequence Ala-Glu-Thr-Asn-Ala-Cys-Ser...). The molar ratio of these two Der p 1 forms was equivalent and similar results were obtained with spontaneous ProDer f 1 processing [14].

To correlate IgE binding reactivity of recombinant Der p 1 to that of natural Der p 1, both allergens were directly coated on

ELISA plates. A set of 48 human sera with positive radio-immunosorbent tests to *D. pteronyssinus* extract was used at dilution 1:8. IgE titre determinations clearly showed a close correlation of IgE reactivity with both allergens. Consequently, recombinant Der p 1 has very similar IgE binding characteristics compared with natural Der p 1 ($R^2 = 0.9448$, $P < 0.0001$) (Fig. 2). These results, combined with those previously observed with recombinant forms of ProDer p 1 produced by CHO or S2 cells, clearly indicated that glycosylation levels in Der p 1 did not influence the IgE binding activity of the allergen.

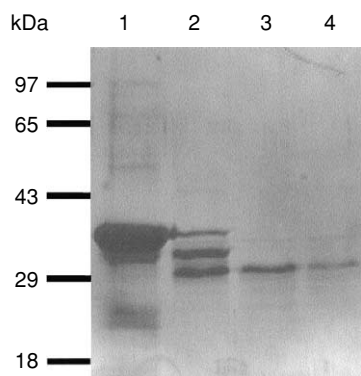


Fig. 1. Der p 1 processing. Purified ProDer p 1 was incubated in acidic conditions for 24 or 48 h. Proteins were treated with glycopeptidase F prior to SDS-PAGE analysis. Lane 1: purified and glycopeptidase F-treated ProDer p 1, Lane 2: deglycosylated ProDer p 1 after a 24-h acid treatment, Lane 3: deglycosylated ProDer p 1 after a 48-h acid treatment, Lane 4: purified natural Der p 1.

Competitive inhibition assays clearly demonstrated that recombinant Der p 1 were able to inhibit the IgE reactivity of natural Der p 1 in solution, with efficiencies similar or slightly weaker to those seen when natural Der p 1 was used as inhibitor (Fig. 3). These results suggested that overall IgE-binding epitopes of natural Der p 1 are present in the recombinant allergen.

The functional activity of recombinant Der p 1 was also investigated by histamine release assay. Basophils from one house dust mite-allergic patient were stimulated *in vitro* with various concentrations of both forms of Der p 1 and the re-

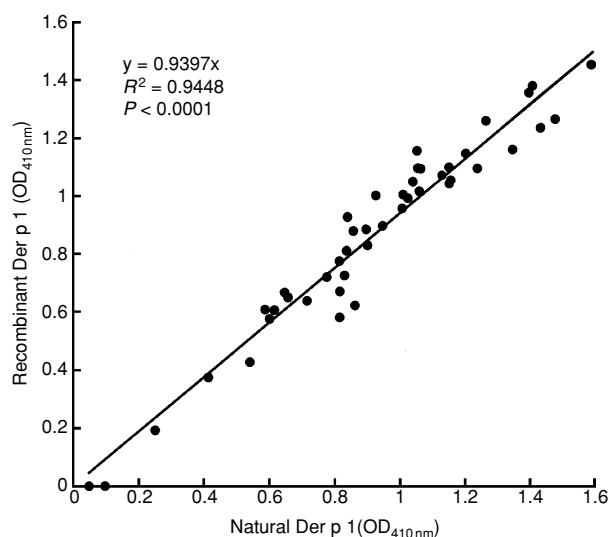


Fig. 2. Correlation between the IgE reactivity of recombinant and natural Der p 1. Immunoplates were coated with 500 ng/well of purified natural or recombinant Der p 1 and incubated with 48 sera (diluted 1:8) radioallergosorbent positive to *D. pteronyssinus*. Bound IgE were detected after incubation with a mouse anti-human IgE and alkaline phosphatase-labelled anti-mouse IgG antibodies, followed by an enzymatic assay. Results are expressed as OD_{410nm} values.

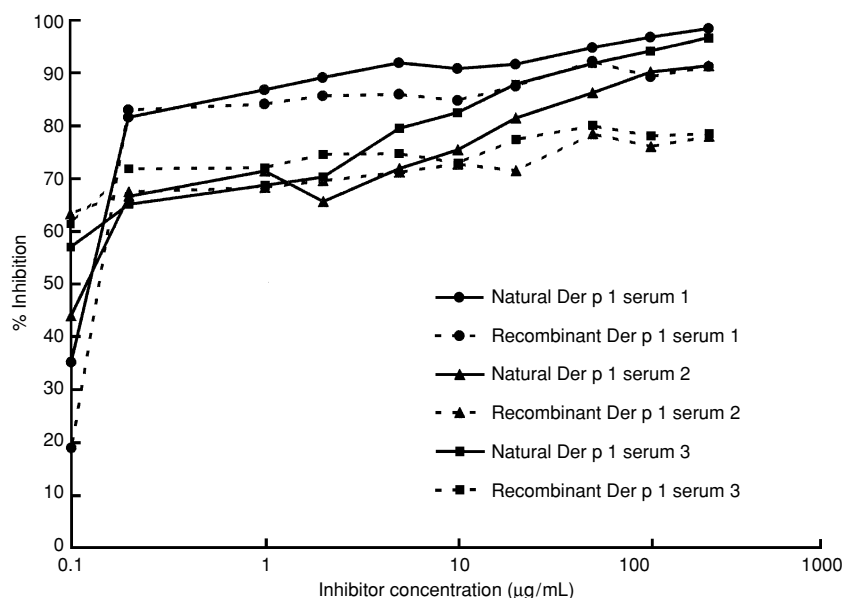


Fig. 3. Inhibition of IgE binding to natural Der p 1. IgE binding to immobilized natural Der p 1 was inhibited by preincubation of sera with increasing concentrations of natural (—) or recombinant Der p 1 (---). A set of three individual sera from dust mite allergic patients was used in these assays. Results are shown as 100%-(IgE binding in the presence of inhibitor/IgE binding in the absence of inhibitor).

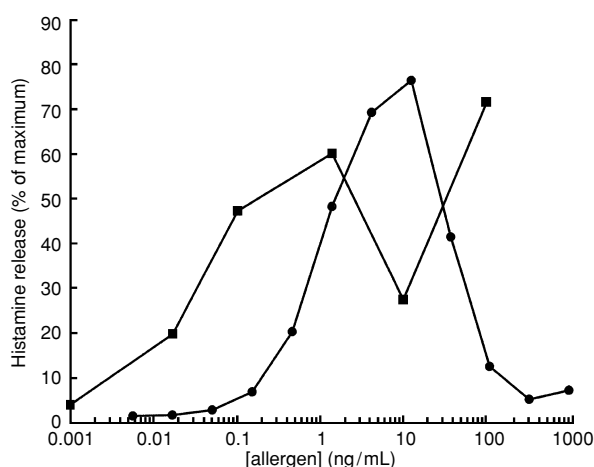


Fig. 4. Histamine release activity of natural or recombinant Der p 1. Basophils isolated from the peripheral blood of one allergic donor were stimulated with serial dilutions of natural (■) or recombinant (●) Der p 1. The histamine released from cells was measured by ELISA. The total amount of histamine in basophils was quantified after cell disruption with the detergent IGEPAL CA-630. Results are shown as the ratio of released histamine by allergens to total histamine.

leased histamine was measured. Recombinant Der p 1 induced slightly weaker release of histamine than natural Der p 1 (Fig. 4). These results correlate well with those obtained by IgE reactivity measurements in direct or competition ELISA.

To demonstrate that recombinant Der p 1 was enzymatically active, assays were performed using the fluorogenic substrate Boc-Gln-Ala-Arg-AMC. This sensitive fluorescent assay was previously reported for investigating proteolytic activity of Der p 1 [16]. Recombinant Der p 1 was shown to be proteolytically active against this fluorogenic substrate (Fig. 5). This enzymatic activity, similar to that of natural Der p 1, was totally inhibited by the irreversible cysteine proteinase inhibitor *L-trans*-epoxy-succinylleucylamido-(4-guanidino)-butane (E-64). It must be pointed out that, contrary to recombinant ProDer p 1 expressed in *Drosophila* or CHO cells, ProDer p 1 secreted by *P. pastoris* was shown to be weakly enzymatically active [11, 12]. This result could be explained by the absence of the first 19 amino acid residues in the prosequence when ProDer p 1 is produced in yeast. Consequently, the inhibitory activity of the truncated propeptide could be weaker than that of full-length prosequence present in ProDer p 1 produced by insect or mammalian cells.

In conclusion, our results suggest that recombinant Der p 1 adopts an appropriate conformation as it displays the biological activity of natural Der p 1. Recombinant Der p 1 represents an attractive alternative to natural Der p 1 in diagnostics and allergen-specific immunotherapy of house dust mite allergy. The very high level of ProDer p 1 production in *P. pastoris* allows us to elucidate the tertiary structure of the immature as well as mature allergen by X-ray crystallography. Moreover, as the *P. pastoris* expression system is faster, easier and less expensive to use than other eukaryotic expression systems such as insect or CHO cells, IgE epitope mapping experiments by site-directed mutagenesis is now made possible for Der p 1.

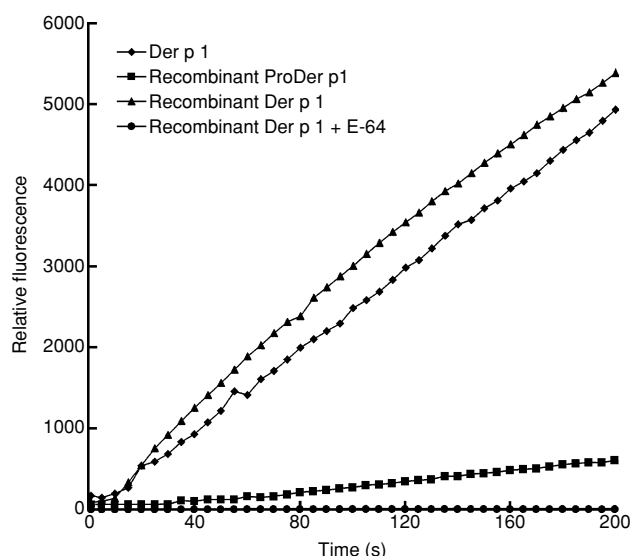


Fig. 5. Proteolytic activity of natural and recombinant forms of Der p 1. Progression curves for the hydrolysis of the fluorogenic substrate Boc-Gln-Ala-Arg-AMC by wild-type Der p 1 (◆), recombinant ProDer p 1 (■), recombinant processed Der p 1 (▲). To demonstrate the cysteine proteinase activity of processed Der p 1, the matured recombinant allergen was incubated with the cysteine proteinase inhibitor E-64 prior to enzymatic assay (●).

Acknowledgements

We thank Dr Michel Vandenbranden (Laboratoire de Chimie-physique des macromolécules aux interfaces, Université Libre de Bruxelles) for performing enzymatic activity assays. Sera from allergic patients to *Dermatophagoides* was kindly provided by Prof J. Duchateau (Hôpital Brugmann, Bruxelles, Belgium). This work was supported in part by GlaxoSmithKline and by the Walloon Region (Direction Générales des Technologies, de la Recherche et de l'Energie) of Belgium.

References

- Chapman MD, Smith AM, Vailes LD, Arruda LK. Defined epitopes: in vivo and in vitro studies using recombinant allergens. *Int Arch Allergy Immunol* 1997; 113:102–4.
- Akdis CA, Blaser K. Regulation of specific immune responses by chemical and structural modifications of allergens. *Int Arch Allergy Immunol*, 2000; 121:261–9.
- Platt-Mills TAE, Chapman MD. Dust mites: Immunology, allergic disease and environmental control. *J Allergy Clin Immunol* 1987; 80:755–75.
- Chapman MD, Rowntree SR, Di Prisco Defuenmajor MS, Platt-Mills TAE. Quantitative assessments of IgG and IgE antibodies to inhalant allergens in patients with atopic dermatitis. *J Allergy Clin Immunol* 1983; 72:27–33.
- Krillis S, Baldo BA, Sutton R, Basten A. Antigens and allergens from the common house dust mite *Dermatophagoides pteronyssinus*. I. Demonstration of multiple allergens by immunochemical and biologic analysis. *J Allergy Clin Immunol* 1984; 74:132–41.
- Chua K, Stewart G, Thomas W et al. Sequence analysis of cDNA coding for a major house dust mite allergen, Der, p. 1. Homology with cysteine proteases. *J Exp Med* 1988; 167:175–82.

- 7 Chua K, Kehal PK, Thomas W. Sequence polymorphisms of cDNA clones encoding the mite allergen Der p I. *Int Arch Allergy Immunol* 1993; 101:364–8.
- 8 Greene WK, Cyster JG, Chua KY, O'Brien RM, Thomas WR. IgE and IgG binding of peptides expressed from fragments of cDNA encoding the major house dust mite allergen Der p I. *J Immunol* 1991; 147:3768–73.
- 9 Chua KY, Kehal PK, Thomas WR, Vaughan PR, Macreadie IG. High-frequency binding of IgE to the Der, p. allergen expressed in yeast. *J Allergy Clin Immunol* 1992; 89:95–102.
- 10 Scobie G, Ravidran V, Deam S et al. Expression cloning of a dust mite cysteine proteinase, Der p1, a major allergen associated with asthma and hypersensitivity reactions. *Biochem Soc Trans* 1994; 22:448S.
- 11 Jacquet A, Haumont M, Massaer M et al. Biochemical and immunological characterization of a recombinant precursor form of the house dust mite allergen Der p 1 produced by *Drosophila* cells. *Clin Exp Allergy* 2000; 30:677–84.
- 12 Massaer M, Mazzu P, Haumont M et al. High-level expression in mammalian cells of recombinant house dust mite allergen ProDer p 1 with optimized codon usage. *International Arch Allergy Immunol* 2001; 125:32–43.
- 13 Yasuhara T, Takai T, Yuuki T, Okudaira H, Okumura Y. Biologically active recombinant forms of a major house dust mite group 1 allergen Der f 1 with full activities of both cysteine protease and IgE binding. *Clin Exp Allergy* 2001; 31:116–24.
- 14 Best EA, Stedman KE, Bozic CM et al. A recombinant group 1 house dust mite allergen, rDer f 1, with biological activities similar to those of the native allergen. *Protein Expr Purif* 2000; 20:462–71.
- 15 Sreekrishna K, Nelles L, Potenz R et al. High-level expression, purification, and characterization of recombinant human tumor necrosis factor synthesized in the methylotrophic yeast *Pichia pastoris*. *Biochemistry* 1989; 28:4117–25.
- 16 Schulz O, Sewell HF, Shakib F. A sensitive fluorescent assay for measuring the cysteine protease activity of Der p 1, a major allergen from the dust mite *Dermatophagoides pteronyssinus*. *Mol Pathol* 1998; 51:222–4.