

Characterization of the house dust mite allergen Der p 21 produced in *Pichia pastoris*



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ABSTRACT

Background: The development of recombinant house dust mite (HDM) allergens opened the way for the in-depth characterization of these molecules but also provided new opportunities to refine the diagnostic procedures of HDM allergy as well as the allergen-specific immunotherapy through tailor-made treatments.

Objective: In the present study, the HDM allergen Der p 21 was expressed in *Pichia pastoris* under a secreted form. The physico-chemical as well as the allergenic characterizations of recombinant Der p 21 (rDer p 21) were performed.

Methods: Purified rDer p 21, secreted from recombinant *P. pastoris* was characterized by CD and MS analysis and the frequency of IgE reactivity was determined by ELISA using 96 sera of HDM-allergic patients from Bangkok. The direct airway epithelial cell activation by rDer p 21 was also evaluated.

Results: rDer p 21 was highly expressed under a secreted form in *P. pastoris*. The physico-chemical characterization of purified rDer p 21 showed that the allergen displayed appropriate α -helix secondary structure content although a two amino acids truncation at the N-terminus of the protein was evidenced by MS. The prevalence of IgE reactivity to rDer p 21 reached 25% in the cohort of the HDM-allergic patients. rDer p 21 could trigger IL-8 production in airway epithelial cells through TLR2-dependent signaling.

Conclusion: Properly folded rDer p 21 produced in *P. pastoris* is appropriate for HDM allergy diagnosis as well for future recombinant allergen-based specific immunotherapy.

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Introduction

House dust mites ²(HDM; *Dermatophagoides* sp.) are one of the commonest sources of airborne allergens worldwide and HDM allergy affects more than 15–20% of the population from industrialized countries [1]. To date, 19 and 27 different HDM allergen groups eliciting specific IgE responses in sensitized patients were identified

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² Abbreviations used: HDM, house dust mites; rDer p 21, recombinant Der p 21; LPS, lipopolysaccharide; GRAS, generally recognized as safe; OD, optical density; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; Pfu, proofreading activity.

in *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* respectively [2,3]. Based on the strict IgE reactivity, group 1 and 2 HDM allergens are the most important allergenic proteins (the prevalence of IgE to these allergens reaches 80–90% in HDM allergic populations) followed by groups 4, 5, 7 and 21 which react with IgE in 30–50% of sera from HDM allergic patients [4].

The characterization of purified natural HDM allergens was roughly limited to groups 1 and 2, more likely because the concentration of other allergen groups is too low to achieve their purification.

With the elucidation of the cDNA sequence, a large panel of recombinant forms of HDM allergens was successfully produced from different heterologous expression systems and purified to homogeneity. The development of recombinant HDM allergens opened the way for the in-depth characterization of these molecules not only at the level of the T- or B-cell epitope mapping but also for the elucidation of their biological activities in order

to identify their allergenic determinants [5]. Strikingly, recombinant HDM allergens provide new opportunities to refine the diagnostic procedures of HDM allergy but also the allergen-specific immunotherapy through tailor-made treatments according to individual sensitivities [6]. In both cases, the well defined and consistent recombinant allergen preparations would replace poorly characterized HDM natural extracts which are difficult to standardize and which contain allergens inducing new sensitizations in treated patients.

Der p 21 is a relatively newly identified HDM allergen and is present in the gut epithelial cells of HDMs [7]. Whereas the Der p 21 cDNA codes for a 14.6 kD mature protein, small angle X-ray scattering experiments demonstrated that the solution structure of recombinant Der p 21 (rDer p 21) is dimeric [7]. Although the allergenic determinants of Der p 21 remain to be identified, this allergen displayed sequence homologies with Der p 5, a putative lipid-binding protein [8]. Consequently, Der p 21 could transport lipids/fatty acids which trigger allergic response through the activation of innate immune pathways. According to the mid-range prevalence of IgE reactivity to Der p 21 (around 30%, [7]), this allergen could be incorporated in the HDM allergen panels used for specific immunotherapy. rDer p 21 was expressed in the *Escherichia coli* bacteria. Although such heterologous expression system was commonly used for a very large panel of recombinant allergens, *E. coli* not only lacks appropriate post-translational modification machinery but also produces large amount of pyrogenic lipopolysaccharide (LPS) which represents important issues particularly when the produced allergens are used for *in vitro* immune cell activation assays as well as *in vivo* applications such as immunotherapy or skin-prick testing. Whereas Der p 21 does not contain disulfide bridges (absence of cysteine residues) or consensus N-glycosylation sites, this protein could carry other post-translational modifications essential for its biological activity. Consequently, the yeast *Pichia pastoris* expression system may be preferred through its “generally recognized as safe” (GRAS) status (notably by the absence of LPS) and its capacity to perform protein modifications at the post translational level.

In the present study, we successfully expressed rDer p 21 in *P. pastoris* under a secreted form. The physico-chemical characterization as well as the IgE reactivity measurements clearly indicated that such method of production is not only appropriate for future Der p 21-based diagnosis and treatment of HDM allergy but also for the determination of its allergenicity.

Material and methods

Human sera and rProDer p 1 and Der p 5

Ninety six sera were obtained from patients followed at Chulalongkorn Hospital, with a positive case history indicative for HDM allergy and *D. pteronyssinus*-specific IgE antibodies (0.98–90 kU/L) as determined with the ImmunoCAP System (Phadia). Each patient expressed written informed consent. As negative controls, sera from non-allergic subjects were also collected (<0.35 kU/L). The sera collection was approved by the Chulalongkorn University Ethic Committee (ref 327/2012). rProDer p 1 was expressed and purified as previously described [9]. rDer p 5 was expressed and purified following the same cloning and purification strategies as used for rProDer p 1.

Cloning of Der p 21 cDNA into *P. pastoris* expression vector

A 363 bp DNA fragment encoding leader-less Der p 21 (aa 20–140 from full length Der p 21 sequence) was generated by PCR amplification from a *D. pteronyssinus* cDNA library and

using specific primers carrying *Xho*I and *Not*I restriction sites (underlined); forward primer: 5'-CTCGAGAAAAGAGAGGCTGAAGTCTTTATTGTTGGTGACAAAAAGAAG-3'; reverse primer: 5'-GCGGCCGCTTAATAATATTCATCCGGATTACAGC-3'. The amplified DNA piece was digested with *Xho*I and *Not*I and cloned into an expression vector pPICZ α A (Invitrogen) restricted by the same enzymes. The recombinant pPICZ α A-Der p21 plasmid (5–10 μ g) was linearized with *Bgl*II to transform the yeast *P. pastoris* strains KM71 and X-33 (Invitrogen) by electroporation using a Gene Pulser Xcell™ (Bio-Rad) at 1500 V, 25 μ F, 200 Ω in 1 mm electrode gap cuvette. The transformants were selected on YPD agar plate (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing 100 μ g/ml Zeocin (Invitrogen) at 30 °C for 2–3 days.

Expression of rDer p 21 in *P. pastoris*

A single Zeocin-resistant colony was inoculated in 20 ml of YPD medium and grown overnight at 30 °C under shaking conditions. Ten percent of the inoculum was transferred in 500 ml of BMGY medium at 30 °C under shaking conditions until the optical density at 600 nm (OD_{600nm}) reached of 5–9 (approximately 14–15 h). The cells were then harvested by centrifugation at 5000g for 5 min and the cell pellet was resuspended in 500 ml of BMMY medium containing 0.5% methanol to induce rDer p 21 expression at 30 °C. Every 24 h, methanol was added to maintain the final concentration of methanol to 0.5%. Aliquots of culture supernatants were collected by centrifugation at 13000g for 10 min stored at –20 °C until used.

Purification of rDer p 21 from *P. pastoris* supernatant

The culture media containing secreted rDer p 21 were diluted ten times with MilliQ water and the pH of the solution was adjusted to pH 4. This material was applied onto a S Sepharose XL column (5 $\times$ 2.6 cm, GE Healthcare Lifesciences) equilibrated with 20 mM sodium acetate pH 4.0. After washing the unbound proteins from the column, elution proceeded through stepwise increase of NaCl concentration in the equilibration buffer (from 0 to 1000 mM NaCl). The fraction containing rDer p 21 (500 mM NaCl elution) was concentrated by ultrafiltration using a Amicon Ultra-15 10 K membrane (Cut-off 10 kD) and then further purified by gel filtration onto a Superdex 75 HR column 10/30 (GE Healthcare Lifesciences), equilibrated with PBS pH 7.3. The fractions containing purified rDer p 21 were pooled and stored at 4 °C for further analysis. Protein concentration was determined using the microBCA protein assay kit (Pierce) with bovine serum albumin as a standard. The endotoxin content in rDer p 21 preparations, determined using LAL Chromogenic Endotoxin Quantitation Kit (Pierce), was lower than 0.05 U/ml.

UHPLC–UV–ESI–MS

Accurate mass measurement was performed on a Maxis 4G mass spectrometer (Bruker Daltonics) coupled with a RSLC Ultimate 3000 (Dionex). Purified rDer p 21 was desalted and concentrated for 4 min onto a Acquity BEH300 C₄ column (Waters) equilibrated at 70 °C with 98% solvent A (0.1% trifluoroacetic acid); 2% solvent B (100% acetonitrile/0.1% trifluoroacetic acid) at a flow rate of 0.4 ml/min. Protein was eluted directly into the mass spectrometer at a flow rate of 0.4 ml/min with a linear gradient from 2% to 60% solvent B over 20 min. Mass spectra were acquired in ESI mode using the following instrument settings: capillary voltage 4500 V; End plate offset voltage: –500 V; Nebulizer: 4.0 L/min; Dry heater: 180 °C; Scan rate: 1 s; Quadrupole collision energy: 8 eV. Mass spectra were acquired in positive mode at *m/z* 300–3000. Mass accuracy was achieved by infusing the ESI-L low concentration tuning mix solution (at 100 μ L/h) for 4 min at the beginning of the

acquisition. Mass spectra deconvolution was performed using MaxEnt algorithm, and the precise average mass of rDer p 21 was determined using Data Analysis software (Bruker Daltonic, version 4.0).

Circular dichroism

A J-815 CD spectrophotometer (Jasco France) was used to assess the secondary structure of purified rDer p 21. Briefly, the sample was analyzed in a 1 mm cell, at a concentration of 190 µg/ml (based on microBCA assay), at 20 °C, with a wavelength range of 250–200 nm, a 2 nm band width and a scanning speed of 100 nm/min. Eight independent spectral scans were acquired and averaged using Spectra manager v2 software (Jasco).

Serum IgE levels to recombinant HDM allergens

ELISA microplates were coated overnight at 4 °C with rProDer p 1, rDer p 5 or rDer p 21 at 500 ng/well. Plates were washed with PBS-Tween 20 0.05% (PBS-T) and subsequently blocked with PBS-T containing 1% BSA (PBS-T-BSA) for 1 h. Serum samples were then incubated at 1/8 dilution in PBS-T-BSA for 1 h at 37 °C. Plates were washed with PBS-T and incubated with biotinylated secondary anti-human IgE (1/1000 dilution, KPL) for 1 h at 37 °C, followed by streptavidin–peroxidase (1/1000 dilution, BD Biosciences) for 30 min at 37 °C. The allergen-antibody complex was detected by addition of TMB substrate (BD Biosciences). The color development was stopped by addition of sulfuric acid and optical density (OD) was measured at 450 nm using iMark microplate reader (Bio-Rad). A serum was considered as positive when the OD value was higher than a cut off value, established as the mean OD values of negative control serum plus 3 standard deviations.

To evaluate cross-reactivities between Der p 21 and Der p 5, ten rDer p 21 and rDer p 5 double-positive sera were used for inhibition experiments. Sera (1/10 dilution) were preincubated overnight with rDer p 5 or rDer p 21 (10 µg/ml) at 4 °C. The IgE reactivities to rDer p 21 and rDer p 5 were measured as described above, coated rDer p 21 and rDer p 5 being incubated with rDer p 5- and rDer p 21-adsorbed sera respectively.

Cell activation assays

The human bronchial epithelial BEAS-2B cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in 24-well plates. When the culture reached 80% confluency, cells were then incubated in serum-free DMEM for further 24 h. One hour before stimulation, the growth medium was replaced by fresh serum-free DMEM. Cells were stimulated with different concentrations of rDer p 21 (0.5–20 µg/ml) for 16 h in serum-free DMEM. As controls, cells were also incubated with medium alone or with the Pam3CSK4 TLR2 ligand (200 ng/ml, Invivogen). When appropriate, BEAS-2B cells were treated for 1 h at 37 °C with anti-hTLR2 Mab (TLR2.5, eBiosciences, 10 µg/ml) or isotype control prior to the addition of rDer p 21 or Pam3CSK4. Culture supernatants were collected, centrifuged for 5 min at 10,000g, and assayed for IL-8 with enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences) according to the manufacturer's protocol. IL-8 concentrations were determined by interpolation from a standard curve performed with purified human IL-8.

Statistical analysis

Student's *t*-test was used to compare data between different treatments. Differences were considered significant for *P*-values < 0.05.

Result

Cloning of Der p 21

Using two specific primers derived from the sequence DQ354124 (GenBank accession number, [7]) and a *D. pteronyssinus* cDNA library, we successfully cloned the cDNA encoding the mature form of Der p 21 (aa 20–140 from full length Der p 21 sequence). The DNA sequence analysis evidenced three nucleotide substitutions in the cloned cDNA compared with the DQ354124, two of them were silent whereas the third one induced the point mutation M19T (mature Der p 21 numbering). It must be pointed out that similar results were obtained using a polymerase with proofreading activity (Pfu) for the PCR amplification. To express rDer p 21 in the yeast *P. pastoris*, the mature Der p 21 cDNA was cloned into the pPICZ α A expression vector and directly downstream to the α -mating factor leader sequence for the secretion of the expressed allergen. This cloning strategy could lead to the production of authentic Der p 21 without any addition of extra amino acids at the N-terminus and any tag at the C-terminus.

Expression of rDer p 21 in *P. pastoris*

The resulting plasmid was used to transform two *P. pastoris* strains: the wild type X33 strain with an active AOX1 gene giving fast growth on methanol (Mut⁺ phenotype) and the strain KM71H with a deletion in AOX1 gene resulting in slow growth on methanol (Mut^s phenotype). After selection on agar plates containing zeocin, the presence of Der p 21 cDNA in recombinant *P. pastoris* was confirmed by colony PCR. Expression of rDer p 21 was induced by addition of methanol in the culture of recombinant *P. pastoris* in shake flasks at 30 °C. Both KM71 and X-33 recombinant strains, cultured and induced at the same cell density, were able to express and secrete rDer p 21 which migrated onto SDS-PAGE as a 14kD protein (Fig. 1A). Such molecular weight estimation was close to the expected mass for mature rDer p 21 (AA 1–120 with M19T mutation: 14564 Da, UniProt accession number Q2L7C5). No statistically significant difference in the rDer p 21 expression level was observed after 24, 48 or 72 h of methanol induction and between the two tested *P. pastoris* strains. The recombinant KM71 strain was used for the subsequent production and purification of the allergen.

Purification and MS analysis of rDer p 21

Recombinant Der p 21 was purified from the medium of a 48 h-induced shake flask culture of the KM71 strain and using a combination of cation exchange and gel filtration chromatographies. The purification yield was estimated to 10 mg purified rDer p 21 per liter of culture medium (Table 1). Onto SDS-PAGE, purified rDer p 21 migrated as a monomeric 14 kD band (Fig. 1B). However, the UHPLC–UV–ESI–MS analysis revealed the absence of intact rDer p 21 with a corresponding mass of 14563.57 Da but the presence of two main forms at 14304.50 and 14205.40 Da (Fig. 2). These two forms could represent truncated rDer p 21 with N-terminal cleavages at residue V3. Several methionine oxidations (1 to 4, +16 Da) were detected, which is consistent with the presence of five methionine residues within the amino acid sequence.

CD analysis of rDer p 21

The far UV CD spectrum of rDer p 21 showed a maximum around 200 nm and double minimum at 208 and 222 nm (Fig. 3), indicating that rDer p 21 is homogeneously folded, essentially through α helix secondary structure. Based either on the Yang or Reed algorithm [10,11], the α helix content in rDer p 21 was

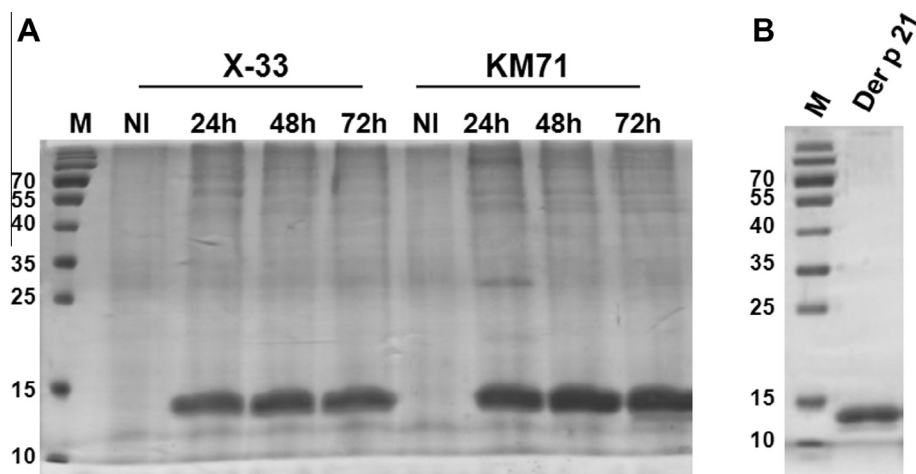


Fig. 1. Der p 21 expression and purification from transformed *P. pastoris* strains. Panel A. X33 and KM71 *P. pastoris* strains were transformed with pPICZ α A vector encoding Der p 21. Recombinant strains were cultured and the Der p 21 expression was induced by methanol for 24, 48 and 72 h. NI: non induced. The secretion of expressed rDer p 21 was monitored by SDS–PAGE and coomassie blue staining. Panel B: SDS–PAGE analysis of purified rDer p 21.

Table 1
Purification of rDer p 21.

Purification step	Total protein (mg)	rDer p 21 (mg)	Yield (%)	Purification factor
Culture medium (100 ml)	90	9.5	100	1
S Sepharose (20 ml)	5	3.4	36	6.6
Ultra 10 kD (5 ml)	3.7	2.4	25	6.1
Sephadex 75 (3 ml)	1.1	1	10	8.7

* The Der p 21 concentration was estimated by densitometric analysis of SDS–PAGE using a Der p 21 protein standard and through the use of the Quantity One 1-D Analysis software (Bio-Rad).

estimated to 72% whereas less than 28% of the structure corresponded to β -sheets. Such secondary structure quantification was consistent with predictions based on the Der p 21 primary structure and using the GOR4 algorithm [12] or the Chou and Fasman [13] method, that both predict that 80% of the residues will be structured as α helices.

IgE reactivity to rDer p 21

Using a panel of 96 sera from HDM-allergic patients, the IgE reactivity to rDer p 21 was determined by direct ELISA and com-

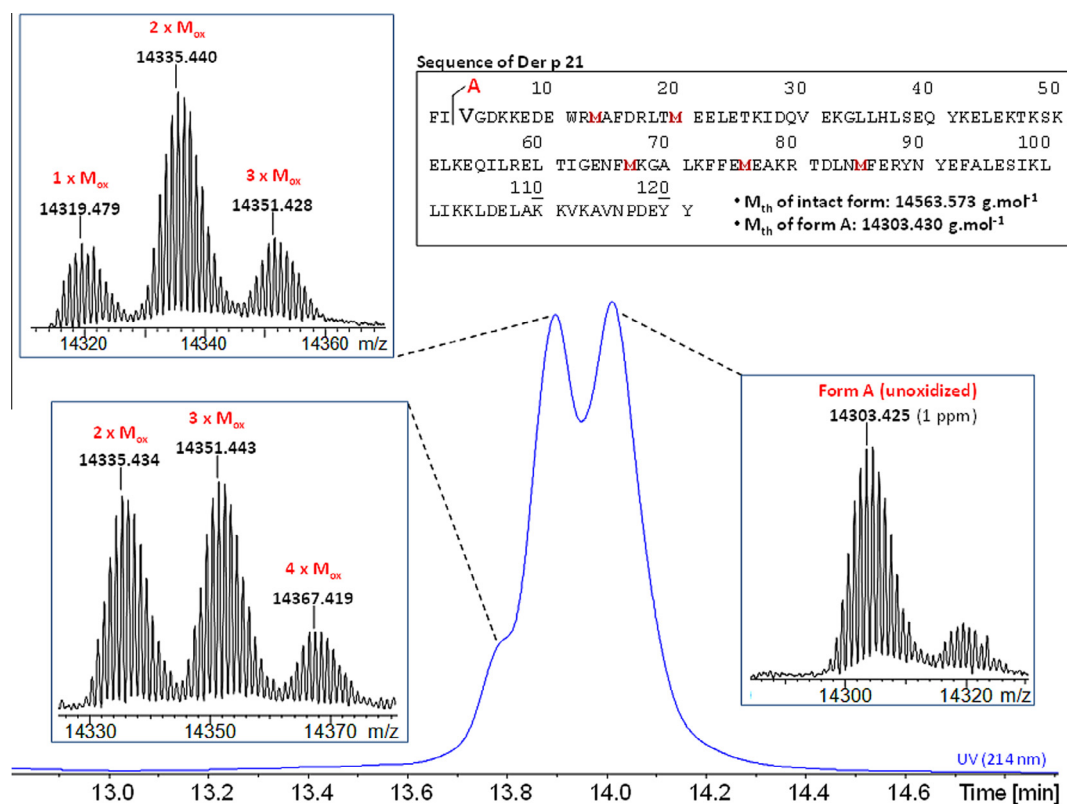


Fig. 2. rDer p 21 UHPLC–UV chromatogram and corresponding mass spectra. Purified allergen was eluted from an Acquity BEH300 C₄ column coupled with a Maxis 4G ESI–Q–ToF MS for accurate mass measurement. Right panel shows the mass spectrum of non-oxidized rDer p 21 (form A) eluted after 14 min with a mass accuracy of 1 ppm. Left panels show the separated oxidized forms. N-terminus truncation and Met oxidation are illustrated within the sequence panel.

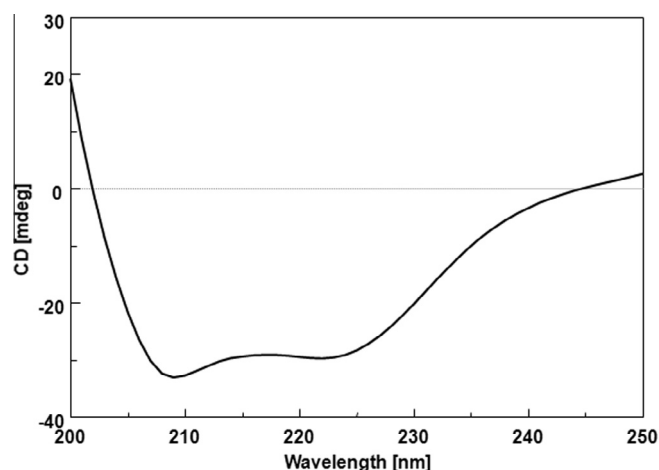


Fig. 3. UV-Circular dichroism spectrum of purified rDer p 21. Purified rDer p 21 was analyzed in a 1 mm cell, at a concentration of 190 $\mu\text{g/ml}$, at 20 $^{\circ}\text{C}$ using a J-815 CD spectrophotometer.

pared with the IgE binding capacity of rProDer p 1 and rDer p 5. Twenty-four patients displayed specific IgE to rDer p 21 (25%) whereas IgE reactivity to rProDer p 1 and rDer p 5 was detected in 69 (72%) and 66 (69%) in mite-allergic individuals. It must be pointed out that more than 85% of the patients developing anti-rDer p 21 IgE displayed reactivity to rDer p 5 (21 sera) and rProDer p 1 (22 sera). Inhibition assays using sera displaying both IgE to rDer p 5 and rDer p 21 clearly confirmed the absence of cross-reactivities between these two allergens. Indeed, whereas serum preincubation with rDer p 5 or rDer p 21 fully inhibited the IgE binding to the corresponding allergen, no inhibition of IgE reactivity to rDer p 21 was induced by rDer p 5 and vice versa (data not shown).

Biological activity of rDer p 21

To confirm that rDer p 21 produced in *P. pastoris* can also be appropriate to characterize its allergenicity, preliminary experiments were conducted using human bronchial epithelial cells as airway epithelium is the first cellular point of contact for airborne allergens. rDer p 21 was shown to trigger IL-8 secretion from BEAS-2B cells in a concentration-dependent manner when compared with control medium (Fig. 4A, $P < 0.05$). As Der p 21 displays homologies with fatty acid/lipid binding proteins, this allergen could trigger cell activation through TLR2 signaling. To evaluate the TLR2 dependence of the rDer p 21-induced IL-8 production in BEAS-2B cells, we compared the IL-8 secretion in cells preincubated

with a blocking antibody to TLR2 [14]. As expected, this pretreatment abolished the IL-8 production trigger by the TLR2 ligand Pam3CSK4 (Fig. 4B). Our results showed also clearly that the cytokine production by BEAS-2B stimulated with rDer p 21 was drastically reduced by the anti-TLR2 blocking antibody (Fig. 4A). Consequently, our preliminary data suggest that rDer p 21 could activate TLR2 signaling.

Discussion

Although numerous recombinant allergens were produced in *E. coli*, the *P. pastoris* expression system could display several advantages over the production in bacteria. First, the downstream processing using *P. pastoris* is facilitated by the localization of the expressed allergen in the supernatant (secretion), contrary to the intracellular production of allergen in bacteria which needs mechanical or chemical cell disruption to initiate the purification. Second, this yeast expression system allows appropriate post-translational modifications of the allergens including disulfide formations, glycosylations and consequently facilitates the appropriate folding [15]. Third, the absence of contaminating endotoxins in yeast reduces drastically the risk of side effects which could occur along allergen-specific immunotherapeutic treatments or allergy diagnosis through skin-prick tests.

In this context, we proceeded to the production, the purification and the characterization of mature rDer p 21 from engineered *P. pastoris*. Using two different *Pichia* strains, we demonstrated that the rDer p 21 expression level was roughly independent on the duration of the methanol induction as comparable production was measured after 24, 48 or 72 h of induction. Consequently, the scaling-up of the rDer p 21 production could be easily shortened through the methanol induction of the allergen expression in 24 h.

The purification of rDer p 21 was successfully achieved by cation exchange and gel filtration chromatographies as estimated by SDS-PAGE analysis. However, the MS characterization of purified rDer p 21 evidenced the absence of authentic protein N-terminus by the truncation of the first two amino acids F1 and I2. N-terminal truncation is a common phenomenon in proteins expressed by *P. pastoris* and likely due to extra cleavages by the yeast proteases Kex2 and STE13 during the α mating factor leader sequence removal [16]. Another modification of rDer p 21 detected by MS was the oxidation of methionine residues. Variability in the amount of methionine oxidation was observed in different preparations as unoxidized rDer p 21 or protein with one to four oxidized methionine residues could be detected. The oxidation phenomenon, commonly observed in proteins produced in *P. pastoris*, could be due to the presence of hydrogen peroxide in the medium produced during the methanol metabolism [17]. Although

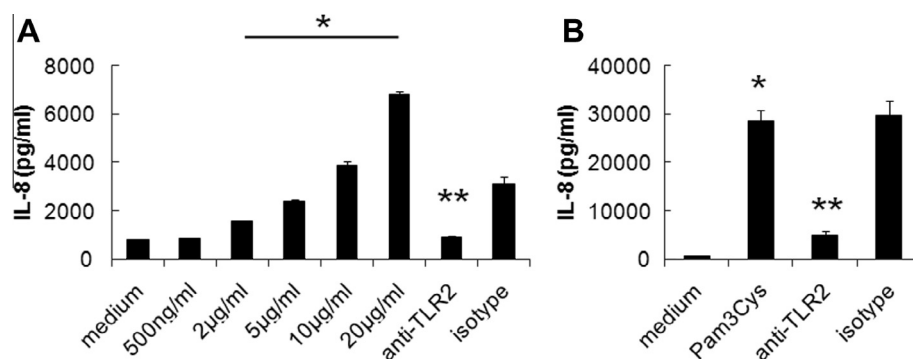


Fig. 4. rDer p 21-induced IL-8 secretion in BEAS-2B cells. Serum-starved BEAS-2B cells were incubated for 16 h with medium alone or with increasing concentrations of rDer p 21 (500 ng–20 $\mu\text{g/ml}$) (Panel A). As positive control, cells were also activated with Pam3CSK4 (200 ng/ml) (panel B). Cells were also preincubated with blocking anti-TLR2 antibody or isotype control before rDer p 21 (10 $\mu\text{g/ml}$) or Pam3CSK4 (200 ng/ml) treatment. IL-8-specific ELISA was used to quantify levels of IL-8 protein in culture supernatants. All data are expressed as the mean $\pm$ S.E. from at least two independent experiments performed in triplicate.

an extensive MS characterization of rDer p 21 produced in bacteria was not achieved, such protein alteration could not be present as rDer p 21 expressed in *E. coli* displayed a molecular mass in agreement with the theoretical mass from the deduced amino acid sequence [7]. However, it must be pointed out that rDer p 21 produced in bacteria does not carry authentic N-terminus too through the presence of initiation Methionine. We speculate that these two rDer p 21 modifications when produced in *P. pastoris*, N-terminal truncation as well as methionine oxidation, do not interfere with the protein stability and folding. First, CD analysis confirmed that rDer p 21 displayed appropriate exclusive and homogenous α -helix content which was comparable with that measured with rDer p 21 produced in *E. coli* and consistent with the secondary structure predictions [7,12,13]. Second, rDer p 21 produced in *P. pastoris* or *E. coli* exhibited comparable IgE binding capacities (25%) using a panel of sera from HDM allergic patients in Thailand. This frequency is in agreement with the measured IgE reactivity to this allergen performed in Austria (26%) [7] and Singapore (20%) [18] and using rDer p 21 prepared from *E. coli* [7]. These results not only confirmed that the rDer p 21 preparation from *P. pastoris* is similar to that produced in bacteria at the level of IgE binding epitope content but that Der p 21 is an important HDM allergen which could be incorporated into the HDM allergy diagnosis and treatment. Our data from IgE inhibition assays confirmed also that rDer p 21 produced in yeast, similarly to the form produced in bacteria does not share common IgE binding epitopes with Der p 5, although the amino acid sequence of Der p 21 and Der p 5 exhibit 30% identity [8]. Through small angle X-ray scattering experiments, rDer p 21 produced in *E. coli* displayed a dimeric structure, similarly to Der p 5 [7]. Under native conditions, gel filtration chromatography of rDer p 21 produced in *P. pastoris* clearly indicated that the recombinant allergen was monomeric. Our results are in agreement with those recently obtained from the comparison between Der p 5 and Der p 21 through modeling and bioinformatics analysis [8]. Notably, according to the Der p 5 X-ray structure, the glycine at position 30 is critical for the formation of a kink in the N-terminal helix leading to an entangled dimeric structure whereas a valine zipper formed by residues 70 and 73 contributes to the dimer stability [19]. The G30V, V70A and V73F substitutions in the Der p 21 sequence could impair the dimeric formation. Nevertheless, our data clearly demonstrated that rDer p 21 was able to activate airway epithelial cells through TLR2 signaling suggesting that the Der p 21 allergenicity is mediated by interactions with lipid/fatty acid ligands triggering innate immunity. It must be pointed out that Der p 2, a LPS-binding protein [20], as well as Der p 5 were shown also to directly activate airway epithelial cells [21,22]. Moreover, the TLR2-dependence of airway smooth muscle activation by Der p 2 was also evidenced [23]. Although further experiments are needed to confirm the lipid binding activity of rDer p 21 as well as the innate immune activation by this HDM allergen, these preliminary results validated once again the quality of recombinant HDM allergen production in *P. pastoris*.

In conclusion, *P. pastoris* is at least as efficient as *E. coli* to produce monomeric and correctly folded rDer p 21. Through the convenient and effective downstream processing and the absence of endotoxins, the *P. pastoris* expression system could be a generic method for the production of recombinant HDM allergens.

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