

Production and Proteomic Characterization of Pharmaceutical-Grade *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* Extracts for Allergy Vaccines

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Key Words

Allergy vaccine • *Dermatophagoides farinae* •
Dermatophagoides pteronyssinus • House dust mite

Abstract

Background: House dust mites (HDM) such as *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* represent a major cause of type 1 allergies worldwide. Hence large quantities of well-characterized HDM extracts are needed to prepare pharmaceutical-grade allergy vaccines. To this aim, the present study was undertaken to define optimal conditions for large-scale cultures. **Methods:** *D. pteronyssinus* and *D. farinae* were grown on different media combining various proportions of wheat germ, yeast and synthetic amino acids (the latter resembling the composition of the human stratum corneum). Extracts thus obtained were analyzed for their total allergenic activity, as well as major allergen and protein contents, using immunosorbent assays, HPLC, immunoblotting, two-dimensional electrophoresis and peptide mass fingerprinting. **Results:** An optimal culture medium (Stalmit APF®) based on wheat germ, yeast and amino acids in defined proportion (42, 42 and

15% w/w, respectively) was selected to grow various HDM species with high yields. A detailed proteomic analysis revealed that *D. pteronyssinus* extracts generated under such conditions did not contain allergens originating from culture medium components and that major prevalent HDM allergens (i.e. groups 1, 2, 7, 10, 13 and 20) are found among the most abundant proteins in the *D. pteronyssinus* extract. Semiquantitative dot-blot assays confirmed the presence of Der p 3–10 as well as Der p 13 and 14 allergens within the extracts. **Conclusions:** We developed a well-defined medium allowing to grow various HDM species at an industrial scale in a highly reproducible manner. Extracts from mites produced under such pharmaceutical conditions contain all the relevant allergens for desensitization purposes and in vivo diagnosis.

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Introduction

House dust mites (HDM) represent a major cause of type 1 allergies throughout the world [1, 2]. Most particularly *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*

goides farinae, both belonging to the Pyroglyphidae family, are the main species involved in Europe and North America. They usually co-inhabit, with a peak of exposure in summer/early fall and a drop during winter [1]. *D. pteronyssinus* predominates in more humid areas, whereas *D. farinae* is rather found in drier habitats (e.g. central Europe, center of the USA) [3–6]. Today allergenic desensitization, also referred to as specific immunotherapy, is broadly recognized as the only etiological treatment for IgE-mediated allergic diseases [7, 8]. This approach has been used for almost a century [9, 10], and its efficacy has been documented in multiple double-blind placebo-controlled studies, including those for HDM allergies [8, 11]. Hence there is a crucial need for high-quality allergenic extracts to prepare therapeutic vaccines as well as in vivo diagnostic products. Importantly such extracts should comprise the various allergens to which most patients are naturally exposed. At least 17 allergens have been described for *Dermatophagoides* spp.: groups 15–18 specifically for *D. farinae*; group 20 specifically for *D. pteronyssinus*; groups 1–11, and 13 and 14 for both *D. pteronyssinus* and *D. farinae*. Some of them display a very high level of cross-reactivity between both species, such as group 2 and 10 allergens [6, 12] [see allergen nomenclature of the International Union of Immunological Societies (IUIS) available at <http://www.allergen.org/List.htm>]. Among *Dermatophagoides* spp. allergens group 1 and 2 allergens seem to be the most important ones, as (i) they are recognized by ~70–100% of HDM-sensitized patients [2, 13–23]; (ii) they bind substantial amounts of anti-mite IgE (i.e. 30–40% each on average) in patients' sera [15, 19–21, 24]. A recent study demonstrated that a mixture of group 1, 2, 5, 7, 8 and 10 allergens binds an average of 76% of *D. pteronyssinus*-specific IgE [25].

The expression of dust mite allergens has been shown to be compartmentalized. For example, while Der p 1 is preferentially found within mite fecal particles [26], Der p 2 is rather associated with mite bodies [17, 27, 28], most particularly in the gut, and is present in lower quantities within fecal pellets [29]. Since both mite bodies and feces can become airborne and contribute to patient sensitization [27], HDM allergenic extracts intended for allergy diagnosis and immunotherapy should ideally be made from material containing both bodies and feces, harvested from spent culture medium.

Mites naturally feed on human skin scales (i.e. dead keratinocytes sloughed off from the stratum corneum, that is, the outermost layer of skin) present in beds, carpets and upholstery [2, 27]. However, in order to totally preclude any potential contamination with adventitious

infectious agents, materials of human or animal origin cannot be used in order to produce mite extracts for in vivo utilization in humans. Thus safe culture conditions need to be defined to produce mites with high yields and batch to batch consistency in order to support industrial-scale manufacturing of HDM extracts. Also, the ideal culture medium must not introduce nonmite allergens while allowing the growth of mites expressing all major allergens involved in HDM sensitization.

Herein we describe the development of such a pharmaceutical culture medium for growing *D. pteronyssinus* and *D. farinae* as well as the characterization of biological extracts obtained under such conditions.

Materials and Methods

Culture Medium

The media tested in the present study were made by combining wheat germ (Sigma, St. Louis, Mo., USA), baker's yeast (Biospringer, Maisons-Alfort, France) and synthetic amino acids with the following percentages w/w in total mass of synthetic amino acids: *L*-aspartic acid (5.8), *L*-glutamic acid (12.9), *L*-arginine (4.7), *L*-cysteine hydrochloride monohydrate (1.9), glycine (3.0), *L*-histidine (3.5), *L*-isoleucine (5.6), *L*-leucine (7.4), *L*-lysine (5.6), *L*-methionine (2.7), *L*-phenylalanine (7.9), *L*-proline (11.5), *L*-serine (10.7), *L*-threonine (4.3), *L*-tryptophan (1.5), *L*-valine (6.2; Ajinomoto Co. Inc., Kawasaki, Japan), *L*-alanine (4.3), *L*-tyrosine (0.6), D-biotin/vitamin H or B₇ and cyanocobalamin/vitamin B₁₂ (Sigma). Parallel control cultures were performed using autoclaved human skin scales instead of synthetic amino acids. None of the free amino acids used were of human or animal origin, nor had they been in contact with human or animal material. To prevent any risk of an allergic reaction due to components from the medium itself, the wheat germ was autoclaved at 121°C for 20 min (and subsequently ground and passed through a 250-μm sieve), while the baker's yeast was heated at 122°C for 2–3 min and at 100°C for 15 min.

Production and Extraction of HDM

Mites (10% w/w) were seeded in culture medium in plastic flasks and incubated at 25°C and a humidity level of 75 ± 5% for *D. pteronyssinus* and 30°C and a humidity level of 65 ± 5% for *D. farinae*, which were found to be optimal conditions [2, 30]. The whole cultures (i.e. bodies and spent medium) were harvested at various time points, freeze-dried, and extracts were prepared at 5% (w/v) in 50 mM of ammonium bicarbonate for 24 h at +4°C under stirring conditions followed by centrifugation at ~6,000 g for 15 min.

Assessment of Protein and Mite or Nonmite Allergen Contents

The total allergenic activity was determined by RAST inhibition and expressed as an index of reactivity per milliliter (IR/ml) [31]. The protein contents were measured by the Bradford technique [32], using a protein assay kit (BioRad Laboratories, Hercules, Calif., USA). Der p 1 and 2, as well as Der f 1 and 2 allergen concentrations were determined using 2-site monoclonal-based ELISA kits (Indoor Biotechnologies, Charlottesville, Va., USA) according to

the manufacturer's instructions. The levels of group 3–10, 13 and 14 (Mag 1) [33] Der p allergens were quantified in two independent *D. pteronyssinus* culture batches by immunodot blotting using specific polyclonal antibodies from the National University of Singapore.

To assess the presence of residual nonmite allergens (i.e. from wheat germ or yeast), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of mite extracts was carried out with homogeneous 12.5% SDS ExcelGel (GE Healthcare, Uppsala, Sweden) for 80 min at 16°C at 50 mA and 600 V using a Multiphor II apparatus (GE Healthcare). The samples were diluted in 6% Tris buffer containing 1% SDS, pH 7.5, and incubated for 5 min at 95°C with 5% β -mercaptoethanol. The proteins were electroblotted onto 0.45- μ m nitrocellulose for 1 h using a Novablot system (GE Healthcare). The immunoblots were incubated with individual sera from patients monosensitized to either wheat (10 sera with specific IgE levels between 1.3 and 10.1 IU/ml) or yeast (4 sera with specific IgE levels comprised between 1.7 and 3.64 using the Pharmacia CAP system, Uppsala, Sweden), as appropriate. To detect residual wheat proteins, blots were incubated with anti-agglutinin polyclonal antibodies (Sigma), then with a HRP-conjugate and revealed with a chemiluminescent substrate (SuperSignal West Pico chemiluminescent substrate, Pierce, Rockford, Ill., USA). The quantification of residual yeast proteins in HDM extracts was performed by reversed-phase HPLC (Symmetry 300 C18 column, 4.6 $\times$ 250 mm, 5 μ m, Waters, Milford, Mass., USA; gradient elution: 0.06% trifluoroacetic acid (TFA) 30% acetonitrile, 0.06% TFA; flow rate: 0.8 ml/min for 50 min; detection wavelength: 210 nm).

Preparation of Dust Mite Extracts for Proteomic Analysis

D. pteronyssinus mites (100-mg batches) were ground with a mortar and pestle in liquid nitrogen and quartz sand. The powder was homogenized and incubated with 10% w/v trichloroacetic acid and 0.07% v/v β -mercaptoethanol in acetone for 1 h at –20°C. The precipitated proteins were centrifuged and the pellet was washed with acetone containing 0.07% v/v β -mercaptoethanol (precooled to –20°C) until all pigmentation was removed. The proteins were then freeze-dried and resuspended in urea sample buffer containing 9 mol/l of urea, 4% CHAPS, 1% DTT, 1% pH 3–10 ampholytes and 35 mM of Tris base. The insoluble mite debris was removed by centrifugation at 45,000 g for 15 min. The supernatants predominantly contained soluble and membrane-associated proteins. The protein concentration was determined by spectrophotometry (SmartSpec, BioRad) using the BioRad protein assay kit according to the manufacturer's instructions. All protein extracts were aliquoted and stored at –80°C.

Two-Dimensional Gel Electrophoresis

The protein extracts were mixed with a rehydration buffer (9 M of urea, 4% CHAPS, 100 mmol/l of DTT, 0.2% pH 3–10 ampholytes). For the 17-cm immobilized pH gradient (IPG) strip (BioRad), 500 μ g of protein was applied. IEF focusing was performed using the Protean IEF cell system (BioRad). To improve the entry of high-molecular-weight proteins into IPG strips, low voltage (50 V for 12 h at 20°C) was applied during rehydration. The IPG strips were then focused at 250 V for 15 min, followed by a linear ramp to 10,000 V and up to a total of 90,000 V thereafter. Following electrophoresis, the proteins in strips were treated with 2% DTT and 2.5% iodoacetamide in an equilibration base buffer (6 M of

urea, 2% SDS, 0.05 M of Tris-HCl, pH 8.8, 20% glycerol) for 10 min. Second dimension electrophoresis was performed on a 12% gel. Subsequently protein spots were silver-stained, and image analysis was performed with a BioRad GS-710 calibrated imaging densitometer, using the BioRad PDQuest software.

Protein Peptide Mass Fingerprinting-Mass Spectrometry Analysis

Protein spots from Coomassie stained gels were excised and transferred to microcentrifuge tubes. Gel fragments were washed with 50% v/v acetonitrile containing 25 mM of ammonium bicarbonate to remove any dye bound to proteins. The samples were dried under speed vacuum and incubated for 16 h at 37°C with 10 μ l of 12.5 μ g/ml modified porcine, sequencing grade trypsin (Promega, Madison, Wisc., USA) in 25 mM of ammonium bicarbonate (pH 8.5). The resulting tryptic fragments were eluted by diffusion into 50% v/v acetonitrile and 0.5% v/v trifluoroacetic acid. Ultrasonication was used to facilitate the diffusion of the peptides. For MALDI-TOF mass spectrometry each sample (0.5 μ l) was mixed with α -cyano-4-hydroxycinnamic acid (0.5 μ l at 1% concentration) as a matrix on the plate and analyzed with a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Framingham, Mass., USA). Spectra were obtained in a reflectron-delayed extraction mode over a mass range of 800–3,500 Da. Spectra from 100 shots at several positions were combined to generate an average peptide mass fingerprint for each protein sample. The peptide masses were externally calibrated using peptide standards (1296.6853 for angiotensin I and 2465.1989 for ACTH). Data analysis and database searching were performed with Auto MS-FIT from PE Biosystems, Proteomics Solution 1 software version 1.0.0 with data explorer version 3.5.0.0, combined with the Protein Prospector package (University of California, San Francisco, Calif., USA).

Quadrupole Time-of-Flight Mass Spectrometry

The above trypsin-digested peptide mixtures were desalted, concentrated using C18 ZipTip (Millipore, Bedford, Mass., USA) and dried. Following dissolution in 2 μ l of 50% acetonitrile and 0.5% formic acid, the samples were loaded into a metallized glass capillary. The capillary was then mounted on the nanoflow Z-spray source of a quadrupole time-of-flight 2 (Q-TOF2) mass spectrometer (Micromass, Manchester, UK). The flow rates usually varied from 8 to 16 nl/min. Instrument operation, data acquisition and analysis were performed using MassLynx/BioLynx 3.5 software (Micromass). The capillary voltage and collision energy were optimized for each sample. The microchannel plate voltage was set to 2,200 V. Data searches against the in-house dust mite expressed sequence tag (EST) databases (produced at the National University of Singapore) [34] were performed using ProteinLynx Global SERVER 2.0 (Micromass).

Data Analysis

For protein identification using peptide mass fingerprints we used the MS-Fit program (<http://prospector.ucsf.edu/ucsfhtm13.2/msfit.htm>) and searched in NCBI nr and the National University of Singapore local house dust mite EST databases. To confirm protein identities we also searched the Swissprot and TrEMBL databases with the Mascot software (<http://www.matrixscience.com/cgi/index.pl?page=../home.html>). The PE Biosystems Proteomics Solution 1 software version 1.0.0 with Data Explorer version 3.5.0.0

Fig. 1. Total allergenic activity and protein, and Der p 1 and 2 contents in 5% (w/v) extracts of *D. pteronyssinus* cultured for 2.5 months on a medium containing 15% (w/w) of a lyophilized synthetic aa formula as compared to human squames. Results obtained from an analysis of 3 independent batches are expressed as mean + standard deviation (SD).

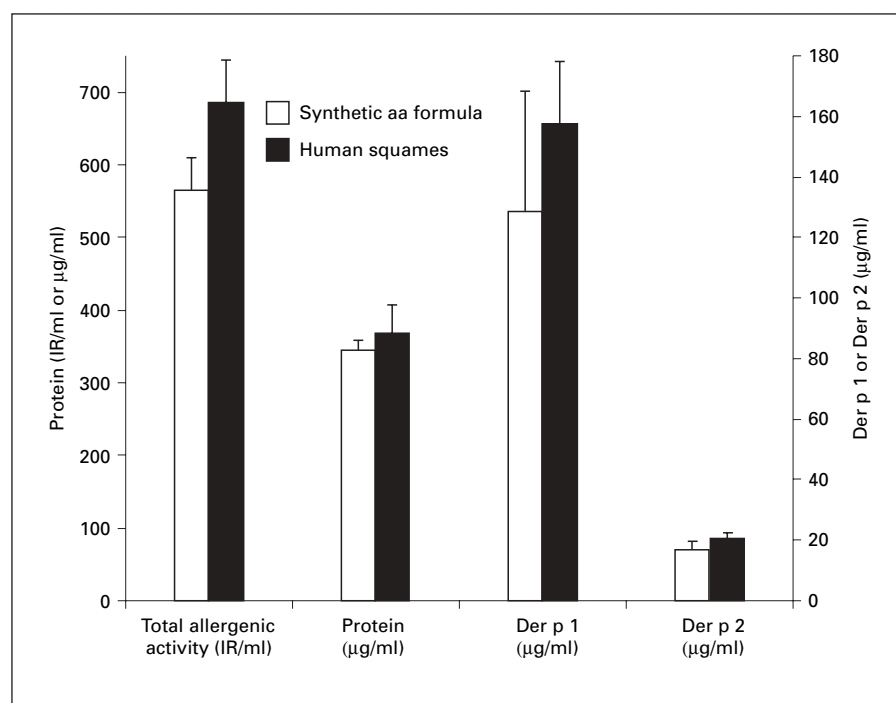


Table 1. Total allergenic activity and Der p 1, Der p 2 and protein contents of 5% (w/v) extracts of *D. pteronyssinus* cultured for 2.5 months on different media

	Reference medium	Wheat germ-free medium	Yeast-free medium
<i>Medium composition</i>			
Wheat germ, %	42	0	84
Yeast, %	42	84	0
Synthetic amino acid formula, %	15	15	15
Biotin + cyanocobalamin, %	1	1	1
<i>Characteristics of extracts obtained</i>			
Total allergenic activity, IR/ml	420	380	230
Der p 1, µg/ml	96	53	16
Der p 2, µg/ml	17	25	12
Proteins, µg/ml	290	220	150

combined with Protein Prospector software was developed to automate the use of the MS-Fit protein identification tools. The data were then collected and placed into a queue. The Proteomics Solution 1 software was subsequently used to process the data in the queue and ascribe identities to the peptides analyzed. The identification of the proteins of interest from the two-dimensional gel profile was facilitated by the incorporation of virtual digested peptides generated from the house dust mite EST database. To enable such an approach raw sequence data were processed with the Phred soft-

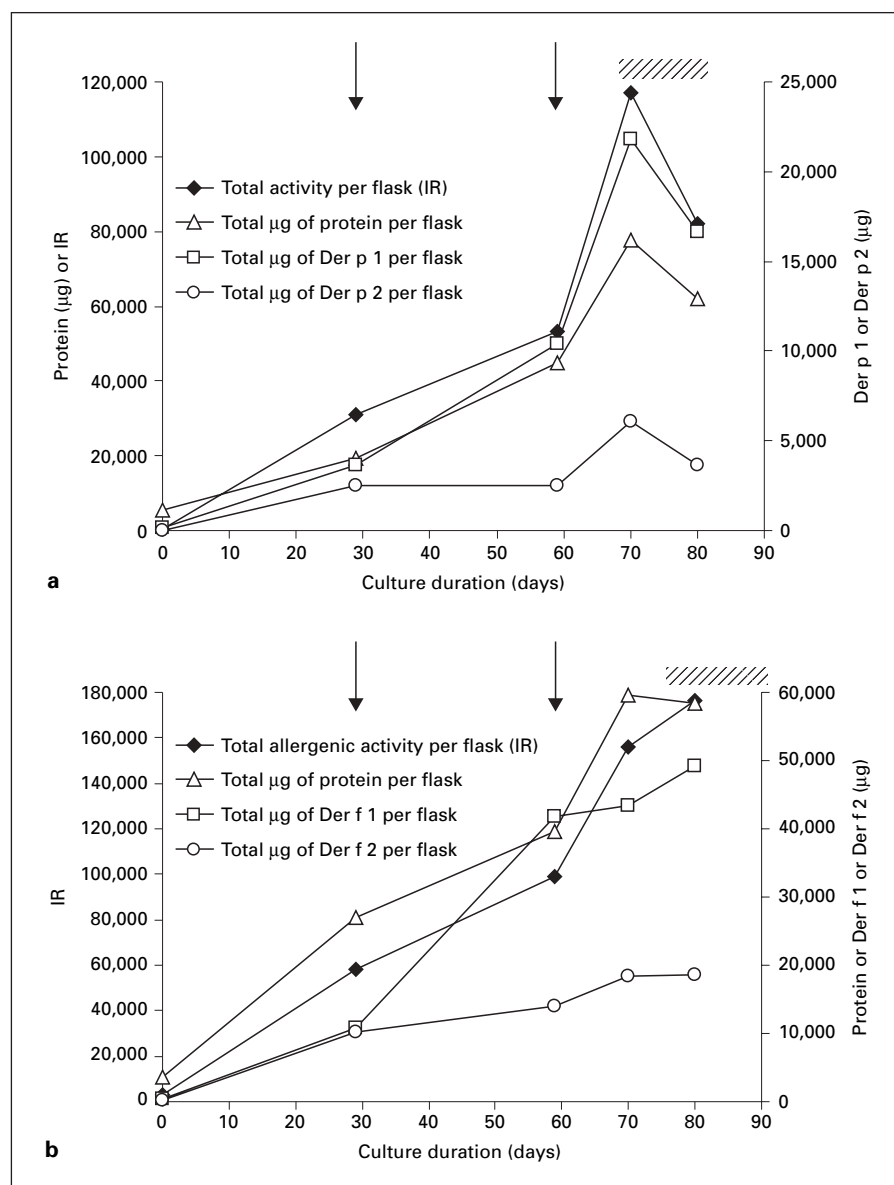
ware (Codon Code, Dedham, Mass., USA) for basecalling, followed by Crossmatch Phrap to remove vector sequences, and then converted to dbEST format for uploading to the ProteinProspector FA index program. Monoisotopic peptide masses were submitted into the MS-Fit program. We used the following parameters for the database search: (i) the peptide mass tolerance was chosen as 100 ppm because external calibration was used; (ii) a minimum of 4 peptides matched (for protein databases) or 3 peptides matched (for searching in the dbEST databases); (iii) 1–4 missed cleavage sites, and (iv) 4 modifications were considered, i.e. conversion of peptide N-terminal glutamine to pyroglutamate, oxidation of methionine, acetylation of protein N-terminus and carboxymethylation or acrylamide modification of cysteine. A first search was performed without selection of any particular species (selection 'all') and molecular weight range (1,000 to ~100,000 Da). When isoforms were identified for some proteins, FindMod and Peptide-Mass tools of ExPASy were used to predict possible protein modifications. Protein similarity was then examined using the BLAST program.

Results

Development of a Pharmaceutical-Grade Culture Medium for HDM

Experiments were performed to determine whether HDM could be grown on a medium containing synthetic well-traced amino acids. To define the optimal amino acid combination to use, we analyzed human skin scales by

Fig. 2. Total allergenic activity and protein and major allergen contents per flask of *D. pteronyssinus* (a) and *D. farinae* (b) cultured for different times with the 'standard medium'. The hatched area represents harvest period. Arrows indicate addition of medium (each flask starts with 6 g of medium.)



HPLC. The amino acid composition of human scales was found to compare with data available from the literature, as it relates to the human stratum corneum [35, 36] (data not shown). On the basis of this analysis, for the subsequent cultures we selected a mix of amino acid referred to as 'synthetic aa mix' (see 'Materials and Methods'), commonly used in humans for parenteral nutrition. Figure 1 shows that using *D. pteronyssinus* as a model, the synthetic aa mix (15% w/w) provided yields comparable to those obtained with human scales, both in terms of total allergenic activity (assessed by RAST inhibition), protein contents and major allergen (i.e. Der p 1 and 2) levels.

Although not shown, we found that 15% (w/w) of the synthetic aa mix is an appropriate amount to support both *D. pteronyssinus* and *D. farinae* growth. Our data also indicated that in addition to amino acids, wheat germ and yeast are necessary to reach optimal yields, both in terms of total allergenic activity and major allergen levels (table 1). Collectively our results established that a culture medium associating amino acids (15% w/w), wheat germ (42% w/w) and yeast (42% w/w) allows to grow *D. pteronyssinus* efficiently and reproducibly, but also *D. farinae* [37] (see below).

Table 2. Variability of the *D. pteronyssinus* and *D. farinae* cultures obtained with the standard medium after 75 ± 5 and 84 ± 7 days, respectively

	Total allergenic activity	Major allergen content	
		group 1	group 2
<i>D. pteronyssinus</i>			
Mean $\pm$ SD	630 ± 151^a	91 ± 31^b	22 ± 7^b
Industrial batches, n ^c	167	238	238
<i>D. farinae</i>			
Mean $\pm$ SD	804 ± 297^a	495 ± 248^b	267 ± 43^b
Industrial batches, n ^c	103	153	20

Mean $\pm$ SD and number of batches studied for the total allergenic activity and the group 1 and 2 major allergen contents in 5% (w/v) extracts.

^a Values represent IR/ml.

^b Values represent μ g/ml.

^c Industrial batches are defined as large-scale lots intended for diagnostic or therapeutic applications in humans.

Optimization of Mite Culture Conditions

Kinetic studies were performed on cultures of *D. pteronyssinus* and *D. farinae*. At various time points (between 0 and 3 months) concentrations in protein or major allergens (i.e. Der p 1 and 2 or Der f 1 and 2, as appropriate) as well as total allergenic activities were measured. As shown in figure 2a the optimal time for harvesting *D. pteronyssinus* cultures was between 70 and 80 days, on the basis of all the tested parameters. Extending the culture duration over 85 days led to a decrease in allergenic activity and Der p 2 contents (results not shown). With respect to *D. farinae* the optimal culture duration was found to be around 80 days or more (fig. 2b). Extending the cultures to ~80–90 days led to significantly more protein and major allergen contents, although the increase was not significant for the total allergenic activity (results not shown). Altogether the culture duration retained was 75 ± 5 days for *D. pteronyssinus* and 84 ± 7 days for *D. farinae* (fig. 2a, b, respectively). In order to document batch to batch consistency, when using the conditions defined above, the outcome of several *D. pteronyssinus* or *D. farinae* cultures was evaluated both in terms of total allergenic activity and major allergen content. As shown in table 2, based on information available from numerous independent batches, the total allergenic activity, as well as group 1–2 concentrations were found to be highly reproducible both for *D. pteronyssinus* and *D. farinae*.

Absence of Contaminating Wheat Germ and Yeast Allergens

Within mite cultures we subsequently assessed the presence of potential contaminating allergenic and nonallergenic proteins which could originate from culture medium components (i.e. wheat germ or yeast). Using individual sera from patients monosensitized to either wheat germ or yeast, respectively, we analyzed the potential residual IgE reactivity within both *D. pteronyssinus* or *D. farinae* extracts. As shown in figure 3a and b, no residual allergenicity due to wheat germ or yeast, respectively, could be detected in either *D. pteronyssinus* or *D. farinae* extracts. This result can in part be explained by the denaturation of potential wheat germ and yeast allergens during the heat treatment applied to those medium constituents. We observed that the reactivity of anti-wheat germ IgE to solid-phase coated wheat germ extracts was reduced by more than 90% when the wheat germ was autoclaved prior to extraction (data not shown). Similarly heat treatment totally denatured the major yeast allergen enolase, as confirmed by immunoblotting and detection with an anti-yeast IgE-containing serum (data not shown).

Having excluded the presence of wheat- or yeast-derived allergens, we nevertheless proceeded to quantify the presence of potential nonallergenic residual proteins from those sources within HDM extracts. We first assessed the reactivity of rabbit polyclonal antibodies specific for wheat agglutinin, an abundant wheat germ protein used as a marker, within HDM extracts. As shown in figure 3c, whereas agglutinin is detectable in as little as 0.4 ng of autoclaved wheat germ proteins, it could not be detected in 1 μ g (1,000 ng) of *D. pteronyssinus* or *D. farinae* extracts (fig. 3c). On this basis the dilution factor of residual wheat germ proteins in mite extracts is at least 2,500 (~1,000 ng/0.4 ng).

In the absence of available antibodies specific for yeast marker proteins we set up an HPLC-based assay. Using this approach 3 peaks corresponding to major yeast marker proteins were identified in the culture medium (arrows in fig. 4a). Importantly none of these peaks was detected in *D. pteronyssinus* and *D. farinae* extracts prepared from 3-month cultures (fig. 4b, c). We cannot exclude the presence of yeast proteins at levels below the detection threshold of the HPLC method.

Proteomic Analysis of *D. pteronyssinus* Extracts

Having defined optimal culture conditions for HDM, we subsequently performed an extensive proteomic analysis of mites thus obtained, with a focus on *D. pteronyssinus*. Extracts from *D. pteronyssinus* harvested after 70-

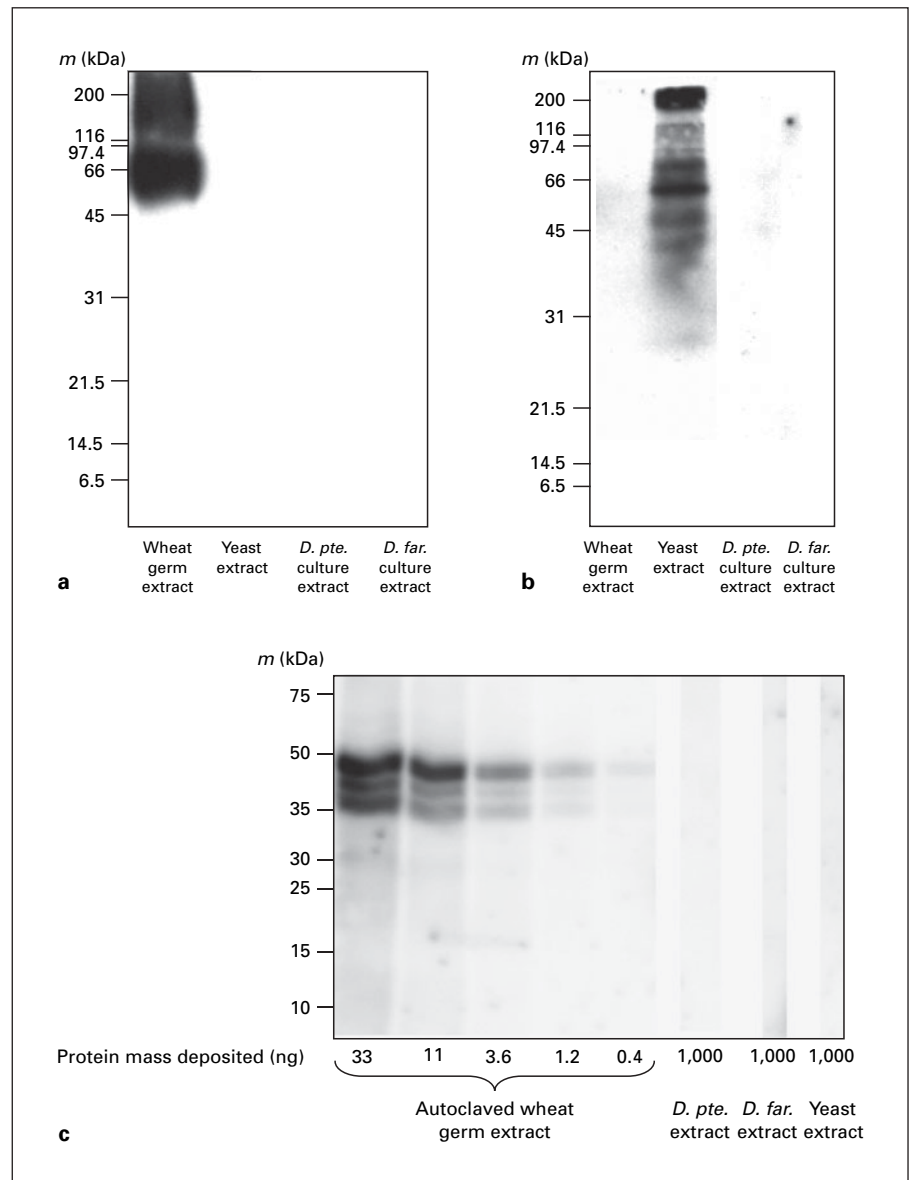


Fig. 3. SDS-PAGE followed by immunoblotting and detection with an individual antiwheat germ (**a**) or antiyeast (**b**) IgE-containing serum, or antiwheat germ polyclonal antibodies (**c**). Results from **a** and **b** are representative of experiments conducted with 10 and 4 sera from patients monosensitized to wheat germ or yeast, respectively. *m* = Molecular weight.

day cultures were analyzed by two-dimensional gel electrophoresis and peptide mass spectrometric analysis as described in 'Materials and Methods'. As shown in figure 5 a total of more than 700 clear spots were detected after silver staining and image analysis (using the BioRad PD-Quest software). Replicate gels gave a spot reproducibility range between 84 and 89% (*n* = 7 replicates each). Using a Coomassie brilliant blue (R-250) stain, approximately 250 clear and reproducible spots could be detected (not shown).

The top 50 most abundant Coomassie-stained spots from *D. pteronyssinus* grown with our standard medium

and culture conditions were excised and subjected to trypsin digestion. Using a MALDI-TOF mass spectrometer, high-quality mass spectra were obtained for these spots. When peptide mass fingerprints were compared with computed mass values from GenBank database entries, actin, tubulin, ferritin, group 1 (cysteine protease), group 2 and group 10 (tropomyosin) mite allergens could be identified. The identification process was subsequently facilitated by the use of an in-house database comprising cloned full-length allergens from *Dermatophagoides* and other species available at the National University of Singapore. This enabled the identification of 10 additional

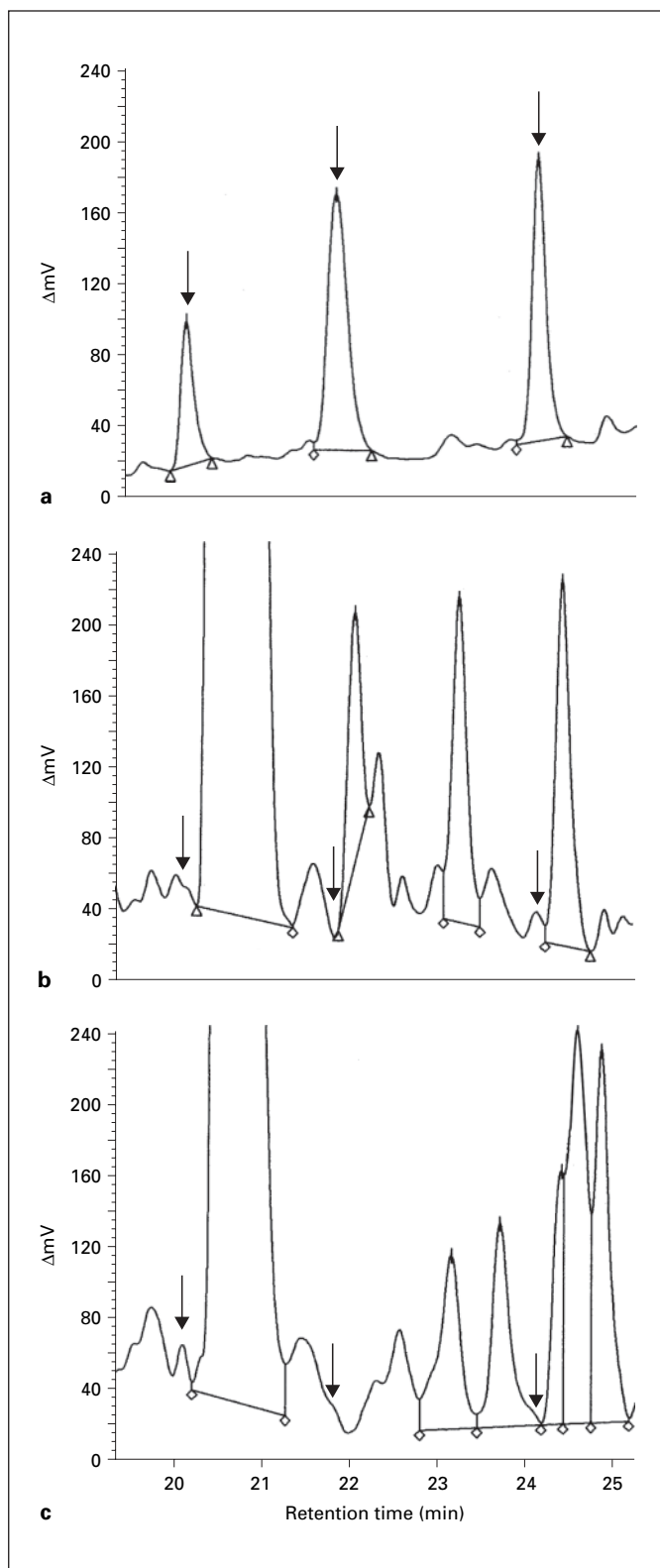


Fig. 4. HPLC profile of yeast extract (a), *D. pteronyssinus* (b) and *D. farinae* (c) culture extracts. Arrows indicate the yeast marker peaks (a) and the corresponding retention times in *D. pteronyssinus* (b)

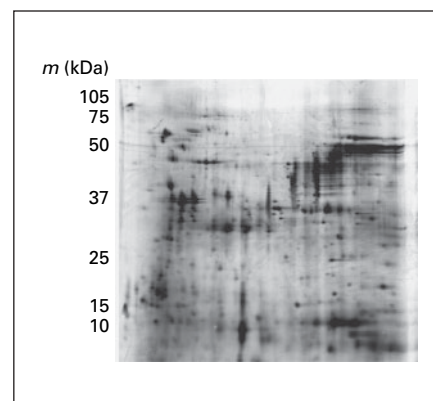


Fig. 5. Two-dimensional gel electrophoresis of a *D. pteronyssinus* extract obtained from a 70-day culture on the standard medium. m = Molecular weight.

spots. Lastly, with the use of a house dust mite EST database at the National University of Singapore, all the 50 excised protein spots were identified based on the EST contigs available. Among the proteins identified, more than 80% matched entries in the *Dermatophagoides* EST database [34], while the remaining peak maps were identified in comparison with databases from other dust mites. Table 3 recapitulates the most abundant proteins corresponding to the largest 50 spots found in *D. pteronyssinus* extracts.

Of specific interest, several important major allergens are represented in this list (marked with an asterisk in table 3). These include the group 13 allergen, the Der p 20/arginine kinase and Der p 10/tropomyosin homologs. The group 1 and 2 allergens were also represented among these abundant proteins, while several variant forms of allergenic components (such as group 7, 10 and 13 allergens) were also present. Noteworthy many of the spots analyzed corresponded to either polymorphic, paralogous or posttranslational forms of similar proteins.

To confirm whether other known groups of allergens, particularly group 3–10 and group 14 (Mag 1) [33] allergens, were present in the extracts, two batches of *D. pteronyssinus* extracts were analyzed by semiquantitative immunodot blotting. Figure 6 shows that all these allergens

and *D. farinae* extracts (c). Spiking experiments indicated that yeast marker peak retention times did not change in *D. pteronyssinus* and *D. farinae* extracts.

were also present at varying quantities in both extracts, with a good consistency observed between independent extracts.

Discussion

Large quantities of the common HDM species *D. pteronyssinus* or *D. farinae* are needed to prepare both in vivo diagnostic and immunotherapeutic products. Culture conditions should be defined to fulfill regulatory requirements in terms of product quality and characterization, while being applicable at an industrial scale, most particularly as it refers to yield and batch to batch consistency issues. Whereas HDM naturally feed on human skin scales, in the current regulatory framework pharmaceutical-grade culture media for the production of allergenic extracts should not contain any human or animal material.

In the present study we have identified a combination of synthetic amino acids resembling the composition of the human stratum corneum, which is equivalent to human skin scales for HDM cultures, with no appreciable impact on total allergenic activity and major allergen content. A combination of such a synthetic amino acid mix with yeast and wheat germ proteins in defined proportions (15, 42 and 42% w/w, respectively) efficiently supports the growth of both *D. pteronyssinus* and *D. farinae* mite species within 70–90 days. This result is in agreement with another study [38] demonstrating that yeast significantly enhances the growth of *D. pteronyssinus*, while in addition wheat germ improves culture yields. Interestingly the optimal culture duration under our conditions is 35–45 days shorter than the one previously described by Eraso et al. [39] for *D. pteronyssinus* and *D. farinae*. In this latter study [39] it was observed that mite cultures display 3 phases: latency, exponential growth and death, with the exponential growth phase associated with the highest allergenic activity, as corroborated by our own observations (fig. 2).

It was confirmed by two independent experts that extracts obtained from HDM cultured with the defined medium meet current regulatory requirements, with respect to precluding any risk of transmission of conventional (e.g. adventitious viruses) and nonconventional (prion) infectious agents.

No reactivity with IgE from patients monosensitized to yeast or wheat germ could be detected with extracts from both *D. pteronyssinus* or *D. farinae*, confirming that cultures thus obtained are not contaminated by nonmite allergens coming from those sources. This result is not sur-

Table 3. Most abundant proteins corresponding to the 50 largest spots in *D. pteronyssinus* extracts

n	Identity
1–7	Major cuticle-like protein (multiple forms on 7 large clusters of protein spots)
8–11	Cuticle-like protein 2 (multiple spots)
12–14	Adult-specific rigid cuticular-like protein (multiple forms on 3 clusters of protein spots)
15	Novel mite-specific protein (1)
16, 17*	Group 13 allergen/fatty acid-binding protein similar to Blo t 13
18	Homologous to a vacuolar ATP synthase subunit G
19	Homologous to a putative sec14 cytosolic factor family protein
20	Novel mite-specific protein (2)
21, 22	Ferritin-like protein
23*	Group 10 allergen/tropomyosin
24, 25	Troponin T-like protein
26–28	Actin
29, 30*	Group 20 allergen/arginine kinase
31, 32*	Group 1 allergen/cysteine protease
33	Novel mite-specific protein (3)
34	Homologous to acetylcholinesterase
35	A protease inhibitor (ovalbumin type)
36, 37*	Group 13 allergen-like/fatty acid-binding protein (paralogue)
38	A serine peptidase
39	Cytochrome c oxidase, subunit
40	Novel mite-specific protein (4)
41*	Group 2 allergen
42	A gap junction protein
43	Homologous to a GABA(A) receptor-associated protein
44*	Group 7 allergen
45*	Aldehyde dehydrogenase, showing some degree of homology to fungal allergens
46	Homologous to a <i>Drosophila melanogaster</i> gene product
47	Homologous to a prolactin regulatory element binding
48	Acyl CoA synthetase
49*	Group 10 allergen/tropomyosin (isoform)
50	Tubulin-like

* Proteins which matched allergens or allergen-like structures.

prising, since heat inactivation of wheat germ and yeast is systematically performed, which abrogates most of the associated allergenic activity. Also the culture medium is progressively eaten by growing mites, with the potential residual medium components likely being eliminated during the manufacturing process of extracts, for example at the ultrafiltration step. As a matter of fact using wheat germ agglutinin as a marker protein, we determined that the dilution factor of wheat germ proteins in *D. pteronyssinus* and *D. farinae* extracts was greater than 2,500. Sim-

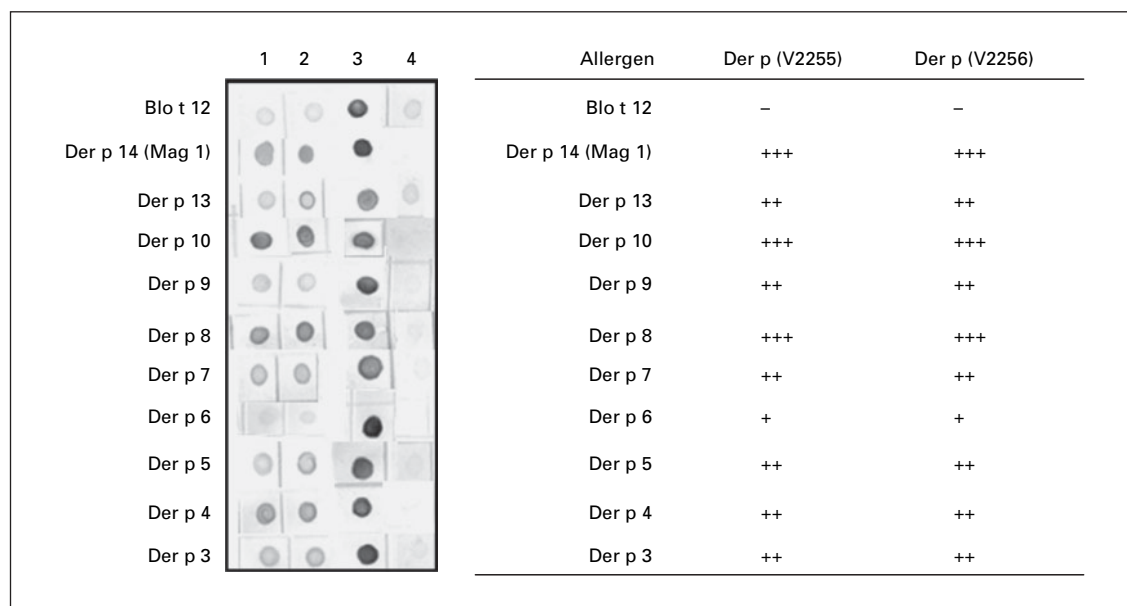


Fig. 6. Immunodot blots of representative *D. pteronyssinus* extracts (batches V2255 and V2256, lanes 1 and 2) assayed for the presence of *Dermatophagoides*-specific group 3–10, 13 and 14 (Mag 1) allergens using specific rabbit polyclonal antibodies raised against the specific recombinant proteins. Additionally a polyclonal antibody against a *Blomia tropicalis* allergen (Blo t 12) was included as a negative control. The corresponding specific recombinant proteins (0.1 µg/dot) and bovine serum albumin (0.1 µg/dot) were included as positive and negative controls, respectively (lanes 3 and 4). Each extract was adjusted to a concentration of 250 µg/ml of total protein prior to the dot blot assay, and 1 µl of the extract was loaded on each dot. Following comparison with specific standard curves we estimate that for 5% (w/v) extract ‘–’ corresponds to allergen levels below the detection limit (~0.60 µg/ml), ‘+’ between 0.61 and 4.8 µg/ml, ‘++’ between 4.9 and 19.1 and ‘+++’ above 19.2.

ilar results were obtained with respect to yeast proteins using an HPLC analysis. We conclude that our mite extracts are safe, even in multiallergic patients, since daily doses of extracts administered to HDM allergic patients (i.e. 100 IR corresponding to 40–70 µg of mite proteins) would at most contain quantities 1,000-fold lower than the ones estimated to trigger a severe reaction to wheat in a highly sensitive allergic individual [40].

These defined culture conditions were found to allow high yields of production of mites over several hundreds of independent industrial batches with a limited variation of allergenic activity, comparable to previous results obtained by others on a much smaller series of *D. pteronyssinus* cultures [41].

The average ratio between group 1 and 2 allergen levels is 4.1 in *D. pteronyssinus* and 1.9 in *D. farinae* cultures (table 2). Interestingly those ratios are similar to the group 1/group 2 ratio observed in air dust (in the range of 2.5–4.7 depending on whether the air is disturbed or not), whereas this ratio appears to be lower (i.e. 0.8–1.6) within sedimented dust [42, 43].

We further characterized the most abundant proteins within *D. pteronyssinus* extracts using comprehensive proteomic methods. Following two-dimensional gel electrophoresis and mass spectrometric analysis, we could confirm that all important major *Dermatophagoides* spp. allergens frequently involved in patient sensitization, namely the group 1, 2, 7, 10, 13 and Der p 20 arginine kinase allergens, were present among the major components in the extract (table 3). In addition we found abundant proteins from the cuticle as well as proteins involved in various metabolic pathways.

Collectively our results indicate that a well-defined medium, Stalmite APF®, can support the growth of common HDM species such as *D. pteronyssinus* or *D. farinae* in large quantities. Obtained biological extracts exhibit an excellent batch to batch consistency in terms of allergenic activity as well as group 1 and 2 allergen levels, while containing all the relevant allergens for desensitization purposes. Thus such extracts are suitable to prepare both safe and efficient diagnostic and immunotherapeutic products for allergic patients.

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