



GATEWAY™ technology and *E. coli* recombinant system produce a properly folded and functional recombinant allergen of the lipid transfer protein of apple (Mal d 3)

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ABSTRACT

The lipid transfer protein of apple fruit, Mal d 3, has been produced as a soluble recombinant protein in transformed *Escherichia coli* cells using the GATEWAY™ technology. Circular dichroism spectra showing the protein essentially consists of α -helices indicate that the rMal d 3 is properly folded. The ¹H NMR spectra also indicates a correct fold for the recombinant allergen. The reactivity of rMal d 3 towards IgE from apple allergic patients and *in vitro* degranulation activity measured on transformed rat basophil leukemia cells expressing the human Fc ϵ RI α -subunit of rMal d 3 is similar to those of the native allergen purified from apple fruits. The expression of active rMal d 3 in *E. coli* is readily feasible and offers an interesting alternative to the production of recombinant allergen in the yeast *Pichia pastoris*. This expression in *E. coli* open the way to the modification of Mal d 3 by site-directed mutagenesis for immunotherapy purposes.

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Introduction

Non-specific lipid transfer proteins (nsLTP)¹ have been identified as major fruit allergens and, especially, in Rosaceae fruits (apple, peach, apricot, and plum) [1–5]. They are widely distributed in plants and are thus considered as important pan allergens [6–8]. The allergenic propensity of these pan allergens most probably depends on the extreme resistance of these small-sized 9 kDa proteins (~90 amino acid residues) to both thermal denaturation and proteolytic digestion [9–11]. All these proteins consist of a single polypeptide chain forming a core of four α -helices extended by a coiled C-terminal tail (see Fig. 5) [12]. Eight conserved cysteine residues form four disulfide bridges that stabilize this compact fold. In European countries apple is one of the most frequent sources of nsLTP-mediated allergic manifestations although food-allergy to peach seems to prevail in Mediterranean populations

[13]. Isolation of nsLTP from apple (Mal d 3) fruits by the classical ion exchange chromatography procedure is a time-consuming task yielding small amounts of proteins that are incompatible with the actual need of pure proteins for diagnosis purposes. The availability of standardized allergens produced in heterologous cell systems has now become a goal to improve the specificity and reliability of clinical tests used for the diagnosis of food-allergy. The peach Pru p 3 allergen has been successfully cloned and expressed as recombinant protein in the yeast *Pichia pastoris* in rather good yield (20 mg of recombinant Pru p 3 per liter of culture supernatant after 4 days) [14,15]. Recombinant Pru p 3 was shown to display a strong immunological activity equivalent to that of its nPru p 3 counterpart that suggests a correct folding of the recombinant allergen in *P. pastoris*. Apple Mal d 3 has been successfully expressed in *P. pastoris* but no data is available on the yield of recombinant protein [16] or possible addition of N- and O-glycosylation that can modify IgE-binding of patient sera. In addition, no CD spectra were performed to detect some possible misfolding of the recombinant allergen. Since Mal d 3 is apparently devoid of any N- and O-glycan chain (no putative N-glycosylation signal occurs along the amino acid sequence of Mal d 3 and the native protein is not retained on a column of immobilized Con A) [17], we decided to produce recombinant Mal d 3 in *Escherichia coli* using the flexible GATEWAY™ technology [18].

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¹ Abbreviations used: CD, circular dichroism; Mal d 3, nsLTP allergen from *Malus domestica* (apple); nsLTP, non specific lipid transfer protein; Pru p 3, nsLTP allergen from *Prunus persica* (peach).

Although Mal d 3 was expressed in modest amount in *E. coli* using the GATEWAY™ technology, both the structural (CD and ^1H NMR spectra) and functional (IgE-binding ability, and degranulation test) analyses suggest an overall three-dimensional fold of rMal d 3 similar to that of the native allergen.

Materials and methods

Mal d 3 was purified from the skin of mature apple (Golden delicious cv.) fruits as previously described in details elsewhere [19].

Polyclonal antibodies against native Mal d 3 were raised in rabbits in the presence of Freund's complete adjuvant (Calbiochem) as previously reported [19].

Blood samples were drawn after informed consent of three patients allergic to Rosaceae fruits (peach and apple) [patients experiencing urticaria and facial angioedema upon fruit consumption, exhibiting up to 540 kU/L total IgE and positive skin prick tests (44–63 mm²) to apple] and the patient sera were used for both Western-blotting, ELISA measurements and degranulation assay of rat basophil leukemia cells expressing the human FcεRI α-subunit.

Cloning and expression of rMal d 3 in *E. coli*

Total RNA was extracted from the peel of mature apple fruits using the Qiagen extraction kit in the presence of sarkosyl as detergent. The reverse transcription was performed from 1 ng of total RNA previously treated at 70 °C for 3 min and the resulting cDNA was amplified by PCR using the oligonucleotide 5'-primer 5'-ATGA-TAATATGTGGCCAAGT-3' and the oligonucleotide 3'-primer 5'-TTACTTCACGGTGGCGCAGT-3' for the reverse direction. Two additional PCR steps have been performed for introducing the recombination sites attB1 and attB2 (underlined) at both 5'- (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATG-3') and 3'-end (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTA-3') of the cDNA. The PCR product was inserted in a pDONR201 vector (Invitrogen) to obtain the "pEntry" construct used to transform DH5α *E. coli* competent cells, and the DNA from several Kan^r clones were sequenced. Insertion of the cDNA into a pDEST14 vector (Invitrogen) used as a "destination vector" was performed through a second recombination step in DH5α cells to select the Amp^r "expression clones" producing Mal d 3 in *E. coli* BL21-DE3 cells. The selected transformants were cultured in LB medium containing 0.1% (w/v) glucose and 0.1 mg mL⁻¹ Ampicillin at 37 °C, and diluted to obtain a 0.1 OD at 600 nm. Induction was obtained by adding 2 mM IPTG to the culture during the exponential phase of growth (0.5–0.7 OD at 600 nm) and cells were harvested 2.5 h later. The cell pellets obtained by centrifugation were kept frozen at –20 °C until used. After several cycles of freezing–thawing, cells were resuspended at 4 °C in 50 mM Tris buffered saline buffer (pH 7.3) supplemented with 0.5% Triton X-100, 1 mM DTT, 0.1 mM PMSF, 1 μg/mL DNase, 20 μg/mL lysozyme, 5 mM EDTA and 1 mM MgCl₂. After an incubation time of 30 min on ice, cells were lysated using French press and bacterial pellets were discarded after centrifugation at 12,000g during 5 min at 4 °C. Supernatants were extensively dialyzed against 50 mM Phosphate buffer (pH 7.3) for more than 3 days. The rMal d 3 protein was then purified on a cation exchange SP FF column (Amersham) and eluted with 200 mM NaCl. rMal d 3 was further purified on the cation exchange column SP FF and, after an extensive dialysis against distilled water, rMal d 3 was lyophilized. This procedure yielded 3 mg pure protein per liter of culture medium. Checking for lower temperatures of 30 and 32 °C, respectively, yielded lower amounts of rMal d 3.

Analytical methods

The protein content of the Mal d 3 samples was estimated using the bicinchonic acid kit reagent (Pierce) [20] with bovine serum albumin and nMal d 3 as standards.

The purity of the nsLTP preparations was checked by SDS–PAGE in 15% polyacrylamide gels using Tris–taurine as trailing ion [21] and stained with Coomassie blue and silver nitrate. Coomassie blue stained bands were digested with trypsin in the gel and mass mapped by MALDI-TOF analysis as previously described [22]. The software Protein Prospector was used for the identification of the protein using the NCBI non-redundant database.

Far-UV circular dichroism (CD) spectra of nMal d 3 and rMal d 3 were recorded at room temperature with a spectropolarimeter Jobin and Yvon CD6. The light path for all measurements was 1 mm. A concentration of 250 μg mL⁻¹ in 50 mM phosphate buffer (pH 7.5) was used for both protein samples. The proportion of α-helical segments was estimated from the CD spectra [23].

^1H NMR spectra were recorded at 298 K on a Bruker AVANCE 600 MHz NMR spectrometer (Wissembourg, France) using a purified 0.3 mM rMal d 3 solution in 10 mM phosphate buffer (pH 7.5). The one dimensional ^1H NMR spectra were recorded at a nominal field of 600.13 MHz from 16 individual scans, 16 K points acquired in 7184 Hz with a repetition time set to 1 s. Water suppression was achieved using the WATERGATE W5 pulse cycle [24]. An apodization function of the exponential type of 0.5 Hz was applied to the free induction decay (FID) before the Fourier transform.

ELISA measurements

ELISA measurements were performed on standard 96-wells microtitration plates (Limbro) using whether rabbit polyclonal antibodies (Ab) against Mal d 3, polyclonal antibodies raised against synthetic epitopes or IgE-containing sera from allergic patients. Routinely, the wells were loaded with 50 μL of 10 mM PBS (pH 7.5) containing 0–100 ng mL⁻¹ of nMal d 3/rMal d 3. The plates were incubated overnight at 4 °C and washed 3-times with PBS (pH 7.5). One hundred μL of PBS (pH 7.5) containing 0.2% Tween 20 (v/v) and 3% BSA (w/v) (PBSTB) were added to each well and the plates were incubated at room temperature for 2 h. Fifty μL of 200-fold diluted Ab in PBSTB (pH 7.5) were added and the plates were incubated for 1 h 30 at room temperature under constant stirring. After three washes with PBSTB, 50 μL of either 1/2000 (v/v) diluted sheep anti-rabbit IgG coupled to alkaline phosphatase (Dako) or 1/300 (v/v) diluted mouse monoclonal anti-human IgE coupled to alkaline phosphatase (Sigma) were added and, after incubation at room temperature for 45 min, three washes were performed with PBSTB. One hundred μL of alkaline phosphatase substrate (Sigma) were added and after incubation for 45 min in the dark, 50 μL of 3 M NaOH were added to stop the color reaction. The absorbance at 405 nm was recorded on a Titertek Multiscan spectrophotometer. Each value is the mean of three separate experiments. Appropriate blank and substrate controls were performed under the same conditions.

For inhibition experiments, the wells were loaded with 50 μL of 10 mM PBS (pH 7.5) containing 40 ng mL⁻¹ of nMal d 3. The plates were incubated overnight at 4 °C and washed three-times with PBS (pH 7.5). One hundred μL of PBS (pH 7.5) containing 0.2% Tween 20 (v/v) and 3% BSA (w/v) (PBSTB) were added to each well and the plates were incubated at room temperature for 2 h. Fifty μL of 10-fold diluted patient serum in PBSTB (pH 7.5) that has been previously incubated for 30 min with increasing concentrations up to 10 mg mL⁻¹ of rMal d 3, were added and the plates were incubated for 1 h 30 at room temperature under constant stirring. Measurements were performed as above described.

Degranulation of human FcεRI-expressing cells

Checking of rMal d 3 for *in vitro* degranulation assay was performed on transfected rat basophilic leukemia cells expressing the human FcεRI α-subunit (RBL-2H3E5.D12.8) [25] cultured as a monolayer in DMEM-Glutamax medium (Gibco BRL) containing 10% (v/v) foetal calf serum (FCS), 100 U/mL penicillin, 100 U/mL streptomycin, 1 mg/mL G-418 (Gibco) and 20 mM Hepes (pH 7.3), after adding IgE and the corresponding allergens.

After a 72 h culture in the absence of G-418, adherent cells were washed twice in FCS-free medium, treated with trypsin-EDTA (Gibco), and incubated for 5 min at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were then collected, washed and resuspended for counting using a Malassez counting chamber. Cells were replated at 5×10^4 cells/well (200 μL) in a standard 96 well plate in G-418-free medium containing human IgE (patient sera diluted 1/200). After an incubation of 48 h at 37 °C in a humidified atmosphere at 5% CO₂, the supernatant was discarded and the cells were washed with the G-418-free medium, a 1:1 mixture of Tyrode-D₂O (pH 7.4) (Sigma), and then flooded by 100 μL of a 1:1 mixture of Tyrode-D₂O (Sigma) containing increasing concentrations of the protein to be assayed (from 5 to 1000 ng/mL). After an incubation of 45 min at 37 °C and centrifugation at 150g for 5 min, 50 μL of supernatant from each well was used for immediate measurement of α-hexosaminidase activity. Cell samples were similarly incubated with Tyrode-D₂O buffer free of patient serum for measuring the spontaneous release of α-hexosaminidase. Samples for measuring the α-hexosaminidase content of cells were treated with 3 μL of 10% (v/v) Triton X-100 for 3 min before centrifugation.

The 50 μL samples corresponding to the tested cell samples were distributed in a 96 well plate and 50 μL *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide at 1.3 mg mL^{-1} in 0.05 M citrate buffer (pH 4.5) were added. After incubation at 37 °C for 90 min, the enzymatic reaction was stopped by adding 150 μL of 200 mM glycine buffer (pH 10.7) and optical density at 410 nm was estimated. Results are expressed as the percentage of α-hexosaminidase released from the IgE-stimulated cells in the presence of nMal d 3

or rMal d 3 after correction for spontaneous release from unstimulated cells. Values are the means of triplicate cell incubations.

Results

Expression of rMal d 3 was successfully performed in *E. coli* using the GATEWAY™ technology [17]. Although the amount of protein produced in *E. coli* remains rather modest with an average yield of 3 mg rMal d 3 per liter of culture medium, the recombinant protein is favourably produced as a soluble protein (Fig. 1) that avoids performing the often unreliable recovery of some correctly folded recombinant protein from inclusion bodies. However, the expression level of rMal d 3 should be improved by carefully selecting more appropriate culture conditions for the transformant cells.

The purity of rMal d 3 checked by MALDI-TOF spectrometry gave a M_r of 9.052 (vs. a M_r of 9.049 for nMal d 3) corresponding to the entire amino acid sequence (91 amino acid residues) of the Mal d 3 allergen (Fig. 2).

The far-UV CD spectra of rMal d 3 and nMal d 3 readily show the same shape and signal from 208 to 222 nm, showing the exclusive

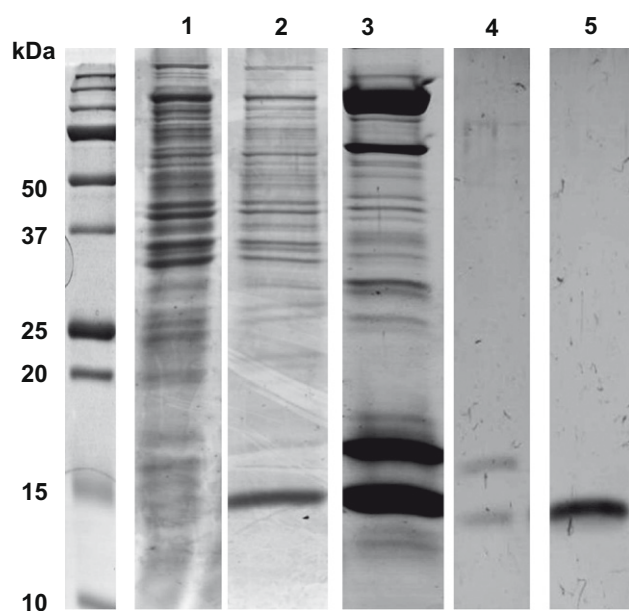


Fig. 1. Silver nitrate-stained SDS-PAGE analysis of protein extract from non-induced bacteria (line 1), induced bacteria (line 2), the first (line 3) and second (line 4) cation exchange isolation step of rMal d 3 and the purified rMal d 3 (line 5). Left hand line corresponds to the molecular weight markers (kDa).

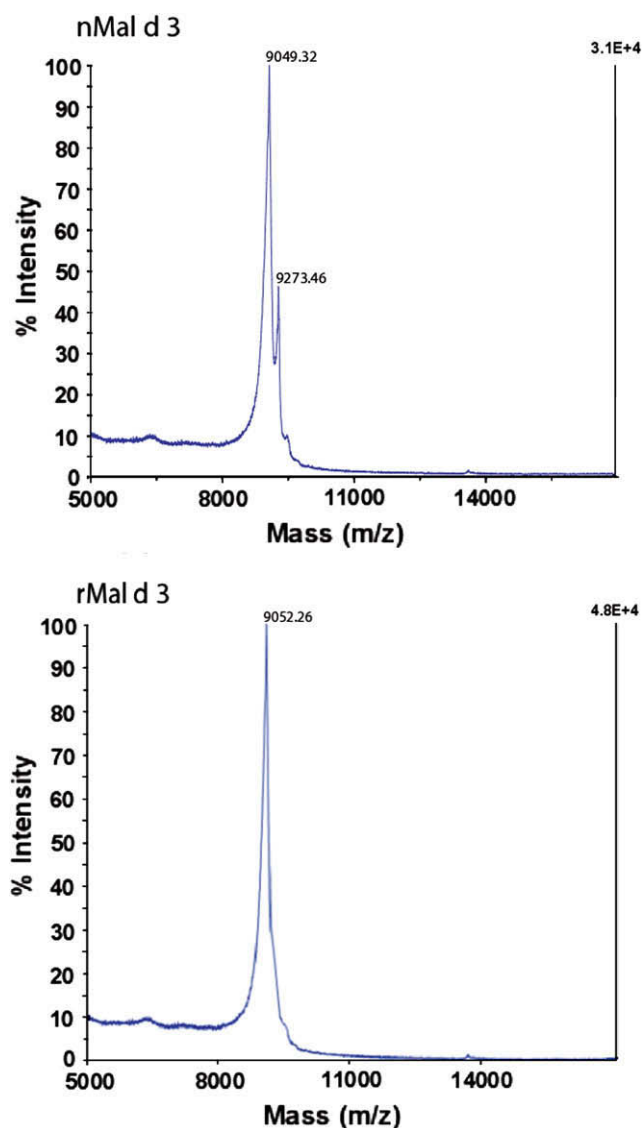


Fig. 2. Mass spectra of the native (nMal d 3) and recombinant (rMal d 3) nsLTP. The major ion (¹H⁺) of nMal d 3 has a mass of 9049.32 Da. The major (¹H⁺) ion of rMal d 3 exhibits a mass of 9052.26 Da.

occurrence of α -helical and coiled secondary structures in both allergens (Fig. 3). These results agree with the putative three-dimensional model built for Mal d 3 from the coordinates of both Pru p 3 [26] (RCSB PDB code 2AGL) and another nsLTP, Zea m 14 from maize (*Zea mays*) [27] (RCSB PDB code 1MZL) showing the occurrence of α -helices and coil structures but the lack of β -sheet structures (result not shown). Additionally, the ^1H NMR spectrum is typical of a well folded protein, displaying well resolved NH resonances at a chemical shift higher than 8.5 ppm, as well as methyl resonances at chemical shift lower than 0.5 ppm.

Western-blotting experiments using IgE-containing sera from allergic patients indicated that both rMal d 3 and native Mal d 3 specifically interacted with the IgE of sensitized individuals (results not shown) suggesting that rMal d 3 was produced in a fully active form in *E. coli*. Moreover, ELISA measurements indicated a very similar response for both rMal d 3 and native Mal d 3 towards polyclonal rabbit anti-nMal d 3 IgG (Fig. 4A). However, at higher concentration ($>50 \text{ ng mL}^{-1}$), rMal d 3 appeared as slightly less reactive than its native counterpart, most probably because of a slightly reduced solubility in phosphate buffer. A pre-incubation of the IgE-containing patient sera in the presence of increasing amounts of rMal d 3 significantly inhibited and finally abolished the interaction of nMal d 3 with the IgE (Fig. 4B). In addition, the synthetic peptide 39RTTADRQTA46 of Mal d 3, that has been previously characterized as a consensual epitopic region of fruit nsLTP [27], inhibited the Ab-rMal d 3 interaction performed with either rabbit polyclonal IgG or IgE-containing sera from allergic patients (Fig. 4C). Finally, no significant difference occurred between rMal d 3 and native Mal d 3 in their ability to trigger the *in vitro* degranulation of a Fc ϵ RI-expressing rat basophilic leukemia cell line as estimated by the α -hexosaminidase release from IgE-stimulated cells previously treated by the allergens (Fig. 5). This common immunoreactivity suggests that natural and recombinant allergens contain similarly surface-exposed IgE-binding epitopes which, in turn, favours the occurrence of a similar fold for both proteins.

Discussion

Expression of rMal d 3 in *E. coli* using the GATEWAY™ technology yielded a soluble recombinant protein exhibiting a CD spectrum that suggests a correct folding for the recombinant protein.

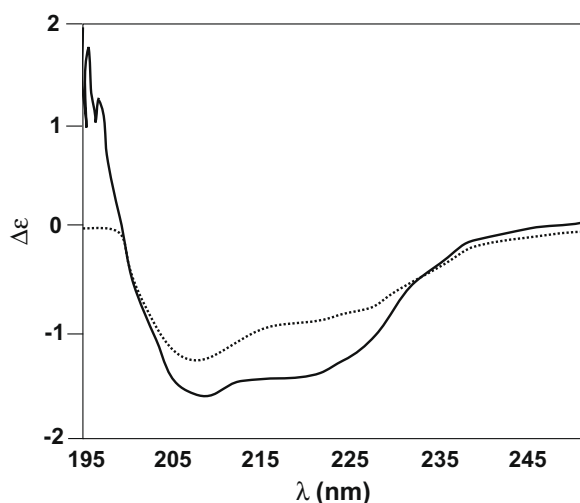


Fig. 3. CD spectra of nMal d 3 (black) and rMal d 3 (dashed) recorded in the far-UV region (190–260 nm). nMal d 3 and rMal d 3 were used at concentrations of 250 and $150 \mu\text{g mL}^{-1}$ in 50 mM phosphate buffer (pH 7.5), respectively.

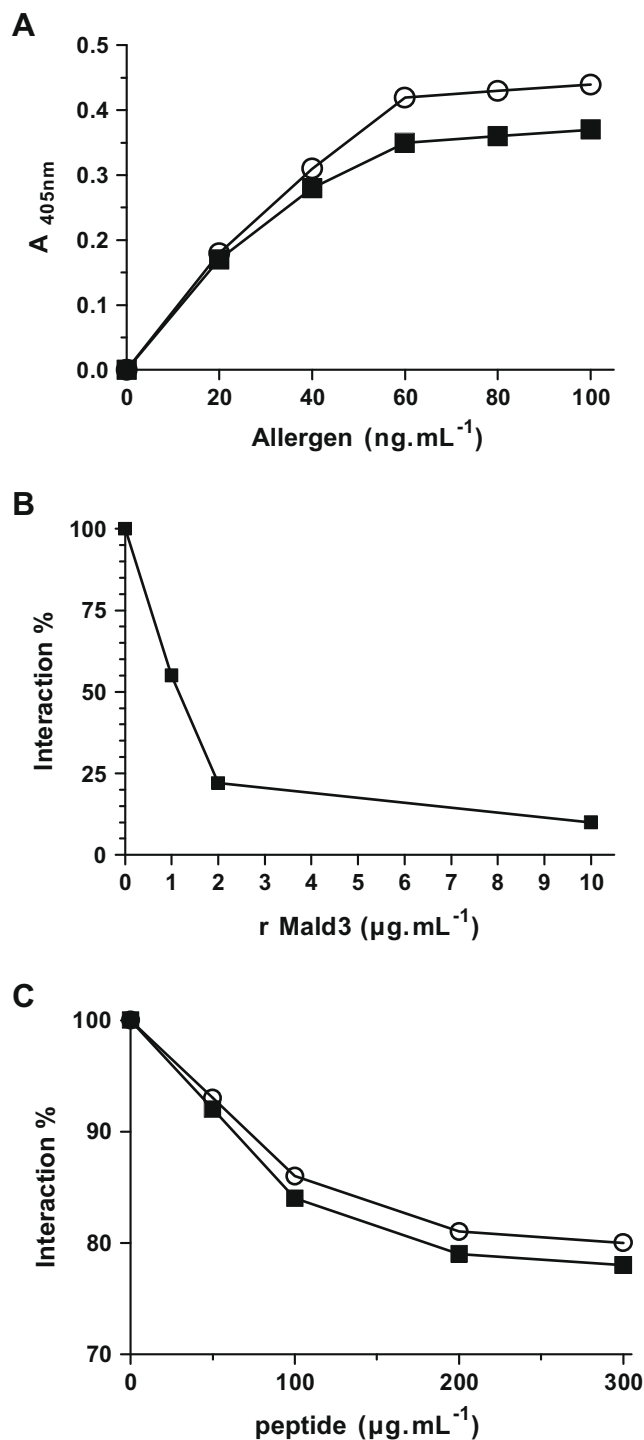


Fig. 4. (A) ELISA measurement (absorbance at 405 nm) showing the interaction of increasing concentrations of nMal d 3 (○) and rMal d 3 (■) with rabbit polyclonal antibodies against nMal d 3. Values are means of two separate ELISA measurements. (B) Inhibition of the interaction of nMal d 3 with IgE by increasing concentrations ($\mu\text{g mL}^{-1}$) of rMal d 3. (C) Inhibition by increasing concentrations ($\mu\text{g mL}^{-1}$) of the synthetic peptide 39RTTADRQTA46 of Mal d 3 of the interaction of nMal d 3 (○) and rMal d 3 (■) with IgE from patient sera. All the values are means from triplicate experiments.

In addition, the far-UV CD spectra of nMal d 3 and rMal d 3 are readily superimposable and suggest the exclusive occurrence of α -helical and coiled secondary structures in both proteins. These results are in full agreement with the predominant α -helical fold

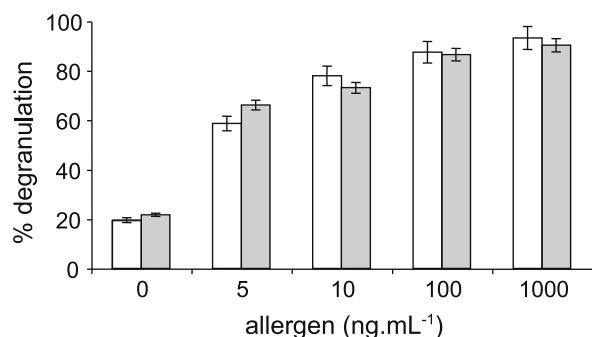


Fig. 5. Degranulation, expressed as percentage, of transformed rat basophil leukemia cells expressing the FcεRI α-subunit previously treated with IgE from apple allergic patients in the presence of increasing concentrations of nMal d 3 (white) and rMal d 3 (grey). Note that some degranulation occurs even in the absence of native or recombinant allergen.

of plant nsLTP which consist of a tightly packed core of four α-helices connected to an extended coiled C-terminal tail. Besides these physico-chemical similarities, nMal d 3 and rMal d 3 similarly interacted with IgE-containing sera of allergic subjects in Western-blotting experiments, degranulation experiments and ELISA measurements with both rabbit anti-Mal d 3 IgG and IgE from allergic patients, that suggests a close immunoreactivity (and a closely-related fold) for both nsLTP. However, additional *in vitro* (expression of interleukins by stimulated lymphocytes) and *in vivo* (skin prick testing) biological tests will be necessary to check that nMal d 3 and rMal d 3 correspond to virtually identical allergenic proteins. This is a prerequisite to use rMal d 3 as a substitute for nMal d 3 for both structure–function relationship investigations or screening purposes such as the component-resolved diagnosis of food-allergy.

Although produced in rather modest yield (~3 mg pure protein per liter of culture supernatant) the expression of Mal d 3 in *E. coli* is readily feasible and offers an interesting alternative to the production of recombinant nsLTP allergens in the yeast *P. pastoris*. However, a screening of different *E. coli* strains and corresponding growth conditions is under way in order to improve the amount of rMal d 3 as a correctly folded and active allergen expressed in transformed bacterial cells.

The availability of rMal d 3 paves the way to the production of hypoallergenic Mal d 3 by site-directed mutagenesis of the amino acid residues recognized as immunodominant along the four IgE-binding epitopic stretches previously predicted on the molecular surface of the protein [19]. Two of these epitopic regions consist of consensual epitopes readily conserved among the Rosaceae fruit LTP [28]. Accordingly, the hypoallergenic rMal d 3 tailored for the treatment of apple allergy should also apply to other fruit allergies, e.g. peach, apricot and plum allergies.

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