

Che a 1: Recombinant Expression, Purification and Correspondence to the Natural Form

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Key Words

Che a 1 · *Chenopodium album* pollen · Ole e 1 · *Pichia pastoris* · Recombinant allergen

Abstract

Background: Pollinosis to chenopods is one of the main causes of allergy in desertic regions and it is increasing in the South of Europe and Western USA. Che a 1 is a major allergen for chenopod-allergic subjects and belongs to the Ole-e-1-like family of proteins. **Methods:** *Pichia pastoris* yeast has been used as expression system to produce the recombinant form of Che a 1 (rChe a 1). The allergen was isolated using a gel permeation column and reverse-phase/high-performance liquid chromatography. Molecular characterization was performed using Edman degradation, mass spectrometry and concanavalin A staining. Sera from patients allergic to chenopod pollen, as well as polyclonal and monoclonal antibodies raised against Ole e 1, were used in immunoblotting, ELISA and inhibition assays for immunological characterization of rChe a 1. **Results:** The allergen was purified to homogeneity with a final yield of 15 mg/l of cell culture and showed a glycosylated character. N-terminal amino acid sequence of rChe a 1 and molecular mass were according to those of the protein isolated

from chenopod pollen. The recombinant allergen maintained the IgG and IgE epitopes of the natural allergen deduced from the immunological assays. **Conclusions:** Structural and in vitro immunological properties of rChe a 1 produced in *P. pastoris* were equivalent to those of the natural form of the allergen and, thus, it could be used in testing patients allergic to chenopods.

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Introduction

Chenopod (*Chenopodium album*, commonly named Lamb's quarter) pollen allergy has been reported as a significant problem in Western USA and temperate areas of Europe [1–4]. This pollinosis is a severe problem in desert countries such as Saudi Arabia, Iran and Kuwait where Chenopodiaceae pollens are the main cause of sensitization because of its recent use in greening programs [5–7]. Allergic sensitizations associated to Chenopodiaceae pollen are also increasing probably as a result of the climatic change that produces an expansion in desert areas where these plants grow [8, 9]. A remarkable feature of chenopod pollinosis is its concomitant occurrence with sensitivities to other taxonomically or non-taxonomically related pollens [10, 11].

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Recently, our group has reported the existence of Che a 1, Che a 2 (profilin) and Che a 3 (polcalcin) as relevant allergens in *C. album* pollen [12–14], weed that defines the name of the Chenopodiaceae family. Che a 1 and Che a 2 have been described as major allergens, since these proteins are recognized by the IgE from at least 50% of patients suffering from chenopod sensitization. Che a 3 was found to be a very relevant allergen since 46% of the patients had IgE against this protein.

Che a 1 belongs to a family of proteins specifically expressed in pollen tissue [15], and consists of a single glycosylated polypeptide chain of around 145-amino-acid residues and for which a role in the control of pollen tube growth has been reported [16]. Several members of this family of proteins have been recognized as relevant allergens: Che a 1 affects >70% of patients sensitized to chenopod pollen, Ole e 1 >70% of patients allergic to olive pollen, Lol p 11 >65% of ryegrass-allergic subjects and Pla l 1 >80% of English plantain-allergic patients [12, 17–19]. Che a 1 displays very low cross-reactivity with Ole e 1 as well as with Pla l 1 [12, 20].

The scarce cross-reactivity between these proteins warrants the collection of different homologues belonging to non-taxonomically related plants in order to analyze the possible use of these allergens as markers for the sensitization of individual patients to a specific pollen. Because of the low yield obtained during the purification of the natural allergen (nChe a 1) from the pollen [12], the production of rChe a 1 with immunological and structural features of the natural form can be useful for several purposes. *Pichia pastoris* yeast was used in the production of different members of the Ole e 1-like family such as Ole e 1, Lig v 1, Syr v 1, Pla l 1 [18, 21, 22] and Fra e 1 [unpubl. data], which were secreted as soluble and active allergens with high yields. These results led us to select this heterologous expression system for the production of rChe a 1.

The aim of this work was the production of biologically active Che a 1 using the heterologous *P. pastoris* system that facilitates the isolation of the allergen. A comparison of the recombinant protein with its natural counterpart has also been performed.

Materials and Methods

Preparation of Lamb's Quarter Pollen Extract and Purification of nChe a 1

C. album pollen extract was prepared using pollen from ALK-Abelló (Madrid, Spain) as described previously [12]. nChe a 1 was isolated according to Barderas et al. [12].

Subcloning and Expression of rChe a 1

The coding region of Che a 1 was cloned as described [12] using degenerate primers. The sense primer (CH3) incorporated an *Xho*I restriction site and a sequence that enables fusion of the Che a 1-encoding region in-frame with the preprosequence corresponding to the α -mating factor present in plasmid pPICZaA. The antisense primer (CH2) contained a *Sac*II restriction site together with a stop codon. The pCR2.1/Che a 1 plasmid [12] was digested with *Xho*I and *Sac*II and subcloned into the same sites of the plasmid pPICZaA (Invitrogen, Groningen, The Netherlands) rendering the pPICZaA/Che a 1 construction. Recombinant DNA methods other than those reported above were carried out by standard procedures. pPICZaA/Che a 1 was linearized with the *Sac*I restriction enzyme, and the purified fragment was used to electroporate the KM71 strain of *P. pastoris* (Invitrogen) cells by a replacement-type DNA integration as described [21].

Transformed cells were incubated with YPDS/zeocin at 30 °C for 2–4 days until colonies appeared. To produce rChe a 1, selected His⁺ Mut^s-transformed strains were cultured for 48 h at 30 °C in 50 ml buffered minimum glycerol medium. Cells were then collected by centrifugation and resuspended in one fifth of the original volume of buffered methanol minimum medium for induction of the AOX1 promoter. This culture was maintained for 4 days and supplemented daily with methanol (0.5% of the total volume). As the recombinant construction facilitates the secretion of the protein into the extracellular medium, KM71-induced cells were collected at different times and sedimented by centrifugation at 4 °C. The presence of rChe a 1 in the supernatant was analyzed by SDS-PAGE. Large-scale production of rChe a 1 was performed under similar conditions using the colony that rendered the best yield in the small-scale experiments.

Purification of rChe a 1 and Analytical Procedures

After the induction, the supernatant with the recombinant protein was dialyzed against 20 mM ammonium bicarbonate at pH 8.0, lyophilized, and used as starting material. The purification of rChe a 1 was performed as described for nChe a 1 [12]. rChe a 1 was visualized by Coomassie brilliant blue staining and IgG reactivity with a monoclonal antibody (mAb) raised against Ole e 1.

Protein concentration of purified samples was determined by amino acid analysis after hydrolysis with 5.7 N HCl at 105 °C for 24 h in sealed tubes under vacuum [17]. Hydrolyzed samples were analyzed on a 6300 amino acid analyzer (Beckman Instruments, Palo Alto, Calif., USA). N-terminal Edman degradation of the protein was performed automatically using a 477A sequencer (Applied Biosystems, Foster City, Calif., USA) [17].

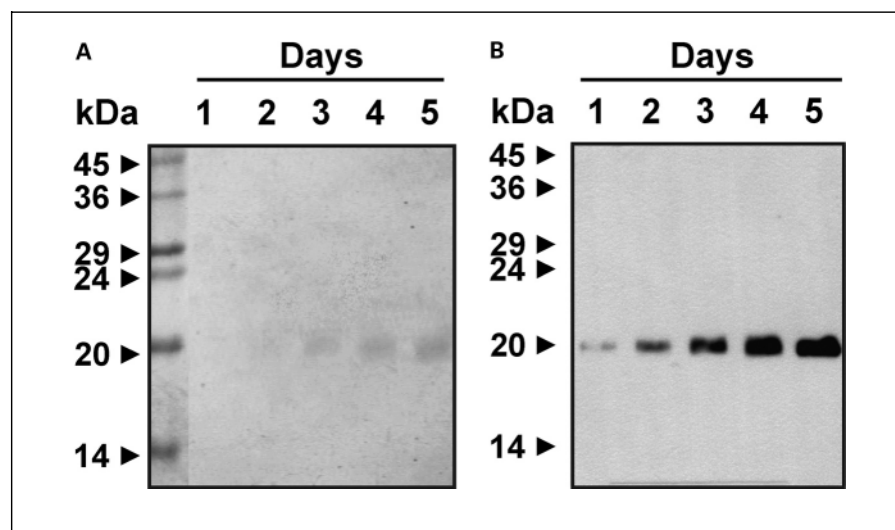
Sera and Antibodies

Sera from patients sensitized to *C. album* pollen, RAST class 1–6 (determined by CAP, Amersham Pharmacia Biotech, Uppsala, Sweden), and a pool of sera from non-atopic subjects were used in this study. Patients were selected because of a well-known sensitivity to chenopod pollen [12, 14]. All patients and control individuals provided informed consent. Polyclonal antiserum (pAb) against Ole e 1 was prepared according to Huecas et al. [21]. Ole e 1-specific mAb was kindly provided by Dr. Lombardero (ALK-Abelló). This Ole e 1-specific mAb recognized nChe a 1, as previously reported [12].

Electrophoresis and Immunological Characterization

SDS-PAGE was performed according to the method of Laemmli [23] in 15% polyacrylamide gels. Proteins were stained with Coomas-

Fig. 1. SDS-PAGE analysis of rChe a 1 expression. Extracellular medium (0.01%, v/v, of the total sample) at different times after induction. **A** Coomassie blue staining. **B** Reaction with the Ole e 1-specific mAb after Western blot.



sie blue or electrophoretically transferred to nitrocellulose membranes. Immunodetection was performed as described [12] using sera from patients allergic to chenopod pollen (diluted 1:10), a mAb (diluted 1:5,000) or a pAb (diluted 1:5,000). The signal was developed by the ECL-Western blotting reagent (Amersham Pharmacia Biotech) [12]. For immunoblotting inhibition assays, strips with 0.5 µg of nChe a 1 or rChe a 1 were incubated with sera from a pool of *C. album* pollen-sensitized patients preadsorbed with PBS or 2 µg of nChe a 1 or rChe a 1 as inhibitors. Alternatively, strips with 50 µg of *C. album* pollen extract were incubated with the sera preadsorbed with PBS or 5 µg of rChe a 1.

IgE quantitation was performed by ELISA in microtiter plates coated with 100 µl/well of protein (1 µg/ml) [12]. The plates were incubated with sera from allergic individuals (diluted 1:10). The binding of human IgE was detected as indicated above. Peroxidase reaction was developed using fresh enzyme substrate and measuring optical density (OD) at 492 nm [12]. Each value was calculated as mean of two determinations. OD values under 0.1 were considered as negative responses. ELISA inhibition assays were performed as described [12]. After being coated with 100 µl nChe a 1 (1 µg/ml), the plates were incubated with the serum pool (diluted 1:10), previously adsorbed with different concentrations of rChe a 1 or nChe a 1 (1 µg to 10 pg) as inhibitors. The percentage of inhibition was calculated as $[(OD - OD_{inh})/OD] \times 100$, where OD and OD_{inh} indicate the absorbance values after pre-incubation without and with the inhibitor, respectively.

Carbohydrate Detection and Deglycosylation Treatment

Carbohydrate detection of protein transferred to nitrocellulose membranes was performed as described [24] using a biotinylated concanavalin A (ConA) solution. The staining was developed by horseradish peroxidase reaction with 0.05% (w/v) 3,3'-diaminobenzidine/HCl, 0.03% (v/v) of 30% H_2O_2 in Tris/NaCl (pH 7.5) reaction buffer. Enzymatic deglycosylation was performed with PNGase F endoglycosidase (Boehringer-Mannheim, Mannheim, Germany) [25].

Mass Spectrometry (MS) and Circular Dichroism (CD) Analyses

Samples were analyzed on a Bruker Reflex II matrix-assisted laser-desorption ionization time-of-flight mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany), as described [12]. The equipment was externally calibrated employing singly, doubly and triply charged signals from cytochrome c (12,360 Da).

CD spectra were obtained in the far-UV range on a Jasco J-715 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) as described [26], with minor modifications. The protein concentration was 0.20 mg/ml in 20 mM ammonium bicarbonate (pH 8.0). Mean residue mass ellipticity was calculated based on 110.84 as the average molecular mass/residue, obtained from the amino acid composition, and it was expressed in terms of θ (degree $\times$ cm² $\times$ dmol⁻¹).

Results

Expression of Che a 1

A cDNA encoding the allergen Che a 1 was obtained by digestion of pCR2.1/Che a 1 with *Sac*II and *Xho*I, and was subcloned into the pPICZaA expression plasmid. pPICZaA/Che a 1 inserted downstream of the AOX1 promoter was used to transform *P. pastoris* KM71 cells obtaining the efficient extracellular secretion of rChe a 1. The time course of the expression was examined in the extracellular medium after separation in SDS-PAGE by protein staining with Coomassie blue and immunoblotting with mAb (fig. 1A and B, respectively). A major band with an apparent molecular mass of 20.9 kDa can be visualized, and the highest level of production was obtained after 5 days. After selection of the growth conditions, one of the best colonies produced was selected for the protein isolation.

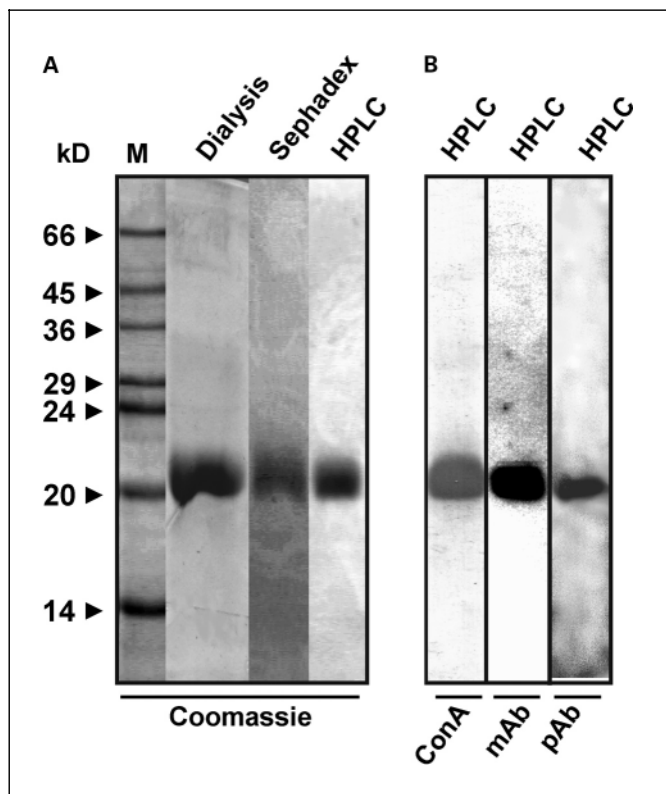


Fig. 2. rChe a 1 purification procedure. **A** Coomassie blue staining after SDS-PAGE of aliquots of the sample containing the recombinant protein after dialysis (0.14%, v/v, of the total sample; lane Dialysis), chromatography in Sephadex G-75 (0.125%, v/v; lane Sephadex) and RP-HPLC column (1 µg of protein; lane HPLC). **B** Reaction of 1 µg of purified rChe a 1 (sample after HPLC column) after Western blotting with ConA, mAb and pAb.

Purification of rChe a 1 and Molecular Properties

A two-step procedure consisting of a size exclusion chromatography and an RP-HPLC column was used to purify rChe a 1. The extracellular medium of the culture obtained after centrifugation was dialyzed. Afterwards, the lyophilized medium was loaded onto a Sephadex G-75 column. The elution profile was examined with the Ole e 1-specific mAb, and fractions containing the reactive protein rChe a 1 were pooled and lyophilized. Afterwards, the material was loaded onto an RP-HPLC C₁₈ column. rChe a 1 was then obtained with high purity (fig. 2A), and the final yield was 15 mg of purified protein per liter of cell culture. rChe a 1 has an apparent molecular mass of 20.9 kDa in SDS-PAGE and is glycosylated as demonstrated by the reaction with ConA lectin (fig. 2B). rChe a 1 was recognized by both IgG antibodies (pAb and mAb) raised against Ole e 1. These results suggest that rChe a 1

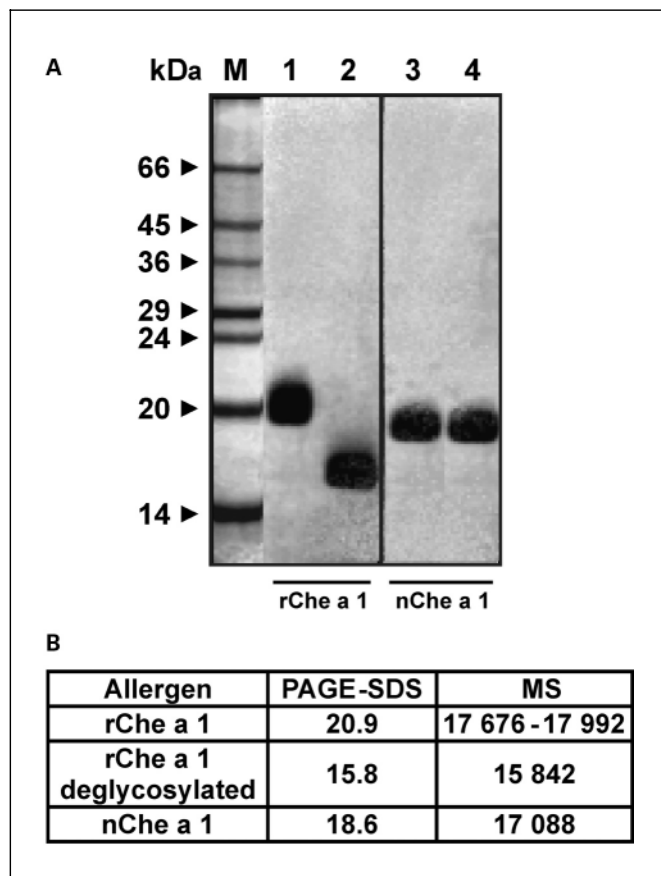


Fig. 3. **A** Coomassie blue staining of glycosylated (lanes 1 and 3) and deglycosylated Che a 1 with PNGase F (lanes 2 and 4), after SDS-PAGE. M = Mass markers. **B** Molecular mass of Che a 1; SDS-PAGE values are given in kDa and MS results in Da.

was well folded, since rChe a 1 maintained the IgG epitopes of the protein isolated from the pollen.

Coomassie blue staining after SDS-PAGE was performed with native and recombinant Che a 1 after treatment with PNGase F endoglycosidase (fig. 3A), in order to determine the size of the glycan group and as a purity test. In figure 3B, the molecular mass of the different species of native and deglycosylated Che a 1 are also shown after the treatment with PNGase F. rChe a 1 was successfully deglycosylated, and the apparent loss of molecular mass of 4.1 kDa corresponds to the glycan group linked to the polypeptide chain. However, nChe a 1 did not respond to the treatment by PNGase F, as no change was detected in the electrophoretic mobility. MS analysis of rChe a 1 gave peaks at molecular masses of 17,676, 17,830 and 17,992 Da (fig. 4A). The difference between these values is close to 160 Da, which corresponds to one mannose

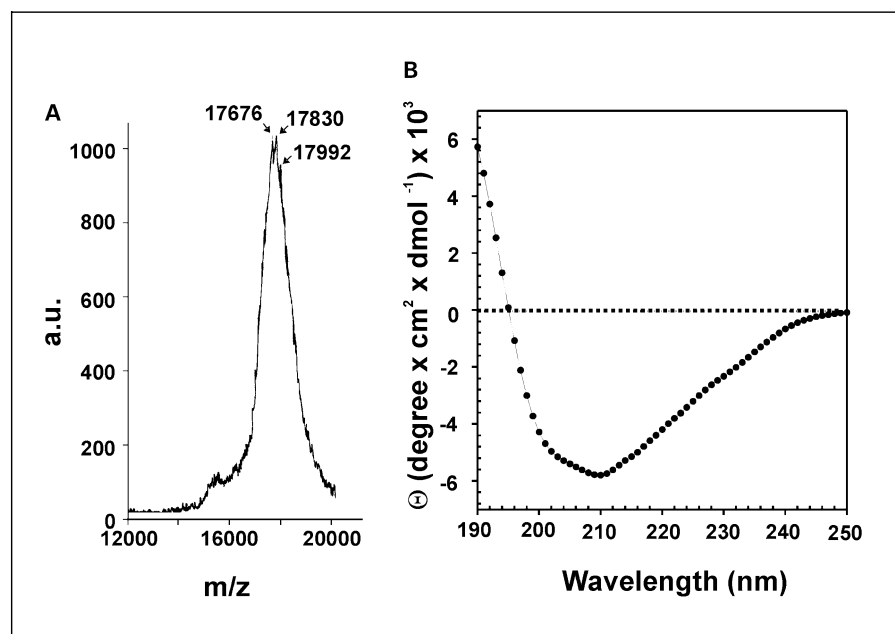


Fig. 4. MS and CD analyses of rChe a 1. **A** Intensity of the spectrum in arbitrary units (a.u.). **B** CD spectrum in the far-UV range, at 25 °C and pH 8.0.

residue. After deglycosylation with PNGase F, the obtained molecular mass was 15,842 Da, which closely approaches the value deduced from the sequence of the Che a 1 clone, 15,849 Da [12]. *P. pastoris* produces glycosylation at N-consensus Asn sites adding mannose-rich glycans with around 10 mannose residues linked to the N-acetylglucosamine (GlcNAc) core. Thus, the molecular weight of the glycosylated protein is in agreement with the polypeptide chain plus three major glycans: Man₉GlcNAc₂, Man₁₀GlcNAc₂ and Man₁₁GlcNAc₂. The amino acid sequence of the N-terminal end of rChe a 1 was determined by Edman degradation. The sequence obtained was AENHF, which is in agreement with the sequence of nChe a 1 obtained from the pollen [12], indicating the correct removal of the secretion peptide.

CD analysis of rChe a 1 was performed in the far-UV range (fig. 4B). Calculation of the contribution to the ellipticity of the secondary structure elements of the protein from the spectrum gave: 9% α -helix, 37% β -sheet, 23% β -turn and 36% non-regular conformation.

Immunological Properties

A comparison of the IgG- and IgE-binding activities of rChe a 1 and nChe a 1 was performed by immunoblotting and ELISA using sera from chenopod-allergic individuals and Ole e 1-specific mAb. Eight sera from chenopod-allergic patients showed comparable IgE-binding to both proteins after SDS-PAGE and Western blotting (fig. 5A).

Moreover, the mAb exhibited similar reactivity to both proteins in ELISA (fig. 5B). The slight difference observed in this assay could be due to a high capability of the expressed clone of Che a 1 to bind to the mAb; the presence of different isoforms in nChe a 1 can lead to a lower IgG reactivity for the whole natural protein.

IgE-binding competition between rChe a 1 and nChe a 1 was evaluated by means of ELISA and immunoblotting inhibition experiments with an equivolumetric pool of the same eight sera used above. nChe a 1 was added to the wells, and natural and recombinant forms of Che a 1 were used as inhibitors. The two proteins raised similar level of inhibition (fig. 6A). In this case, the Che a 1-specific IgE from the sera were induced by the natural protein and, thus, the presence of isoforms in nChe a 1 would lead to a more complete inhibition of the IgE binding. In immunoblotting, >95% of inhibition was obtained in all cases (fig. 6B). Inhibition experiments of the IgE binding to pollen extract in the presence of rChe a 1 as inhibitor were also performed with the same pool of sera from chenopod-allergic individuals. As shown in figure 6C, the response of the protein band corresponding to Che a 1 was notably reduced. These results are in agreement with the structural analyses indicating that rChe a 1 is a well-folded protein which conserved the capacity of binding to IgG and IgE antibodies.

To confirm that both nChe a 1 and rChe a 1 share IgE epitopes and display equivalent recognition, 50 sera from

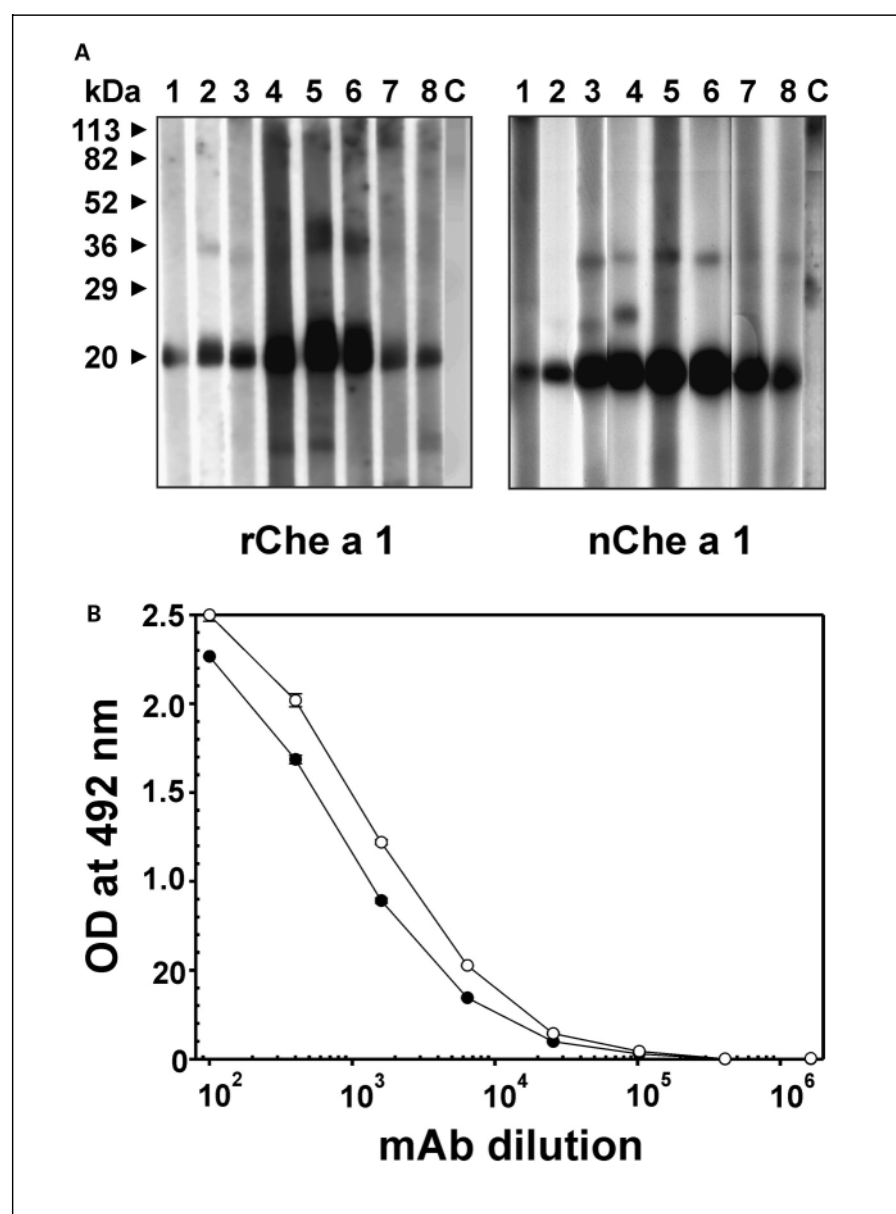


Fig. 5. IgE- and IgG-binding analysis by immunoblotting and ELISA. **A** Assay for 8 individual sera from patients sensitized to nChe a 1 (lanes 1–8). A pool of sera from non-atopic subjects was used as a negative control (lane C). **B** Reaction of nChe a 1 (●) and rChe a 1 (○) with Ole e 1-specific mAb.

chenopod-allergic patients were used in ELISA. Estimation of IgE levels, given as OD at 492 nm in figure 7, showed that Che a 1 isolated from the pollen and the recombinant protein expressed in *P. pastoris* are equivalent. A correlation value close to 1 was obtained, with a similar value in the slope of linear regression (slope = 0.92, $r = 0.95$ and $p < 0.01$).

Discussion

The members of the family of proteins to which Che a 1 belong display low amino acid sequence identities excluding those which are taxonomically related. This fact may explain the limited cross-reactivity detected so far among them. Consequently, the allergens of this family could constitute specific allergens of their respective pollen sources, and cannot be used to diagnose sensitization against other members of the family. The recombinant production of these allergens may extend the knowledge

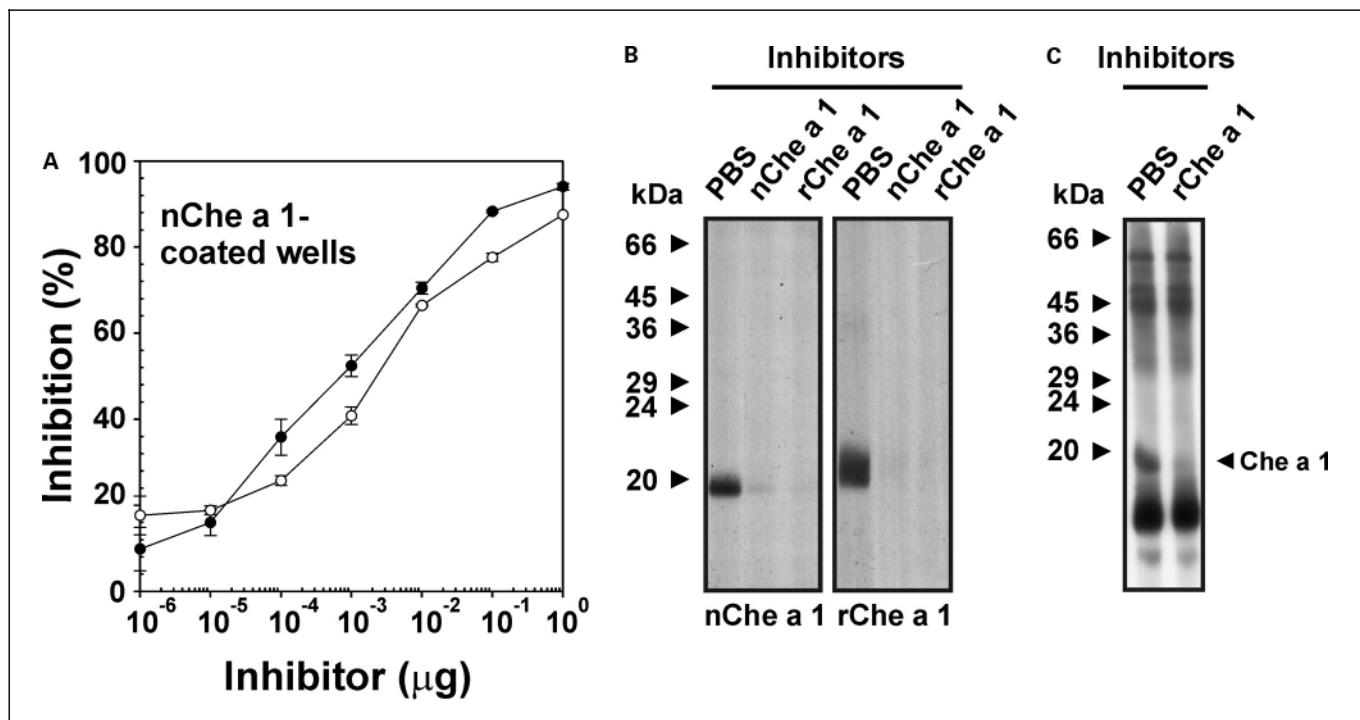


Fig. 6. IgE-binding inhibition assays of natural and recombinant Che a 1. **A** ELISA plates were coated with nChe a 1 and assayed against a pool of sera preadsorbed with rChe a 1 (○) or nChe a 1 (●). **B** Immunoblotting assay. Strips with nChe a 1 or rChe a 1 (0.5 μg/lane) were incubated with a pool of sera preadsorbed with PBS, nChe a 1 (2 μg) or rChe a 1 (2 μg) as inhibitors. **C** Inhibition of the IgE binding of sera to chenopod pollen extract (50 μg of total protein) in the absence (lane PBS) and the presence (lane rChe a 1) of rChe a 1 (5 μg).

on their immunological activity and may help to produce high protein amounts which may be used as diagnostic and therapeutic agents [27].

P. pastoris yeast cells have been chosen to produce Che a 1 because this glycoprotein has a complex folding stabilized by three disulfide bridges. This eukaryotic expression system for the production of recombinant proteins is capable of introducing such a posttranslational modification. cDNA encoding Che a 1 has been efficiently expressed in *P. pastoris* cells and secreted in the extracellular culture medium, which has low levels of contaminants and facilitates the isolation of the allergen. The amount of purified rChe a 1 from the *P. pastoris* culture is in agreement with the yield reported for other proteins of the Ole e 1-like family in the same expression system [18, 22], which provides a well-defined soluble protein that would be suitable for clinical and scientific purposes.

To complete the characterization of Che a 1 and to confirm the IgG and IgE correspondence between rChe a 1 and nChe a 1, their responses to a rabbit antiserum and

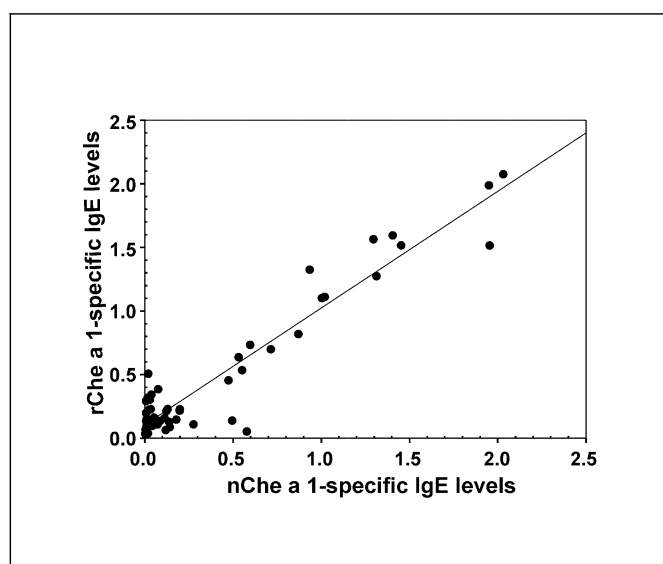


Fig. 7. Analysis of the IgE-binding levels to purified nChe a 1 and rChe a 1 using 50 sera from patients allergic to chenopod pollen. Their responses at 492 nm are shown in OD arbitrary units.

to a mouse antibody, both raised against Ole e 1, and to sera of patients allergic to chenopods were evaluated in ELISA and immunoblotting, as well as by inhibition assays. No significant differences in the recognition were found by both IgG antibodies and sera of the patients, as has been demonstrated in the different experiments. Moreover, the IgE-binding correlation between both proteins and the slope of the linear regression of the experiment, which was close to 1, affirm that both allergens are similar at IgE-binding level. These results indicate that IgG and IgE epitopes of the natural allergen are conserved in the expressed protein and were preserved during the purification steps.

Asn-39 is the single N-glycosylation consensus site detected in the amino acid sequence of Che a 1 [12]. ConA treatment demonstrated that the recombinant protein as well as the natural form is glycosylated. By MS analysis, a heterogeneous glycosylated form was observed for rChe a 1. A similar result was demonstrated to occur for the recombinant forms of Ole e 1, Lig v 1 and Syr v 1 [21, 22] as a consequence of the glycosylation machinery of *P. pastoris*, which is specialized in producing high-mannose-type N-glycans [28]. The glycan composition, Man₉₋₁₁GlcNAc₂, is in agreement with those reported for other glycosylated proteins since *P. pastoris* links a glycan group at the N-consensus site with 8–15 mannose residues [28]. The glycan moiety of rChe a 1 is bigger than that of nChe a 1, as the apparent molecular mass (fig. 3) and MS data of both glycosylated proteins showed. From molecular mass calculations of glycosylated and deglycosylated

forms of these proteins, a composition of 4–5 C₆-monosaccharides plus 2 GlcNAc can be deduced for the natural glycan of Che a 1. Moreover, this glycan group should contain a α 1→3 fucose residue linked to the innermost N-GlcNAc, according to the specificity of PNGase F [29].

The carbohydrate moiety of Ole e 1 was reported to be involved in the recognition by IgE and IgG antibodies [25, 30]. The relevance of the carbohydrate moiety of Che a 1 in IgE binding has been investigated in this work. Indirect evidence demonstrated that the glycan group of Che a 1 does not constitute a relevant epitope, since glycans in recombinant and natural Che a 1 are different, and IgE inhibition assays in immunoblotting attained similar values when inhibited with natural or recombinant forms (fig. 6). Analogous results were reported for Pla 1 [31]. The lack of IgE epitopes in the carbohydrate moiety of Che a 1 reduces the possibility of cross-reactivity with other members of this family of allergens.

In conclusion, this work shows that rChe a 1 expressed in yeast behaves similarly to the natural form of the allergen.

Acknowledgments

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