

Date Palm Pollen Allergoid: Characterization of Its Chemical-Physical and Immunological Properties

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Key Words

Date palm pollen allergy · Autochthonous palm pollen
extract · Monomeric allergoid · Hypoallergenic extract ·
Potassium cyanate

Abstract

Background: Date palm (DP) pollen can cause allergic symptoms in people living in different countries. Specific immunotherapy with allergenic extracts by subcutaneous route is effective to cure allergic people. However, the risk of side effects has led to explore safer therapeutic modalities. The aim of our work was to evaluate IgE cross-reactivity between DP and autochthonous palm (European fan palm, EFP) pollen extracts, to chemically modify DP extract with potassium cyanate in order to obtain an allergoid, and to characterize it. **Methods:** By radioallergosorbent test inhibition, immunoblotting (IB) and skin prick test, in vitro and in vivo allergenic activities of native and modified DP extracts were compared. By SDS-PAGE and IB, we compared the protein profile and IgE-binding capacity of both native and modified DP, as well as of EFP extracts. By IB inhibition, IgE cross-reactivity of native DP and EFP extracts was evaluated. By ELISA, the capacity of modified DP-induced IgG to react with native DP extract was determined. **Results:** Radioallergosorbent test inhibition, IB and skin prick test results demonstrated that modified DP was significantly less allergenic than

native DP extract. The SDS-PAGE profile showed that potassium cyanate treatment of DP extract did not alter the molecular weight of its components. In addition, no difference was observed between native DP and EFP extracts. Subsequent IB inhibition data evidenced the existence of a strong IgE cross-reactivity between native DP and EFP extracts. ELISA results indicated that the administration of modified DP in mice was able to induce specific IgG also recognizing native DP extract. **Conclusions:** Modified DP extract (allergoid) seems to be a good candidate for immunotherapy of patients affected by specific allergy.

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Introduction

The importance of date palm (DP, *Phoenix dactylifera*) pollen and pollen from other members of the palm family as potent inhalant allergens has been reported from different areas of the world where these palms grow [1–11]. The intense influx of immigration from these countries to Italy where there is an abundance of autochthonous palm tree species, such as the European fan palm (EFP, *Chamaerops humilis*), could in the near future increase the risk for sensitized migrating people of developing allergic symptoms.

According to recent estimates, the prevalence of palm pollen allergy ranges from 13% in the United Arab Emirates to over 44% in India and Saudi Arabia [3, 9, 10]. The existence of date palm cultivars which are differentially allergenic can create problems [12–14], making it difficult to devise precise in vitro and in vivo diagnosis, a factor which in turn creates gross underestimates of the real incidence of this kind of allergy [15]. The use of diagnostic extract prepared from highly allergenic cultivars which contain all major date pollen allergens is fundamental to identifying people affected by such a specific allergic disease and to develop a vaccine for specific immunotherapy [14, 16, 17].

In the last 10 years, an approach based on the chemical modification of allergens with potassium cyanate (KCNO), under conditions that promote the selective substitution of the ϵ -amino groups of the lysine residues of the protein molecules, has been found useful for the development of peculiar vaccine for therapeutic purposes [18]. Such allergoids are characterized by a significant reduction in allergenic potency (probably as the result of an alteration in IgE epitopes of allergenic molecules) while still maintaining their immunogenic properties (capacity to induce IgG antibodies able to interact with native extract). Furthermore, unlike polymeric allergoids (such as those obtained by reaction with aldehydes) which are characterized by a strong increase in molecular dimension of their components due to inter-/intramolecular cross-linking phenomena [19, 20], they maintain a native molecular (monomeric) dimension of their own components [18].

The aims of the present study are: (1) to evaluate cross-reactivity between DP and EFP pollen extracts, (2) to modify DP pollen extract by chemical reaction with KCNO, (3) to characterize the chemically modified DP extract for its allergenic and immunogenic potential as well as its physical-chemical features, and (4) to compare the in vivo allergenic potential of modified DP extract in respect to nonmodified DP extract.

Data are discussed with the perspective of developing specific products to be used for immunotherapy of patients affected by date pollen and related allergy.

Materials and Methods

Preparation of Palm Pollen Extracts

Extracts were prepared using pollen collected in Riyadh, Saudi Arabia, from highly allergenic DP *P. dactylifera* trees [12, 14] and from pollen of EFP *C. humilis*, provided by Dr. Raddi (Istituto per la Protezione delle Piante, CNR, Sesto Fiorentino, Florence, Italy). Pollens were defatted in diethyl ether before being

submitted to 5% (w/v) aqueous extraction in 0.125 M NH_4HCO_3 for 4 h at 4°C under continuous stirring. The suspensions were centrifuged at 20,000 g for 1 h at 4°C and supernatants were extensively dialyzed against distilled water.

To further purify the proteins, ammonium sulfate precipitation of DP and EFP extracts was performed by slowly adding ammonium sulfate to obtain an 80% saturated solution. After stirring for 4 h at 4°C, the precipitated proteins were recovered by centrifugation at 20,000 g for 1 h at 4°C, redissolved in 1/5 of the initial volume with water, dialyzed extensively against deionized double-distilled water to eliminate the residual salt, and lyophilized.

Chemical Modification of DP Pollen Extract

Lyophilized DP pollen extract was reconstituted in 20 mM phosphate buffer (pH 7.2), and for the chemical modification, the protein concentration was adjusted to 1 mg/ml according to the Bradford method [21]. A volume of 2.5 ml of the extract was passed through a Sephadex G-25 column (PD10, Amersham Pharmacia Biotech, Milan, Italy) which was previously equilibrated in the same buffer. A fraction of 3.5 ml of excluded peak was collected according to the manufacturer's instructions and then submitted to chemical modification as previously described [18]. Briefly, solid sodium tetraborate and potassium cyanate were added to 2.5 ml of gel-filtered sample at final concentrations of 0.1 and 1 M, respectively. A control sample (native) treated with 1 M KCl instead of KCNO was also prepared using the same procedure described above. After 20 h at 40°C, salts from both extracts were eliminated by gel filtration on a G-25 column (PD10, Amersham Pharmacia Biotech). Native and modified DP extracts were analyzed for total protein content by the Lowry method [22] and used for all subsequent experiments.

Preparation of Solid Phases

Polystyrene beads (6.4 mm diameter; Precision Plastic Balls, Chicago, Ill., USA) were coated with DP pollen extract according to the following procedure. Beads were pretreated with 0.2 M of glutaraldehyde in PBS for 5 h at room temperature. After washing out glutaraldehyde excess, they were incubated overnight in end-over-end agitation with 150 μl /bead of native extract at a concentration of 10 $\mu\text{g}/\text{ml}$ in PBS. After several washings with PBS, the beads were stabilized with NaHBO_3 0.37% in PBS for 1 h and finally saturated with 5% BSA in PBS for 24 h.

Radioallergosorbent Test Inhibition Analysis

Radioallergosorbent test (RAST) inhibition analysis was performed according to Ceska et al. [23] in order to compare allergenic activity of native DP and KCNO DP extracts.

Briefly, several two-fold dilutions of the two considered extracts (starting from the same protein concentration of 2 mg/ml) in 1% BSA in PBS were preincubated for 3 h with a pool of sera from DP pollen-allergic patients. Subsequently, one extract-coated bead was added to each tube and incubated overnight. Bound specific IgE was detected after further incubation for 8 h with ^{125}I -labelled goat anti-human IgE (KLH, Celbio, Milan, Italy) diluted in PBS containing 1% BSA in order to obtain 30,000 cpm/50 μl . After further washings, residual radioactivity on each bead was measured by a gamma scintillation counter. Data were expressed as percentage of inhibition in respect to a maximum binding obtained without inhibitor, and the C_{50} value was defined as the

amount (in micrograms) of extract needed to give 50% of inhibition of the response.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed to compare the protein profiles of both native and modified *P. dactylifera* and *C. humilis* pollen extracts. Experiments were carried out in 10% polyacrylamide precast Nupage Bis-Tris gels according to the manufacturer's instructions (Novex, Prodotti Gianni, Milan, Italy). Thirty micrograms of extracts were loaded per centimeter of gel and then ran at 180 mA for 1 h. The resolved proteins were stained with Coomassie Brilliant Blue (Colloidal Blue staining kit, Amersham) and subsequently destained with water.

Immunoblotting and Immunoblotting Inhibition

The protein components of both native and modified *P. dactylifera* and *C. humilis* pollen extracts, resolved by SDS-PAGE as previously described, were transferred onto nitrocellulose membrane (0.45 mm; Protran BA 85, Schleicher & Schuell, Whatman, Dassel, Germany) according to the method of Towbin et al. [24]. The membrane was saturated in Tris-buffered saline containing 0.05% Tween-20 and 5% defatted dry milk (saturating buffer), before incubation with positive or negative human sera diluted 1:2 in saturating buffer. Bound specific IgE was detected by 1:2,000 peroxidase-conjugated goat anti-human IgE antiserum (KPL, Celbio) in saturating buffer, using the ECL Blotting Kit (Amersham) as substrate of peroxidase. In immunoblotting (IB) inhibition studies, the IgE reactivity was inhibited by preadsorption of the human positive sera pool (800 μ l) with EFP extract (200 μ l), putting them in a tube. After 4 h of precontact, all was transferred onto native DP blotted membrane which was submitted to the same procedure described before.

Immunization Protocol

Three Balb/c mice (Charles River, Calco, Como, Italy) were injected subcutaneously with 20 μ g of chemically modified DP pollen extract. The first injection was performed with extract emulsified in complete Freund's adjuvant. It was followed by 3 subsequent injections, at weekly intervals, with extracts emulsified in incomplete Freund's adjuvant. Three days after the last booster, mice were bled by the tail vein, their sera were pooled and tested for the ability to recognize both native and modified DP extracts by ELISA.

ELISA Procedure

Specific IgG antibody titer against native and modified DP pollen extracts was determined by the ELISA method. Briefly, microtiter plates (Maxisorp, Nunc, Mascia Brunelli, Milan, Italy) were coated with 0.1 μ g/well of native or modified DP extracts. Hundred microliters of several two-fold dilutions of the pooled mouse sera in PBS/2% BSA were added to the wells. Bound specific IgG was detected by adding a 1:2,000 dilution of peroxidase-conjugated goat anti-mouse IgG serum (Sigma, Milan, Italy); the subsequent colorimetric reaction was developed using TMB/ H_2O_2 as substrate, and data were expressed as optical density values by reading at 450 nm.

Human Sera

Twelve human sera from patients allergic to DP pollen, provided by Dr. Harfi (National Center of Allergy, Asthma and Im-

munology, Riyadh, Saudi Arabia), were pooled and used to perform RAST inhibition and IB experiments. Sera derived from patients with skin prick test (SPT) were positive to highly allergenic cultivars as previously described [16].

SPT Experiments

All patients selected for SPT experiments were atopic and have had a long-standing history of date pollen sensitivity. In addition, they were all SPT positive to the native DP extract prepared for the present study. Patients had a mean age of 26 years and a male:female ratio of 10:12. All of them gave written consent as approved by the Basic, Clinical and Ethics Research Committees of the King Faisal Specialist Hospital and Research Centre, and the institutional guidelines were strictly followed throughout the study.

Twenty-two patients were enrolled in the study and told not to use antihistamines for at least 4 days before being submitted to the test. SPT was carried out with 3 different dilutions (1:1, equal to 1 mg/ml as in the Lowry method, 1:3, and 1:9) of both native and modified DP extracts on the volar surface of the forearm with sterile 1-mm-tip lancets. Negative and positive controls were performed using a glycerin solution and histamine phosphate (10 mg/ml), respectively. In addition, SPT was performed on 3 nonallergic individuals. Readings were taken after 15 min and reactions were expressed as the mean wheal diameter in millimeters (adding the longest diameter to the orthogonal one and dividing it by 2). A wheal diameter of 3 mm or more was considered positive.

Statistical analysis of wheal sizes was done using the paired t test, performed using Unistat Light for Excel.

Results

Characterization of Both Native and Modified DP and EFP Pollen Extracts

Protein contents of both native and modified DP and EFP extracts assayed by the Lowry method were 2.3, 2.15 and 3.7 mg/ml, respectively.

As no significant difference in terms of allergenic potency has been observed between not-KCl-treated and KCl-treated native DP extracts (data not shown), KCl-treated native DP extract has been used as control sample (defined as 'native') for all subsequent experiments. RAST inhibition experiments comparing allergenic potency of native and modified DP extracts are shown in figure 1. The C_{50} value (defined as micrograms of extract able to inhibit 50% of IgE binding) for the native DP extract was 0.08 μ g compared with 16.03 μ g for the modified DP extract. This observation indicated that modified DP extract was about 200 times less allergenic than the unmodified one.

SDS-PAGE analysis of native DP extract showed the presence of several protein bands with molecular weights ranging from 10 to about 100 kDa. In particular, bands at about 14, 25, 28–30, 37–40, 55–70 and 90–100 kDa, corresponding to the main allergens reported from the literature [16, 25], were present (fig. 2a). No qualitatively

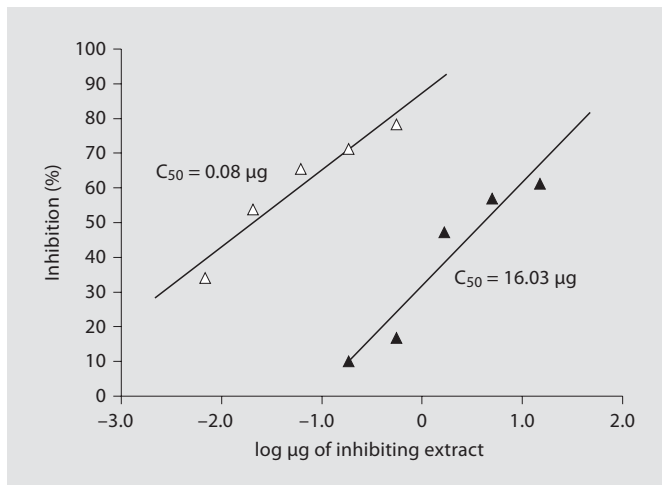


Fig. 1. RAST inhibition experiments. Serial dilutions of native ($\triangle$) and modified ($\blacktriangle$) DP pollen extracts (starting concentration at 2 mg/ml) were used to inhibit the binding of specific IgE to solid phases coated with native palm pollen extract. The graph shows the percent of inhibition obtained as function of protein amounts of the used inhibitors.

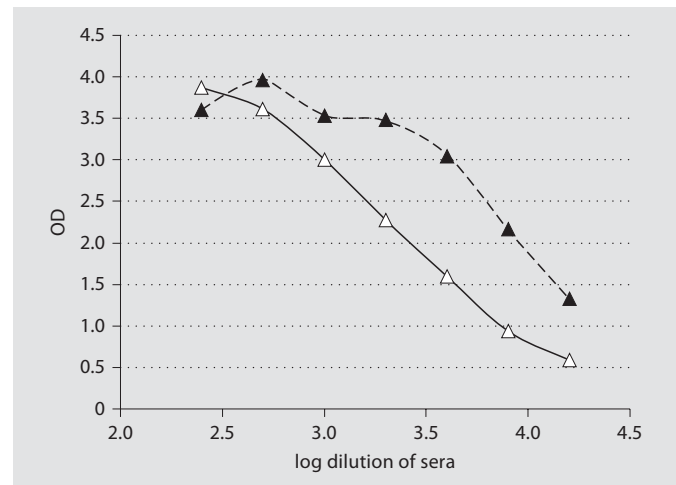


Fig. 3. ELISA experiments. A group of 3 mice was immunized with modified DP pollen extract. Their sera were pooled, and serial dilutions of the pool were tested by ELISA using both native ($\triangle$) and modified ($\blacktriangle$) DP extracts as antigen.

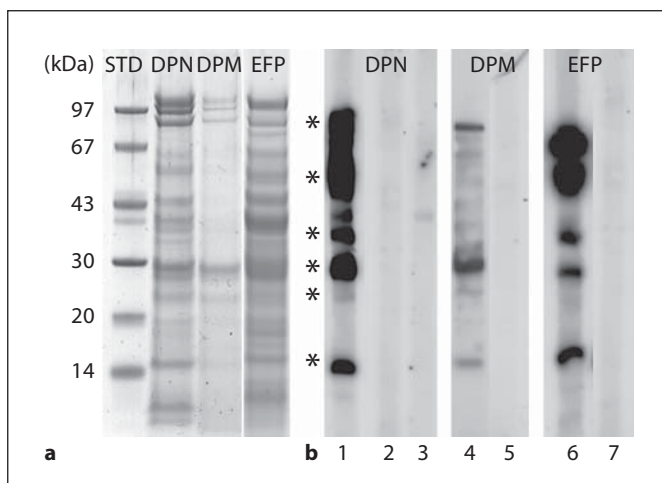


Fig. 2. **a** SDS-PAGE of native (DPN) and modified (DPM) DP pollen and EFP pollen extracts. Thirty micrograms of each extract were loaded per centimeter of gel and then ran under reducing conditions. The gel was stained with colloidal Coomassie Brilliant Blue. STD = Molecular weight standards: phosphorylase B (97 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) trypsin inhibitor (20 kDa) and lactalbumin (14 kDa). **b** IB and IB inhibition experiments. Lanes 1, 4 and 6 show IgE reactivity of pooled positive sera against native and modified DP and EFP extracts, respectively; lanes 2, 5 and 7 show IgE reactivity of negative pooled sera against native and modified DP and EFP extracts, respectively; lane 3 shows inhibition of IgE reactivity against DP by preincubation of pooled positive sera with EFP extract.

significant differences in SDS-PAGE profile were seen using modified DP extract, except that protein bands in the modified DP extract stained faintly. The less intense stain of the modified proteins is most probably due to a lower capacity of Coomassie dye to bind them [26]. A very similar protein profile was observed between DP and EFP extracts.

IB experiments using a pool of sera from allergic patients are shown in figure 2b. Bands corresponding to major allergens were well evidenced in both native DP and EFP extracts. The chemical modification of DP extract caused a wide reduction in its IgE-binding capacity, which is more evident for the 14-kDa component and for those between 40 and 100 kDa; a residual IgE reactivity was preserved by 30-kDa allergen. The pool of sera from nonallergic patients did not show any IgE reactivity.

Finally, IgE reactivity to native DP extract was totally abolished by preabsorption of sera with EFP extract.

ELISA Experiments

In order to verify the capacity of the chemically modified DP extract to elicit an IgG response against its native, nonmodified counterpart, mice were immunized with modified DP extract, and their pooled sera were tested by ELISA using both native and modified DP extracts as antigen. As shown in figure 3, mice sera, as expected, showed a strong IgG response against modified

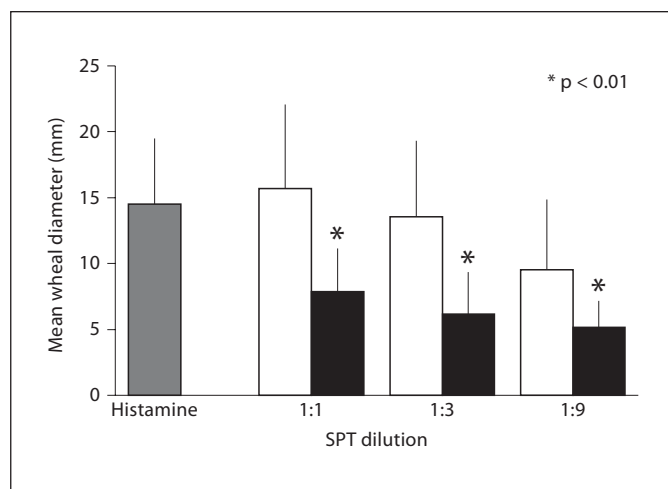


Fig. 4. SPT results. Comparison between native (□) and modified (■) DP pollen extracts by SPT at different dilutions (1:1, 1:3 and 1:9). SPTs were performed on 22 palm pollen-allergic subjects, and the response was calculated as mean wheal diameter. Bars represent the mean of results for each dilution and positive control (histamine). Error bars represent standard deviation.

DP extract; in addition, such antibodies were able to recognize native, nonmodified DP extract, even if at a lower level.

SPT Results

In vivo allergenic activity of modified DP extract was significantly different ($p < 0.01$) from native DP extract at any considered dilution (fig. 4). In particular, the mean wheal diameters were 15.7, 14.3 and 10.3 mm for the native and 8.6, 7.6 and 6.8 mm for the modified extract at 1:1, 1:3 and 1:9 dilution, respectively. The mean wheal diameter induced by histamine was 14.5 mm. The SPT in non-allergic individuals using 1:1 dilution of native and modified DP extracts did not reveal any estimable skin reaction.

Discussion

Pollen from members of the Arecaceae (Palmaceae), in particular the date palm (*P. dactylifera* L.), can cause allergic symptoms, especially among people living in Middle Eastern, North African and some Mediterranean countries, as well as in India [1–11]. Previous papers evidenced the presence of several allergens in DP pollen extract, the more clinically relevant being those at 14, 25, 28–30, 37–40, 55–70 and 90–100 kDa [12, 16, 17]. In addition,

a different allergenicity of pollen derived from different cultivars has been observed [12, 14]. However, until now, no study has been performed on the eventual IgE cross-reactivity between *P. dactylifera* palm tree and European autochthonous palm tree (*C. humilis*) pollen extracts. The present paper represents the first study exploring such a possibility. IB and IB inhibition studies using a pool of human sera from patients allergic to extract derived from *P. dactylifera* pollen clearly demonstrated a strong IgE cross-reactivity between the latter and extract prepared from *C. humilis* pollen. The existence of such cross-reactivity shows the risk for DP pollen-allergic people migrating from North Africa to Italy or to other Southern European countries of developing allergic symptoms during exposure to pollen of autochthonous palm trees.

To date, no specific DP extract used for therapeutic purpose has been developed. Evidences demonstrated that a therapeutic approach based on the administration of extracts by the subcutaneous route is able to control the allergic symptoms of patients [27]. However, the risk of inducing side effects, sometimes associated with the injection of the above products [28], has led allergen manufacturers to explore safer therapeutic modalities. KCNO-modified allergens have proven useful for developing original, safe and effective products such as monomeric allergoids [18, 29–36]. An analogous approach has been performed on DP pollen extract. The extract was extensively characterized by using different physical-chemical and immunological techniques. Results of these investigations revealed some interesting aspects of chemically modified DP extract compared with the unmodified extract. Our comparative RAST inhibition experiments indicated that treatment of DP extract with KCNO significantly reduced its allergenic potency. This is probably due to the fact that KCNO, reacting with the ϵ -amino groups of the lysine residues of allergen molecules, is able to reduce the interaction of specific IgE antibodies and B cell epitopes present on the above allergen molecules. The importance of lysine residues in the expression of IgE epitopes was also confirmed by a recent paper of Gehlhar et al. [37]. A substitution of 10 out of 12 lysine residues with alanine of Phl p5b allergen was enough to significantly reduce the IgE-binding capacity of the molecule. At the same time, the capacity of specific IgG to react against modified molecules was not diminished. This last observation agreed with our ELISA experiments with sera from animals immunized with palm tree pollen allergoid extract. In fact, these experiments demonstrated that modified DP extract (allergoid) was able to induce specific IgG antibodies also recognizing the native, unmodified DP

extract, suggesting that treatment with KCNO did not alter the immunogenic properties of the derived extract.

In the present study, we did not evaluate the direct effects of the DP allergoid on T cell reactivity. However, considering that immune response to allergens is a T-lymphocyte-dependent reaction, the above observation can constitute indirect evidence that the allergoid is able to interact with T cells.

The reason why KCNO treatment would preferentially alter IgE in respect to IgG epitopes is not known. We can hypothesize that the number of IgE epitopes can be significantly lower than that of IgG epitopes, or alternatively, that the modification of few critical lysine residues can have a greater effect on IgE-binding capacity, in view of their high level of affinity, than on IgG epitopes. The possibility to alter the IgE reactivity of an allergen without significantly compromising its capacity to be recognized by specific IgG antibodies opens new perspectives for immunotherapy (SIT) of people suffering from a specific allergy.

In addition, another important feature was preserved in the DP allergoid. As shown in figure 2, the SDS-PAGE profile of native and modified DP extracts appears to be very similar, suggesting that the molecular dimension of various components of the modified extract was not altered by reaction with KCNO. To underline this feature, we coined the term 'monomeric allergoid' for the derived product. In the above figure, a difference in color intensity of corresponding bands of native and modified DP extracts was observed. As the same amount of proteins has been run, the reduced staining of bands of the DP allergoid should be the result of KCNO treatment. In fact, Tal et al. [26] reported a striking correlation between the intensity of reaction with Coomassie dyes and the presence in a sample of basic charged amino acid residues such as lysine, histidine and arginine. Reaction of extract proteins with KCNO induces a transformation of lysine residues into the ureido group [38] shifting the net charge of proteins involved towards more acidic pI. Therefore, it is highly probable that a sample of modified DP extract is less stainable by Coomassie dye than the native DP extract.

The preservation of the molecular size of KCNO-treated DP allergens (monomeric allergoid), associated with the other characteristics mentioned above, represents a very important element in the development of a vaccine to be used for SIT by the sublingual route. Other monomeric allergoids have been successfully used in clinical trials, including dibromochloropropane studies [33–36], and absorption of allergens through the mucosal membrane seems to be important in the expression of their therapeutic effects [39, 40].

The above peculiarity of KCNO-treated allergens allows the possibility to distinguish monomeric from polymeric allergoids such as those obtained by the reaction of allergens with glutaraldehyde [19, 20]. Polymeric allergoids are characterized by a significant increase in the molecular size of their components (reaching more than 1,000 kDa) as result of inter-/intramolecular cross-linking. As the molecular dimension of a substance can in general hinder its ability to cross the mucosal membrane, it is reasonable to imagine that polymerized allergoids could encounter some obstacles in passing through mucosal tissue and be adsorbed. This fact raised many doubts about the potential usefulness of administering polymeric allergoids by the sublingual route in the SIT approach; moreover, it constitutes the reason why polymerized allergoids are nowadays administered only by the subcutaneous route to express their optimal clinical effectiveness [41–43]. The existence of a unique paper that dates back more than 20 years, describing the clinical efficacy of polymerized allergoids when administered by the intranasal route, does not seem enough to eliminate existing doubts about their use by ways of administration different from the subcutaneous one [44].

In conclusion, the reduced allergenic potency of the monomeric DP allergoid as evidenced by in vitro (RAST inhibition and IB) and in vivo (SPT on allergic volunteers) experiments, associated with a preservation of the molecular size of its own components, makes it a good potential candidate to be used via the sublingual route for the SIT of patients allergic to palm tree pollens, including those who develop allergic symptoms by exposure to pollen from autochthonous species such as *C. humilis*.

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