

Role of carbohydrate moieties in IgE binding to allergenic components of *Cupressus arizonica* pollen extract

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Summary

Background A reduction of IgE immunoreactivity after periodate-treatment has been previously reported for various glycoprotein allergens.

Objective The aim of this study was to investigate the role of glycan moiety of a *C. arizonica* extract in the binding of patients' IgE and to identify the carbohydrates possibly involved.

Methods The reactivity of IgE with *C. arizonica* extract, before and after periodate-treatment, was evaluated by immunoblotting and ELISA inhibition. The specificity of carbohydrate-reactive IgE was evaluated by ELISA using unrelated glycoproteins with known sugar composition and structure, such as pineapple bromelain, honeybee venom phospholipase A₂, and ovalbumin, before and after periodate treatment.

Results When periodate-treated *C. arizonica* extract was probed after SDS-PAGE and immunoblotting with patients' IgE, no reactivity could be detected. Furthermore, a very poor inhibitory activity of the periodate-treated *C. arizonica* extract as compared with the untreated sample could be observed in the ELISA inhibition experiments performed using *C. arizonica* extract as antigen. When phospholipase A₂ and bromelain were used as antigens in ELISA, they were recognized by patients' IgE, whereas ovalbumin was negative. Treatment of phospholipase A₂ and bromelain with periodate completely abolishes the IgE reactivity.

Conclusion A large portion of the IgE reactivity of Cupressaceae-allergic subjects appears to be associated with sugar moieties of *C. arizonica* extract which appear to be shared by bromelain and phospholipase A₂, thus suggesting that the IgE of patients reacting with such epitopes probably react with β 1 $\rightarrow$ 2 xylose, α 1 $\rightarrow$ 3 fucose and/or α 1 $\rightarrow$ 6 fucose.

Keywords: cross-reactivity, *Cupressus arizonica*, glycan epitopes, IgE

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Introduction

Species of the Cupressaceae family have been identified as responsible for an increasing number of cases of allergy over the winter period [1–9]. *Cupressus arizonica* plants account for the majority of the *Cupressus* genus currently spread in the Mediterranean area [7], due to its use not only in the reforestation programs but also for gardening and

park. Thus, we focused our attention on the preparation and characterization of extracts from this species [10] and we demonstrated by SDS-PAGE and immunoblotting [10] that *C. arizonica* extract (CaE) contains several glycoprotein components that are able to react with a panel of plant lectins [10], with monoclonal antibodies specific for periodate-sensitive epitopes [11], and with IgE of allergic patients.

Since recent data suggest that carbohydrate moieties are involved in the IgE binding to allergens [12–15], in the present paper we evaluated the role of glycan moieties of

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CaE allergenic components in the binding of IgE from Cupressaceae-allergic subjects.

Materials and methods

Antigens

Pollen from *C. arizonica* was purchased from Allergon (Angelholm, Sweden). The extract was prepared as previously described [10]. Pineapple stem bromelain (BRO), phospholipase A₂ (PLA₂) from honeybee venom and ovalbumin (OVA) were purchased from Sigma Chemical Company, Milan, Italy. Protein content was measured according to the method of Bradford [16].

Human sera

Fifteen human sera were obtained from allergic patients sensitive to species of the Cupressaceae family, as proven by results of skin prick tests and specific *in vitro* IgE detection (0.2–2.8 optical density range in the ELISA for *C. arizonica*). Out of these 15 sera, samples from three subjects with highly positive results (1.8–2.8 optical density) and containing IgE able to recognize most of the components as proven by immunoblotting were pooled and stored in aliquots at –20 °C.

Subjects were free from allergen-specific immunotherapy and their informed consent to participate in the study was obtained.

Serum samples to be used as negative controls were collected from two nonatopic volunteers.

Monoclonal antibodies

Two monoclonal antibodies, named 2D5 and 4A6, raised against *C. arizonica* components [17] were used to evaluate the quality of the batch of periodate-treated *C. arizonica* extract used throughout the experiments.

Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE and electrotransfer were carried out as previously described [18,19] in a mini-system (Bio-Rad, Richmond, CA, USA). CaE (10 µg protein/well) was reduced by 50% v/v 2-mercaptoethanol before application to the 15% w/v polyacrylamide gel. The gel was stained with 0.05% Coomassie Brilliant Blue in water: methanol: acetic acid (50:40:10), for protein detection. Immunoblotting was performed as described by Barletta *et al.* [18].

Carbohydrate analysis

Carbohydrate content and composition was evaluated in the extract by the phenol-sulphuric acid method [20], and by the High Performance Liquid Chromatography (HPLC) method, as described in [21], respectively.

Direct ELISA and ELISA inhibition

Direct ELISA was performed as described by Di Felice *et al.* [10]. Polyvinyl chloride plates (Falcon 3912; Becton Dickinson, Oxnard, CA, USA) were coated with 0.2 mL/well of 20 µg/mL of CaE, and with either BRO (13.2 µg/mL), or PLA₂ (9.6 µg/mL), or OVA (26.4 µg/mL) in 0.1 mol/L carbonate buffer, pH 9.6. Plates were blocked with PBS/Tween-20 0.1% v/v.

ELISA inhibition was performed according to the procedure previously developed in our laboratory [19]. Antigen concentrations were the same as for direct ELISA. Inhibition was carried out by incubating for 3 h at room temperature the pooled human sera, prediluted 1:2.5 in PBS-Tween-1% w/v gelatine, with an equal volume of fivefold serial dilution of CaE as inhibitor (concentrations ranging from 0.03–100 µg protein/mL).

Periodate treatment

Deglycosylation was performed by periodate treatment of soluble CaE (S-CaE), carried out essentially according to Hansen *et al.* [22]. Treatment of CaE and of unrelated glycoproteins adsorbed onto the ELISA plate was performed essentially according to Woodward *et al.* [23].

In the case of treatment in solution of the extract, 6.3 mg of sodium meta-periodate were added to 7 mg of CaE dissolved in 3 mL of 0.01 mol/L acetate buffer pH 4.4, to give a final concentration of 0.01 mol/L. This solution was kept under gentle shaking at 4 °C for 6 h and 30 min in the dark. Two drops of ethylene glycol and 3 mg of sodium borohydride were added to the solution, which was further incubated under gentle shaking for 19 h at 4 °C. Finally, drops of 0.2 mol/L acetic acid were added to the solution to bring the pH to 5. The mixture was then extensively dialysed against distilled water, concentrated to final volume of 200 µL and dialysed against ammonium bicarbonate 0.125 mol/L with collodium membranes by Sartorius system (Sartorius AG, 37070, Goettingen, Germany). A 'mock control' sample was prepared by treating in the same way an equal amount of extract using in all the steps identical buffers and reagents but omitting periodate.

In the method involving the treatment of the antigen already adsorbed onto the plate, 0.2 mL/well of a 10-mmol/L solution of sodium periodate in 0.05 mol/L, pH 4.5, acetate buffer were added for 1 h at room temperature in the dark to

antigen-coated plates previously blocked as described in direct ELISA.

After two washings with acetate buffer, 0.2 mL/well of a 0.05-mol/L sodium borohydride solution in PBS were added for 30 min at room temperature in the dark and after further washings with PBS-Tween the treated plates were incubated as in direct ELISA. All buffers and reagents without periodate were used for the treatment of control wells (mock).

Results

Results obtained by SDS-PAGE with the periodate-treated S-CaE are reported in Fig. 1. From a qualitative point of view, the whole extract, the mock and the periodate-treated extract showed a comparable electrophoretic pattern after Coomassie staining (lanes a–c). To quantify the extent of the effect of the periodate treatment on the carbohydrate moieties of CaE, total sugar estimation by phenol-sulphuric acid was carried out before and after treatment. A reduction of total carbohydrate content from 74% w/w of freeze-dried extract and from 72% of the mock sample down to 45% for the periodate-treated sample was reported. Moreover, the analysis of individual sugar content performed by HPLC

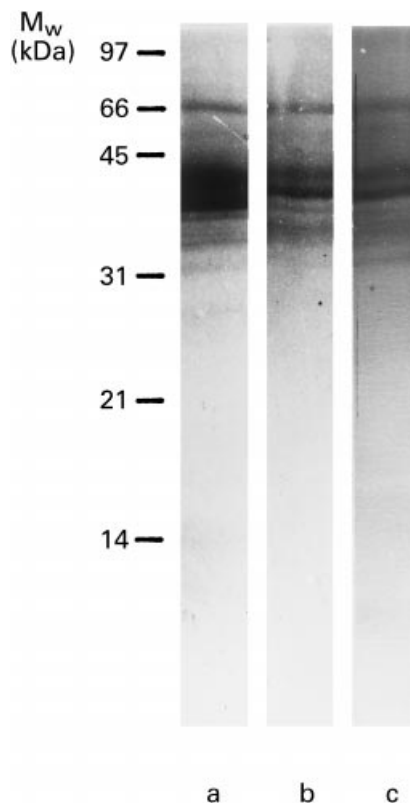


Fig. 1. SDS-PAGE pattern after Coomassie staining of untreated CaE (lane a), mock control (lane b), and S-CaE (lane c).

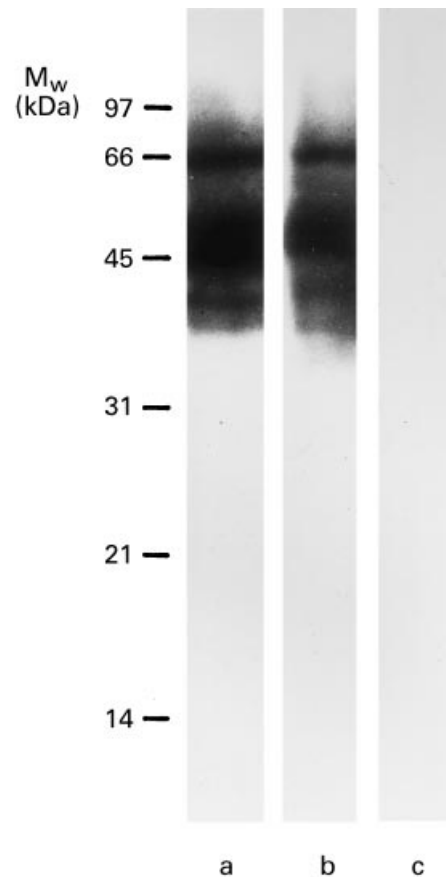


Fig. 2. Immunoblotting performed with pooled IgE from three Cupressaceae-allergic subjects of untreated CaE (lane a), mock control (lane b), and S-CaE (lane c).

showed that the treated extract had a content of galactose, glucose, mannose and fucose of about 73%, 58%, 75% and 60%, respectively, of the untreated extract, showing the mock sample values comparable with those obtained with the untreated extract.

When human sera, pooled as described in Materials and methods section, were tested in immunoblotting with native, mock and periodate-treated CaE (Fig. 2), IgE reactivity with the periodate-treated extract was completely abrogated for all components, thus indicating that IgE reactivity of the patients' sera included in the pool is essentially associated with periodate-sensitive epitopes. Native extract and mock control showed a typical pattern of reactivity. To confirm that the periodate treatment essentially does not alter the protein structure of the mostly represented allergenic components, the treated extract was probed with two monoclonal antibody specific for two different allergenic epitopes. Figure 3 reports the results obtained when the two monoclonal antibodies were titrated against native and periodate-treated CaE. It is clearly evident that no

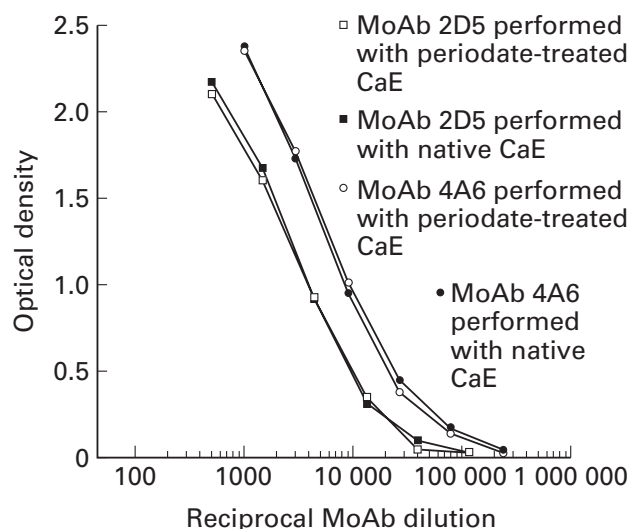


Fig. 3. Direct ELISA titration of MoAb 2D5 and MoAb 4A6 performed with either native or periodate-treated CaE.

differences could be detected between treated and untreated samples.

To investigate whether the behaviour of the pool of three sera could be regarded as representative of a larger patient population, sera from 15 patients were individually tested by direct ELISA using as antigen adsorbed onto the plate native, mock and periodate-treated CaE. The results obtained clearly indicate that all of them had at least 70% of their IgE antibodies specific for periodate-sensitive epitopes, ranging the residual reactivity after treatment between 0 and 30%.

To quantitatively evaluate the reactivity of IgE specific

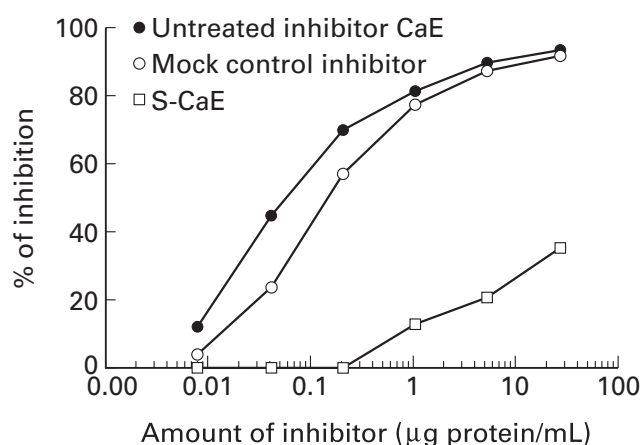


Fig. 4. ELISA inhibition of pooled IgE binding. Filled symbol represents untreated inhibitor CaE, open symbols represent 'mock control' inhibitor, or S-CaE.

for periodate-sensitive epitopes and present in the sera pool, the ELISA binding to untreated CaE was inhibited by S-CaE and mock control (Fig. 4). The inhibition curves show that IgE reactivity was better inhibited in a comparable way by untreated and mock CaE (50% inhibition was obtained with 0.055 and 0.14 µg of protein/mL, respectively). On the contrary, the reactivity of the deglycosylated extract hardly reached 35% of inhibition at the highest concentration tested (25 µg of protein/mL).

To better characterize the carbohydrate moieties involved in the IgE binding, some commercially available glycoproteins with known sugar composition and structure, PLA₂, BRO and OVA were used. Such molecules, which

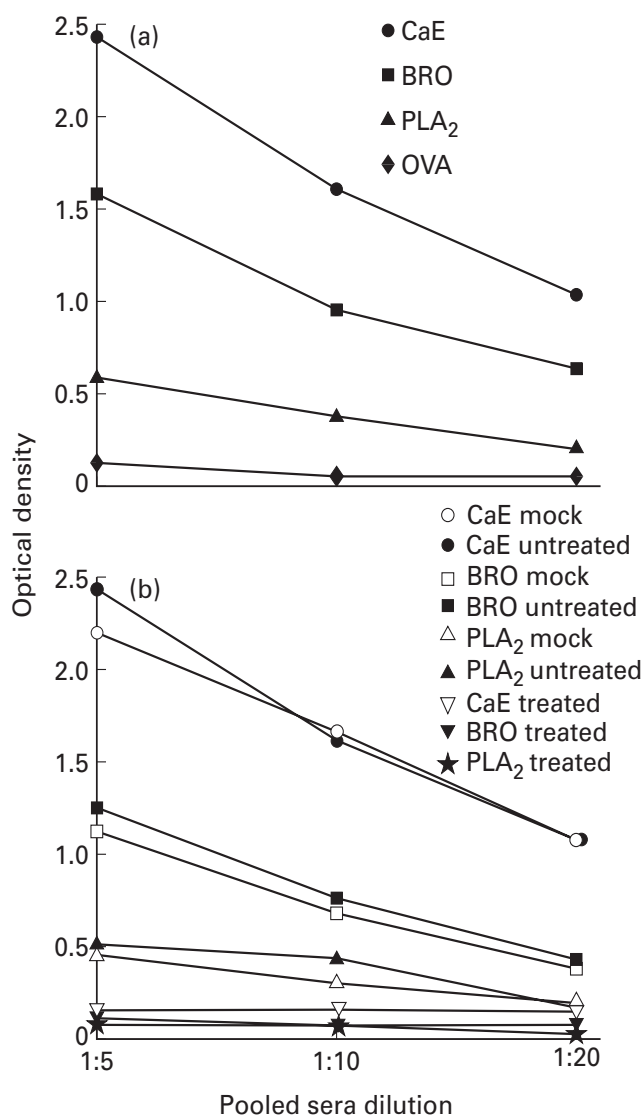
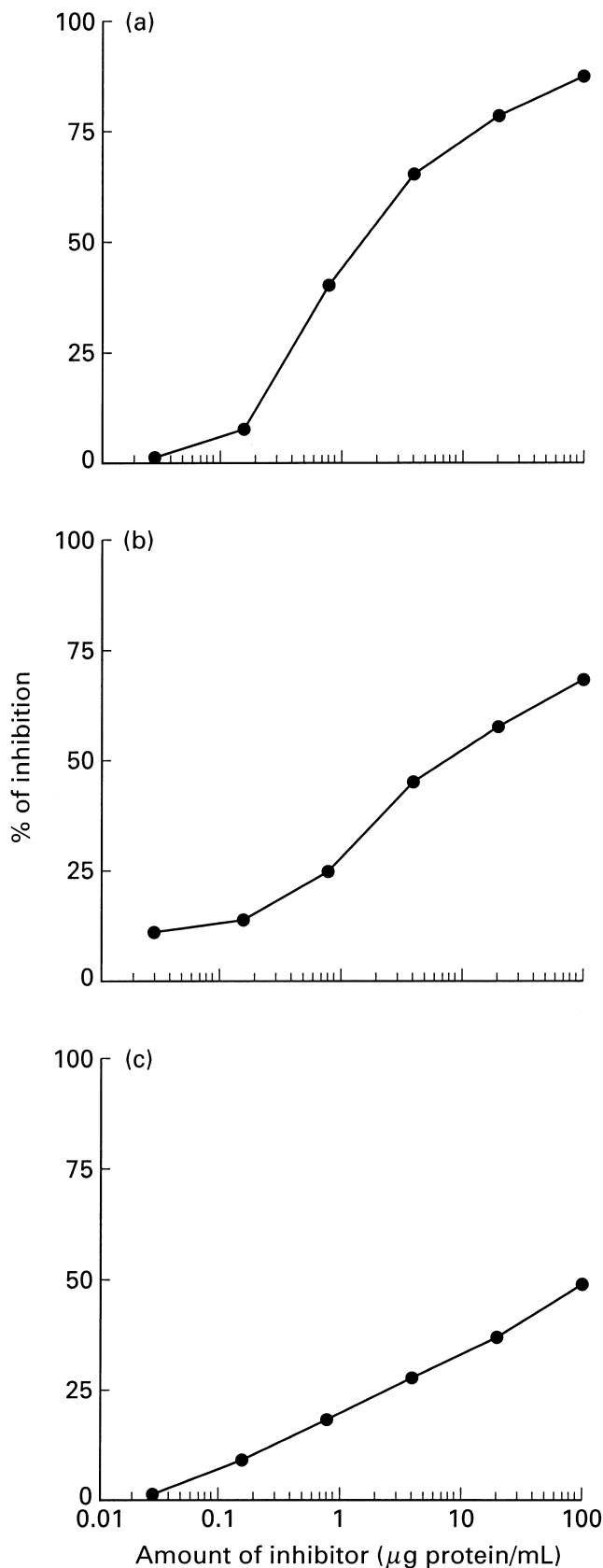


Fig. 5. ELISA titration of pooled IgE on (a) untreated antigens: CaE, BRO, PLA₂ and OVA; (b) periodate-treated antigens, 'mock control' antigens, untreated antigens as controls.



contain mannose, fucose and *N*-acetylglucosamine, were chosen on the basis of preliminary data on the sugar composition of CaE extract, as determined by data obtained by lectins analysis [10] as well as by HPLC analysis.

Thus, we firstly evaluated the reactivity of the human allergic pool against untreated BRO, PLA₂ and OVA by direct ELISA. As reported in Fig. 5, part A, IgE binding for BRO and PLA₂ was observed when the plates were coated with the untreated glycoproteins, whereas no significant binding could be demonstrated for OVA. This reactivity was completely abolished when the sugar moieties were destroyed in the adsorbed glycoproteins by periodate treatment (Fig. 5, part B). A similar result was obtained with CaE adsorbed on the plate, thus confirming the finding obtained with S-CaE. Further investigations, reported in Fig. 6, indicated that the IgE reactive with PLA₂ and BRO recognize cross-reacting epitopes shared at various extent by CaE. In fact, CaE was able to inhibit the IgE binding to BRO up to 50% with the highest (100 μg/mL) amount of inhibitor used, whereas 6 μg/mL of CaE was required to produce 50% inhibition when PLA₂ was used as antigen. Moreover, when CaE was used as antigen on the plate, 1.4 μg/mL of CaE as inhibitor was required to reach 50% of inhibition. The fact that the inhibition curves did not reach a plateau is probably due to the maximum amount of inhibitor used, since doses higher than 100 μg/mL protein were never tested due to solubility problems of CaE.

Discussion

This paper reports that periodate-sensitive epitopes of allergenic components of *C. arizonica* pollen extract do exist and are involved in the binding of IgE from allergic subjects.

As previously reported, most of the IgE-binding components of *C. arizonica* pollen extract are glycosylated since they are reactive in blotting with a panel of lectins, able to recognize mannose, glucose, fucose, galactose and *N*-acetylglucosamine [10]. Data reported in the present paper suggest that epitopes on the major allergen of CaE and on the other most reactive allergenic components of CaE [10] lose their reactivity for IgE after deglycosylation with periodate, as identified by immunoblotting analysis. These data could be hardly explained on the basis of amino acids oxidation by periodate, since it has been reported that such oxidation normally occurs when periodate is used at high concentrations, alkaline pH and room temperature [24,25]. On the basis of this assumption, we performed this step under mild conditions (10-mmol/L periodate

Fig. 6. ELISA inhibition by CaE of pooled IgE binding on CaE and two unrelated glycoproteins. Antigens adsorbed onto the plate were CaE (a), PLA₂ (b), and BRO (c).

concentration, pH 4.5, low temperature, and darkness), which are known to affect the carbohydrate groups only [26,27]. These mild conditions were experimentally confirmed by total and individual sugar analysis of S-CaE performed by HPLC, showing that the periodate treatment produced a reduction of approximately 30% only of total sugar content. Moreover, two monoclonal antibodies, raised against major allergenic components of CaE, were still able to recognize in an identical fashion native as well as treated-extract, further confirming that the treatment does not appear to alter the allergenic structures involved.

On the other hand, the existence of sugar-associated IgE epitopes is further demonstrated by the reactivity of pooled IgE from subjects allergic to Cupressaceae pollen with BRO and PLA₂, and by the abrogation of such reactivity by periodate treatment of the antigens adsorbed on the plate, thus confirming the existence of sugar epitopes shared by CaE, BRO and PLA₂.

BRO and PLA₂, as well as OVA, have one Asn-N-linked oligosaccharide per polypeptide chain, sharing a core of $\beta 1 \rightarrow 4$ mannose, $\beta 1 \rightarrow 4$ GlcNac, $\beta 1 \rightarrow$ GlcNac-N-Asn. BRO, which bears $\alpha 1 \rightarrow 6$ mannose, $\beta 1 \rightarrow 2$ xylose and $\alpha 1 \rightarrow 3$ fucose, and PLA₂, which bears $\alpha 1 \rightarrow 6$ mannose, $\alpha 1 \rightarrow 3$ mannose, $\alpha 1 \rightarrow 6$ fucose and $\alpha 1 \rightarrow 3$ fucose, were able to bind IgE from patients [28]. On the contrary, OVA, having only branched mannose on the common core [28], was not able to display any reactivity with the sera tested. Therefore, from the knowledge of the molecular structure of BRO and PLA₂ it can be suggested that $\alpha 1 \rightarrow 3$ fucose, $\alpha 1 \rightarrow 6$ fucose and $\beta 1 \rightarrow 2$ xylose are probably involved in the IgE binding with *C. arizonica* glycoproteins.

The presence of similar oligosaccharide structures on different allergens, such as Ole e 1 [28,29], Fel d 1 [30–32], and honeybee venom PLA₂ [33,34], has been recently reported and their role in the IgE cross-reactivity between related as well as unrelated allergens has been suggested. Such glycan structures are frequent on glycoproteins from plants and insects [35], and can be easily accepted as a good candidate to explain the broad allergenic cross-reactivity among various allergens from insect and plants [36–42].

Reduced IgE reactivity after periodate treatment has previously been reported for potato extract [43], timothy pollen extract [44], a glycoprotein from the yeast *Saccharomyces cerevisiae* [45], two allergens of *Aspergillus fumigatus* [22] and allergens from *Cladosporium herbarum* [26] which partially lose their IgE-binding ability after periodate treatment. More recently, Taniai *et al.* [14] showed that IgE were able to recognize only a glycosylated fragment, when tested with enzyme-digested Cry j 1, the major allergen of *Cryptomeria japonica*. Moreover, Ogawa *et al.* [15] showed that IgE carbohydrate epitopes of Cry j I were represented by a branch containing $\alpha 1 \rightarrow 6$ fucose and

a core containing fucose and xylose, confirming the idea that carbohydrate epitopes recognized by IgE do exist.

Thus, our data further indicate that IgE specific for carbohydrates play a role in the patients' reactivity with *C. arizonica* pollen components. Moreover, they support the idea that common carbohydrate epitopes, as well as panallergens such as profilin [46] and calcium-binding proteins [47,48] could influence *in vitro* and *in vivo* diagnosis and immunotherapeutic strategies.

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