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Decrease of the IgE-binding by Mal d 1, the major apple allergen, by means of polyphenol oxidase and peroxidase treatments

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Abstract

Mal d 1, the major apple allergen, is heat labile and easily oxidized. Oxidative reactions catalyzed by polyphenol oxidase (PPO) and/or peroxidase (POD), present in apple, may be involved in decreasing its allergenicity. PPO and POD convert phenolic compounds into *o*-quinones.

In this study the effect of PPO and POD, the polyphenol catechin and the antioxidant DIECA on IgE-binding by Mal d 1 was analyzed. Golden Delicious peel was selected for its high PPO and POD contents. IgE-binding was analyzed by competitive ELISA.

IgE-binding by Mal d 1 decreased by adding oxidative enzymes, this decrease was most pronounced when PPO was used. Catechin induced a reduction in IgE binding when POD was used. The combination of catechin and PPO causes the strongest decrease of the allergenicity of Mal d 1. DIECA protected the IgE-binding by the allergen, protection being less strong in the presence of exogenous PPO and POD. The decrease of immunoreactivity is likely to be due to *o*-quinones, as active species or other intermediates modifying the tertiary structure of the allergens and cross-linking of the proteins, thus reducing their allergenicity.

Keywords: Mal d 1; Polyphenol oxidase; Peroxidase; Antioxidants; Competitive ELISA

1. Introduction

Apple allergy affects up to 2% of Northern and Central European populations. Apple (*Malus domestica*, fam. Rosaceae) allergy is associated with type 1 allergy to birch (*Betula verrucosa*, fam. Betulaceae) pollen, which is an important source of inhalant allergies in the Northern hemisphere. Patients with allergy to birch pollen frequently show adverse clinical reactions to fruits, vegetables and nuts (Schöning, Vieths, Petersen, & Baltes, 1995). Allergic reactions to birch pollen-related foods are due to a primary sensitization to pollen allergens and a subsequent IgE cross-reaction with homologous molecules in food (Dreborg & Foucard, 1983).

Mal d 1, the major apple allergen (Vanek-Krebitz et al., 1995), is an 18 kDa protein which belongs to a group of so-called pathogenesis-related proteins (PR) in plants, subgroup 10 (Breiteneder & Ebner, 2000; Hoffmann-Sommergruber, 2005). These proteins are induced in response to environmental stress such as pathogen attack (Midoro-Horiuti, Brooks, & Goldblum, 2001). Mal d 1 shows a high degree of sequence homology with Bet v 1, the major allergen of birch pollen (Son, Scheurer, Hoffmann, Haustein, & Vieths, 1999). Because of this homology the proteins share allergenic epitopes, which is the basis of their cross-reactivity. IgE antibodies specific for Bet v 1 cross-react with its homologue in apple, Mal d 1. The major apple allergen is expressed in peel and pulp of the fruit (Marzban et al., 2005). It has also been detected in mature flowers of Fuji apple (Sung, Jeong, Nam, Kim, & An, 1998), its amount showing considerable variation between apple cultivars (Marzban et al., 2005; Vieths, Jankiewicz, Schoning, &

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Aulepp, 1994). Mal d 1 is a proteolysis sensitive allergen, so symptoms are mainly limited to the oral cavity (Sicherer, 2001; Ortolani, Ispano, Pastorello, Bigi, & Ansaloni, 1988), causing the so-called Oral Allergy Syndrome (OAS). Also, Mal d 1 is thermolabile and may participate in oxidation reactions (Vieths et al., 1998). Hence, it has been hypothesized that the allergenicity of cut apples decreases, most probably due to oxidation reactions between apple phenols and the allergen (Rudeschko et al., 1995a, 1995b). A reduction in allergenicity could be due to the masking of IgE binding sites on the allergenic proteins through cross-linking of proteins induced by oxidative enzymes (Chung, Maleki, & Champagne, 2004), causing a decrease in IgE binding capacity of the protein.

Polyphenol oxidase (PPO) is the main factor involved in enzymatic browning in fruits (e.g. apple) and vegetables. PPO is a copper enzyme, that can catalyze two different reactions under oxygen atmosphere: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) (Espin, Morales, García-Ruiz, Tudela, & García-Cánovas, 1997). The formed quinones are highly reactive, and rapidly polymerize into red, brown or black pigments (Prota, 1988). Other enzymes which contribute to these reactions are peroxidase and laccase which are both present in apple. Peroxidase (POD) is a heme-containing enzyme catalyzing the oxidation of a variety of phenolic compounds to *o*-quinones in the presence of H_2O_2 (Witaker, 1972). Generated *o*-quinones can react with other phenolics and with amino or sulfhydryl sites of proteins giving rise to cross-linked products (Figueroa-Espinoza et al., 1999). POD induces the cross-linking of proteins (Stahmann, Spencer, & Honold, 1977). The allergenicity of Mal d 1, containing several tyrosine residues (Atkinson, Perry, Matsui, Ross, & MacRae, 1996), could be directly reduced by enzymatic oxidation of these residues. Laccase can, similarly to PPO, catalyze the oxidation of *o*-diphenols to *o*-quinones.

Interactions of oxidized plant polyphenols with allergenic proteins are supposed to reduce their allergenicity (Björkstén, Halmepuro, Hannuksela, & Lahti, 1980; Rudeschko et al., 1995a, 1995b). Therefore, the allergenicity of birch-pollen-related fruits may decrease by the action of polyphenol oxidase activity. A decrease of IgE binding activity by the recombinant cherry (*Prunus avium*) major allergen, as a consequence of PPO catalyzed oxidation, has been recently reported (Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004). Reactive intermediates formed by enzymatic polyphenol oxidation are suggested as the active chemical reactants responsible for modifying nucleophilic amino acid side chains of proteins, thus inducing an irreversible change in the tertiary structure of the protein and resulting in a loss of conformational epitopes of the allergen (Gruber et al., 2004). POD reduces the IgE-binding by roasted peanuts, supposedly by inducing the cross-linking of its main allergens Ara h 1 and Ara h 2 (Chung et al., 2004).

The use of antioxidants such as sulphite can inhibit the decrease of allergenicity of proteins. This is likely related to its capacity to inhibit the conversion of *o*-diphenols to quinones. (Pastorello & Trambaioli, 2001). Similarly, diethyldithiocarbamic acid (DIECA) inhibits reactions between proteins and phenolic compounds and seems to preserve the IgE-binding capacity of Mal d 1 (Björkstén et al., 1980).

The aim of this work was to study the effect of the oxidative enzymes, PPO and POD, the antioxidant DIECA and the polyphenol catechin, on the IgE-binding capacity of native apple major allergen Mal d 1 in its native environment, the apple tissue.

2. Materials and methods

2.1. Apple samples

Apple cultivars: Golden Delicious, Royal Gala, Granny Smith, Appelstar, Goudreinet, Cox's orange, Santana and Jonagold were purchased at a local market. After peeling of the fruits, pulps and peels were treated separately, frozen under liquid N_2 and lyophilized. Subsequently the samples were ground to a fine powder in a mortar and stored at −20 °C. The humidity of apple flesh and peel ranged from 82% to 85% and 75% to 78% respectively.

Unless stated otherwise, all reagents used were of analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Enzymatic activity measurement

Enzyme extraction (from peel and pulp of both PPO and POD) from each apple cultivar was performed as follows: 100 mg apple powder/ml in phosphate buffer (PB): 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, pH 6.5, containing 0.05% (v/v) Tween 20 (Sigma, St. Louis, MO) were stirred on a vortex and centrifuged during 1 min at 12,000*g*. The supernatant was used as enzyme source. Each extraction was carried out in triplicate. All procedures were conducted on ice. PPO and POD activity were measured in each enzyme extract at 25 °C. PPO activity of apple extract was spectrophotometrically measured (Espin et al., 1997), using 3, 4 dihydroxyphenylalanine (L-DOPA) as substrate in PB, and 3-methyl-2-benzothiazolinone hydrazone (MBTH) (Fluka, Steinheim, Germany) as nucleophilic reagent, resulting in a coloured product. Tropolone was used as PPO inhibitor and sodium dodecyl sulfate (SDS) was used as enzyme activator. The final volume of reaction was 1 ml. Total polyphenol oxidase activity was measured in a reaction mixture containing 14 mM L-DOPA, 5 mM MBTH and 3.5 mM SDS. Active PPO and laccase activity were measured in a similar reaction mixture without SDS. Laccase activity was assayed in a similar reaction mixture without SDS and with 6.67 mM tropolone, The colorimetric reaction was monitored at $\lambda = 484$ nm. The values of total PPO, active PPO and latent PPO were calculated.

Total PPO = active PPO + latent PPO + laccase, active PPO = active PPO + laccase. The value for active PPO was used as endogenous enzyme PPO amount in the subsequent experiments.

POD activity of apple extract was measured spectrophotometrically (Castillo, Stenstrom, & Ander, 1994). The nucleophilic reagent MBTH interacts with 3-(methyl-2-benzothiazolinone hydrazone) (DMAB, Sigma) generating a coloured reaction product in the presence of H_2O_2 (Sigma). Total peroxidase and laccase activity were assayed in a mixture reaction containing 0.07 mM MBTH, 1 mM DMAB, 0.1 mM H_2O_2 (Sigma) and 0.06 mM manganese sulfate in succinic–lactic buffer, pH 4.5 (SLB): 0.1 M lactic acid, 0.1 M succinic anhydride. Laccase activity was measured in a similar reaction mixture without H_2O_2 or manganese sulfate. The final volume of reaction was 1 ml. The colorimetric reaction was monitored at $\lambda = 590$ nm. Total POD = peroxidase + laccase. The values of POD were calculated: POD activity = total peroxidase − laccase. Absorption spectra were recorded in a UV/VIS Perkin Elmer Lambda-16 spectrophotometer (Germany) at 25 °C using a circulating thermostatic water bath. Reference cuvettes contained all the components except the enzyme. Enzyme activity was expressed in nkatals. One nkatal was defined as the amount of enzyme that consumed 1 nmol of substrate (L-DOPA or MBTH) per second under the assay conditions.

2.3. Treatments of apples

Apple powder (100 mg/ml) from Golden Delicious peel was treated according to different protocols. Commercial mushroom tyrosinase (50.000 U/mg, Sigma) and horseradish peroxidase (25.000 U/mg, Sigma) were added in the treatments in order to increase the inherent PPO and POD activity in apple five-fold. The following treatments were applied: addition of commercial PPO, addition of the substrate catechin (0.01 g/g apple powder), addition of catechin combined with PPO, addition of 2 mM DIECA and addition of 2 mM DIECA combined with PPO, in PB, pH 6.5. Similar treatments were done using commercial POD in SLB, pH 4.5 in the presence of H_2O_2. Controls without enzyme, catechin or DIECA were included. Samples were stirred on a vortex during 1 min at room temperature. Treated samples were taken at time 0, 1, 2, 5 and 24 h of incubation, frozen in liquid nitrogen and lyophilized. Protein extracts from lyophilized treated samples were obtained using an extraction solution (100 mg dry treated sample/ml) containing 0.05% (v/v) Triton X-114 (Sigma) in Milli-Q water, shaken 1 min on a vortex and centrifuged during 1 min at 12,000*g*. Supernatants were used as Mal d 1 source.

2.4. Protein concentration

Protein concentration of samples was assessed using a colorimetric method (Bradford, 1976). Interpolation was performed using a standard curve with bovine serum albumin (BSA, Sigma) at $\lambda = 595$ nm.

2.5. Immunodetection

Human Sera from 15 Birch pollen-allergic patients which showed cross-reactivity with the apple major allergen Mal d 1, were obtained from the Laboratory for Primary Health Care (SHO), Velp, The Netherlands. Specific IgE was determined for each serum by the ImmunoCAp method (Pharmacia, Uppsala, Sweden). Sera containing a specific IgE antibody concentration >100 kU were pooled.

Enzyme Linked ImmunoSorbent Assay (ELISA). Immunodetection was performed by means of competitive ELISA. Ninety six-well microplates (Greiner Bio-one, Frickenhausen, Germany) were coated with 1 μg/ml of recombinant Mal d 1 (Biomay, Austria) in coating buffer: 40 mM $NaHCO_3$, 9 mM Na_2CO_3, pH 9.6, 100 μl/well, and incubated for 1 h at 37 °C. All following incubations were performed at room temperature in a microplate shaker. Coating solution was removed and 200 μl/well of blocking buffer: 2% (w/v) BSA in PBS: 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 130 mM NaCl, pH 7.4, was added and incubated for 1 h. Microplates were washed in a microplate washer (Anthos Fluido, Anthos Labtec Instruments GmbH, Austria) with 4×400 μl/well of washing buffer: 0.05% (v/v) Tween 20, 0. 1% (w/v) BSA in PBS, pH 7.4 after each incubation. During incubation, microplates were sealed. Mal d 1 extracted from treated apple samples (Mal d 1 source) was diluted three times in dilution buffer: 0.05% (v/v) Tween 20, 0.1% (w/v) BSA in PBS, pH 7.4. Of this dilution, 50 μl were pipetted in each well as "free" Mal d 1. Competition was performed by adding 50 μl/well of 1:5 serum in PBS. The time of incubation was 3 h. Wells were sequentially incubated with 100 μl of 1:1000 mouse anti-human IgE (DakoCytomation, Denmark) in dilution buffer, and 100 μl of 1:1000 goat anti-mouse IgG antibodies conjugated with alkaline phosphatase (Sigma) in dilution buffer, during 1 h respectively. Two hundred microliter per well of freshly prepared substrate solution was added, consisting of 1 mg/ml 4-nitrophenylphosphate disodium salt in carbonate buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, 1 mM $MgCl_2$, pH 9.6). Colour development was measured at $\lambda = 405$ nm using a microplate reader (ThermoLab Systems, Franklin, Mass.). Water for solutions was obtained from a MilliQ Plus Purification System. Calibration curves and a negative control were included in each microplate. Standard curve samples were performed in triplicate. Fifty microliter per well of a serial dilution (50–0.02 μg/ml) of recombinant Mal d 1 was pipetted as "free" allergen in dilution buffer. Competition was performed by adding 50 μl/well of 1:5 serum diluted in PBS, and followed by the same procedure as the test samples. Negative control was performed with negative sera from patients non allergic to birch pollen, as primary antibody, with concentration for specific IgE antibody <0.35 kU.

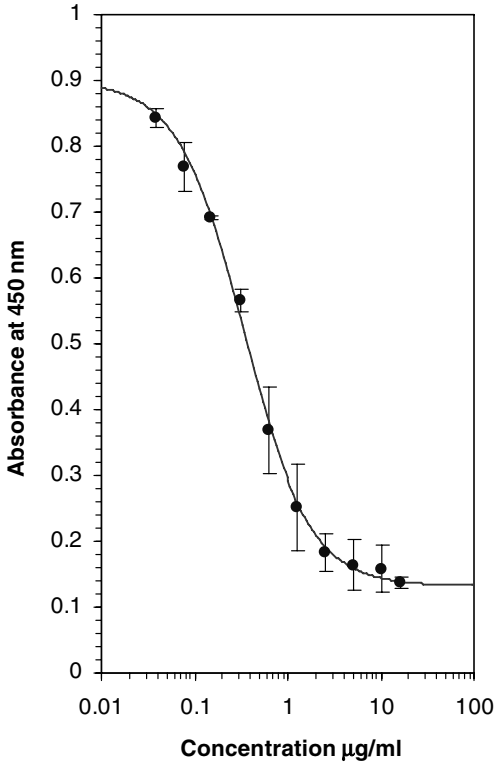

Fig. 1. Competitive ELISA standard curve, performed using a serial dilution of recombinant Mal d 1, as "free" allergen, concentration given as µg/ml. $r^2 = 0.998$.

Data were analyzed with the LSW Data Analysis Package plug-in for Excel (Microsoft). Mal d 1 standard curve was obtained by plotting absorbance against the logarithm of standard recombinant Mal d 1 concentrations. Sigmoidal curve was fitted using the following equation:

$$y = (a - d)/[1 + (x/c)^b] + d$$

where x = concentration of Mal d 1 (µg/ml), y = corresponding absorbance at $\lambda = 405$ nm, a = asymptotic maximum (maximum absorbance in absence of analyte, A_{max}), b = slope parameter at the inflexion point, c = inflection point (I_{50} value), d = asymptotic minimum. Mal d 1 concentration in samples was calculated by interpolation of measured absorbance in the standard curve of the same microplate (Fig. 1).

3. Results and discussion

3.1. Analysis of oxidative enzymes

The amount of oxidative enzymes PPO and POD was analyzed in peel and pulp of eight apple varieties. This was done in order to determine which variety and which part of the apple was richest in oxidative enzymes, and then to ascertain the amount of these respective enzymes that had to be added to tissue samples, in order to achieve a five-fold excess of the endogenous amount, accelerating a possible oxidative effect on Mal d 1.

3.2. PPO activity measurement

Table 1 shows total polyphenol oxidase activity and PPO activities in the eight apple varieties. Golden Delicious and Goudreinet showed the highest levels of active PPO in peels, of 9.3 and 9.0 nk/g dry apple respectively. In pulps, Golden Delicious, Granny Smith and Goudreinet showed the highest values of 14.1, 7.3, and 5.4 nk/g dry apple respectively. Cox Orange, Appelstar and Jonagold showed the lowest total PPO activity. Peels displayed a more heterogeneous activity level than pulps.

It must be noticed that Golden delicious is an apple variety with high PPO activity, however it also contains a high concentration of the allergen Mal d 1 (Son et al., 1999; Marzban et al., 2005).

3.3. POD activity measurement

Table 2 shows POD activities in eight apple varieties. Santana, Golden Delicious and Goudreinet showed highest activity of POD in peels of 2.4, 2.2 and 2.2 nk/g dry apple respectively. Pulps of Goudreinet, Royal Gala and Golden Delicious displayed major values: 1.3, 0.9 and 0.8 nk/g dry apple respectively. The value for POD activity was used as endogenous enzyme POD level in incubation experiments with additional enzyme.

Table 1
Total polyphenol oxidase and PPO activities (nk/g dry apple) in peel and pulp of eight apple cultivars

Apple cultivar	Total polyphenol oxidase		Active PPO		Latent PPO		Laccase	
	Peel	Pulp	Peel	Pulp	Peel	Pulp	Peel	Pulp
Golden delicious	43.4 (3.2)[a]	29.2 (1.5)	9.3 (0.7)	14.1 (0.7)	19.3 (1.6)	12.3 (0.7)	14.8 (0.9)	2.8 (0.1)
Royal gala	34.4 (1.3)	25.9 (3.0)	0.1 (0.5)	4.8 (0.3)	17.4 (0.2)	14.1 (2.1)	16.9 (0.6)	7.0 (0.5)
Granny smith	8.2 (0.6)	12.4 (1.9)	0.6 (0.2)	7.3 (0.9)	4.8 (0.1)	3.1 (0.8)	2.8 (0.3)	2.0 (0.2)
Appelstar	4.7 (0.9)	10.1 (1.0)	0.2 (0.1)	3.4 (0.4)	2.8 (0.5)	5.3 (0.4)	1.7 (0.2)	1.4 (0.2)
Goudreinet	13.1 (1.3)	21.1 (0.5)	9.0 (0.7)	5.4 (0.1)	1.6 (0.3)	14.3 (0.3)	2.5 (0.3)	1.4 (0.1)
Cox orange	4.3 (0.5)	14.1 (1)	0.5 (0.1)	2.2 (0.1)	2.7 (0.3)	8.5 (0.3)	1.1 (0.1)	3.4 (0.6)
Santana	8.5 (0.4)	13.3 (0.9)	nd [b]	4.9 (0.4)	5.5 (0.1)	6.4 (0.1)	3.0 (0.3)	2.0 (0.4)
Jonagold	5.5 (0.9)	8.2 (0.7)	0.8 (0.2)	2.9 (0.1)	1.4 (0.2)	3.4 (0.2)	3.3 (0.4)	1.9 (0.1)

[a] Standard deviation of three analyses.
[b] Not detected.

Table 2
POD activity (nk/g dry apple) in peel and pulp of eight apple cultivars

Apple cultivar	Total peroxidase	
	Peel	Pulp
Golden delicious	2.2 (0.1)[a]	0.8 (0.1)
Royal gala	1.1 (0.1)	0.9 (0.1)
Granny smith	1.7 (0.1)	0.5 (0.1)
Appelstar	0.3 (0.1)	0.1 (0.1)
Goudreinet	2.2 (0.4)	1.3 (0.2)
Cox orange	0.2 (0.1)	0.1 (0.1)
Santana	2.4 (0.1)	0.7 (0.1)
Jonagold	2.0 (0.2)	0.6 (0.1)

[a] Standard deviation of three analyses.

3.3.1. Treatments

On the basis of data (Tables 1 and 2), Golden Delicious peel was selected to perform the incubation experiments with combinations of additional exogenous oxidative enzymes, DIECA and catechin.

3.3.2. Treatment with additional exogenous enzyme

Fig. 2 shows the evolution of the reactivity of Mal d 1 towards IgE in samples in the presence and absence of exogenous PPO. Oxidation due to endogenous enzymes (control) gives rise to reduction of the IgE binding capacity with 9% in the first hour, and with 51% in 5 h of treatment; IgE-binding by Mal d1 decreased faster when PPO was added. Accordingly in the first hour of treatment a decrease of 46% was found, in 5 h the reactivity of Mal d 1 was decreased by 89%. Whilst the allergen was still quite immunoreactive in the control after 24 h of treatment, residual

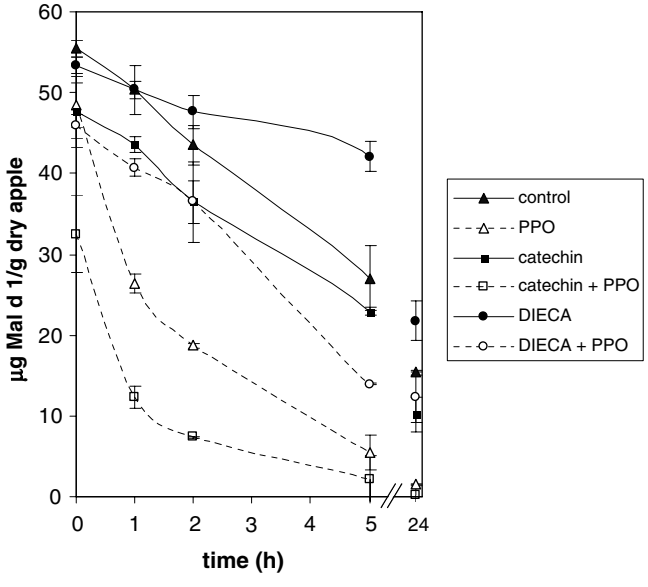

Fig. 2. Influence of the treatments performed on apple peel samples (cv. Golden Delicious) in PB, pH 6.5, on Mal d 1 concentration (µg Mal d 1/g dry apple), determined by competitive ELISA using a pool of sera. The treatments consisted of: additional PPO, additional substrate catechin, additional catechin combined with PPO, additional DIECA and additional DIECA combined with PPO. The incubation times were 0, 1, 2, 5, and 24 h at room temperature.

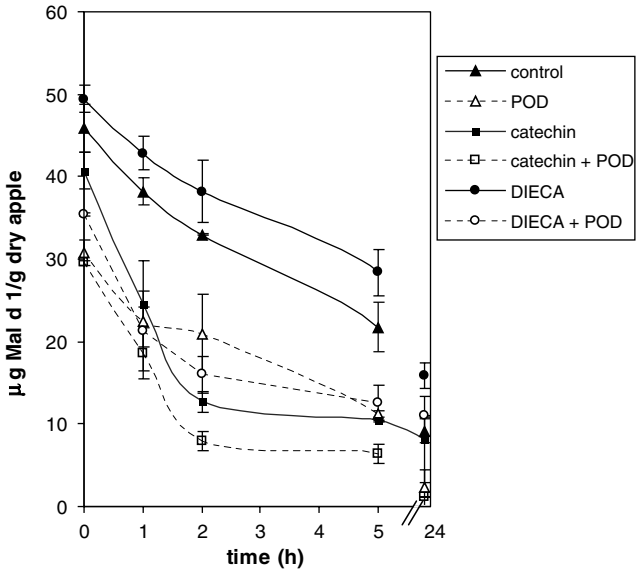

Fig. 3. Influence of the treatments performed on apple peel samples (cv. Golden Delicious) in SLB, pH 4.5, on Mal d 1 concentration (µg Mal d 1/g dry apple), determined by competitive ELISA using a pool of sera. The treatments consisted of: additional POD, additional substrate catechin, additional catechin combined with POD, additional DIECA and additional DIECA combined with POD. The incubation times were 0, 1, 2, 5, and 24 h at room temperature.

levels of IgE-binding by Mal d 1, measured in PPO treated samples, had almost disappeared completely after 24 h.

Fig. 3 shows the decrease of IgE-binding by the allergen Mal d1 during the treatment with exogenous POD. After 5 h of treatment, natural oxidation (control) was able to cause a 53% reduction in the initial IgE-binding activity of the allergen, whereas in treated samples this reduction is higher. After 24 h, 20% of allergenicity was still present in the control. In contrast, only 8% of Mal d 1 was detected in POD treated samples. Additional enzyme treatments performed with PPO and POD showed a similar outcome; in both cases, inhibition of IgE-binding capacity is compared to the control. The inhibitory effect of enzymatic oxidation on IgE-binding capacity is stronger when PPO was used.

3.3.3. Treatment with additional substrate

Apples are quite rich in catechin, an *o*-diphenol which has been identified as substrate of enzymatic oxidative reactions catalyzed by PPO and POD (Rocha & Morais, 2001), so it was used as additional substrate. Fig. 2 shows the effect of the addition of catechin in PB pH 6.5. The addition of the substrate did not cause significant changes in the IgE-binding by the allergen, compared to the control. It is concluded that endogenous PPO activity limits the oxidative reaction. In contrast, the incubation with exogenous PPO in presence of additional substrate provokes a strong decrease of IgE-binding by Mal d 1 (Fig. 2). In the first hour of incubation, IgE-binding was reduced six times compared to the control. After 24 h of incubation, a total depletion of Mal d 1 reactivity was

found whereas the control retains 28% of IgE-binding capacity.

When the treatment was performed with additional catechin incubated in SLB pH 4.5 (Fig. 3), the amount of Mal d 1 during the two first hours was decreased 2.4 times compared with the control. Accordingly, in these conditions additional catechin was used by endogenous POD, and oxidative residues resulting of endogenous enzyme activity give rise to reduction of the IgE-binding by Mal d 1.

3.3.4. Treatments with additional DIECA

Fig. 2 shows the effect on the IgE-binding by Mal d 1 when DIECA was used as antioxidant during the incubations. After 5 h of incubation in PB pH 6.5 the IgE-binding by Mal d 1 did not decrease in the DIECA treated sample as much as in the control sample possible due to the inhibition of endogenous enzymes. After 24 h of treatment Mal d 1 displayed still 41% of its IgE binding capacity. The protective effect of DIECA is also observed when PPO was added (Fig. 2). After 5 h of incubation the protective effect was lower, probably because of DIECA depletion. Stabilization of IgE-binding is also strong in the treatments performed with antioxidant in SLB pH 4.5 (Fig. 3): 58% of the allergen remains reactive after 5 h of treatment. When additional POD is added, IgE-binding capacity is also stabilized after 2 h. This decreasing level is then maintained. Up to 31% of immunoreactivity is still measured after 24 h of treatment.

The experiments show that treatment of apple extracts with the oxidative enzymes PPO and POD, provokes a decrease in the IgE-binding by Mal d 1. PPO shows the strongest effect in this respect. Apple peel treated with a combination of additional exogenous oxidative enzyme and the polyphenol catechin showed a drastic inhibition of IgE-binding by Mal d1. A recent study about the major allergen in strawberries Fra a 1, homologous to Bet v 1, has revealed that an up-regulation of certain oxidative enzymes is related to a low allergenicity in fruits. These results seem to corroborate our data (Hjerno et al., 2006).

Incubations with catechin showed an effective decline of IgE-binding capacity in SLB pH 4.5. DIECA showed to be a potent protecting agent for the IgE-binding by Mal d 1, even in the presence of additional exogenous POD. It must be noticed that buffers exert some influence on these reactions, in fact control in SLB gave rise to a reduction of the IgE binding capacity of 17% and control in PB just only 9%.

The decrease of immunoreactivity is likely to be due to the effect of reactive chemical compounds derived of oxidative catalytic reactions, as also evidenced for recombinant Pru av 1 from cherry (Gruber et al., 2004). The mechanisms by which Mal d 1 is affected by PPO and POD oxidation are not elucidated. Subsequent reactions and cross-linking of proteins may affect directly tyrosine residues of the allergen or elsewhere in the protein. Covalent interactions formed may cause permanent changes in the tertiary structure of Mal d 1. Conformational epitopes can be lost or masked and the IgE-binding capacity of the allergen is then decreased or depleted. In conclusion, in this work, it has been demonstrated that the incubation of apple with the oxidative enzymes PPO and POD decreases its allergenicity. The antioxidant DIECA is a potent protector of Mal d 1 allergenicity. These results should have significance in relation to the processing and biotechnology of apples.

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