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Maillard Reaction and Enzymatic Browning Affect the Allergenicity of Pru av 1, the Major Allergen from Cherry (*Prunus avium*)

PATRICK GRUBER,[†] STEFAN VIETHS,[‡] ANDREA WANGORSCH,[‡] JÖRG NERKAMP,[§] AND THOMAS HOFMANN*,[||]

Deutsche Forschungsanstalt für Lebensmittelchemie, Lichtenbergstrasse 4, D-85748 Garching, Germany; Department of Allergology, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, D-63225 Langen, Germany; Lehrstuhl für Biopolymere, Universität Bayreuth, D-95440 Bayreuth, Germany; and Institut für Lebensmittelchemie, Universität Münster, Corrensstrasse 45, D-48149 Münster, Germany

The influence of thermal processing and nonenymatic as well as polyphenoloxidase-catalyzed browning reaction on the allergenicity of the major cherry allergen Pru av 1 was investigated. After thermal treatment of the recombinant protein rPru av 1 in the absence or presence of carbohydrates, SDS−PAGE, enzyme allergosorbent tests, and inhibition assays revealed that thermal treatment of rPru av 1 alone did not show any influence on the IgE-binding activity of the protein at least for 30 min, thus correlating well with the refolding of the allergen in buffer solution as demonstrated by CD spectroscopic experiments. Incubation of the protein with starch and maltose also showed no effect on IgE-binding activity, whereas reaction with glucose and ribose and, even more pronounced, with the carbohydrate breakdown products glyceraldehyde and glyoxal induced a strong decrease of the IgE-binding capacity of rPru av 1. In the second part of the study, the effect of polyphenoloxidase-catalyzed oxidation of polyphenols on food allergen activity was investigated. Incubation of rPru av 1 with epicatechin in the presence of tyrosinase led to a drastic decrease in IgE-binding activity of the protein. Variations of the phenolic compound revealed caffeic acid and epicatechin as the most active inhibitors of the IgE-binding activity of rPru av 1, followed by catechin and gallic acid, and, finally, by quercetin and rutin, showing significantly lower activity. On the basis of these data, reactive intermediates formed during thermal carbohydrate degradation as well as during enzymatic polyphenol oxidation are suggested as the active chemical species 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.

KEYWORDS: Food allergy; cherry allergen; Pru av 1, Maillard reaction; polyphenoloxidase; nonenzymatic browning

INTRODUCTION

Pollen-related food allergy is the most frequent form of food hypersensitivity in the adult population, and the prevalence of pollen-induced allergic reactions is increasing. Patients with allergy to tree pollen, in particular birch pollen, frequently show adverse reactions to fruits, vegetables, and nuts. Members of the *Rosaceae* (apple, pear, cherry, peach), *Betulaceae* (hazelnut), and *Apiaceae* (celery, carrot) plant families are most frequently involved (reviewed in refs *1*−*4*). Cherry is an important allergenic food within this cluster of pollen-related food allergies. For example, from 380 birch-pollen-allergic subjects 53% were found to be allergic to hazelnut, 47% to apple, 34% to peach, 29% to cherry, and 27% to almond (*5*). In a recent study conducted in Japan, 61% of 54 birch-pollen-allergic patients reported adverse reactions to foods, and cherry allergy had a prevalence of 58% in the food-sensitive group (*6*). The main symptom of cherry allergy is relatively mild oral allergy syndrome, but more severe reactions have been reported in a subgroup of patients (*7*, *8*). 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. Bet v 1, the major birch pollen allergen (*9*), shares common IgE epitopes with the major food

* To whom correspondence should be addressed. Phone: (49) 251-83-33-391. Fax: (49) 251-83-33-396. E-mail: thomas.hofmann@uni-muenster.de.
[†] Deutsche Forschungsanstalt für Lebensmittelchemie.
[‡] Paul-Ehrlich-Institut.
[§] Universität Bayreuth.
[||] Universität Münster.

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allergens, for example, Mal d 1 from apple (*10*, *11*), Pru av 1 from cherry (*12*, *13*), Pyr c 1 from pear (*14*), Cor a 1.0401 from hazelnut (*15*), Api g 1 from celery (*16*), and Dau c 1 from carrot (*17*). Similar to Bet v 1, the homologous proteins in foods share between 40% and 60% amino acid sequence identity with members of the PR-10 family of pathogenesis-related proteins which are inducible in many food plants in response to environmental stress such as microbial attack (*17*, *18*).

Patients with birch-pollen-related allergy to fruits such as apple, pear, and cherry usually tolerate these fruits after heat treatment. This corresponds to our in vitro data which show that the apple allergen Mal d 1 and the cherry allergen Pru av 1 become rapidly inactivated during heat processing of the fruit (*19*; unpublished data). By contrast, we found that the allergenicity of the major celery allergen Api g 1 is not affected by a heat treatment of 30 min at 100 °C when heating is applied to semipurified allergen extracts. Even the extremely labile Mal d 1 showed a residual IgE-binding potential after 30 min of heating of apple extract (*19*). By contrast, the peanut allergens Ara h 1 and Ara h 2 were recently reported to bind higher levels of patient IgE after heating in the presence of carbohydrates (*20*). Taken together, these observations point to interactions of the allergens with matrix constituents of the fruits, leading to allergen modification during food processing and food preparation. Moreover, it is known that the allergenicity of pollen-related fruits decreases during grinding of the fruit, and when allergen extracts are prepared by simple buffer extraction procedures. Endogenous polyphenoloxidase activities, in particular interactions of oxidized plant polyphenols with allergenic proteins, have been supposed to cause these effects (*21*−*23*). However, the factors responsible for the instability of fruit allergens during processing have not been studied under controlled conditions, or at the molecular level.

The immunological and biochemical properties of the major cherry allergen Pru av 1, a lysine-rich 17.7 kDa protein, have been studied intensively (*7*, *12*, *13*). After heterologous expression in the procaryotic host *E. coli*, the recombinant protein completely resembled the allergenic activity of its natural counterpart. Ninety-six percent of 101 patients with pollen-related cherry allergy presented IgE antibodies specific for this protein, whereas other cherry allergens such as the profilin Pru av 4 bound IgE from less than 20% of the patients (*7*). The tertiary structure of recombinant Pru av 1 has recently been determined by heteronuclear NMR spectroscopy (*24*), showing that the cherry protein shares an almost identical backbone structure with the major birch pollen allergen. A recent study which included a combination of structural and immunological analyses of rPru av 1 and several variants of the allergen generated by site-directed mutagenesis clearly demonstrated that the IgE reactivity of Pru av 1 is strongly dependent on its intact conformation. Moreover, it was shown that the so-called P-loop region around amino acid Glu 45 is involved in a conformational IgE epitope of this allergen (*25*). In the present study, we have chosen recombinant Pru av 1 as a typical representative of the Bet v 1 allergen family to study the influence of heat treatment in the presence and absence of carbohydrates as well as enzymatic browning on the allergenic reactivity of pollen-related food allergens under well-defined conditions. Our data show that the pure allergen has persistent allergenic activity after thermal treatment due to complete refolding, whereas heating in the presence of certain carbohydrates and incubation in the presence of polyphenols and polyphenoloxidase lead to a drastic decrease of the allergenic potential.

EXPERIMENTAL PROCEDURES

Chemicals. The following chemicals were obtained from commercial sources: isopropyl d-thiogalactopyranoside (IPTG), (+)-catechin, and rutin trihydrate were obtained from Sigma (Steinheim, Germany). Coomassie Brilliant Blue R250, D-ribose, DL-glyceraldehyde, glyoxal (40% solution in water), and tyrosinase (EC 1.14.18.1) were purchased from Aldrich (Steinheim, Germany). Maltose monohydrate, D-glucose, caffeic acid, and starch (soluble acc. to Zulkowsky) were from Merck (Darmstadt, Germany). (−)-Epicatechin and gallic acid monohydrate were purchased from Fluka (Buchs, Switzerland) Quercetin dihydrate was from Lancaster (Muelheim, Germany).

Production and Purification of Recombinant Pru av 1 (rPru av 1). Expression and purification of Pru av 1 was done following the protocol reported recently (*24*). Briefly, the coding region of the cDNA of Pru av 1 (*12*) was cloned into the expression plasmid pET11a (Novagen, Madison WI) coding for a nonfusion protein with an authentic N-terminus. After transformation in *E. coli* BL21 (DE3) host cells, protein synthesis was induced with IPTG (1 mM) and the native target protein was purified on the basis of chromatofocusing followed by anion exchange chromatography. Purified rPru av 1 was freeze-dried and stored at −20 °C in a desiccator until use.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed under reducing conditions in a Mini Protean cell (Bio-Rad, Munich, Germany) according to the literature (*26*). The total acrylamide content of the gels was 12.5% (w/v), and the cross-linker concentration was 2.7% (w/w). The sample load on the gel was 3 μg of protein per analytical slot. Protein bands were visualized by Coomassie Brilliant Blue R250 staining.

Enzyme Allergosorbent Test (EAST). Native as well as thermally treated rPru av 1 was covalently coupled to cyanogen bromide activated filter paper disks (*27*) as described in the literature for plant food extracts (*22*, *28*). The protein concentration in the coupling solution was 1.5 μg/mL, corresponding to 0.25 μg of rPru av 1 per disk. A serum pool was prepared from equal volumes of sera from five patients with pollen-related allergy to cherry. For determination of allergen-specific IgE the allergen disks were incubated overnight with a human serum pool (50 μL) at room temperature. After washing, the amount of IgE specifically bound to the immobilized allergens was determined by a commercial EAST using the calibration system of the manufacturer (Allergopharma, Reinbek, Germany).

Inhibition Assay Using Paper Disks. A quantitative estimation of the allergenic potency of rPru av 1 prior to and after treatment in the presence of carbohydrates was performed by means of a competitive ELISA inhibition assay. The human serum pool was diluted in a ratio of 1:2 with an aqueous solution of Tris buffer (0.05 mol/L, pH 7.4), sodium chloride (0.15 mol/L), bovine serum albumin (BSA; 3 g/L), and Tween 20 (5 mL/L). The rPru av 1 preparations were diluted in ratios of 1:5, 1:25, 1:125, and 1:725 in an aqueous mixture containing Tris buffer (0.05 mol/L, pH 7.4), sodium chloride (0.15 mol/L), BSA (3 g/L), and Tween 20 (5 mL/L). Diluted pool serum (50 μL) and inhibitor solution (50 μL) were mixed and incubated with an allergen disk overnight at room temperature. Subsequently, an EAST was performed as detailed above. The results were expressed as percent inhibition. Generally, all experiments were carried out in duplicate.

Circular Dichroism (CD) Spectroscopy. A CD spectrum of Pru av 1 was recorded after dialysis with ammonium actetate buffer (5 mmol/L, pH 7.0) on a Jasco J-600 spectropolarimeter with a step width of 0.2 nm and bandwidth of 1 nm and within a spectral range from 185 to 250 nm. The CD spectrum of Pru av 1 was measured 10 times at a concentration of 7.4 μM at 22 °C. These results were accumulated, and the ratio of secondary structure elements was calculated using the Jasco Secondary Structure Estimation program provided by the supplier (Jasco, Tokyo, Japan). Thermal transition of 2.2 μM Pru av 1 was measured in 10 mM potassium phosphate buffer, pH 7.0, at 222.6 nm in a temperature range between 22 and 95 °C with a temperature slope of 1 °C/min. Molecular ellipticity was calculated according to the literature (*29*), and the data were adjusted by a temperature-fitting equation (*30*). The cooling graph was recorded after a delay time of 5 min.

Model Experiments with Pru av1 and Carbohydrates. rPru av 1 was dissolved in a PBS buffer (pH 6.8) containing sodium phosphate (150 mmol/L) and sodium chloride (150 mmol/L) to give a final concentration of 1 mg/mL. An aliquot (150 μL) of that stock solution was mixed with an aqueous solution (150 μL) of maltose, glucose, ribose, glyceraldehyde, or glyoxal (100 mmol/L each) or starch (18 mg/mL) in a SafeLock Cap (500 μL) (Eppendorf, Germany). After the mixtures were incubated for different time periods in a UNO-Thermoblock (Biometra, Göttingen, Germany) at 100 °C, the preparations were cooled in an ice bath. As the control, either the protein or the carbohydrate component was displaced by PBS. The preparations were directly used for SDS−PAGE and EAST assays as described above.

Model Experiments with Pru av1 and Polyphenols. rPru av 1 was dissolved in PBS buffer (pH 6.8) containing sodium phosphate (150 mmol/L) and sodium chloride (150 mmol/L) to give a final concentration of 1 mg/mL. An aliquot (150 μL) of that stock solution was mixed with a solution (150 μL) of epicatechin, catechin, gallic acid, caffeic acid, quercetin, or rutin (5 mmol/L each) in PBS. After the mixtures were incubated in the absence or presence of tyrosinase (10 μg in 10 μL of PBS) for 30 min, the preparations were directly used for the enzyme allergosorbent test as described above. In a control experiment either the protein or epicatechin was displaced by PBS.

RESULTS AND DISCUSSION

To gain the first insight into the influence of nonenzymatic as well as enzymatic browning reactions on the activity of fruit allergens, the recombinant protein of the major cherry allergen Pru av 1 was prepared and purified, and after thermal treatment with carbohydrates or incubation in the presence of polyphenols and polyphenoloxidase the IgE-binding activities of the modified proteins were compared to that of the nonreacted protein. Prior to the model experiments, the correct folding of rPru av 1 was assessed by CD spectroscopy, and a thermal denaturation curve was recorded.

Secondary Structure Analysis. The far-UV CD spectrum of rPru av 1 is shown in **Figure 1A**. The spectrum was identical to the spectrum of correctly folded Pru av 1 reported in the literature and highly superimposed to the spectrum of the homologous birch pollen allergen Bet v 1 (*13*), thus indicating that the protocol applied to generate the material used for the model experiments resulted in correctly folded recombinant protein. A thermal transition graph was recorded at 222.6 nm between 22 and 95 °C (**Figure 1B**). The resulting curve clearly indicated thermal unfolding of the allergen occurring at a calculated melting temperature of 66.3 °C. Cooling of the sample resulted in an exactly identical shape of the curve, demonstrating complete refolding of the allergen under the applied experimental conditions (data not shown).

Influence of Nonenzymatic Browning Reactions. To gain the first insight into the influence of nonenzymatic browning reactions, rPru av 1 was thermally treated for 30 min at 100 °C in the presence of either the polysaccharide starch, the disaccharide maltose, the monosaccharides glucose, fructose, and ribose or glyoxal and glycolaldehyde, which are well-known carbonyl compounds formed during carbohydrate fragmentation (*31*). After the reaction, the protein was analyzed by SDS−PAGE and the proteins bands were stained with Coomassie Brilliant Blue R250 (**Figure 2**). Lane 5 showed the band of the rPru av 1 heated in the absence of any carbonyl compound to be close to the molecular mass marker at 20.1 kDa, thus fitting well with the molecular mass of 17.7 kDa of Pru av 1. The reaction of Pru av 1 with starch (lane 9), maltose (lane 3), glucose (lane 1), and fructose (lane 2) did not result in a decrease of the intensity of the protein band. After reaction with ribose, the band became more weak and diffuse (lane 4 in **Figure 2**),

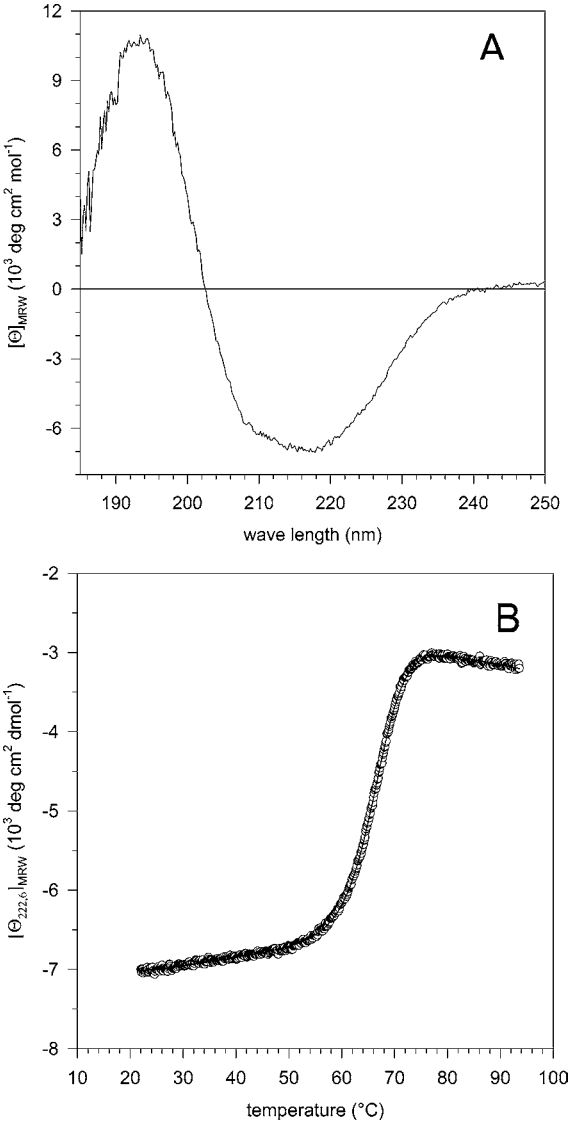

Figure 1. (**A**) Far-UV CD spectrum of recombinant Pru av 1 in ammonium acetate (5 mmol/L). (**B**) Thermal transition curve of rPru av 1 recorded at 222.6 nm. An identical curve was obtained when the solution was cooled again.

thus showing modification of Pru av 1. In particular, the carbohydrate breakdown products glyoxal (lane 8) and glyceraldehyde (lane 9) induced a rapid modification of the protein; no defined protein band was detectable any more. As expected, controls of glucose and glyoxal, respectively, heated in the absence of Pru av 1 did not show any band (lanes 6 and 10).

To gain further insight into the IgE-binding properties of the carbohydrate-modified protein, EAST and inhibition assays were performed. After 30 min of thermal treatment, equivalent amounts of the protein were covalently coupled to cyanogen bromide activated filter paper disks and incubated with pooled sera of persons showing an allergy to sweet cherry. The amount of IgE specifically bound to the immobilized allergens was then visualized enzymatically. As control, an aqueous solution of rPru av 1 was heated in the absence of any carbonyl compound. As shown in **Figure 3**, thermal treatment of rPru av 1 in the absence of any carbonyl compound did not show any influence on the IgE-binding activity of the protein, perfectly correlating with the experimentally observed refolding of the allergen in buffer solution. From this result, it was concluded that interaction with matrix constitutents represents the most likely cause

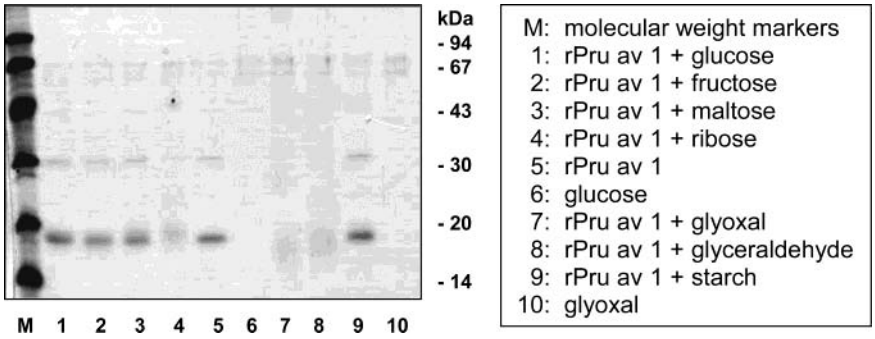

Figure 2. SDS–PAGE of rPru av 1 (500 μg/L) heated for 30 min at 100 °C in the absence or presence of carbohydrates and carbohydrate breakdown products (0.05 M each). Protein staining was performed with Coomassie Brilliant Blue R250.

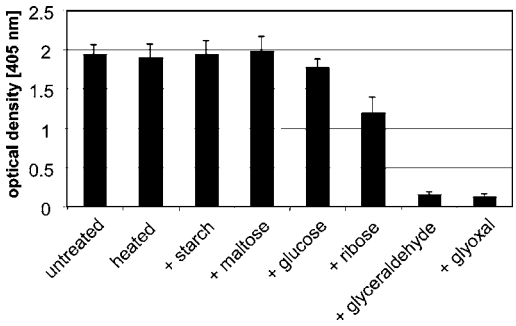

Figure 3. EAST assay on the influence of carbohydrates and carbohydrate cleavage products on the IgE-binding activity of rPru av 1. The results are given as the means of triplicate measurements.

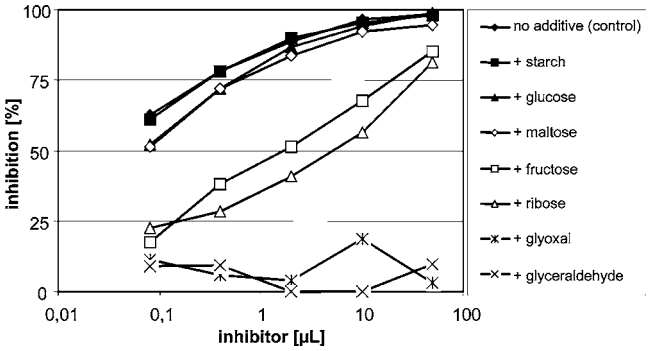

Figure 4. Inhibition assay on the influence of carbohydrates and carbohydrate cleavage products on the IgE-binding activity of rPru av 1. The results are given as the means of triplicate measurements.

of the instability of Pru av 1 and its homolgues in fruits during heating of foods. Incubation of the protein with starch and maltose also showed no effect on IgE-binding activity, whereas reaction with the monosaccharides glucose and ribose induced a partial decrease of the IgE-binding ability of rPru av 1 (**Figure 3**). In contrast to the carbohydrates, the carbohydrate breakdown products glyceraldehyde and glyoxal induced a depletion of any IgE-binding activity of the protein. Control experiments performed with sera obtained from nonallergenic subjects did not show any IgE binding at all (**Figure 3**). In addition, nonspecific binding was not detectable.

To further strengthen these findings, an inhibition assay was performed with the protein/carbohydrate solutions heated for 45 min. To achieve this, untreated rPru av 1 was covalently bound to filter disks and the reaction mixtures were added in dilution series as inhibitor. As outlined in **Figure 4**, heating of rPru av 1 in the presence of starch induced no change in IgE-binding activity. In the presence of glucose as well as maltose, the inhibition curves are shifted slightly toward higher inhibitor

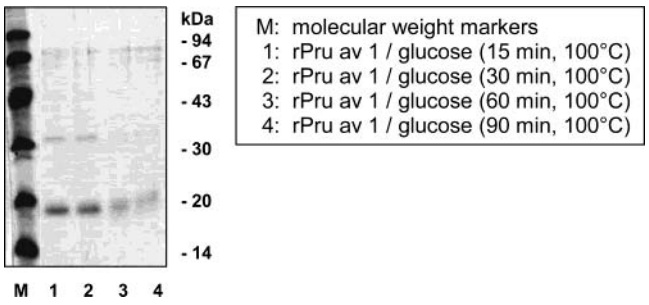

Figure 5. SDS–PAGE of a mixture of rPru av 1 (500 μg/L) and glucose (0.05 M) heated for 15, 30, 60, and 90 min at 100 °C. Protein staining was performed with Coomassie Brilliant Blue R250.

concentrations, demonstrating that this glycation reaction induced a slight decrease in IgE-binding activity of the rPru av 1. In comparison, the pentose ribose as well as the ketohexose fructose showed a significantly stronger reduction of the IgE-binding activity of the protein than the aldohexose. Being well in line with the results of the EAST experiments, the reaction of rPru av 1 with the carbohydrate cleavage products glyoxal and glyceraldehyde induced a total depletion of IgE-binding activity. Even in high concentrations, the protein heated in the presence of glyoxal or glyceraldehyde did not showed any inhibitory effect toward the binding of antibodies to the untreated protein. Substitution of rPru av 1 with other proteins such as, e.g., BSA, did not show any inhibitory influence on IgE binding to rPru av 1, demonstrating that the effects observed are relying on specific allergen−antibody interaction (data not shown).

In a further experiment, the influence of the reaction time on carbohydrate-induced protein modification was investigated by SDS−PAGE. To achieve this, rPru av 1 was thermally treated in the presence of glucose at 100 °C for 15, 30, 60, and 90 min, and then analyzed by SDS−PAGE with Coomassie Brilliant Blue R250 staining (**Figure 5**). After the reaction mixture was heated for 15 and 30 min, respectively, the rPru av 1 was still detectable at 17.1 kDa as a strong band (lanes 1 and 2). Increasing the reaction time, however, led to a loss of the intensity of the stained protein, which became very diffuse after 60 min (lane 3). After 90 min of heating, the protein band nearly disappeared, thus demonstrating significant carbohydrate-induced structural changes of the protein (lane 4).

To investigate how the carbohydrate-induced IgE-binding activity of the cherry allergen depends on the reaction time, purified rPru av 1 was heated in aqueous solution in the presence of glucose or ribose and, in comparison, in the absence of any carbohydrate. After thermal treatment of the model mixtures for 15, 30, 45, 60, and 90 min, EAST assays were performed as detailed above. Being well in line with the data given in **Figure 2**, the allergen showed high stability when heated in

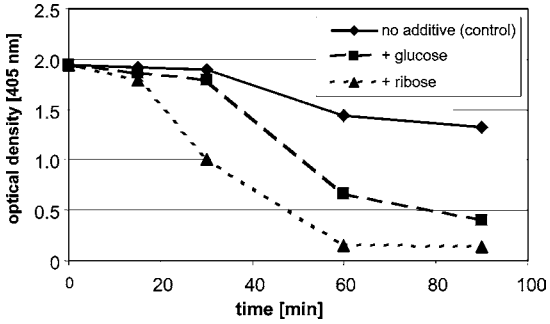

Figure 6. EAST assay on the time course of a carbohydrate-induced decrease of the IgE-binding activity of rPru av 1. The results are given as the means of triplicate measurements.

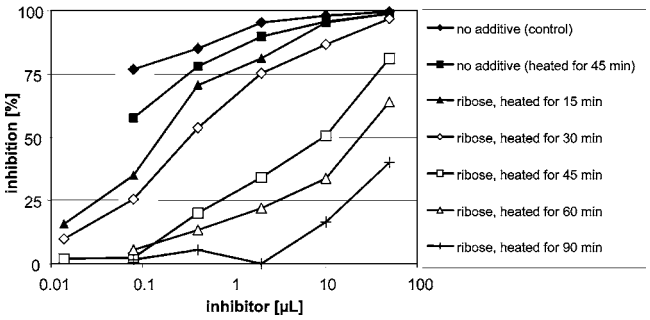

Figure 7. Inhibition assay on the time course of a ribose-induced decrease of the IgE-binding activity of rPru av 1. The results are given as the means of triplicate measurements.

the absence of carbonyls; for example, no change in IgE binging was observed within the first 30 min of heating, and about 70% of the IgE-binding activity was still detectable after rPru av 1 was heated for 90 min at 100 °C (**Figure 6**). The presence of carbohydrates strongly accelerated the depletion in IgE-binding activity; for example, no activity of the protein was detectable after rPru av 1 was heated for 60 min in the presence of the pentose. Also glucose decreased the binding activity of the cherry allergen, but with somewhat lower activity (**Figure 6**).

To further strengthen these findings, an inhibition assay was performed with untreated rPru av 1 covalently bound to filter disks and a dilution series of the protein/ribose solutions as inhibitor (**Figure 7**). Controls of untreated rPru av 1 as well as the protein heated in the absence of ribose showed a significantly higher inhibitory activity than any sample incubated with ribose. The ribose-induced decrease of inhibitory activity strictly went in parallel with the duration of thermal treatment.

Influence of the Enzymatic Browning Reaction. Besides Maillard-type reactions, also enzymatic browning reactions running during processing of plant foods might influence the IgE-binding properties of food allergens. To investigate the effect of polyphenoloxidase-catalyzed oxidation of polyphenols on food allergen activity, as an example, binary mixtures of rPru av 1 and phenolic compounds were incubated at room temperature, heated at 100 °C, or incubated in the presence of tyrosinase, and IgE binding was investigated by means of an EAST assay (**Figure 8**). Neither the incubation of rPru av 1 with epicatechin at room temperature nor that at 100 °C showed any influence on the IgE-binding activity of the recombinant cherry allergen. Also single addition of tyrosinase showed no change in specific IgE binding. In contrast, incubation of rPru av 1 with epicatechin in the presence of the enzyme led to a drastic decrease in IgE-binding activity of the protein. Variations of the phenolic title compound revealed caffeic acid and epicatechin as the most active inhibitors of the IgE-binding

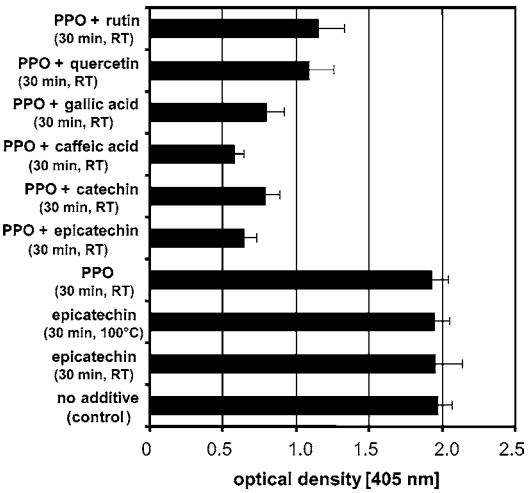

Figure 8. EAST assay on the influence of polyphenols on the IgE-binding activity of rPru av 1. The results are given as the means of triplicate measurements.

activity of rPru av 1, followed by catechin and gallic acid with somewhat lower activity. Although the biological activity of the protein was decreased also in the presence of quercetin as well as the quercetindiglycoside rutin, these flavon-3-ols showed significant lower inhibitory activity than the flavan-3-ols catechin and epicatechin.

Taking all these data into consideration, we hypothesize that reactive carbonyl intermediates formed during thermal carbohydrate degradation as well as during enzymatic polyphenol oxidation are the active chemical species responsible for modifying nucleophilic amino acid side chains of proteins, thus inducing a significant and irreversible change in the tertiary structure of the protein and resulting in a loss of conformational epitopes of the allergen.

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