

# Impact of Glutathione on the Allergenicity of the Peach Lipid Transfer Protein Pru p 3

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## ■ Abstract

*Background and Objective:* The allergenic potential of proteins can be altered under various physicochemical conditions. Glutathione (GSH) is a reducing agent that is used as an antioxidant in food products. We aimed to characterize the natural folding of peach proteins and test the allergenicity of reduced and natural Pru p 3, the major peach allergen.

*Methods:* Pru p 3 was purified from peach, and its conformation was analyzed by means of circular dichroism. Using a thiol fluorescent probe, reduced proteins were detected in fresh peach. GSH-reduced Pru p 3 was tested in vitro for T-cell proliferation and in vivo using skin prick testing.

*Results:* GSH-reduced Pru p 3 produced variable skin prick reactions in peach-allergic patients. The proliferative response of peripheral blood mononuclear cells from allergic patients to reduced Pru p 3 tended to be less intense, whereas secretion of the cytokines IFN- $\gamma$ , IL-5, and IL-10 was comparable. In a pool of sera from peach-allergic patients, reduction hardly impaired IgE-binding. Moreover, the stability of reduced Pru p 3 to gastrointestinal digestion was similar to that of the natural form.

*Conclusions:* GSH can at least transiently reduce Pru p 3. We found that the effect of reduction on the allergenicity of Pru p 3 varied. Therefore, as an additive, GSH does not seem to eliminate the risk of reactions for peach-allergic patients.

**Key words:** GSH. Pru p 3. Peach allergy. Reducing agent. IgE-binding.

## ■ Resumen

*Antecedentes:* El potencial alérgico de las proteínas puede alterarse mediante modificaciones fisicoquímicas. El glutatión (GSH) es un agente reductor utilizado como antioxidante en productos alimentarios.

*Objetivo:* Este estudio pretende caracterizar el plegamiento natural de las proteínas de melocotón y cuantificar la alergenicidad del alérgeno mayor del melocotón, Pru p 3, natural y reducido.

*Métodos:* Para ello, se purificó Pru p 3 y se analizó su conformación mediante dicroísmo circular (DC). Mediante el análisis con tiol fluorescente, se detectaron las proteínas reducidas en melocotones frescos. Pru p 3 reducido por GSH fue analizado mediante un ensayo in vitro de proliferación de células T e in vivo mediante prueba cutánea.

*Resultados:* Pru p 3 reducido produjo reacciones variables en las pruebas cutáneas de los pacientes alérgicos a melocotón; sin embargo, su estabilidad a la digestión gastrointestinal fue similar a la de la forma natural. La respuesta proliferativa de las células mononucleares de los pacientes alérgicos frente a Pru p 3 reducido mostró una tendencia a ser inferior, mientras que la secreción de citocinas IFN $\gamma$ , IL5 e IL10 fue similar a la producida con la forma natural. La reducción alteró la unión de la IgE a Pru p 3 en un pool de sueros de pacientes alérgicos a melocotón.

*Conclusión:* En conclusión, el glutatión es capaz de reducir Pru p 3, al menos de forma transitoria. En nuestro estudio, la reducción no afectó a la alergenicidad de Pru p 3, de forma que dicho aditivo no parece resolver el riesgo de alergia en pacientes alérgicos a melocotón.

**Palabras clave:** GSH. Pru p 3. Alergia a melocotón. Agente reductor. Unión a IgE.

## Introduction

Peach allergy is one of the most prevalent plant food allergies in Mediterranean countries. It is caused mainly by the major allergen Pru p 3 [1-3], a nonspecific lipid transfer protein (nsLTP) generally found in peach peel [4,5], although also in pollen. Sensitization to nsLTP is associated with manifestations ranging from oral allergy syndrome (OAS) to anaphylaxis [2,6]. The structure of Pru p 3 consists of 4  $\alpha$  helices stabilized by 4 disulfide bridges [7], which are highly resistant to gastrointestinal digestion [8] and heat [9]. The stability of Pru p 3 enables it to reach and sensitize the gastrointestinal immune system and thus elicit allergic reactions in sensitized individuals upon ingestion. The allergenic potential of food proteins can be altered by processing (eg, heating), treatment with enzymes, or sulfurization [10-16].

Sulfurization is commonly used for preservation of peaches. Sulfur dioxide is an antioxidant that protects color and flavor in dried fruits and soft drinks. It can induce asthma when inhaled or ingested by sensitized individuals. [17]. The tripeptide glutathione (GSH) plays a role in detoxification of sulfur dioxide through sulfitolysis of glutathione disulfide (GSSG) to S-sulfogluthathione (GSSO<sub>3</sub><sup>-</sup>) [18]. We previously showed GSH to be a potential reducing agent [19].

GSH is the most abundant nonprotein thiol compound in living organisms that can neutralize free radicals and reactive oxygen compounds [20,21]. It serves as an immune booster, antioxidant, and detoxifier of xenobiotics and has been used in medicine and as a food additive [22,23]. The added GSH content in food ranges from 0.004% to 6.667% [23].

Dietary GSH is found in some raw fruits and vegetables [24]. Its levels can fluctuate diurnally [25,26] depending on the developmental stage of the plant [27] and environmental factors [28]. Cooking and freezing reduce GSH content considerably [28,29].

We investigated the impact of GSH occurring naturally or added as antioxidant on Pru p 3 folding and monitored its effects on the allergenicity of Pru p 3.

## Materials and Methods

### Biological Samples

We analyzed sera from 5 peach-allergic patients at the Allergy Service of the Jimenez Diaz Foundation, Madrid, Spain. All patients had experienced immediate allergic reactions after peach ingestion (urticaria, angioedema, or anaphylactic symptoms), a positive response in the skin prick test (SPT) using a commercial peach peel extract (ALK-Abelló), and a positive response to peach by open oral challenge.

### Skin Prick Tests

SPTs were performed by trained personnel at the Allergy Service of Jimenez Diaz Foundation. The wheals induced by Pru p 3 in native and reduced form at different concentrations were measured twice after 20 minutes [30,31]. A wheal diameter >3 mm was considered a positive result [31,32]. Written informed consent was obtained from all patients. The

study was approved by the Ethics Committee of Jimenez Diaz Foundation and Polytechnic University of Madrid, Madrid, Spain.

### Tissue Printing

Slides of commercial ripe peaches (*Prunus persica* cultivar Calante, Spain [33]) were blotted onto a preactivated polyvinylidene difluoride (PVDF) membrane for 30 seconds. The membrane was air-dried and incubated with a solution of 125  $\mu$ g/mL 5-iodoacetamidofluorescein (5-IAF) (Molecular Probes I30451, Life Technologies Ltd) for 2 hours at room temperature with shaking in darkness. The membrane was then washed with distilled water to remove the unlabeled probe. Fluorescence was visualized using a UV transilluminator (Herolab).

### Peach Extract and Purification and Treatment of nPru p 3

Proteins from the peel of natural peaches (*Prunus persica* cultivar Calante, Spain [33]) were extracted (1 hour at room temperature in phosphate-buffered saline with 0.5 M NaCl), and natural Pru p 3 was isolated as previously described [34]. Pru p 3 used for cell experiments was checked for purity and endotoxin content using standard methods (Thermo Fisher Scientific). Thirty micrograms of peach peel extract and 10  $\mu$ g of nPru p 3 in distilled water were incubated for 2 hours at 38°C with the fluorescent probe 5-IAF (1.25 mg/mL DMSO; Molecular Probes, Life Technologies) bound to free sulfhydryl groups in the absence or presence of 2 mg of GSH (Sigma-Aldrich). GSH alone was used as a control. Samples were desalted using MicroSpin G25 Columns (GE Healthcare) and loaded onto a 15% SDS-PAGE gel for silver staining. Additionally, samples were blotted onto PVDF membranes, blocked, and incubated with sera from peach-allergic patients (undiluted) and peroxidase-conjugated goat antihuman IgE antibody (1:3000 dilution, Biosource) using enhanced chemiluminescence (Thermo Fisher Scientific).

### Circular Dichroism Analysis

Circular dichroism measurements of nPru p 3 and GSH-reduced nPru p 3 were taken in water at a concentration of 1 mg/mL on a Jasco J-180 spectropolarimeter (Jasco) using 1-mm path-length quartz cuvettes equilibrated at 20°C. Spectra were recorded from 190 nm to 260 nm at a resolution of 0.5-nm and a scan speed of 50 nm/min. The final spectra were baseline-corrected by subtracting the corresponding solvent spectra obtained under identical conditions. The data were fitted using the Dichroweb analytical tool [35,36]. The results were expressed as mean residue ellipticity, reflecting that the peptide bond is the absorbing species for a given wavelength [37].

### Protein Digestion

Ten micrograms of nPru p 3 was incubated with/without 2 mg of GSH (Sigma-Aldrich) for 2 hours at 38°C as described above. Samples were digested with pepsin (1% wt/vol, Calbiochem) in 100 mM HCl pH 2 simulated gastric fluid (SGF) and gastrointestinal tract (GIT) fluid pH 6.5 containing 0.1% wt/vol trypsin (Sigma-Aldrich) and 0.4% wt/vol  $\alpha$ -chymotrypsin

(Sigma-Aldrich) at different time points (30 and 60 minutes), as previously described [38,39]. Before loading onto a 4-12% Tris-tricine gradient gel (Novex) for Coomassie staining, unbound GSH was removed from samples using MicroSpin G25 Columns (GE Healthcare). In parallel, samples were blotted onto PVDF membranes, blocked, incubated with polyclonal anti Pru p 3 antibody (provided by Carlos Pastor from Jimenez Diaz Foundation), and detected with goat antirabbit IgG, alkaline phosphatase conjugate (1:5000, Biosource), and enhanced chemoluminescence (Thermo Scientific). Undigested proteins (nPru p 3 and GSH-treated nPru p 3) were used as controls. Experiments were performed in duplicate.

#### Peripheral Blood Mononuclear Cell Proliferation Assays

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from whole blood from peach-allergic patients, as previously described [40]. Briefly, PBMCs from 50 mL of blood underwent density gradient centrifugation on Lymphoprep medium (Axis-Shield). The proliferation analysis was performed in triplicate with 200 000 cells per well cultured in 96-well plates (Costar) in 200  $\mu$ L Roswell Park Memorial Institute medium (Invitrogen) supplemented with 10% (vol/vol) fetal calf serum (Invitrogen) and 2 mM of glutamine (Invitrogen) in the presence of nPru p 3 (5  $\mu$ g/mL), reduced Pru p 3 (5  $\mu$ g/mL + 25  $\mu$ g/mL GSH), and GSH (25  $\mu$ g/mL) at 37°C in a 5% CO<sub>2</sub> humidified atmosphere for 48 hours. Concentrations were chosen based on

previous determinations [41]. [<sup>3</sup>H]-thymidine (0.5 mCi/well) was added during the last 16 hours, and the incorporated radioactivity was measured by scintillation counting. Phytohemagglutinin-L (PHA) from *Phaseolus vulgaris* (1 mg/mL; Roche) was used as a positive control.

#### Cytokine Measurements

Fifty microliters of supernatant/well of PBMCs stimulated in the presence of nPru p 3 (5  $\mu$ g/mL), reduced Pru p 3 (5  $\mu$ g/mL + 25  $\mu$ g/mL GSH), and GSH (25  $\mu$ g/mL) were recovered in triplicate to quantify IFN- $\gamma$ , IL-5, and IL-10 levels by ELISA using matched antibody pairs (eBioscience). Cultures containing PBMCs alone served as a negative control.

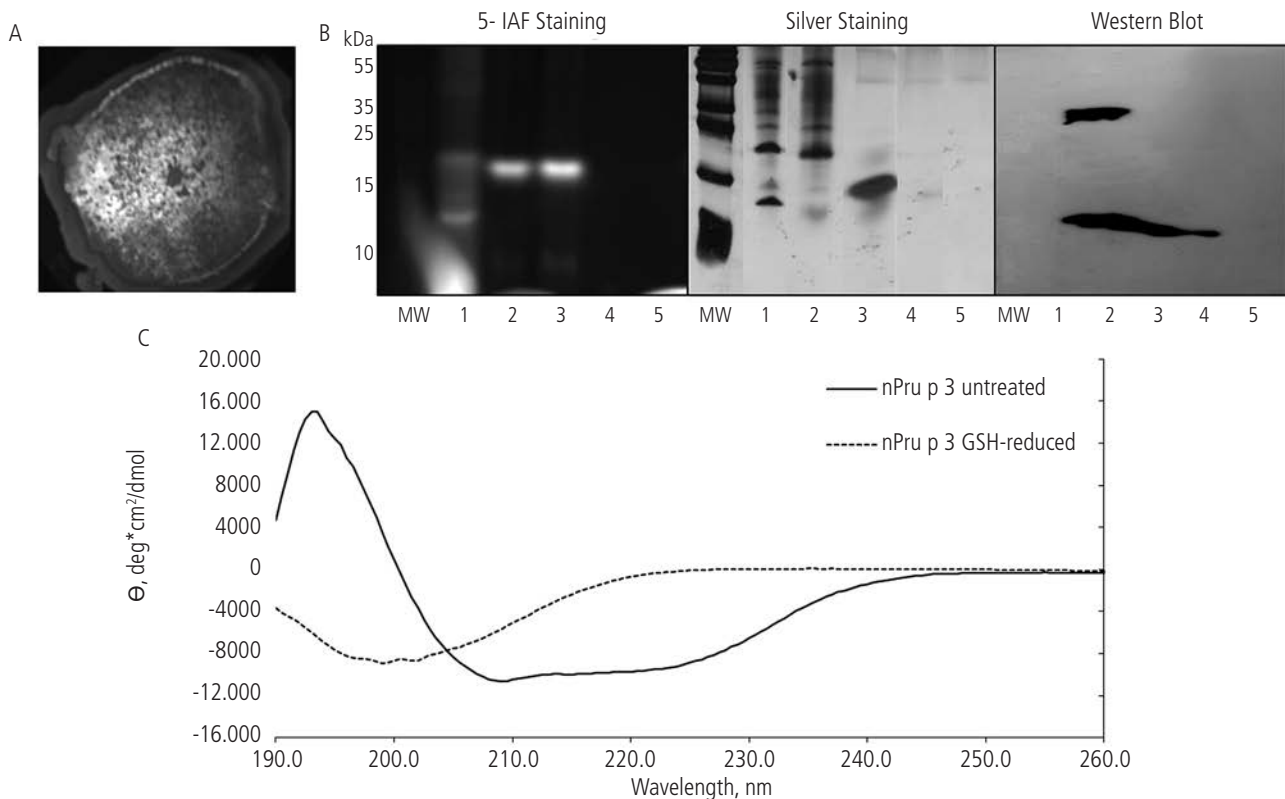
#### Statistical Analysis

The Kruskal-Wallis test and 2-way analysis of variance were performed with correction for multiple comparisons when appropriate using GraphPad 6.01. *P* values <.05 were considered significant.

## Results

#### GSH Reduces Peach Proteins In Vitro

Reduced peach proteins were detected using tissue printing and SDS-PAGE with the fluorescent probe 5-IAF (Figure 1A and



**Figure 1.** Characterization of nPru p 3 in peach extract and isolated form (GSH-reduced and nonreduced [untreated]). A, Tissue print of a commercial peach fruit blotted onto a PVDF membrane and stained with 5-IAF (fluorescent probe) for assessment by reduction. B, 5-IAF staining, silver staining, and Western blot of peach extract and nPru p 3 (untreated and GSH-reduced). MW markers: 1, peach extract; 2, peach extract + GSH; 3, nPru p 3 + GSH; 4, nPru p 3; 5, GSH. C, Circular dichroism spectroscopy analysis of nPru p 3 (GSH-reduced and nonreduced [untreated]). GSH indicates glutathione; MW, molecular weight; PVDF, polyvinylidene difluoride; 5-IAF, 5-iodoacetamidofluorescein.

B, 5-IAF staining). We failed to specifically detect Pru p 3 in the tissue prints using a polyclonal antibody. SDS-PAGE revealed 2 protein bands with free thiol groups at around 10 and 20 kDa, whereas only the upper band was visible when GSH was added to the peach extract or Pru p 3 [42]. nPru p 3 was not detected with the fluorescent probe. Interestingly, reduction by GSH did not seem to affect IgE binding to Pru p 3 (Figure 1B, Western blot). Circular dichroism analysis enabled us to confirm that nPru p 3 can be reduced by GSH in vitro (Figure 1C).

### **GSH-Reduced nPru p 3 Is Resistant to Gastrointestinal Digestion**

nPru p 3 and its GSH-reduced counterpart were digested in vitro with SGF and GIT for 30 and 60 minutes and loaded onto a 4-12% Tris-tricine gel under nonreducing conditions for Coomassie staining. nPru p 3 was not affected and appeared as a single band in the gel. Pru p 3 treated with GSH was also found to be resistant, even after 60 minutes of gastrointestinal digestion (Figure 2). Higher molecular bands correspond to the enzymes used for gastrointestinal digestion.

### **Reduced Pru p 3 Produced a Reaction Similar to That of Its Native Counterpart in SPT**

The clinical response of patients to both natural and reduced Pru p 3 forms was assessed using SPT at the Allergy Service of Jimenez Diaz Foundation. Three out of 5 patients had a stronger reaction towards the 20 µg/mL of reduced Pru p 3 than towards its natural counterpart, although the reaction was significant for only 2 of them. One patient (patient 4) experienced a stronger reaction to natural Pru p 3 (Figure 3), and another patient (patient 2) experienced similar reactions to both variants. Therefore, the patients' responses were heterogeneous. Representative photographs of SPT in patients 4 and patient 5 are shown in Figure 4.

### **PBMCs Are Less Proliferative When They Are Cultured With Reduced Pru p 3**

PBMCs from all the patients tended to be less proliferative when incubated with Pru p 3 + GSH than with nPru p 3, although significant differences in the stimulation index were not found for individual patients (Kruskal-Wallis 1-way analysis of variance with a Dunn correction for multiple comparisons,  $P$  value < .05). The rate of T-cell proliferation in the presence of GSH alone was comparable to or in some cases even higher than in the presence of reduced protein (Figure 5). The experiment was performed in triplicate.

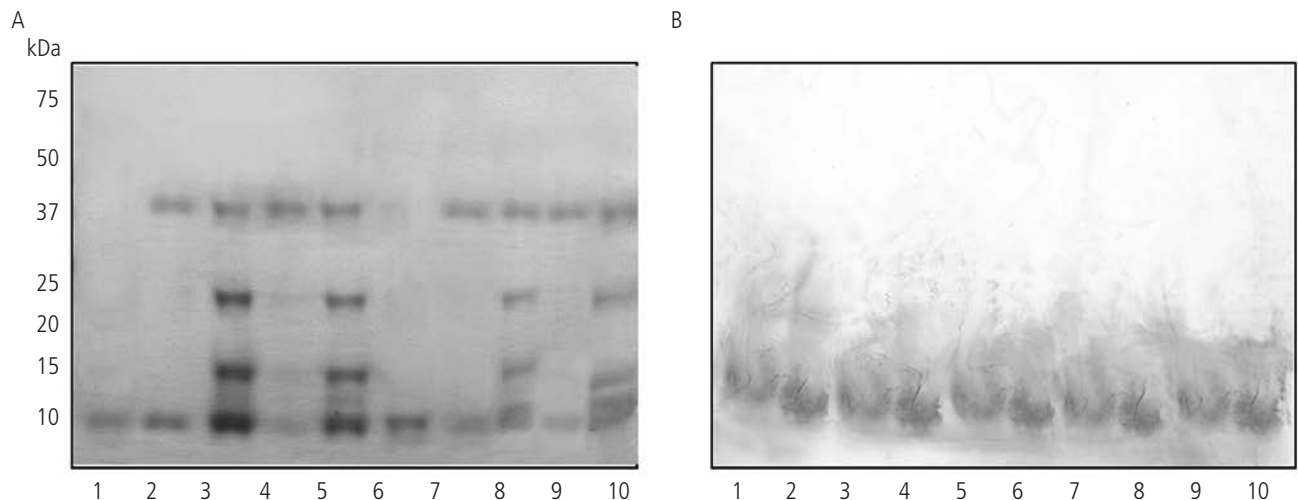
### **Cytokine Profiles of Stimulated PBMCs of Allergic Patients Correlate With Clinical Reactivity**

Secretion of IFN- $\gamma$ , IL-5, and IL-10 in PBMCs cultured in the presence of reduced or nonreduced Pru p 3 was assessed using ELISA. No differences were detected in cytokine levels between natural and reduced Pru p 3 when individual levels were combined. However, PBMCs responded heterogeneously to the natural and reduced forms of Pru p 3, especially in the case of IL10 (Figure 6). The analysis was based on 2-way ANOVA using a Bonferroni correction for multiple comparisons ( $P$  value < .05).

## **Discussion**

Peach allergy is one of the most prevalent allergies in the Mediterranean area. It affects more than 40% of food-allergic patients [43] and is caused mainly by the nsLTP Pru p 3, which is abundant in peach peel [1-5].

Studies on alterations in the allergenic potential of food proteins during processing report an increase [44,45] or decrease in IgE-binding [10,11,13] due to exposure to otherwise hidden linear epitopes [14,15], differential routes of gastrointestinal



**Figure 2.** Digestion of nPru p 3 and GSH-reduced Pru p 3 at different time points (30 and 60 minutes) with SGF and GIT fluid. A, Three micrograms of digested proteins was loaded on a 4-12% Tris-tricine gel and stained with Coomassie. B, Replicas were electrotransferred and incubated with a polyclonal anti-Pru p 3 antibody. Undigested proteins were used as controls. MW markers: 1, undigested Pru p 3; 2, Pru p 3 digested for 30 minutes with SGF; 3, Pru p 3 digested for 30 minutes with SGF and GIT; 4, Pru p 3 digested for 60 minutes with SGF; 5, Pru p 3 digested for 60 minutes with SGF and GIT; 6, undigested GSH-reduced Pru p 3; 7, GSH-reduced Pru p 3 digested for 30 minutes with SGF; 8, GSH-reduced Pru p 3 digested for 30 minutes with SGF and GIT; 9, GSH-reduced Pru p 3 digested for 60 minutes with SGF; 10, GSH-reduced Pru p 3 digested for 60 minutes with SGF and GIT. GSH indicates glutathione; SGF, simulated gastric fluid; GIT, gastrointestinal tract; MW, molecular weight.

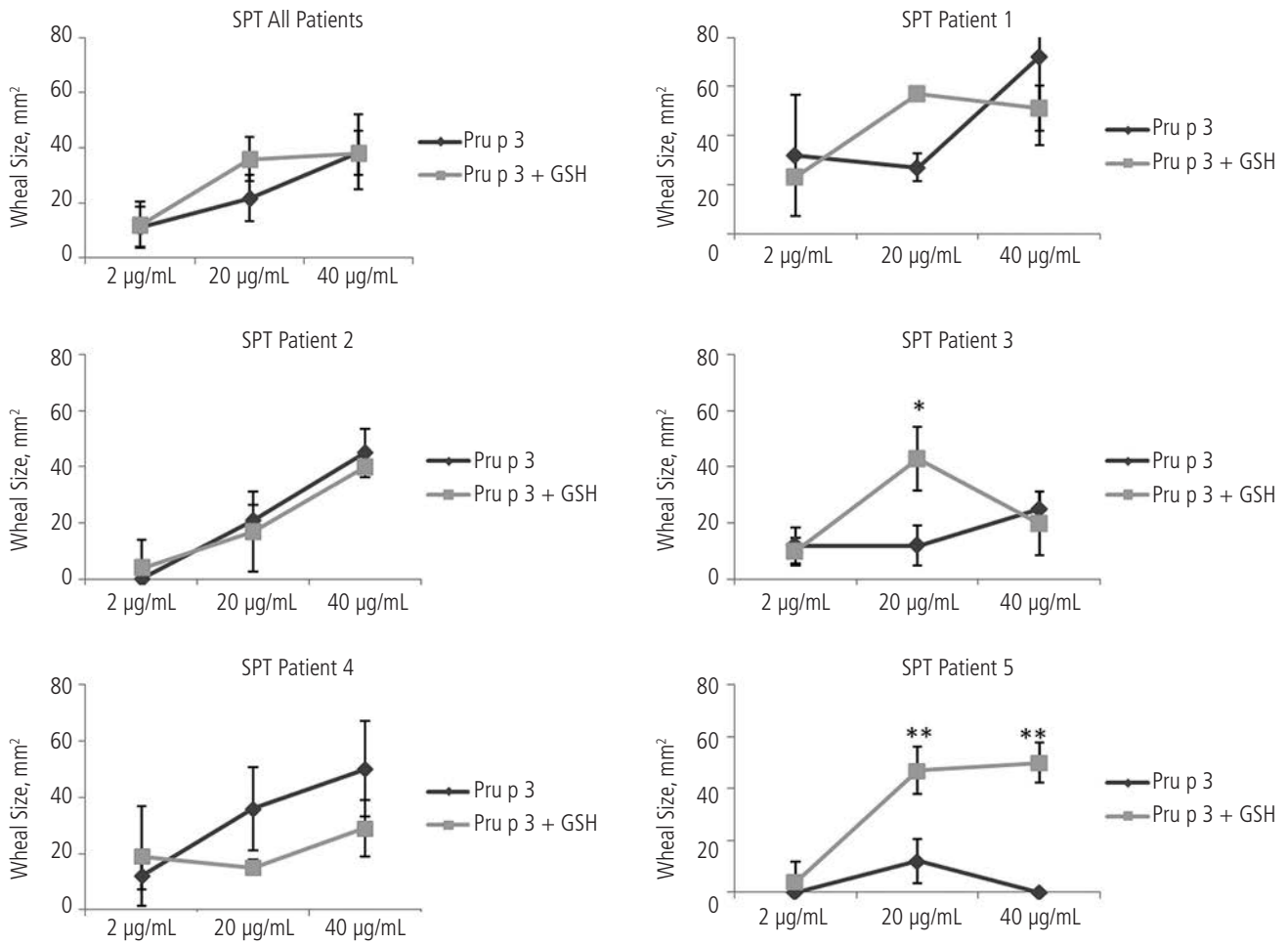


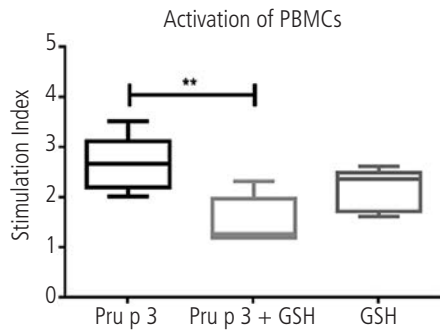
Figure 3. Individual and collective SPT reactions to GSH-reduced and untreated nPru p 3. Two measurements for each tested concentration were taken and compared with their homologous counterpart (nPru p 3 vs nPru p 3 + GSH) by 2-way analysis of variance with a Bonferroni correction for multiple comparisons. *P* values <.05 were considered significant. SPT indicates skin prick test; GSH, glutathione.

**A**

Patient	Age, y	Sex	Test, mm <sup>2</sup>					
			Pru p 3			Pru p 3 + GSH		
			2 µg/mL	20 µg/mL	40 µg/mL	2 µg/mL	20 µg/mL	40 µg/mL
1	38	F	32	27	72	23	57	51
2	55	M	0	21	45	4	17	40
3	39	M	12	12	25	10	43	20
4	45	F	12	36	50	19	15	29
5	43	M	0	12	0	4	47	50



Figure 4. A, Summary of patients' data. B, Two representative pictures of skin prick tests with GSH-treated and untreated nPru p 3. GSH indicates glutathione.



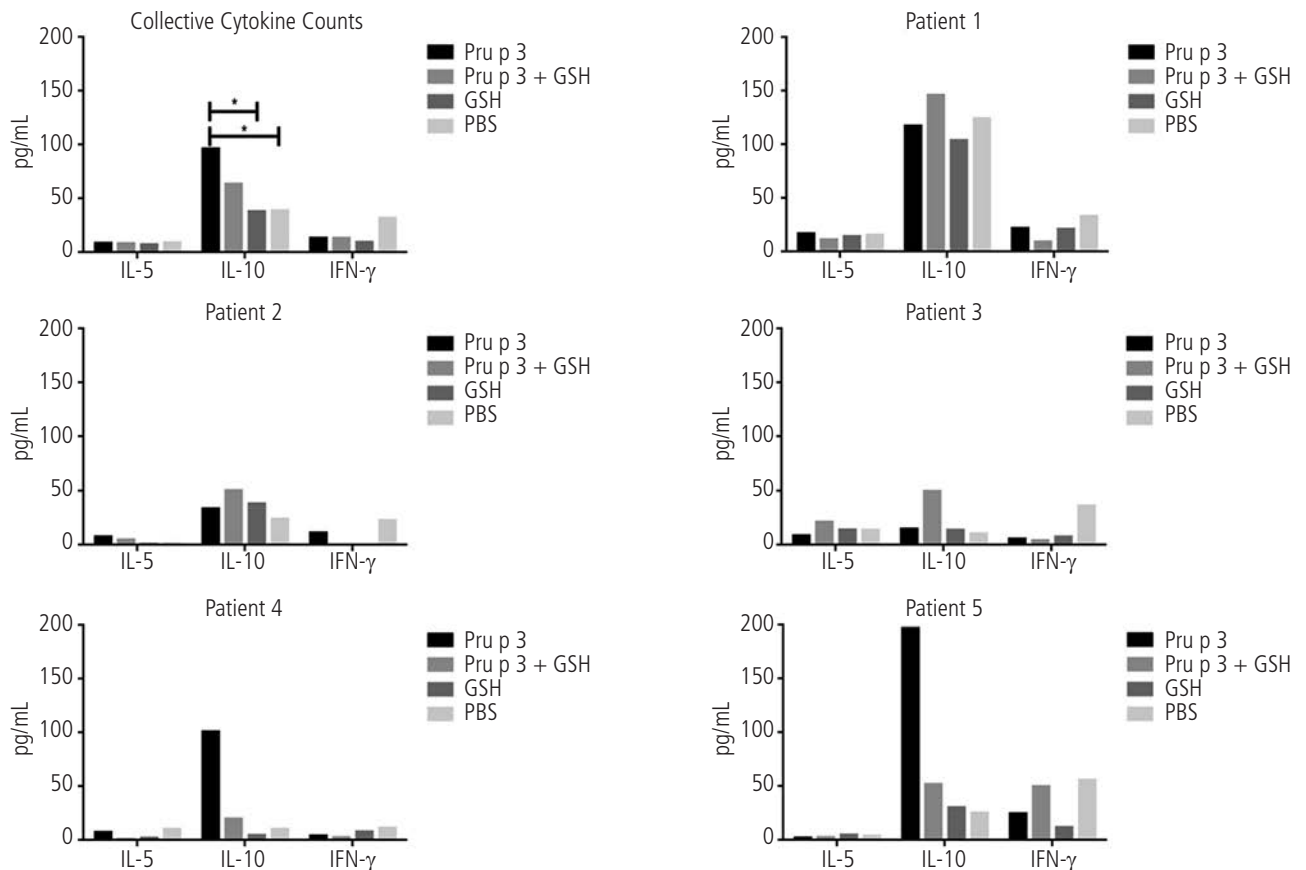
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Pru p 3 (5 µg/mL)	2.67±0.41	3.54±0.46	2.40±0.27	2.02±0.85	2.72±0.44
Pru p 3 (5 µg/mL) + GSH (25 µg/mL)	2.33±0.61	1.27±0.62	1.19±0.24	1.60±0.40	1.23±0.44
GSH (25 µg/mL)	2.38±0.02	1.63±0.60	2.62±0.41	1.83±0.38	2.36±0.14

**Figure 5.** Freshly isolated PBMCs from peach-allergic patients were cultured in the presence of 5 µg/mL of nPru p 3, reduced Pru p 3 (+25 µg/mL GSH), and GSH (25 µg/mL) for 48 hours. Proliferation was measured by [<sup>3</sup>H]-thymidine incorporation. A response was considered positive when the stimulation index was over 2. Medians are represented. Results were analyzed using a Kruskal-Wallis 1-way analysis of variance with a Dunn correction for multiple comparisons. *P* values <.05 were considered significant. PBMC indicates peripheral blood mononuclear cell; GSH, glutathione.

uptake, or a loss of conformational epitopes [46]. Dietary GSH is found in moderate amounts in some raw fruits and vegetables, mostly in its reduced form [24,47]. In the food industry, GSH is used as an antioxidant in plant protein products, processed fruits and fruit juices, and processed vegetables and vegetable juices [23].

We investigated whether disruption of the disulfide bridges of the Pru p 3 molecule—and thus linearization—might affect allergenicity. We showed that GSH was able to reduce Pru p 3 in vitro when added in amounts similar to those present in food products [23]. Reduction by GSH in vitro led to linearization of Pru p 3 and loss of 3D structure. Using tissue printing, we demonstrated that some peach proteins are reduced naturally; however, we were not able to state whether Pru p 3 was one of them. Failure to detect proteins in plant tissue using specific anti-Pru p 3 antibody (data not shown) could be due to low protein concentrations or antigen masking by matrix components.

We also investigated whether GSH-reduced Pru p 3 affected allergic reactivity. In SPT, patients reacted diversely to reduced Pru p 3, possibly owing to partial refolding processes during the in vivo test. Reduction of Pru p 3 led to a lower cytokine response, but the effects on in vitro IgE binding in our patient population were, in contrast to previous reports, very weak [16]. The unfolded allergen in a stabilized form has even been suggested as a safer treatment option for peach allergy [16,48]. Moreover,



**Figure 6.** Collective and individual cytokine production (IL-5, IL-10, and IFN $\gamma$ ) by PBMCs cultured in the presence of GSH-reduced and untreated nPru p 3. Means of 2 measurements for each condition and cytokine are represented. No significant differences were observed using 2-way analysis of variance with Bonferroni correction for multiple comparisons (*P* value <.05). PBMC indicates peripheral blood mononuclear cell; GSH, glutathione; PBS, phosphate-buffered saline.

in the presence of GSH, Pru p 3 persisted in our in vitro digestion experiment. Similarly, Vassilopoulou et al [49] showed that grape LTP digestion fragments were still IgE-reactive.

Although the PBMCs of the peach-allergic patients in the present study showed lower proliferation levels when incubated with the unfolded protein, there were no differences in cytokine production by PBMCs cultured in the presence of nPru p 3 alone or combined with GSH. SPT reactions correlated with cytokines secreted by Pru p 3-stimulated PBMCs of allergic individuals. Similar results were previously observed by Starkl et al [48] for the major peanut allergen Ara h 2, which also belongs to the prolamin plant allergen superfamily. In addition, consistent with the findings of Novaes et al [50], PBMCs in the presence of GSH alone proliferated at a rate comparable to or higher than those incubated with the reduced protein.

In conclusion, the natural reducing agent GSH leads to conformational changes in Pru p 3, but the reactivity of patients to reduced or nonreduced allergen was heterogeneous. Importantly, IgE reactivity was not lost upon reduction, and Pru p 3 stability in the gut was not impaired. Therefore, given its transient effects on Pru p 3 conformation, GSH does not seem to eliminate the risk of reactions in peach-allergic patients.

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### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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