

Reducing peanut allergens by high pressure combined with polyphenol oxidase

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Polyphenol oxidase (PPO) has been shown to reduce major peanut allergens. Since high pressure (HP) can increase enzyme activity, we postulated that further reduction of peanut allergens can be achieved through HP combined with PPO. Peanut extracts containing caffeic acid were treated with each of the following: (1) HP; (2) HP + PPO; (3) PPO; and (4) none. HP was conducted at 300 and 500 MPa, each for 3 and 10 min, 37 °C. After treatment, SDS-PAGE was performed and allergenic capacity (IgE binding) was determined colorimetrically in inhibition enzyme-linked immunosorbent assay and Western blots, using a pooled plasma from peanut-allergic patients. Data showed that HP alone had no effect on major peanut allergens. However, HP at 500 MPa combined with PPO (HP500/PPO) induced a higher (approximately twofold) reduction of major peanut allergens and IgE binding than PPO alone or HP300/PPO. There was no difference between treatment times. We concluded that HP500/PPO at 3-min enhanced a twofold reduction of the allergenic capacity of peanut extracts, as compared to PPO itself.

Keywords: high pressure; polyphenol oxidase; PPO; peanut allergens; Ara h 1 and Ara h 2; IgE antibodies

Introduction

High pressure (HP) is a non-thermal technology for food preservation. It inactivates pathogens and maintains the nutritional and sensory properties of foods and fruit/vegetable juices.[1,2] Because of its ability to denature proteins and dissociate protein complexes,[3,4] HP has been applied to food allergens for reduction of their immunoreactivity. For instance, allergens from such foods as soybean,[5] apple,[6] carrot,[7] and beef [8] are reported to have to a lower immunoreactivity after HP treatment. Additionally, HP has been used to increase the susceptibility of proteins/allergens to hydrolysis by enzymes such as trypsin and chymotrypsin. In this case, a higher degree of hydrolysis was seen with proteins/allergens (*e.g.* ovalbumin, bovine and soybean whey proteins) treated first with HP and then with proteolytic enzymes.[9–11] One study reports that HP has no effect on peanut allergens and their structures at room temperature or 80°C.[4] However, when HP is applied in conjunction with a homogenization process, the degree of hydrolysis of peanut proteins/allergens by digestive enzymes was seen to increase,[9] suggesting that a combined

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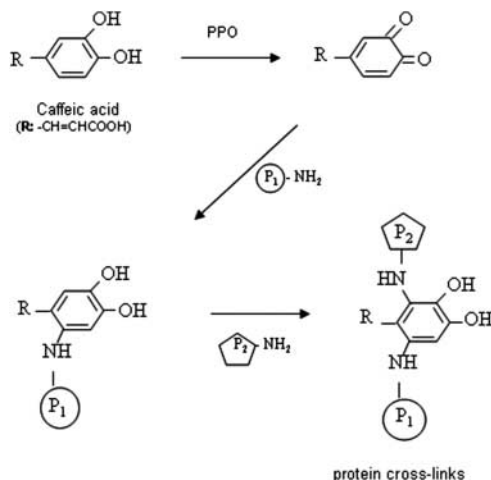


Figure 1. Formation of protein–protein cross-links in a PPO-catalyzed reaction. Proteins (P_1 and P_2) are cross-linked as shown in the presence of PPO and CA.

method (*i.e.* HP and homogenization) helps to make peanut proteins/allergens more susceptible to hydrolysis by enzymes. Also, a new HP technology called HP microfluidization that includes homogenization is reported to induce changes in structure and antigenicity of peanut allergens.[12]

Additionally, a combined HP/enzyme method (*i.e.* HP and enzymes applied at the same time) has been reported. Several studies have demonstrated that the combined method can lead to an increase in the degree of hydrolysis of allergens from milk [13,14] and soy [15] and subsequently a decrease in allergenic capacity of the allergens. An *in vivo* study with mice has also confirmed that the combined HP/enzyme system helps reduce the allergenicity of whey proteins from milk.[16] While protein denaturation may be a contributing factor in the degree of hydrolysis, an increase in enzyme activity due to HP may also be responsible for the increased hydrolysis in the combined HP/enzyme system. Indeed, increase of enzyme activities due to HP has previously been reported.[17,18] In those studies, enzymes such as chymotrypsin and thermolysin under an HP condition are reported to increase 30–45 times higher than those at atmospheric pressure. Also, HP microfluidization is reported to induce activation of polyphenol oxidase (PPO).[19]

In this study, we hypothesized that a combined HP/PPO may perform much better than PPO alone in terms of reducing the allergenic capacity of peanut extracts. PPO or a tyrosinase is a copper-containing enzyme widely distributed in fruits and vegetables. It catalyzes the oxidation of phenols such as caffeic acid (CA) to *o*-quinones, which react with the amino groups of proteins to form cross-links or protein polymers (Figure 1).[20] We previously have shown that PPO together with CA reduces the allergenic capacity of peanut allergens by cross-linking and masking the immunoglobulin E (IgE)-binding sites of peanut allergens.[20] With a combined HP/PPO system, we postulated that the allergenic capacity of peanut allergens would further be reduced or, in other words, the reduction of allergenic capacity is enhanced as a result of the HP/PPO system. Our objective was to determine if the combined HP/PPO system is a better method than PPO itself in the reduction of the allergenic capacity of peanut extracts. If confirmed by clinical studies, the research could lead to the development of less allergenic peanut-based beverages and products.

Materials and methods

Materials

PPO (25,000 units), 96-well microtiter plate (Corning), rabbit anti-human immunoglobulin E (IgE)-peroxidase, o-phenylenediamine, Tween 20, 4-chloro-1-naphthol, and phosphate-buffered saline were purchased from Sigma Co. (St. Louis, MO). 4–20% Tris-glycine pre-cast gels were purchased from Invitrogen (Carlsbad, CA). Immobilon-P membrane was obtained from Millipore Corp. (Bedford, MA). Human plasmas from three patients with peanut allergy (determined by CAP-FEIA assay for IgE antibodies) were obtained from PlasmaLab International (Everett, WA). Superblock blocking buffer, GelCode Blue Stain Reagent and bicinchoninic acid (BCA)-protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL). Raw high-oleic peanut seeds (SunOleic) were obtained from the University of Florida, Gainesville, FL.

Preparation of peanut extracts

Peanut extracts were prepared as previously described from defatted meals of raw peanuts.[20] Briefly, defatted peanut meals (0.2 g) were stirred in 20 mM sodium phosphate buffer (1.5 mL), pH 8 for 30 min and then centrifuged at $8000 \times g$ for 10 min. The supernatants (extracts) were retrieved and analyzed for protein concentration, using the BCA kit assay.

Treatment with PPO

Treatment of peanut extracts with PPO was performed according to the method of Chung et al.[20] Briefly, peanut extracts (2 mg/mL) containing CA (20 mM) were incubated with PPO (40 units) at 37°C for 60 min. The samples were then frozen until further analyses.

Treatment with HP and combined HP/PPO

HP treatment with an isostatic press (Zdas CYX 6/0103) was performed according to the method of Houska et al.[21] Drinking water was used as the pressure-transmitting medium. The vessel was preheated to the final temperature as planned. For HP treatment without PPO, peanut extracts (2 mg/mL) containing CA (2 mM) were treated with HP at 300 and 500 MPa, respectively, for 3 and 10 min at 37°C. For a combined HP/PPO treatment, samples were treated with PPO (40 units) and HP simultaneously at the energy and time indicated above. After HP and HP/PPO treatment, the samples were frozen until further analyses.

SDS-PAGE and Western blots for IgE binding

SDS-PAGE and Western blots on treated and untreated samples (5 μ L, 2 mg/mL) were performed according to the method of Chung and Reed.[22] After SDS-PAGE, gels were stained in GelCode Blue Stain and destained with deionized water. For Western blot, proteins from the gel (without staining) were transferred to a polyvinylidene difluoride membrane, blocked with a SuperBlock solution, and treated with a pooled plasma (1:10) from peanut-allergic patients. IgE binding was detected, using a rabbit anti-human IgE peroxidase conjugate (1:500) and a substrate solution of 4-chloro-1-naphthol (0.5 mg/mL) containing 0.03% hydrogen peroxide.

Inhibition ELISA for IgE binding

Competitive inhibition enzyme-linked immunosorbent assay (ELISA) was carried out as previously described.[23] Briefly, treated and untreated peanut extracts (50 μ L) (0.01–100 μ g/mL) were mixed with a pooled plasma (50 μ L) (1:20) and then incubated for 30 min in a plate coated with the untreated peanut extract (20 μ g/mL), followed by IgE detection, using a rabbit anti-human IgE peroxidase conjugate (1:500) and a substrate containing *o*-phenylenediamine (0.5 mg/mL), 0.03% hydrogen peroxide in 0.1 M citrate buffer, pH 5.5. The absorbance was read at 490 nm with a CERES 900C plate reader (Bio-Tek Instruments, Inc., Winooski, VT), following the addition of 4N sulfuric acid (50 μ L). Percent inhibition was determined and defined as $(1 - A/A_0) \times 100$, where A is the absorbance value of sample mixed with the plasma, and A_0 is the absorbance value of the control mixed with the plasma. Values are means of three determinations. Statistical analyses were performed using a Student's *t*-test at a $p = .05$ level of significance.

Results and discussion

Effect of HP treatment only

Figure 2 shows the effect of HP itself (without PPO) on peanut allergens in the extract. As shown in SDS-PAGE, bands corresponding to peanut allergens (Ara h 1 and Ara h 2) remained unchanged after treatments at 37°C with HP at 300 and 500 MPa, respectively, for 3 and 10 min. The results suggest that HP under these conditions had no effect on peanut allergens. Our data are in agreement with the finding of Van der Plancken et al.,[4] in which purified peanut allergens (Ara h 2 and Ara h 6) were found to be stable to HP between 150 and 800 MPa (10 min), and their structures remained unchanged even during HP treatment at 80°C. By contrast, allergens from apples treated with HP at 80°C exhibited structural changes and a reduction in immunoreactivity.[4,6]

The high stability of peanut allergens to HP as shown in this study and that of Van der Plancken et al. [4] is unusual because HP has previously been shown to induce protein denaturation, dissociation, or aggregation [8,15] and as a result, the HP-treated proteins are more easily digested and have a lower immunoreactivity. Also, HP can destabilize hydrophobic interactions,[24] which

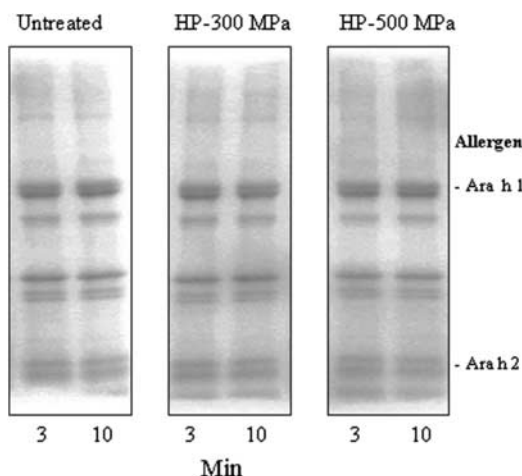


Figure 2. SDS-PAGE profiles of HP-treated and untreated peanut protein extracts at 3 and 10 min, 37°C. HP was conducted at 300 and 500 MPa, respectively.

usually play a major role in the stabilization of protein tertiary structure. Another factor associated with protein destabilization is hydration. According to Balny et al.,[25] HP can induce hydration changes which in turn may cause protein structural changes and denaturation, and this phenomenon may explain why proteins in a solution are more unstable to HP than those in the dry state. However, in contrast to those studies, our current study and that of Van der Plancken et al. [4] showed a different HP effect – that is, peanut allergens prepared in solutions were stable to HP rather than undergoing structural changes.

Effect of PPO versus a combined HP/PPO

Figure 3 shows the SDS-PAGE protein profiles of the untreated, PPO-, HP300/PPO- and HP500/PPO-treated peanut extracts at 3 and 10 min. HP300 and HP500 refer to HP conducted at 300 and 500 MPa, respectively. Results showed that treatment times (3 and 10 min) did not differ in the protein profiles for each treatment. In this case, 3 min was considered to be sufficient for the treatments. Compared to the untreated, PPO-treated extracts exhibited a distinct reduction in the density of protein bands, in particular those corresponding to peanut major allergens Ara h 1 and Ara h 2. The reduction was accompanied by the appearance of high-molecular weight protein cross-links (shown as dark smears). They were formed because of the reactions catalyzed by PPO via CA as illustrated in Figure 1. The results are in line with our previous study [20] and other studies [26,27], where protein cross-links were seen as a result of PPO and laccase-catalyzed reactions. When PPO was combined with HP at 300 MPa (HP300/PPO) and applied to the peanut extracts, there appeared to be no difference in the protein profiles between the PPO-treated and combined HP300/PPO, regardless of treatment time. However, when HP was increased to 500 MPa and combined with PPO (*i.e.* HP500/PPO), further reduction in the density of protein bands including major peanut allergens was seen. In this case, most protein bands appeared as smears (cross-links) in the SDS-PAGE profile. Therefore, of the three treatments, the combined HP500/PPO was the most effective in the reduction of major peanut allergens.

The above finding appears to indicate that HP at 500 MPa may have increased the susceptibility of peanut allergens to PPO in the formation of cross-links. In many cases, increased susceptibility

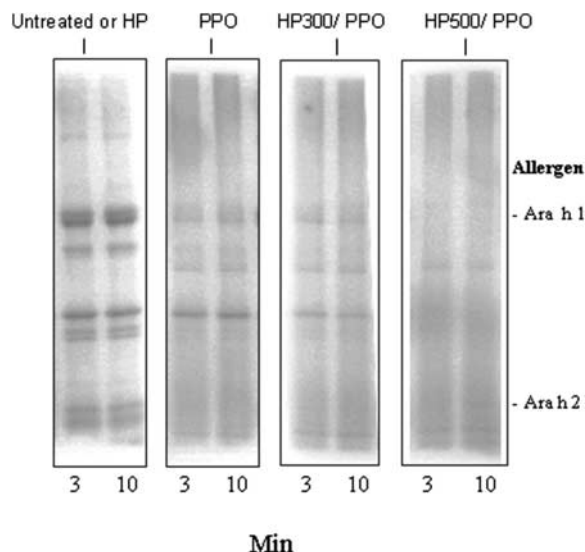


Figure 3. SDS-PAGE profiles of PPO-, HP300/PPO-, and HP500/PPO-treated peanut extracts versus untreated/or HP at 3 and 10 min, 37°C. HP300 = HP at 300 MPa; HP500 = HP at 500 MPa.

of proteins to enzymes during HP treatment is mostly due to the unfolding or dissociation of proteins by HP,[16] which therefore allows the accessibility of enzymes to the peptide bonds or functional groups of proteins that were hidden prior to HP treatment. For example, when proteolysis is conducted under HP, proteins such as β -lactoglobulin become more susceptible to enzymes like pepsin, trypsin, and chymotrypsin, and, therefore, its hydrolysates can be obtained in 5 or 20 min instead of 24 h at atmospheric pressure.[28] Also, Penas et al.,[10] observed a similar increase in hydrolysis for soybean whey proteins treated by HP. All these studies pointed to protein unfolding (caused by HP) being responsible for the increased hydrolysis. However, Van der Plancken et al. [4] reported that HP had no effect on protein unfolding or the structure of peanut allergens under the conditions of 800 MPa, 80°C, and 10 min.[4] In that study, a spectroscopic measurement was used. The potential problem with the spectroscopic technique is that it does not always show an explicit unfolding of a protein, and as a matter of fact, there may always be some fraction of a protein (*e.g.* a few percent or even a fraction of a percent) denatured or unfolded.[29] In such a case, even if there is no obvious denaturation apparent, a repeated action of the enzyme PPO on an increased fraction of the denatured protein under pressure (though “invisible”) may cause a sizable amount of the reaction product accumulated with time. As the stability of a globular protein is generally lowered by pressure, pressure is always expected to increase the reactivity (whether proteolysis or PPO reaction) of a globular protein at elevated pressures.[29] Therefore, in the current study, the increased reduction of peanut allergens by the combined HP500/PPO can be explained by such a mechanism [29]: the conformation of the allergen changes from a folded state to a PPO-susceptible unfolded state, the result of which is the enhanced accessibility of the unfolded allergen to PPO rather than an enhancement of enzyme activity as reported by other studies.[17,18] In one study, the enzyme alcohol dehydrogenase was reported to lose activity in the HP range of 100–400 MPa.[30]

Effect of PPO and HP/PPO on IgE binding

Figure 4 shows the inhibition of IgE binding to the plate by peanut extracts treated at 3 min with PPO and HP/PPO, respectively, in ELISA. IgE binding was reduced by the PPO-treated extract with an IC_{50} (inhibitor concentration at 50% inhibition) value of 0.9 $\mu\text{g/mL}$, compared to 0.5 $\mu\text{g/mL}$ for the control, which means the reduction was almost twofold (*i.e.* $0.9/0.5 \approx 2$). Extracts treated by HP300/PPO exhibited the same degree of inhibition as PPO itself, indicating

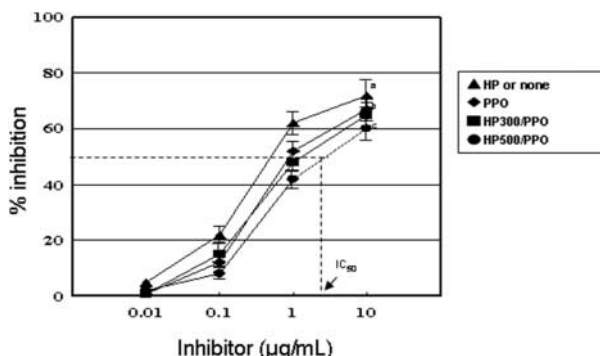


Figure 4. Inhibition ELISA of HP/or untreated, PPO-, HP300/PPO-, and HP500/PPO-treated peanut extracts at 3 min, 37°C. Samples (0.01–100 $\mu\text{g/mL}$) were each mixed with a pooled plasma (1:20) from peanut-allergic individuals and incubated in a microtiter plate coated with an untreated extract (20 $\mu\text{g/mL}$). IgE binding was detected, using a rabbit anti-human IgE-peroxidase conjugate (1:500) and a substrate solution of *o*-phenylenediamine. Values (on semi-log scale) are means of three determinations. Values between 0.1 and 10 $\mu\text{g/mL}$ with different letters are significantly different from each other ($p < .05$).

that HP at this energy level (300 MPa) along with PPO did not help enhance the reduction of IgE binding. By contrast, HP at 500 MPa combined with PPO (HP500/PPO) enhanced further the inhibition or reduction of IgE binding ($IC_{50} = 2.5 \mu\text{g/mL}$). This is a 2.5-fold reduction, compared to PPO itself. The results were in line with the SDS-PAGE data which showed that peanut major allergens were reduced further by HP500/PPO treatments, compared to PPO or HP300/PPO. Treatment for a longer time (*i.e.* 10 min) did not result in a higher reduction in IgE binding (data not shown). This indicates that 3 min is sufficient enough for the reduction of the allergenic capacity of peanut extracts with HP500/PPO.

Additionally, Western blot analyses were carried out. Results showed (Figure 5) that IgE binding to the peanut major allergens (Ara h 1 and Ara h 2) was reduced as a result of the PPO or HP/PPO treatments. While there was no difference in IgE-binding reduction between PPO and HP300/PPO treatments, a distinct difference was observed between HP500/PPO and HP300/PPO. In this case, HP500/PPO exhibited the most reduction in IgE binding to the peanut major allergens in Western blots. Such reduction was not due to the loss of IgE binding sites on the surface of allergens but the amount of allergens being reduced after they were partially transformed into polymers or cross-links as a result of the PPO reactions (see Figures 1 and 2). Although the cross-links ($>93 \text{ kDa}$) bound IgE (shown as smears in blots), they were thought to have a lower IgE-binding capacity than the untreated peanut allergens. This is because the overall IgE binding of the HP500/PPO-treated extract in ELISA (Figure 4) was lower than that of the untreated. In this case, the cross-links were deemed less allergenic than the native peanut allergens, otherwise, the overall IgE binding would have been the same as or higher than the native. The cross-links had a lower allergenic capacity probably because the IgE-binding sites present on the surface of peanut allergens prior to cross-linking were masked due to the cross-linking and transformation of the allergens into polymers.[20] Several studies have reported similar reductions in allergenicity of cross-links formed from milk allergens such as β -casein [26] and β -lactoglobulin [27] treated with laccase/CA and PPO/CA, respectively.

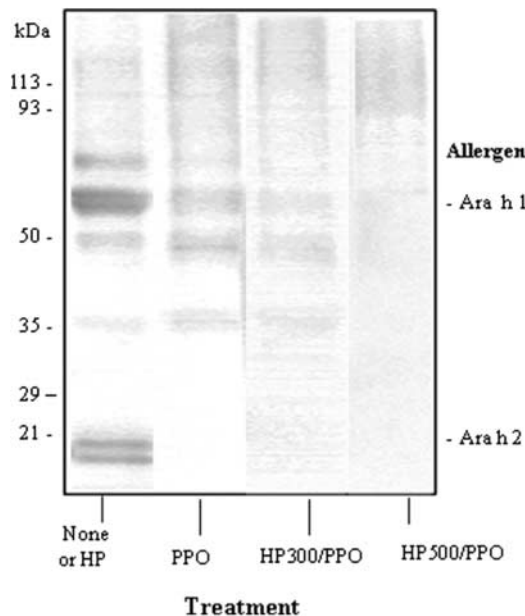


Figure 5. Western blot analyses of PPO-, HP300/PPO-, and HP500/PPO-treated peanut extracts at 3 min, 37°C versus the untreated/or HP, using a pooled plasma from peanut-allergic individuals. IgE binding was detected, using a rabbit anti-human IgE-peroxidase conjugate (1:500) and a substrate of 4-chloro-1-naphthol containing 0.03% hydrogen peroxide.

Without PPO, HP itself had no effect on IgE binding of peanut extracts (in Figure 4). Allergens from other foods such as apples and celery have also been shown to be unaffected by HP.[1,21] However, at a high temperature (115°C), HP is reported to be able to reduce the allergenicity of apple and celery allergens.[6] Peanut allergens are not likely to change at a high temperature in terms of structures and IgE binding because a previous study has shown that HP at a high temperature has no effect on peanut allergens.[4] To date, allergens that have been identified to be affected by HP alone include those from soy.[5,31] The mechanism as to why proteins from different sources react differently toward HP is not clear. Matrix effect probably may have played a role. According to Van der Plancken et al.,[4] it is difficult (quoted) to draw a general conclusion on the effect of HP processing, as there is clearly an effect of the matrix on allergen inactivation and the mechanism seems to differ between allergens.

Conclusions

HP by itself (300 or 500 MPa) had no effect on major peanut allergens (Ara h 1 and Ara h 2) and their allergenic capacity. Cross-linking of the allergens (as smears) and a reduction in IgE binding were observed with PPO treatment of the peanut extracts. When PPO was combined with HP at 300 MPa (HP300/PPO) and applied to peanut extracts, the results were the same as those of PPO itself. However, when a combined HP500/PPO was applied, an increase in cross-linking of allergens and an enhanced reduction (approximately twofold) in IgE binding were observed, as compared to PPO alone or HP300/PPO. A treatment time of 3 min was sufficient for this treatment. In conclusion, only HP at 500 MPa was able to enhance or display a twofold reduction of allergenic capacity of peanut extracts catalyzed by PPO. If confirmed by clinical studies, the research could lead to the development of less allergenic peanut-based beverages and products.

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