

Binding of peanut allergen Ara h 2 with *Vaccinium* fruit polyphenols

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ABSTRACT

The potential for 42 different polyphenols found in *Vaccinium* fruits to bind to peanut allergen Ara h 2 and inhibit IgE binding epitopes was investigated using cheminformatics techniques. Out of 12 predicted binders, delphinidin-3-glucoside, cyanidin-3-glucoside, procyanidin C1, and chlorogenic acid were further evaluated *in vitro*. Circular dichroism, UV–Vis spectroscopy, and immunoblotting determined their capacity to (i) bind to Ara h 2, (ii) induce protein secondary structural changes, and (iii) inhibit IgE binding epitopes. UV–Vis spectroscopy clearly indicated that procyanidin C1 and chlorogenic acid interacted with Ara h 2, and circular dichroism results suggested that interactions with these polyphenols resulted in changes to Ara h 2 secondary structures. Immunoblotting showed that procyanidin C1 and chlorogenic acid bound to Ara h 2 significantly decreased the IgE binding capacity by 37% and 50%, respectively. These results suggest that certain polyphenols can inhibit IgE recognition of Ara h 2 by obstructing linear IgE epitopes.

1. Introduction

Peanut allergy is a worldwide health concern. According to the International Union of Immunological Societies, affected individuals typically show mild to life-threatening adverse reactions triggered by one (or several) of the 16 known peanut allergenic proteins found in the edible seed (according to the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies) (WHO/IUIS; <http://www.allergen.org/search.php?AllergenSource=Arachis+hypogaea>). Peanut allergy is a type I (immediate and IgE-mediated) hypersensitivity disorder. During sensitization, IgE antibodies directed against peanut allergens are produced which ultimately bind to high affinity receptors (FcεRI) on mast cell and basophil surfaces. Upon re-encounter with peanut allergens, certain protein epitopes (protein segments) bind and cross-link peanut-specific IgE antibodies located on mast cells and basophils. Downstream cascade reactions trigger these cells to release preformed granules containing inflammatory mediators like histamine or prostaglandins, which are ultimately responsible for allergic symptoms. Out of all the known peanut allergens, Ara h 2 is one of the most potent (Koppelman, Wensing, Ertmann, Knulst, & Knol, 2004). Ara h 2 is a 2S albumin, seed storage protein, which belongs to the prolamin superfamily. Ara h 2 exists as two isoforms, Ara h 2.01 (16,670 Da) and Ara h 2.02 (18,050 Da), the former lacking 12 amino acids. This short 12 amino acid sequence contains a linear IgE binding

epitope (comprised of a continuous amino acid sequence) between α -helix 2 and 3 (Burks et al., 1992; Chatel, Bernard, & Orson, 2003). Structurally, Ara h 2 contains five α -helices and four disulfide bonds. Along the polypeptide chain of Ara h 2.01, 10 linear IgE binding epitopes have been identified (Stanley et al., 1997). The term Ara h 2 will refer to both isoforms in this text, unless specified as Ara h 2.01 or Ara h 2.02.

In recent studies, we have shown through *in vitro*, *ex vivo*, and *in vivo* experiments that complexing protein-rich peanut flour with polyphenols from certain plant and fruit extracts resulted in the formation of protein–polyphenol complexes with attenuated allergenic potential (Supplementary Fig. 1; Plundrich et al., 2014; Plundrich et al., 2015; Plundrich, Foegeding, Williams, & Lila, 2017; Bansode, Plundrich, Randolph, Lila, & Williams, 2018). When digested *in vitro* with pepsin, some peanut allergens, including Ara h 2, hydrolyzed more rapidly when bound in stable protein–polyphenol complexes, and digestive fragments were less immunoreactive (based on their capacity to bind to peanut-specific IgE from a pool of peanut-sensitive patient plasma and peanut-specific rabbit polyclonal IgG *in vitro*) when compared to uncomplexed peanut flour (Plundrich et al., 2015). Studies to date suggest that peanut proteins formed aggregates with polyphenols stabilized through both non-covalent interactions and covalent bonds (Plundrich et al., 2015; Plundrich, Foegeding, Williams, & Lila, 2017; Bansode et al., 2018). Indication for covalent bonds was found through a

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polyphenol concentration-dependent decreased band density and upward movement of known allergen bands, including Ara h 2, on SDS-PAGE gels accompanied with the formation of high molecular weight protein cross-linked material. The fact that those complexes were not broken down by SDS or β -mercaptoethanol indicated that the bonds formed between the molecules in those complexes must be of covalent nature. Protein-polyphenol complexes electroblotted onto a PVDF membrane and subsequently stained with nitroblue tetrazolium (stains protein-quinone complexes) further indicated at least partial covalent modification of peanut proteins (Plundrich et al., 2017; Bansode et al., 2018). It was hypothesized that the binding of polyphenols either masks or alters the structure of allergenic epitopes, reducing the IgE binding capacity of the aggregate complexes. Experiments in the present study aimed to determine how polyphenols may interface with a primary peanut allergen, Ara h 2, and which polyphenolic classes could be most effective at reducing its allergenic potency or properties. To investigate potential compounds involved in attenuating allergic properties, we used an *in silico* virtual screening of a library of polyphenols using molecular modeling techniques. The compounds were then prioritized according to their predicted binding affinity towards Ara h 2 and experimentally examined to validate the predictions.

For this study, 42 diverse polyphenolic compounds found in cranberries (*Vaccinium macrocarpon* Ait.) and/or lowbush blueberries (*Vaccinium angustifolium* Ait.) including flavonoids and phenolic acids were investigated. We used structure-based computational docking to assess their binding potential to Ara h 2. Four polyphenols predicted to bind to Ara h 2 and Ara h 2 IgE epitopes, belonging to the anthocyanin (delphinidin-3-glucoside and cyanidin-3-glucoside), procyanidin (procyanidin C1), and phenolic acid (chlorogenic acid) polyphenol classes, were selected for further experimental validation. Benzoic acid, an aromatic carboxylic acid, was tested as a negative control due to minimal *in silico* binding results. The compounds were evaluated for their Ara h 2 binding potential and their capacity to induce protein structural changes through spectroscopic methods using purified Ara h 2 (containing both isoforms) *in vitro*. Finally, Ara h 2-polyphenol complexes were tested for their IgE binding potential through immunoblotting. We hypothesized that all proposed polyphenols bind to Ara h 2 as predicted *in silico* and that some of them might be able to induce protein secondary structure changes. We tested some of the formed protein-polyphenol complexes for *in vitro* IgE binding based on the polyphenols' predicted potential to bind with amino acids that are part of linear IgE binding epitopes, and block the binding of epitope-specific IgE antibodies.

2. Methods and materials

2.1. Materials

Ara h 2 was isolated and purified from raw peanuts (Runner variety) as described previously (Sen et al., 2002) and provided by the Maleki laboratory (United States Department of Agriculture-Agricultural Research Service-Southern Regional Research Center, New Orleans, LA, USA). Analytical grade standard procyanidin (PAC) C1 and cyanidin-3-glucoside were obtained from ChromaDex (Irvine, CA, USA). Delphinidin-3-glucoside was purchased from Cayman Chemical (Ann Arbor, MI, USA). Chlorogenic acid (3-O-caffeoylquinic acid) and benzoic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Precast mini TGX 4–20% polyacrylamide gels, 10 $\times$ tris/glycine/SDS running buffer, Precision Plus protein pre-stained standard, and Laemmli sample buffer (2 $\times$) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Chameleon duo pre-stained protein ladder, Odyssey PBS blocking buffer, protein sample loading buffer (4 $\times$), REVERT total protein stain, and REVERT total protein wash solution were purchased from LI-COR (Lincoln, NE, USA). Plasma from seven peanut-allergic individuals were obtained from PlasmaLab International (Everett, WA, USA) and pooled (peanut-specific IgE levels ranged from 53 to > 100 kU L⁻¹ as

determined via ImmunoCAP; Phadia, Uppsala, Sweden). Goat anti-human IgE DyLight 800 conjugated secondary antibody was obtained from Agrisera (Vännäs, Sweden). Polyvinylidene fluoride (PVDF) membranes were purchased from EMD Millipore (Billerica, MA, USA). Tween20 and PBS (10 $\times$) were purchased from Fisher Scientific (Hampton, NH, USA).

2.2. Molecular docking studies

2.2.1. Preparation of the Ara h 2.01 protein structure

An X-ray crystal structure available for Ara h 2.01, PDB ID: 3OB4, (but not for Ara h 2.02) was used for computational binding studies (Mueller et al., 2011). The X-ray crystal structure of Ara h 2.01 (resolution of 2.71 Å) bound with Maltose Binding Protein (MBP) was extracted from the Protein Data Bank. Ara h 2.01 binding to MBP (which facilitated crystal structure formation) resulted in the removal of 27 amino acid residues (Mueller et al., 2011). For this docking study, the MBP protein was removed and Ara h 2.01 was structurally curated using the Schrödinger Suite's Protein Preparation Wizard (Schrödinger Release 2017-2). All bond orders were assigned and explicit hydrogens were added to the original structure. There were no missing side chains or missing loops detected by the Prime software. All water molecules were removed.

The EPIK program was used to determine the protonation state of Ara h 2.01 at pH 7. A restrained minimization was performed with the OPLS3 force field (Schrödinger Release 2017-2). The amino acid sequence of the refined structure is as follows: RRCQSQLERA₁₀NLRPCE-QHLM₂₀QKIQRDEDSY₃₀ERDPYSPSQD₄₀PYSPSPYDRR₅₀GAGSSQH-QER₆₀CCNELNEFEN₇₀NQRCMCEALQ₈₀QIMENQSDRL₉₀QGRQQEQ-QFK₁₀₀RELRNLPQQC₁₁₀GLRAPQRCDL₁₂₀D.

2.2.2. Binding sites identified at Ara h 2.01

The docking of polyphenolic compounds was performed with two different grid generation methods, namely *SiteMap* and *BlindDocking*, using the Schrödinger Suite's Glide docking program. These grid generation methods were used to position and delimitate the potential binding sites of the ligand.

First, the *SiteMap* program was utilized, which identifies the possible binding sites and calculates their physicochemical characteristics (e.g. exposure to solvent, volume, and hydrophobic and hydrophilic spaces) (Schrödinger Release 2017-2). Using these quantitative parameters, a Druggability Score (Dscore) was calculated. A Dscore > 0.8 associated with a binding site indicates a high likelihood that a small molecule ligand can bind to that pocket. By using the default parameters for *SiteMap* (5 Å buffer region, a minimum of 15 site points, restrictive hydrophobicity, and a fine grid), several possible binding pockets were discovered for Ara h 2.01. The *SiteMap* receptor grid was generated with 15 Å in the X, Y, and Z directions centered around the binding pocket with the highest Dscore, Dscore = 0.95.

Second, a *BlindDocking* protocol was performed, that leads to the creation of a receptor grid without specifying a binding pocket. The entire protein was included in the grid in order to calculate all potential binding sites and modes of the polyphenol ligands. This type of docking can only be used on smaller sized proteins because the squared grid can only reach 40 Å in the X, Y, and Z directions. Herein, the *BlindDocking* receptor grid was formed by 25 Å in the X, Y, and Z directions centered around the entire Ara h 2.01 protein.

2.2.3. Preparation of the screening library

The chemical library considered for this study included a series of 42 naturally occurring polyphenols commonly found in lowbush blueberries and/or cranberries (USDA, 2013) (Supplementary Table 1). All these compounds were processed using LigPrep from the Schrödinger Suite with an OPLS3 force field (Schrödinger Release 2017-2). Tautomeric states of each compound were generated at pH 7 using EPIK, while retaining their specified chirality.

2.2.4. Structure-based molecular docking

After protein and ligand curation, the 42 unique compounds were docked using Schrödinger's GLIDE software with both SP (standard precision) and XP (extra precision) scoring functions using both *SiteMap* and *BlindDocking* receptor grids (Schrödinger Release 2017-2). Overall, it represented 168 individual docking calculations with each compound non-covalently interacting with the protein. All docking results were analyzed according to the docking and eModel scores associated with each docking pose. The docking score (DS) is composed of Glide Scores and represents the ligand's predicted binding affinity in the pocket; the eModel score (eM) helps represent the likelihood of the ligand conformation. To consider a compound as "active" in the binding site, $DS \leq -7 \text{ kcal mol}^{-1}$ and $eM \leq -50 \text{ kcal mol}^{-1}$ are typically favored. The DS and eM thresholds were previously discovered through virtual screening protocols of micromolar binders. These thresholds can vary based on the protein or the scoring function and are considered guidelines for what is predicted to bind (Fourches, Muratov, Ding, Dokholyan, & Tropsha, 2013).

2.2.5. Molecular dynamics and dimer binding potential

The dynamic interactions between polyphenols and Ara h 2.01 were studied using Molecular Dynamics Simulations (MDS) and the Desmond software. Each protein-ligand model was built using the best Glide docking pose, which was then explicitly solvated with water molecules. All MDS ran for 50 ns with TIP3P solvation model, an OPLS3 force field energy minimization, and an orthorhombic volume. The calculation time step was fixed at 1.0 fs with an integration output of 1.0 ps (Schrödinger Release 2017-2).

In this study, the MDS can help determine the nature and persistence of dynamic molecular interactions between the Ara h 2.01 protein and a particular polyphenol. Moreover, dimers of Ara h 2.01 were computationally created to analyze the effects of a larger binding pocket on protein-ligand interaction potential (i.e. potential protein-polyphenol-protein interactions). An Ara h 2.01 dimer was generated using the protein-protein interaction software InterEvDock, which resulted in a larger and more complex binding pocket (Yu et al., 2016). InterEvDock calculated 150 dimer combinations using three different criteria: SOAP_PP, FRODOCK, and IES. SOAP_PP (Statistically Optimized Atomic Potentials Protein-Protein docking) is determined by the statistical potentials of protein-protein docking (Dong, Fan, Schneidman-Duhovny, Webb, & Sali, 2013). FRODOCK (Fast Rotational Docking) is determined by different potentials of van der Waals, electrostatics, and desolvation forces, whereas InterEvScore (IES) is generated from multiple sequence alignment docking scores (Andreani, Faure, & Guerois, 2013; Garzon et al., 2009).

2.3. Circular dichroism (CD) spectra and secondary structure calculation

Circular dichroism spectra of Ara h 2 (containing both Ara h 2 isoforms) and its polyphenol complexes were recorded with a Jasco J-815 spectropolarimeter. For measurements in the far-UV region (secondary structure; 260–183 nm), a quartz cuvette with a path length of 1 mm was used. Ara h 2 (provided in PBS 0.5x) was buffer exchanged using PD Mini Trap G-25 columns into MilliQ water (GE Healthcare, Piscataway, NJ, USA). The protein concentration was estimated using Lambert-Beer's law ($A = l * c * \epsilon$) with A being the absorbance at a specific wavelength, l being the path length of the cuvette, c being the concentration of the protein and ϵ being the molar extinction coefficient of the protein at the wavelength of interest. The absorbance of the Ara h 2 solution was measured at 280 nm (Shimadzu UV-2450 spectrophotometer; Columbia, MD, USA) and $\epsilon_{280 \text{ nm}}$ of $13,950.5 \text{ l} * \text{mol}^{-1} * \text{cm}^{-1}$ for Ara h 2 was used (based on sequence analysis software DNASTAR).

Polyphenol stock solutions were prepared in MilliQ water at a concentration of 1.5 mmol L^{-1} . The protein concentration was kept constant ($\sim 0.1 \text{ mg mL}^{-1}$) while each polyphenol was titrated into the protein solution to obtain molar protein:polyphenol ratios of up to 1:20. Four spectra for each titration point were accumulated at a scan speed of 50 nm per min, a data integration time (DIT) of 1 s with a data pitch of 0.5 nm, and were then averaged. Sample spectra were corrected for solvent (MilliQ water) and polyphenol spectra (two accumulations were each run for solvent and polyphenol, and averaged). The CD results were expressed as mean residue ellipticity (MRE) in $\text{deg cm}^2 \text{ dmol}^{-1}$ based on the following equation (Eq. (1)) (Pan, Qin, Liu, & Wang, 2011):

$$\text{MRE}(\text{deg cm}^2 \text{ dmol}^{-1}) = \frac{\theta}{C_p \times n \times l} \quad (1)$$

where θ is the observed CD signal (ellipticity) in mdeg, C_p is the molar concentration of the protein in mol L^{-1} , n the number of amino acid residues (140 amino acid residues used for Ara h 2 based on DNASTAR) and l is the cuvette path length in mm. Spectra were displayed as MRE functions of wavelength (nm). Further, since the secondary structure of Ara h 2 mainly consists of α -helices the % α -helical content of free and polyphenol-combined Ara h 2 was estimated using the following formula (Eq. (2)) (Pan et al., 2011):

$$\alpha - \text{helix}(\%) = \frac{-\text{MRE}_{208} - 4,000}{33,000 - 4,000} \times 100 \quad (2)$$

where MRE_{208} is the observed MRE value at 208 nm, 4000 is the MRE at 208 nm of pure β -form and random coil structures and 33,000 is the MRE value of a pure α -helix at 208 nm (Kandagal et al., 2006; Roy, Utreja, & Badhe, 2015).

2.4. UV-Vis absorption spectroscopy

The interactions between Ara h 2 and polyphenols were further investigated using UV-Vis absorption spectroscopy scans. Ara h 2 (in MilliQ water) spectra were subtracted from sample spectra to evaluate changes in polyphenol absorption upon interaction with the protein. Measurements were performed using a Shimadzu UV-2450 spectrometer (Columbia, MD, USA) in a 10 mm reduced volume quartz cuvette. The UV-Vis spectra were recorded from 200 nm to 900 nm at room temperature. This experiment was performed once for each treatment.

2.5. Protein distribution and IgE binding capacity of Ara h 2-polyphenol complexes

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to observe the profile of Ara h 2-polyphenol complexes and unmodified Ara h 2. Semi-quantitative immunoblotting was performed to evaluate the IgE binding capacity of Ara h 2-polyphenol complex samples compared to un-complexed Ara h 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were completed as previously described (Plundrich et al., 2017) with minor changes. The amount (μL) of Ara h 2-polyphenol complex or Ara h 2 solution needed to obtain equal protein content ($0.7 \mu\text{g}$) was mixed with the respective sample loading buffer containing β -mercaptoethanol. Immunoblotting was performed four times.

2.6. Statistical analysis

All experiments were replicated five times if not stated otherwise and analysis of variance (ANOVA) was performed using JMP 11.0 (SAS, Cary, NC, USA). When significant ($p < 0.05$), means were separated using Student's t test.

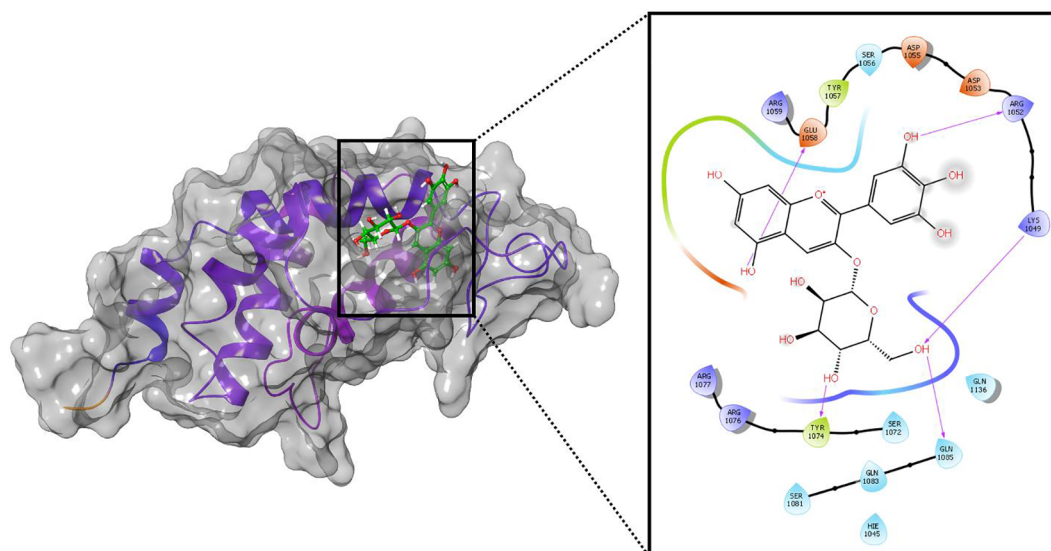


Fig. 1. Docking of delphinidin-3-glucoside to identified binding pocket on Ara h 2.01; Dscore = -8.7 , with SiteMap function at a pH of 7.

3. Results and discussion

3.1. Molecular docking

In this study, potential protein-ligand interactions between Ara h 2.01 and 42 naturally occurring polyphenols found in cranberries (*Vaccinium macrocarpon* Ait.) and/or lowbush blueberries (*Vaccinium angustifolium* Ait.) were computationally examined. Using SiteMap, a binding pocket with a computed volume of $381.5 \text{ \AA}^3$ was identified, which is located between α -helix 2 and α -helix 3 in the extended loop region (Supplementary Fig. 2). All 42 compounds were docked using Glide, two receptor grids, and two scoring functions (see Methods). Full docking results for all 42 compounds at pH 7 are provided in Supplementary Table 1. Out of the 42 compounds, 13 compounds received an average docking score lower than the -7 kcal mol^{-1} activity threshold. However, only 12 compounds were predicted to be likely binders since tannic acid afforded an eM score higher than $-50 \text{ kcal mol}^{-1}$, disqualifying it from the list of possible compounds. The compound affording the best docking score was the anthocyanin delphinidin-3-glucoside (Fig. 1), which was characterized by a docking score as low as $-8.70 \text{ kcal mol}^{-1}$. As shown in Fig. 1, delphinidin-3-glucoside was predicted to interact with five different amino acids Lys22, Arg25, Glu31, Tyr47, and Gln58 throughout the Ara h 2.01 protein between alpha helix 2 and 3 (loop region) via hydrogen bonds. One linear epitope was predicted to be involved in the interactions (epitope 5) which can potentially interfere with IgE binding. A general overview of the location of epitopes and helices is illustrated in Supplementary Fig. 3. The compound that has the lowest overall DS and eM score is also delphinidin-3-glucoside, with a DS of $-8.26 \text{ kcal mol}^{-1}$ and eM score of $-68.9 \text{ kcal mol}^{-1}$ (average of SiteMap and BlindDocking scores at pH 7). To narrow down the results, the top 10 compounds with the lowest docking scores were selected for further analyses (Table 1). When experimentally tested, compounds with docking scores under such threshold values typically correspond to micromolar binders (Fourches et al., 2013).

Molecular Dynamic Simulation results showed that all of the top 10 compounds appeared to remain in the binding pocket. Further examination of the results for dimer position of Ara h 2.01 was performed. Once dimers were obtained using the InterEvDock, each of the top 10 compounds, listed in Table 1, were docked between the two protein molecules. Ten different dimer positions were obtained from the 150 dimers calculated. The top 10 polyphenolic compounds were docked in each of those top-10 dimers, particularly, to evaluate whether new

Table 1

XP Docking and eModel scores for top 10 polyphenolic compounds at pH 7 (average scores for SiteMap and BlindDocking).

Compound	Docking score average (kcal mol^{-1})	eModel score average (kcal mol^{-1})
Delphinidin-3-glucoside	-8.26	-68.9
Quercetin-3-rutinoside	-8.10	-88.3
Delphinidin-3-galactoside	-8.00	-67.0
Cyanidin-3-glucoside	-8.00	-66.7
Cyanidin	-7.95	-57.3
Chlorogenic acid	-7.63	-57.0
Cyanidin-3-arabinoside	-7.45	-63.2
Quercetin-3-arabinoside	-7.42	-56.9
Quercetin-3-rhamnoside	-6.88	-58.3
Procyanidin C1	-6.88	-87.8

protein-polyphenol interactions would be predicted to form. When docked in the binding pocket of the dimer, the docking score of the top 10 compounds slightly decreased resulting in signs of stronger binding (data not shown). A representation of an Ara h 2.01 dimer is shown in Fig. 2, with the two Ara h 2.01 chains, one colored blue and the other green with the polyphenol ligand (PAC C1, purple).

Overall, *in silico* docking results were evaluated for the compounds with the highest predicted potential to bind to Ara h 2, and specifically to IgE binding epitopes of Ara h 2. Compounds further tested *in vitro* were selected based on docking scores (below -7 kcal/mol threshold) and based on binding interactions that were predicted to occur with the residues along the polypeptide chain that are part of IgE binding epitopes. Whether the compounds occurred in cranberries, lowbush blueberries or both (since *Vaccinium* extracts have previously been shown to form complexes with peanut protein) as well as the aim to cover a variety of different polyphenol classes was also taken into consideration to select the top four compounds to be tested in the *in vitro* experiments. It should be noted that flavonol derivatives with a sufficient docking score (Table 1), such as quercetin-3-rutinoside, were not tested further as they were not soluble in water for circular dichroism and UV-Vis experiments.

The top four compounds selected were delphinidin-3-glucoside, cyanidin-3-glucoside, chlorogenic acid, and PAC C1, covering anthocyanin, anthocyanin, phenolic acid, and procyanidin polyphenol classes, respectively. Specific interactions thought to occur between delphinidin-3-glucoside and specific amino acids on the Ara h 2.01

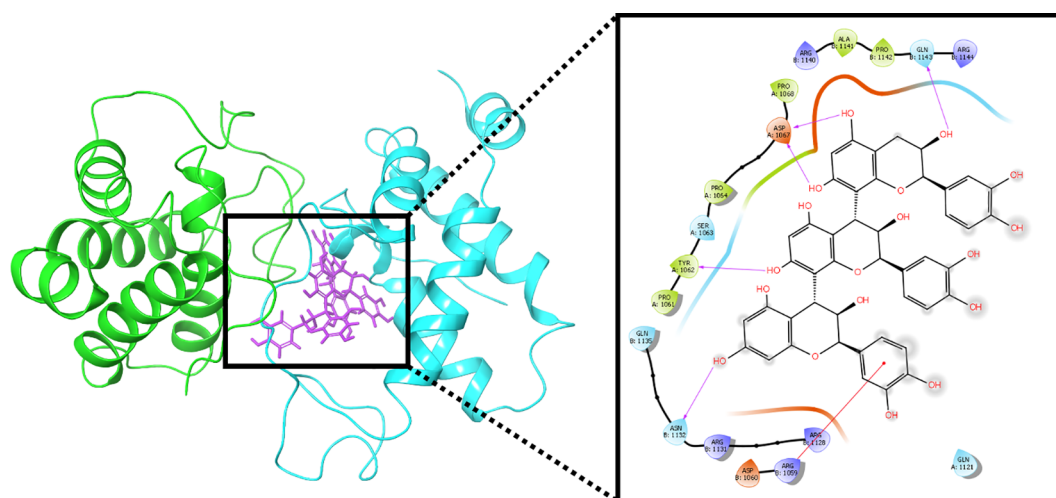


Fig. 2. Dimer representation of Ara h 2.01-Ara h 2.01 (green and blue chains), pictured left. The docked ligand is procyanidin C1 (colored purple in left picture), for which 2D interactions, are shown pictured right, with the respective amino acid. DS = $-8.4 \text{ kcal mol}^{-1}$ at pH 7 on dimer FRODOCK3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

polypeptide chain were discussed above. Cyanidin-3-glucoside docking to Ara h 2.01 showed that the polyphenol may interact with two amino acids, Lys22 and Arg25 via hydrogen bonds, on α -helix 2. Both amino acids, which may take part in the interactions, belong to a known epitope (epitope 5, see [Supplementary Fig. 3](#)). Chlorogenic acid was predicted to interact with four amino acids, Arg25, Arg49, Ser54, and Gln56 between α -helix 2 and 3 via hydrogen bonds, which involved the same epitope (Arg, epitope 5). PAC C1 was predicted to have a total of 12 interactions with amino acids of the polypeptide chain. The amino acids Lys22, Arg25, Asp26, Ser45 and 47, Tyr53, Gly54, and Gln56, via hydrogen bonds, salt bridges, and π - π stacking interactions between α -helix 2 and 3. Two known epitopes on the Ara h 2.01 sequence were directly involved in PAC C1 binding, epitope 5 (Lys, Arg, and Asp) and 7 (Ser).

Benzoic acid, which afforded an average DS of $-4.58 \text{ kcal mol}^{-1}$ and eM score of $-20.3 \text{ kcal mol}^{-1}$, was above the DS and eM thresholds and was predicted to have limited-to-no Ara h 2 binding capabilities. It was predicted to interact with three amino acids, Gln49, Ser54, and Arg58, between α -helix 2 and 3 via salt bridges and hydrogen bonds. No known IgE epitopes were involved in the predicted interactions of this compound. A summary of amino acids and IgE binding epitopes involved in binding with polyphenols is shown in [Table 2](#). Since our predicted interactions are based on Ara h 2.01 which lacks an additional 12 amino acids (containing a duplicate IgE binding epitope “DPYSPS” in epitope 7), compared to Ara h 2.02 ([Supplementary Fig. 3](#)), predicted interactions between Ara h 2.02 and polyphenol ligands may be different, although both protein core structures are the same.

3.2. Protein secondary structure and UV–Vis spectra

Far-UV CD spectroscopy was used to evaluate the influence of polyphenol-binding to Ara h 2 on the secondary structure of Ara h 2.

The CD spectrum of native Ara h 2 showed two minima at 208 nm and 222 nm, respectively, a characteristic footprint for proteins with a mainly α -helical secondary structural feature ([Lehmann et al., 2003](#)). The average % α -helix content of native Ara h 2 was 33 ± 2 (SE) (average of five separate accumulated, and solvent-corrected spectra). This is in agreement with a previous analysis that showed that Ara h 2 has α -helical content between 30% and 60% ([Maleki et al., 2003](#)). Results showed that PAC C1 and chlorogenic acid, but not benzoic acid, delphinidin-3-glucoside, or cyanidin-3-glucoside, resulted in changes in Ara h 2 secondary structures ([Fig. 3](#)). PAC C1 binding to Ara h 2 at a molar protein:polyphenol ratio of 1:8 resulted in an increase in α -helical content by 6% while chlorogenic acid binding at a molar ratio of 1:10 resulted in a decrease in α -helical content by 3%, respectively ([Fig. 3A and B](#)). The increase in α -helical content in the Ara h 2 – PAC C1 complex suggests that PAC C1 binding intensified the protein skeleton as has been described by others ([Gao et al., 2004; Tang, Zuo, & Shu, 2014](#)). In contrast, the decrease in % α -helix observed for the Ara h 2 – chlorogenic acid complex indicates that the binding of chlorogenic acid resulted in the loosening of the protein structure which was also reported by others ([Cui, Fan, Li, & Hu, 2004; Tang et al., 2014](#)). Several other studies have shown protein secondary structural changes upon protein interactions with procyanidins and chlorogenic acid ([Cai, Yu, Xu, Liu, & Yang, 2015; Gonçalves, Mateus, & de Freitas, 2010; Liang et al., 2013; Rawel, Czajka, Rohn, & Kroll, 2002; Rawel, Rohn, Kruse, & Kroll, 2002](#)). Benzoic acid, at a molar ratio of up to 1:19, did not affect Ara h 2 secondary structure ([Fig. 3C](#)) since the overall shape of the Ara h 2 spectra upon benzoic acid addition was similar. Cyanidin-3-glucoside and delphinidin-3-glucoside at molar ratios of 1:15 and 1:11, respectively, had no effect on Ara h 2 secondary structure (data not shown). It should be noted that while protein-ligand binding can have no effect on protein secondary structure, it still can lead to alterations in protein tertiary structure (which was not evaluated in this study).

Protein secondary structural changes are one way to confirm

Table 2

Amino acids and IgE binding epitopes involved in Ara h 2 binding with polyphenols (determined via SiteMap).

Polyphenol	Amino acid	Amino acid position on Ara h 2 sequence	Epitope # involved in polyphenol – Ara h 2 interaction
Delphinidin-3-glucoside	Lys, Arg, Glu, Tyr, Gln	22, 25, 31, 47, 58	5
Cyanidin-3-glucoside	Lys, Arg	22, 25	5
Chlorogenic acid	Arg (2), Ser, Gln	25, 49, 54, 56	5
Procyanidin C1	Lys, Arg, Asp, Ser (2), Tyr, Gly, Gln	22, 25, 26, 45, 47, 53, 54, 56	5, 7
Benzoic acid	Gln, Ser, Arg	49, 54, 58	none

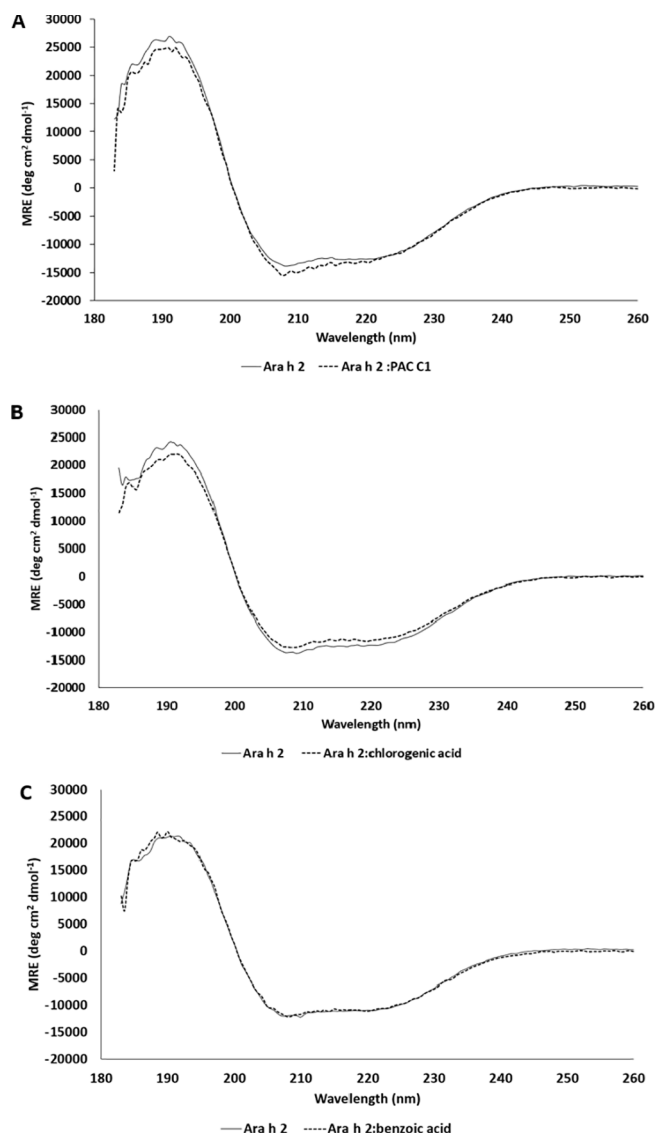


Fig. 3. (A–C) Circular dichroism spectra of unmodified Ara h 2 and Ara h 2-polyphenol mixtures. (A) Spectra of Ara h 2 and Ara h 2-procyanidin C1 (1:8 M ratio); (B) spectra of Ara h 2 and Ara h 2-chlorogenic acid (1:10 M ratio); (C) spectra of Ara h 2 and Ara h 2-benzoic acid (1:19 M ratio).

protein-ligand binding. In addition, UV–Vis absorption measurements provide helpful supportive evidence for protein-ligand interactions. The UV–Vis spectrum of PAC C1 after reaction with Ara h 2 was similar to free PAC C1 (Supplementary Fig. 4A). Since PAC C1 is a trimer consisting of a flavan-3-ol core structure strongly absorbing at 280 nm, the same wavelength at which protein aromatic acid residues Trp and Tyr absorb, it was not possible to observe major spectral changes in this wavelength area. However, it appeared that there was a small hyperchromic shift (increase in absorption intensity) in the UV spectrum of PAC C1 after Ara h 2 addition. Chlorogenic acid, a hydroxycinnamic acid derivative, on the other hand, has an absorption maximum at 324 nm (Supplementary Fig. 4B). The UV–Vis spectrum of chlorogenic acid upon Ara h 2 addition showed a hypochromic (decrease in absorption intensity) and bathochromic (red) shift (*i.e.* shift to longer wavelength). While the absorption maximum of 324 nm was not shifted, the spectrum appeared to be broadened from ~ 370 nm to ~ 425 nm (x -axis intersection). These results indicate interactions between Ara h 2 and chlorogenic acid molecules. Results from our previous study also suggested that chlorogenic acid, and potentially procyanidins, bound to peanut proteins (Plundrich et al., 2017). Benzoic

acid has an absorption maximum at 227 nm, which was not shifted when benzoic acid was combined with Ara h 2. However, there appeared to be a hypochromic and hypsochromic (blue) shift (*i.e.* shift to shorter wavelength) upon reaction with Ara h 2. Caution was taken during the interpretation of those results with regards to potential Ara h 2 - benzoic acid interactions (Supplementary Fig. 4C), since it is well known that small impurities can affect absorption spectra in the UV region of the UV spectrum (Litjens, Quickenden, & Freeman, 1999). No conclusions could be drawn from UV spectra of both cyanidin-3-glucoside and delphinidin-3-glucoside after addition to Ara h 2 (data not shown).

3.3. Protein distribution and IgE binding capacity of Ara h 2 – polyphenol complexes

After SDS-PAGE, two distinct bands (doublet) were observed at approximately 20 kDa (Supplementary Fig. 5). In addition, small molecular weight protein fragments, likely peptides from Ara h 2 breakdown, were also observed at the bottom of the gel. Immunoblotting was performed to evaluate the binding capacity of Ara h 2-polyphenol complexes to peanut-specific IgE from human allergic plasma. Some studies proposed that the diversity of linear IgE binding epitopes is associated with the severity of allergic reaction (Flinterman et al., 2008; Shreffler, Beyer, Chu, Burks, & Sampson, 2004), which suggested that linear epitopes are predominant over conformational epitopes (Shreffler et al., 2004; Shreffler, Lencer, Bardina, & Sampson, 2005).

The SDS-PAGE-separated samples were transferred to a PVDF membrane and linear IgE binding epitopes were tested for their IgE binding potential. Results showed that some polyphenols effectively inhibited IgE binding to Ara h 2 IgE binding epitopes (Fig. 4 and Supplementary Fig. 6). PAC C1, chlorogenic acid and benzoic acid significantly ($p < 0.05$) decreased IgE binding compared to IgE binding potential to the control Ara h 2 (no polyphenols added). For PAC C1, molar protein:polyphenol ratios of 1:5 and 1:20 (PAC C1 concentrations of $29 \mu\text{mol L}^{-1}$ and $106 \mu\text{mol L}^{-1}$, respectively) significantly reduced IgE binding to Ara h 2 by 37% and 37%, respectively. For chlorogenic acid a significant effect was observed for the 1:10 ratio ($56 \mu\text{mol L}^{-1}$ chlorogenic acid; 50% decrease in IgE binding), and for benzoic acid for the 1:10 and 1:20 ratio, $55 \mu\text{M}$ benzoic acid (37% decrease) and $106 \mu\text{mol L}^{-1}$ benzoic acid (36% decrease), respectively. However, no polyphenol dose-dependent effect was seen since the degree of IgE binding to Ara h 2 among the ratios 1:5, 1:10, and 1:20 was not significantly different at $p < 0.05$. It is possible that IgE binding epitope blocking was saturated at the ratios tested and future studies need to evaluate if one polyphenol molecule binding to one Ara h 2 molecule (1:1 M ratio) has the same effect on IgE epitope obstruction. Higher concentrations should also be tested to evaluate if the addition of more polyphenol molecules results in a greater decrease of IgE binding.

The change in IgE binding to Ara h 2 following incubation with polyphenols indicates that these modifications are covalent in nature, particularly because of the harsh conditions of SDS-PAGE and immunoblotting that would disrupt non-covalent interactions. Results from this study suggest that Ara h 2 did *not* form aggregates through binding with polyphenols. This is based on the observation that Ara h 2 bands migrated in the same manner with or without bound polyphenols on the SDS-PAGE gel (Supplementary Fig. 5). This suggests that IgE binding epitopes on Ara h 2 were inhibited by *bound polyphenols* and not by aggregate formation. It is possible that a few polyphenols bound to Ara h 2 molecules, but didn't cross-link them to form bigger particles.

The fact that Ara h 2-PAC C1, -chlorogenic acid, and -benzoic acid complexes were not disassociated under SDS-PAGE conditions (*i.e.* reducing agent, heat, and SDS, which destroy non-covalent bonds and induce protein unfolding) suggests that at least some polyphenol molecules must have remained tightly or covalently bound to Ara h 2, more particularly, bound to or close to linear IgE binding epitopes. It is

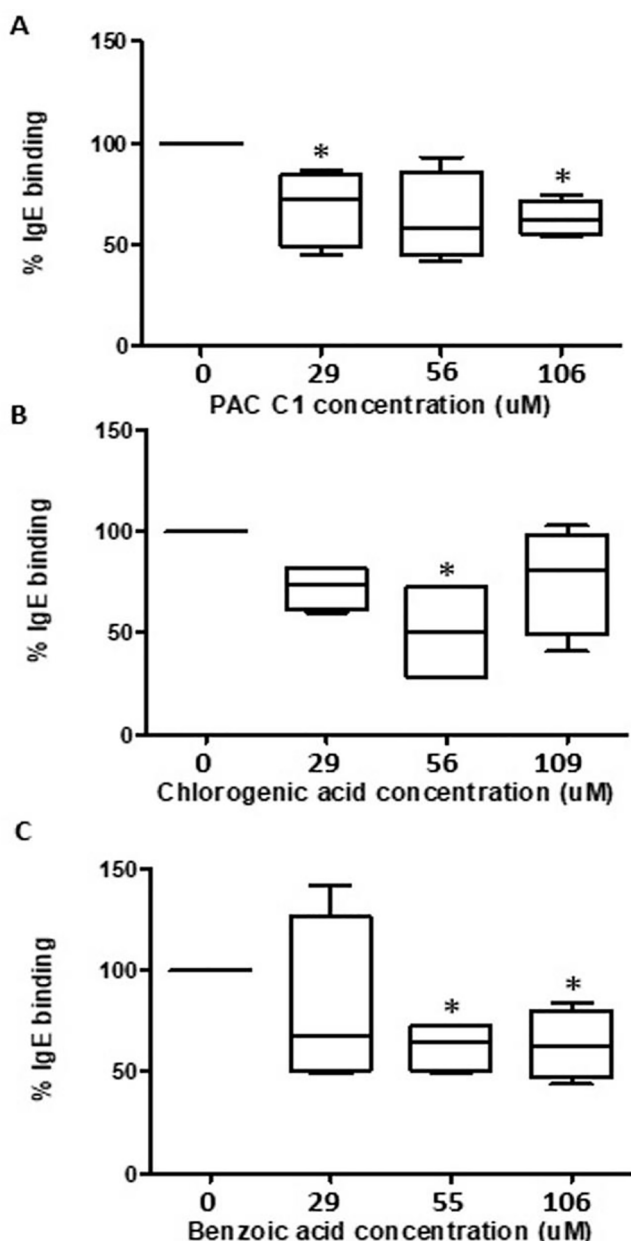


Fig. 4. Semi-quantitative analysis of IgE binding capacity (based on Western blots) of Ara h 2 (0 $\mu\text{mol L}^{-1}$ polyphenol, set as 100% IgE binding) and Ara h 2-polyphenol complexes containing different concentrations of polyphenol ($\mu\text{mol L}^{-1}$) (mean of four replicates with SE; asterisk indicates significant difference compared to the Ara h 2 control at $p < 0.05$).

possible that blocking of one amino acid in an IgE binding epitope sequence is enough to impair sequence-specific binding of IgE antibodies, however, multiple interactions may be involved. Further, binding of a polyphenol in close proximity to an IgE binding epitope can theoretically hamper the steric accessibility by epitope-specific IgE as well. This also may apply to conformational epitopes.

IgE binding capacity to Ara h 2 complexed with delphinidin-3-glucoside and cyanidin-3-glucoside was not significantly different from the control, Ara h 2 alone (data not shown).

Interestingly, benzoic acid was previously predicted as not likely to bind to Ara h 2 (due to a low docking score), and computational docking showed the three predicted amino acid residues involved in the interactions with benzoic acid were not part of or in close proximity to a

linear IgE binding epitope. It was hence expected that benzoic acid may not have the capacity to block linear IgE binding epitopes on Ara h 2. Our results showed that benzoic acid at concentrations used did not change the secondary structure of Ara h 2, but may have interacted with Ara h 2 based on UV-Vis experiments (Supplementary Fig. 4C). Immunoblotting confirmed that benzoic acid *may have bound* to Ara h 2 and reduced Ara h 2-specific IgE antibodies from binding to one or more IgE binding epitopes on Ara h 2 (Fig. 4). It is possible that Ara h 2-bound benzoic acid induced tertiary structural changes but not secondary structural changes. In addition, *in silico* predictions focused on the binding potential of one benzoic acid molecule (through non-covalent interactions). However, benzoic acid is the smallest of the tested polyphenol molecules (122 g mol^{-1}), negatively charged at the investigated pH (pH 7), and hence has a great potential to non-specifically bind at many locations on the Ara h 2 molecule, even covalently (*i.e.*, if benzoic acid oxidized under the experimental conditions) (Cilliers & Singleton, 1991; Friedman, 1997; Haslam, 1996). Further computational testing showed a total of 14 potential binding sites for benzoic acid on the surface of Ara h 2, interacting with various amino acids via H-bonds, π - π stacking, and salt bridges (Supplementary Fig. 7).

Recently, two studies proposed a role for conformational IgE binding epitopes in the allergenicity of Ara h 2 (Chen et al., 2016; Otsu, Guo, & Dreskin, 2015). Interestingly, Chen et al. (2016) investigated such possible conformational epitopes on Ara h 2, which were suggested to be located in the surface-exposed, extended loop area between α -helix 2 and 3, the same area that was predicted to bind polyphenols. Future studies should evaluate conformational epitope blocking by ELISA (Enzyme-Linked Immunosorbent Assay) or cell culture techniques.

In general, there are multiple possible mechanisms by which polyphenol-binding to a food allergen can result in inhibition of IgE antibodies to bind linear and/or conformational epitopes including: 1) binding of one or more polyphenol molecules to portions of the allergenic protein containing conformational and/or linear epitopes without inducing protein structural changes (no protein denaturation); 2) binding of polyphenol molecules to the protein (possibly portions containing epitopes), inducing protein unfolding, and subsequent binding of additional newly exposed IgE binding epitopes by polyphenols; 3) binding of polyphenols to the protein and subsequent aggregation (polyphenols cross-link protein molecules) masking IgE binding epitopes; and 4) any combination of the three proposed scenarios.

Collectively, our results show that the binding of procyanidin C1, chlorogenic acid, and benzoic acid to Ara h 2 leads to the formation of protein-polyphenol complexes with reduced allergenic potential *in vitro*. This study suggests that polyphenols in some way interacted with amino acids on the Ara h 2 polypeptide chain and impaired linear epitope recognition by epitope-specific IgE.

4. Conclusion

Forty-two polyphenols found in cranberries (*Vaccinium macrocarpon* Ait.) and/or lowbush blueberries (*Vaccinium angustifolium* Ait.) were evaluated for their predicted binding potential to Ara h 2 and its IgE binding epitopes through *in silico* structure-based computational molecular docking. Four *in silico* predicted polyphenolic compounds were identified as potential binders that were subsequently evaluated *in vitro* using spectroscopic and immunological methods (UV-Vis spectroscopy, circular dichroism, and immunoblotting, respectively) for their a) Ara h 2 binding potential, b) capacity to induce protein secondary structural changes, and c) capacity to inhibit IgE binding epitopes. Benzoic acid was tested as a control due to minimal *in silico* binding. Ara h 2-polyphenol complexes were further tested for their hypoallergenic potential

through *in vitro* immunoblotting. This study demonstrated that three out of five compounds tested, namely procyanidin C1, chlorogenic acid, and benzoic acid, bound to the major peanut allergen Ara h 2 as shown by spectroscopy experiments (UV and circular dichroism), as well as by immunoblotting *in vitro* methods. Further, the resulting polyphenol-modified Ara h 2 showed decreased Ara h 2-specific IgE binding by *in vitro* immunoblotting. Additional *in vitro* experiments are warranted to investigate the immunomodulatory mechanisms of these Ara h 2-polyphenol complexes/interactions, in particular for Ara h 2-benzoic acid modifications, and to determine the number of Ara h 2-bound polyphenols and the precise polyphenol binding sites on Ara h 2. This study demonstrates that certain polyphenolic compounds are able to modify the major peanut allergen Ara h 2, modulating its native structural conformation and reducing IgE binding.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.01.081>.

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Binding of peanut allergen Ara h 2 with *Vaccinium* fruit polyphenols

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Supplementary Table 1. IgE binding epitopes along the Ara h 2 sequence

Peptide	Amino acid sequence	Epitope position on Ara h 2 sequence
1	<u>HASARQQWEL</u>	NA
2	<u>QWELQGDRRC</u>	NA
3	<u>DR</u> ₁ <u>RCQSQLERA</u> ₁₀	1-8
4	N <u>LRPCEQHLM</u> ₂₀ <u>Q</u>	12-21
5	<u>KIQRDEDSY</u> ₃₀ <u>E</u>	22-29
6	<u>RDPYSP</u>	32-37
7	<u>SQD</u> ₄₀ <u>PYSPS</u>	38-45
8	D <u>RL</u> ₉₀ <u>QGRQQEQ</u>	90-95
9	Q <u>FK</u> ₁₀₀ <u>RELRNLPQQ</u>	100-109
10	<u>QRCDL</u> ₁₂₀ <u>DVESG</u>	116-121

Highlighted in grey are amino acids that were additionally found by Stanley *et al.* (1997) but not recorded by Mueller *et al.* (2011) (amino acids not highlighted). Amino acids that are part of an epitope are underlined. The position of each peptide with respect to the Ara h 2 sequence used in this study is indicated in the right hand column.

Supplementary Table 2. List of 42 polyphenols considered in this study. HBA, hydrogen bond acceptor; HBD, hydrogen bond donor

Compound	Molecular weight (g mol ⁻¹)	HBA score	HBD score
Cyanidin-3-galactoside	449.108	11	8
Cyanidin-3-glucoside	449.108	11	8
Cyanidin-3-arabinoside	454.812	10	7
Peonidin-3-galactoside	463.124	11	7
Peonidin-3-arabinoside	468.838	10	6
Malvidin-3-glucoside	493.135	12	7
Malvidin-3-galactoside	493.135	12	7
Malvidin-3-arabinoside	498.864	11	6
Delphinidin-3-galactoside	465.103	12	9
Delphinidin-3-glucoside	493.135	12	7
Cyanidin	287.056	6	5
Malvidin	331.082	7	4
Quercetin	302.043	7	5
Myricetin	318.038	8	6
Quercetin-3-rutinoside	610.153	16	10
Quercetin-3-arabinoside	434.085	11	7
Quercetin-3-arabinoside	434.085	11	7
Quercetin-3-rhamnoside	448.101	11	7
Quercetin-3-galactoside	464.096	12	8
Kaempferol	286.048	6	4
Quercetin-3-xyloside	434.085	11	7
Quercetin-3-glucoside	464.096	12	8
Chlorogenic acid	354.095	9	6
Caffeic acid	180.042	4	3
P-coumaric acid	164.047	3	2
Ferulic acid	194.058	4	2
Benzoic acid	122.037	2	1
P-hydroxybenzoic acid	138.032	3	2
Tannic acid	1700.173	--	--
Sinapic acid	224.069	5	2
Vanillic acid	168.042	4	2
Gallic acid	170.022	5	4
Resveratrol	228.079	3	3
Catechin	290.079	6	5
Epicatechin	290.079	6	5
Procyanidin B2	578.142	12	10
Procyanidin B1	578.142	12	10
Procyanidin A2	576.127	12	9
Procyanidin C1	866.506	18	15
Epigallocatechin	306.074	7	6

Epigallocatechin gallate	458.085	11	8
Epicatechin gallate	442.09	10	7

Supplementary Table 3. Average of XP Docking and eModel Scores for *SiteMap* and *BlindDocking* at pH 7

Compound	Docking Score	eModel Score
Tannic acid B	-9.38	4905.34
Delphinidin-3-glucoside	-8.26	-68.91
Quercetin-3-rutinoside	-8.10	-88.29
Delphinidin-3-galactoside	-8.00	-66.99
Cyanidin-3-glucoside	-8.00	-66.66
Cyanidin	-7.95	-57.25
Chlorogenic acid	-7.63	-57.00
Cyanidin-3-galactoside	-7.46	-65.83
Cyanidin-3-arabinoside	-7.45	-63.21
Quercetin-3-arabinoside (pyranoside)	-7.41	-56.93
Myricetin	-7.15	-59.33
Quercetin-3-arabinoside (furanoside)	-7.05	-66.51
Quercetin-3-galactoside	-6.92	-57.82
Quercetin-3-rhamnoside	-6.88	-58.33
Procyanidin C1	-6.88	-87.78
Quercetin	-6.84	-58.63
Epigallocatechin gallate	-6.68	-72.08
Epigallocatechin	-6.54	-48.62
Sinapic acid	-6.51	-29.85
Quercetin-3-glucoside	-6.30	-61.15
Peonidin-3-galactoside	-6.20	-59.50
Malvidin-3-arabinoside	-6.03	-66.94
Epicatechin gallate	-5.95	-68.79
Procyanidin B2	-5.90	-53.17
Epicatechin	-5.82	-52.73
Malvidin-3-glucoside	-5.78	-65.93
Quercetin-3-xyloside	-5.74	-51.49
Kaempferol	-5.74	-53.39
Gallic	-5.64	-29.59
(+) Catechin	-5.56	-48.13
p-hydroxybenzoic acid	-5.52	-24.71
Procyanidin A2	-5.38	-62.73
Peonidin-3-arabinoside	-5.32	-57.24
Ferulic acid	-5.25	-29.19
Malvidin	-5.22	-60.26

Procyanidin B1	-5.17	-54.17
Vanillic acid	-5.17	-22.72
Malvidin-3-galactoside	-4.96	-62.50
Caffeic acid	-4.74	-30.49
p-coumaric acid	-4.65	-25.22
Benzoic acid	-4.58	-20.28
Trans-resveratrol	-4.52	-42.86

Supplementary Table 1. Amino acids within 4 Å (determined through the molecular visualization software PyMOL) that have the potential to affect interactions predicted by SiteMap. Potential amino acids and IgE binding epitopes involved in binding with polyphenols are shown

Polyphenol	Amino acid	Amino acid position on Ara h 2 sequence	Epitope # involved in polyphenol – Ara h 2 interaction
Delphinidin-3-glucoside	His, Asp (2), Ser (3), Tyr, Arg (3), Gln (2)	18, 26, 28, 29, 30, 32, 45, 49, 50, 54, 56, 109	4, 5, 6, 7
Cyanidin-3-glucoside	His, Asp (2), Ser (3), Tyr (2), Glu, Arg (3), Gln (4)	18, 26, 28, 29, 30, 31, 32, 39, 45, 47, 49, 50, 54, 56, 58, 109	4, 5, 6, 7
Chlorogenic acid	His, Lys, Asp (2), Ser (2), Tyr (3), Glu, Arg (2), Pro, Gln (2)	18, 22, 26, 29, 30, 31, 32, 33, 34, 35, 39, 45, 47, 50, 58	4, 5, 6, 7
Procyanidin C1	Ser (2), Tyr, Glu, Arg (3), Pro, Ala, Gln	29, 30, 31, 32, 46, 49, 50, 52, 55, 58	5, 6
Benzoic acid	His, Lys, Arg, Tyr, Gln	18, 22, 25, 47, 56	4, 5

Supplementary Figure 1. PDB entry 3OB4: Maltose binding protein (MBP)-fusion protein of Ara h 2. Highlighted with a yellow square is Ara h 2.

Supplementary Figure 2. Positioning of the *SiteMap* binding pocket between α -helix 2 and α -helix 3 of Ara h 2.01. The red, blue and yellow represent different interactions possible with Ara h 2.01. Dscore: 0.95.

Supplementary Figure 3. Illustration of the Ara h 2.01 sequence with indication of the five α -helices (blue) and 10 linear IgE binding epitopes (orange). Cysteines involved in a total of four disulfide bonds are marked in red (adapted from Mueller et al., 2011). Ara h 2.02 has an insertion of 12 amino acids containing one IgE binding epitope (amino acids underlined and in bold) starting at position 45 (Chatel et al., 2003).

Supplementary Figure 4. Chemical structures of delphinidin-3-glucoside (A), cyanidin-3-glucoside (B), procyanidin C1 (C), chlorogenic acid (D), and benzoic acid (E) at pH 7.

Supplementary Figure 5. (A and B) Circular dichroism spectra of unmodified Ara h 2 and Ara h 2-polyphenol mixtures. (A) Spectra of Ara h 2 and Ara h 2-cyanidin-3-glucoside (1:15 molar ratio); (B) spectra of Ara h 2 and Ara h 2-delphinidin-3-glucoside (1:11 molar ratio); (C and D) UV-Vis absorption spectra of polyphenols before and after addition of Ara h 2. (C) spectra of cyanidin-3-glucoside and cyanidin-3-glucoside after Ara h 2 addition (molar ratio Ara h 2:cyanidin-3-glucoside; 1:17); (D) spectra of delphinidin-3-

glucoside and delphinidin-3-glucoside after Ara h 2 addition (molar ratio Ara h 2:delphinidin-3-glucoside; 1:11).

Supplementary Figure 6. Representative Native PAGE of Ara h 2 and Ara h 2:polyphenol complexes at 1:5, 1:10, and 1:20 molar ratios stained with Coomassie Brilliant Blue. M, molecular weight marker.

Supplementary Figure 7. Staining of some polyphenol-bound peanut proteins by NBT (nitroblue tetrazolium) following native PAGE and subsequent electrophoretic transfer to a PVDF membrane. Egg white protein (EWP) and peanut flour (PN) were used as negative controls and peanut flour complexed with 40% cranberry polyphenols (PN CB 40%) was used as a positive control.

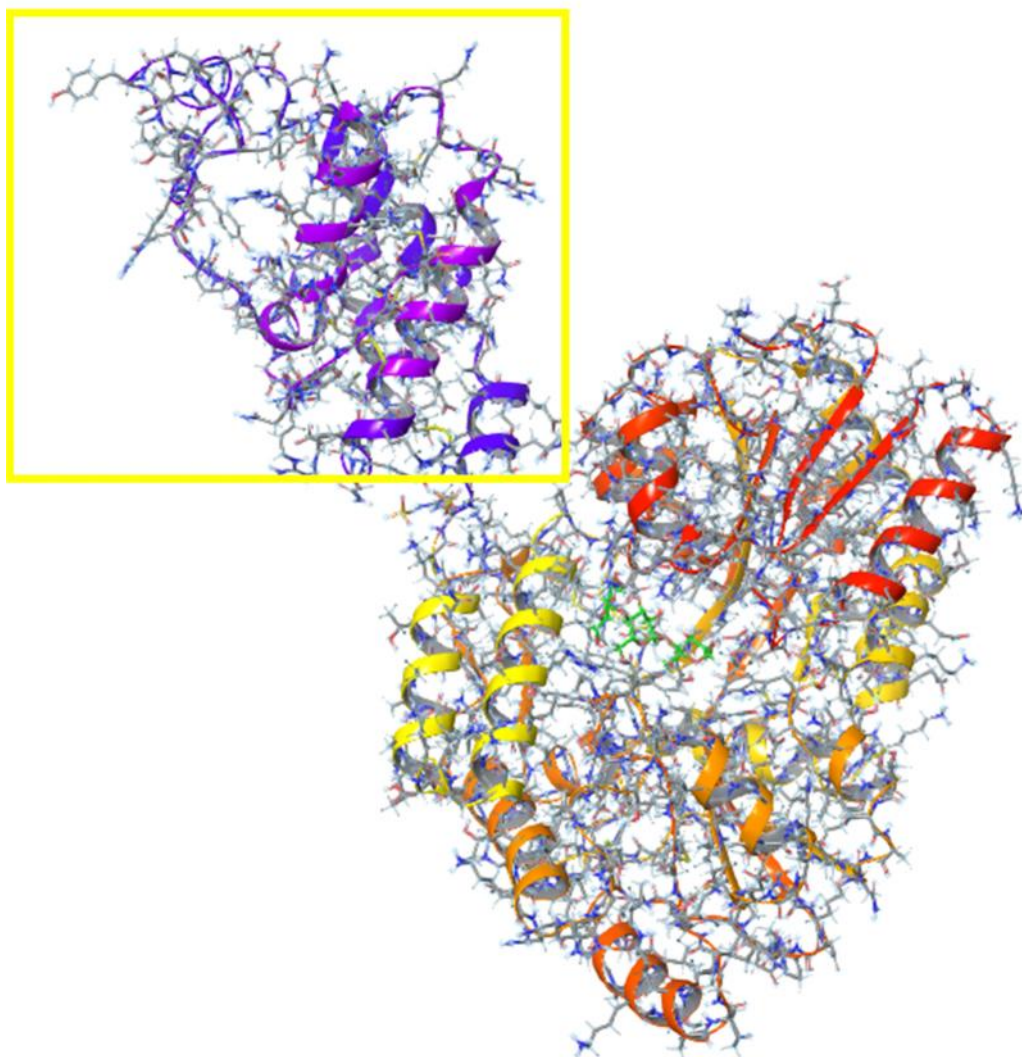
Supplementary Figure 8. Representative SDS-PAGE of Ara h 2 and Ara h 2:polyphenol complexes at 1:5, 1:10, and 1:20 molar ratios stained with Coomassie Brilliant Blue. M, molecular weight marker.

Supplementary Figure 9. Staining of polyphenol-bound peanut proteins by NBT (nitroblue tetrazolium) following SDS-PAGE and subsequent electrophoretic transfer to a PVDF membrane. Peanut flour containing 0.7 µg protein (PN 1) or 5 µg protein (PN 2) were used as negative controls and peanut flour complexed with 40% cranberry polyphenols containing 0.7 µg protein (PN CB 40% 1) or 5 µg protein (PN CB 40% 2) were used as positive controls. M, molecular weight marker.

Supplementary Figure 10. Semi-quantitative analysis of IgE binding capacity (based on Western blots) of Ara h 2 and Ara h 2–polyphenol complexes prepared at molar Ara h 2:polyphenol ratios of 1:5, 1:10, and 1:20 (mean of four replicates with SE; values with different letters are significantly different at $p < 0.05$).

Supplementary Figure 11. Illustration of all possible (14) interactions between benzoic acid and Ara h 2 determined through *BlindDocking* (pH 7).

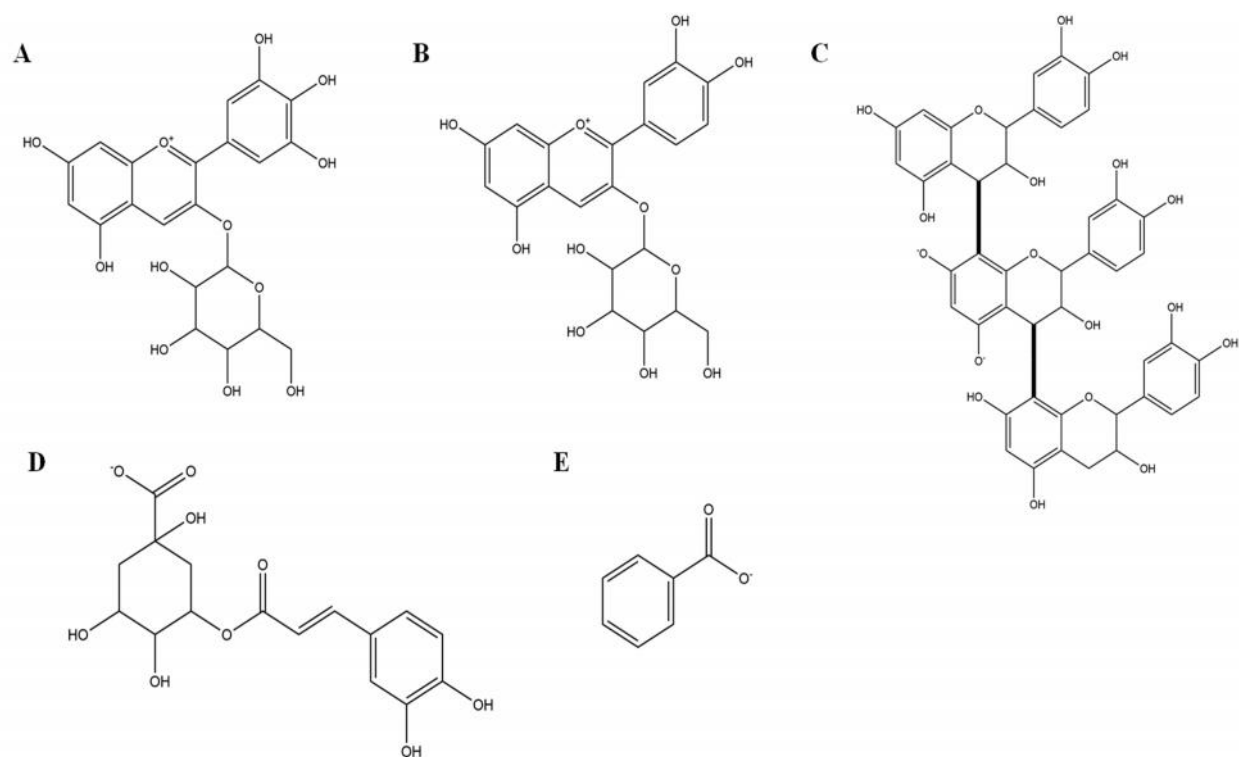
Supplementary Figure 1.



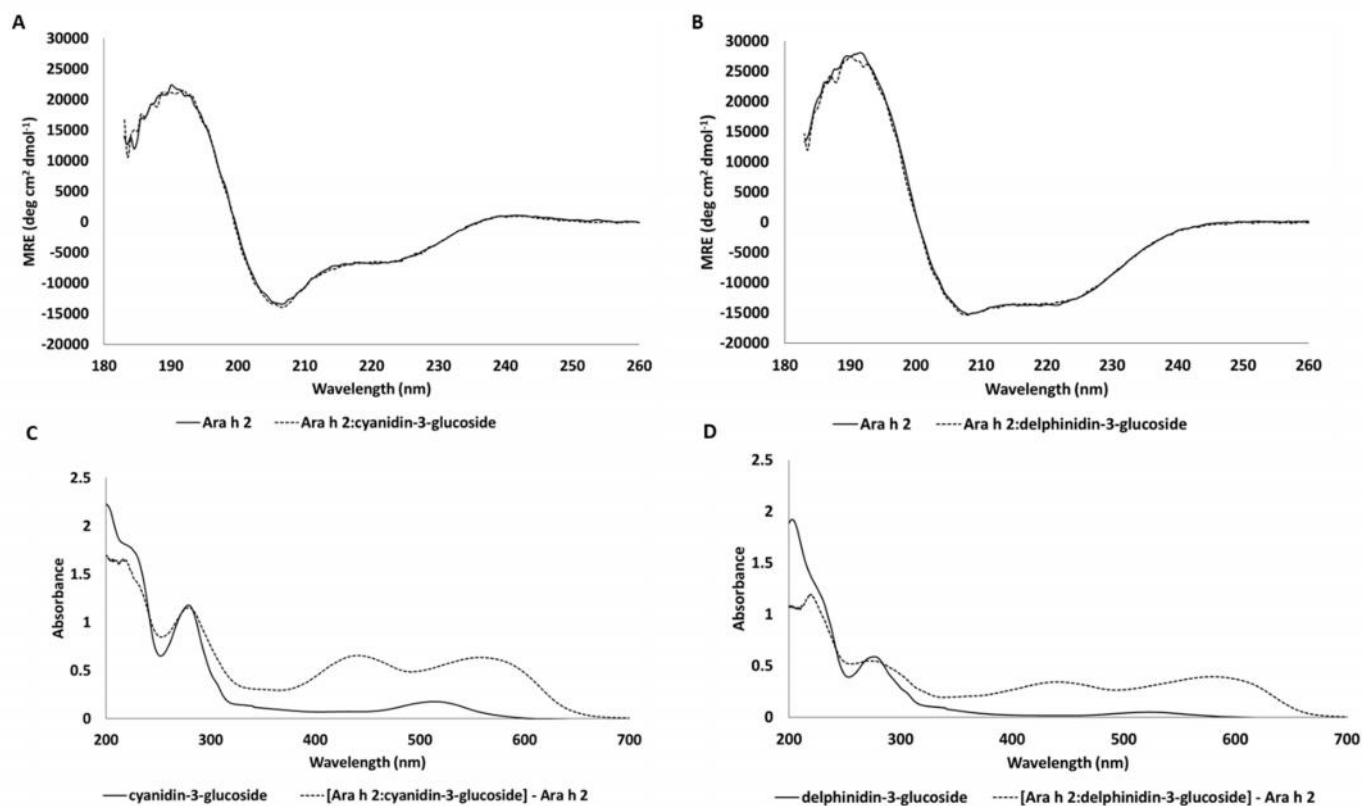
Ara h 2.02: Insertion of QDPDRR**DPYSPS**

The diagram illustrates the protein structure of Ara h 2.02, highlighting the insertion of the QDPDRR**DPYSPS** sequence. The protein is represented as a linear sequence of amino acids with specific residues highlighted in red. The structure is divided into two main regions, each containing alpha helices (α1, α2, α3, α4, α5) and beta strands (E3, E4, E5, E6, E7, E8, E9, E10). The insertion site is indicated by a black arrow pointing to the QDPDRR**DPYSPS** sequence, which is located between the E6 and E7 beta strands. The sequence is as follows: ...RRCQSQLERA₁₀NLRPC**EQ**HLM₂₀QKIQRDEDSY₃₀ERDPYSPSQD₄₀PYSPSPYDRR₅₀GAGSSQHQR₆₀CC NELNEFEN₇₀NQRC**MC**EALQ₈₀QIMENQSDRL₉₀QGRQQEQQFK₁₀₀RELRLNPQQC₁₁₀GLRAPQRC**CD**L₁₂₀D. The red residues are underlined in the original image.

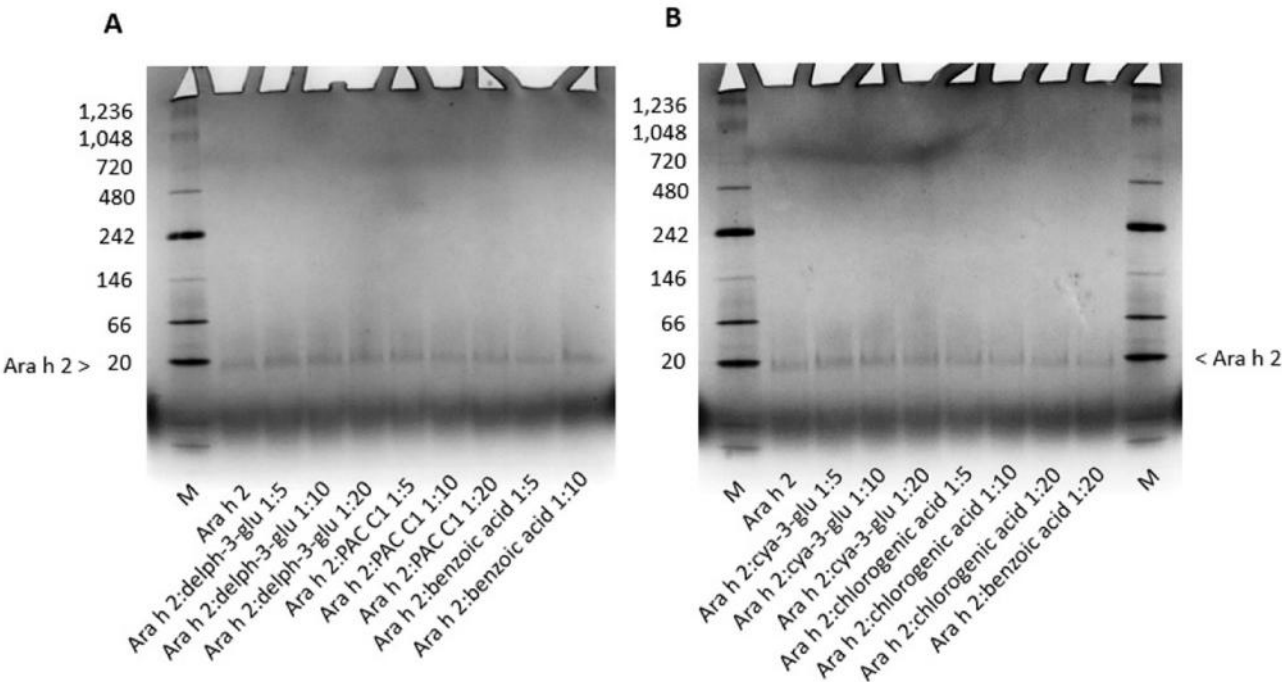
Supplementary Figure 4.



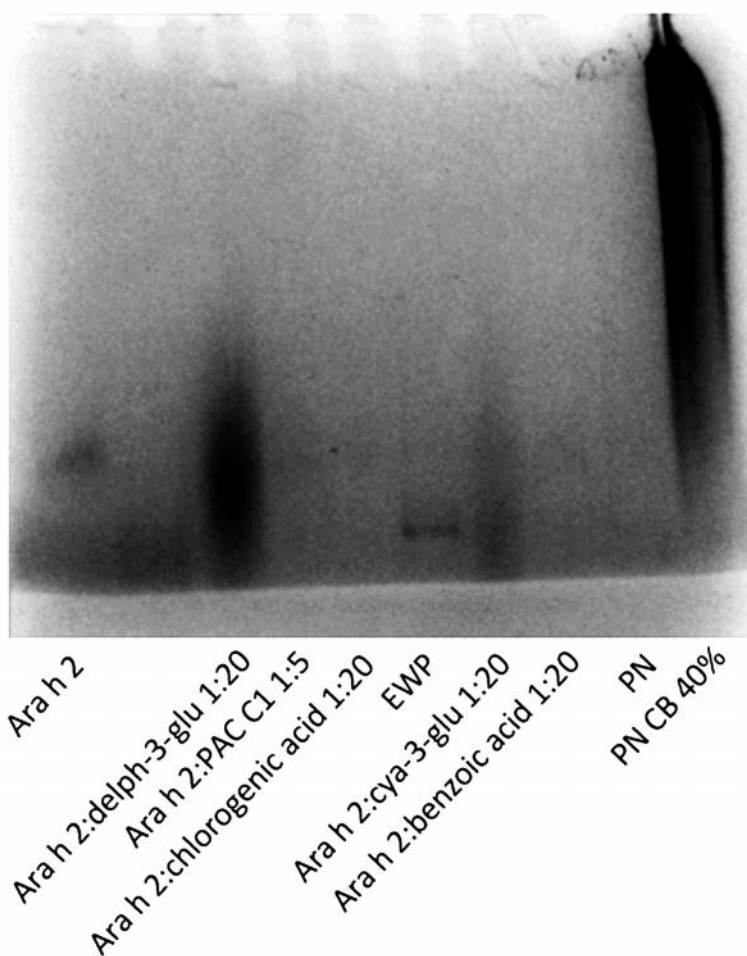
Supplementary Figure 5.



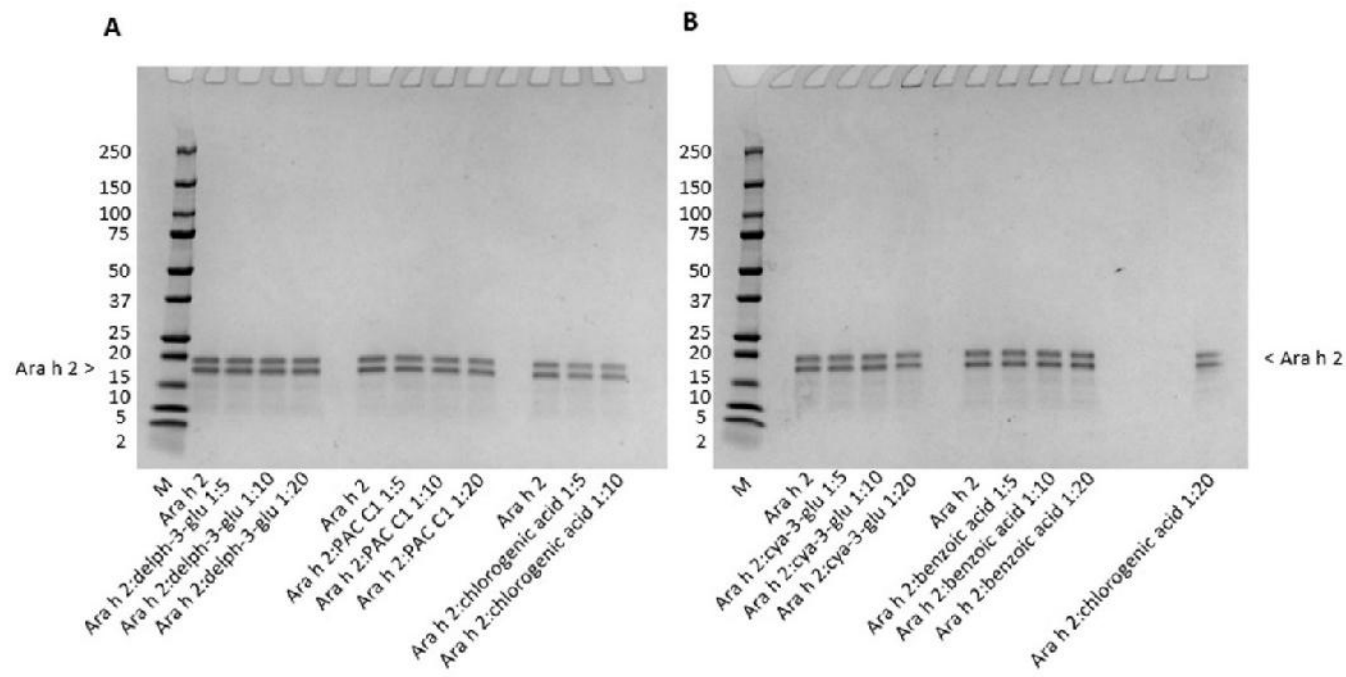
Supplementary Figure 6.



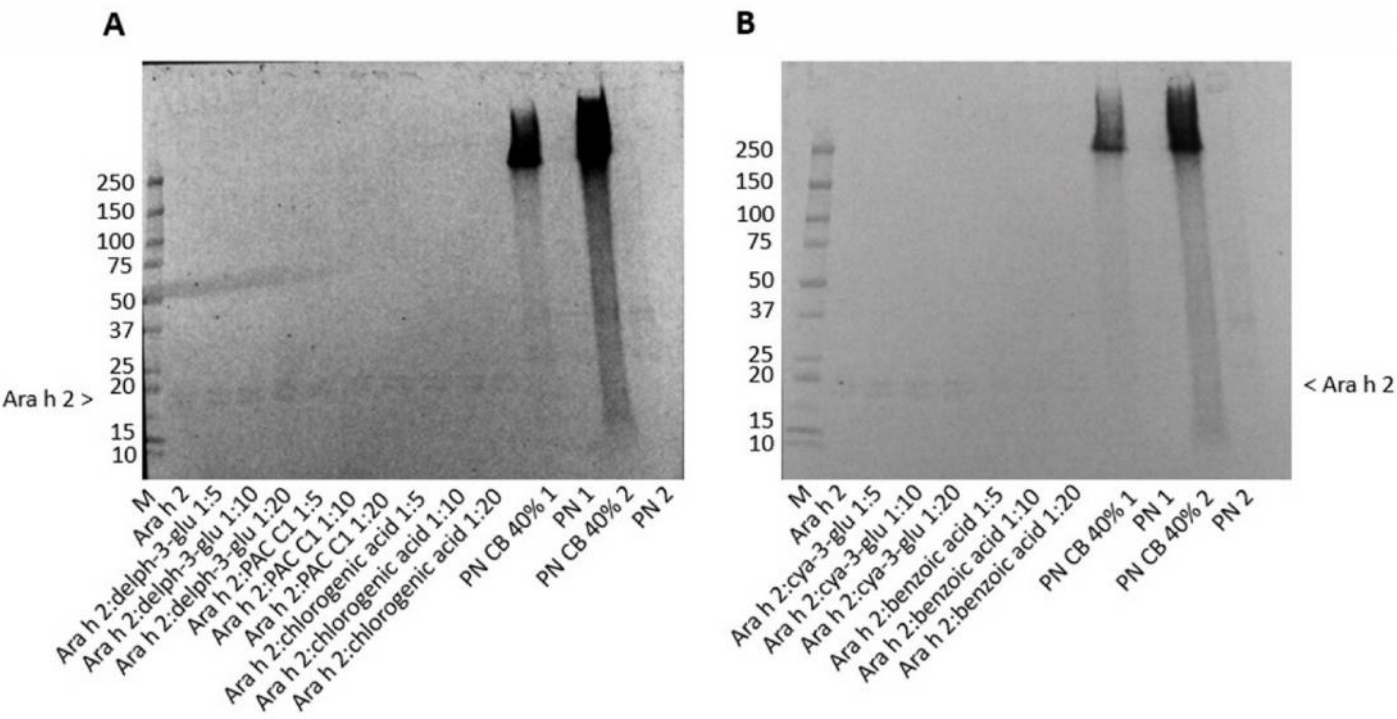
Supplementary Figure 7.



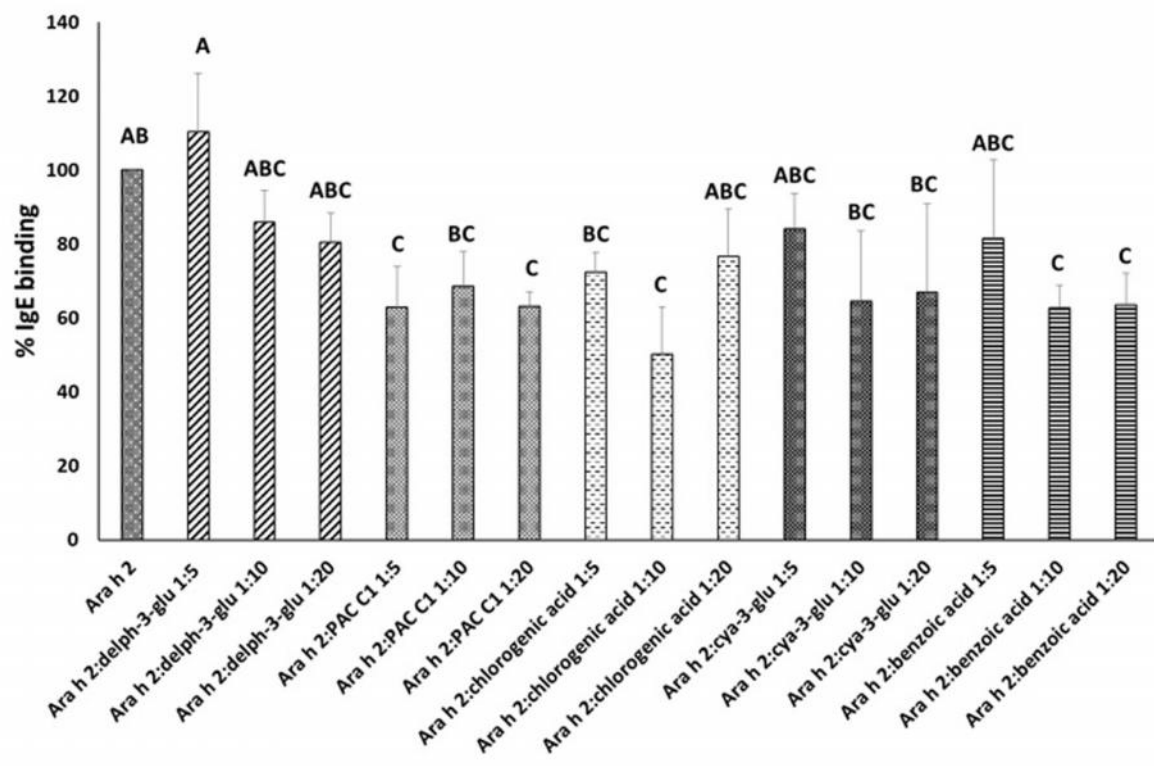
Supplementary Figure 8.



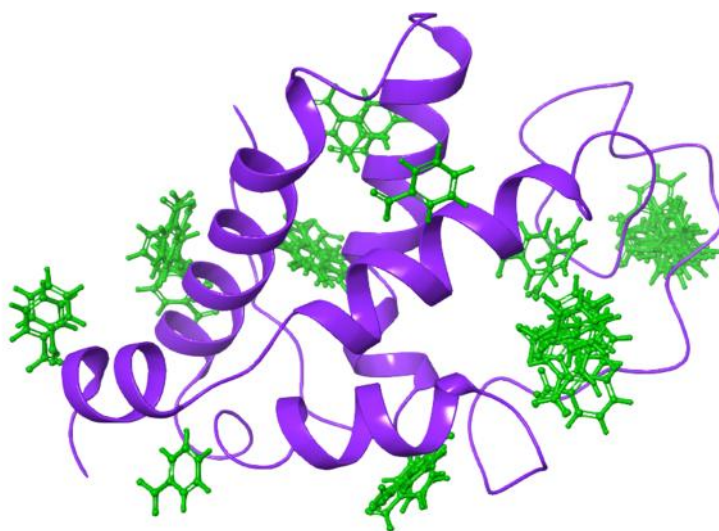
Supplementary Figure 9.



Supplementary Figure 10.



Supplementary Figure 11.



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