ABSTRACT

PLUNDRICH, NATHALIE JENNIFER. Protein-Polyphenol Complexation to Reduce Food Protein Allergenicity. (Under the direction of Drs. Mary Ann Lila and E. Allen Foegeding).

Food allergy is a worldwide health concern. Peanuts are among the "big eight" foods that can cause food allergy, and has been cited as one of the most serious and prevalent. Peanut allergy is an immunoglobulin (Ig) E-mediated hypersensitivity disorder towards certain proteins (allergens) in peanut. Upon initial exposure to peanut allergens, the immune system of a susceptible individual produces peanut-specific IgE that bind to IgE receptors on mast cell and basophil surfaces (sensitization). Upon re-exposure, allergens bind and cross-link with mast and basophil cell-bound peanut-specific IgE and trigger those cells to degranulate and release inflammatory mediators (allergic reaction). Several promising intervention strategies to reduce peanut allergenicity or modulate human immune response have been investigated, but none of these techniques have led to a cure.

Plant polyphenols are known for their natural ability to bind proteins. In previous studies, we have described a new approach to bind fruit juice- and herbal extract-derived polyphenols to peanut proteins (in the form of peanut flour), and demonstrated that some of the resulting protein-polyphenol aggregate particles were hypoallergenic *in vitro*, *ex vivo* and *in vivo*. In this work, we aimed to better understand the *molecular mechanisms* by which such peanut protein-polyphenol aggregate particles are rendered less allergenic.

In the first study, we created peanut protein-polyphenol aggregate particles containing different concentrations of polyphenols derived from cranberry (*Vaccinium macrocarpon* Ait.) and lowbush blueberry (*Vaccinium angustifolium* Ait.) pomaces. Immunoblotting revealed that peanut protein-bound cranberry and blueberry polyphenols significantly

decreased IgE binding to peanut proteins in a polyphenol concentration-dependent manner. In an allergen model system, anti DNP-sensitized RBL-2H3 mast cells challenged with DNP-BSA (antigen) and ionomycin in the presence of aggregate particles showed a significant reduction in histamine and β-hexosaminidase release, also in a polyphenol concentration-dependent fashion. We demonstrated that polyphenolic compounds bound to peanut proteins (reversibly and irreversibly). Quercetin, – in aglycone or glycosidic form- was the main phytochemical identified to be covalently bound to peanut proteins (determined by alkaline hydrolysis and subsequent HPLC analysis). The modification of peanut proteins with cranberry or blueberry polyphenols led to the formation of peanut protein-polyphenol aggregate particles with significantly reduced allergenic potential.

One of the main peanut allergens bound by blueberry and cranberry polyphenols was Ara h 2. While our previous investigations have shown the potential of using polyphenol binding as a way to mediate allergenicity, the activities of individual polyphenols was not investigated. In the second study, we used *in silico* molecular modeling techniques to prioritize polyphenols from cranberry and lowbush blueberry predicted to bind strongly to Ara h 2 in general, and to bind to specific IgE epitopes. Predicted binders delphinidin-3-glucoside, cyanidin-3-glucoside, procyanidin C1, and chlorogenic acid, as well as benzoic acid, a low predicted binder, were selected. UV-Vis spectroscopy suggested that procyanidin C1 and chlorogenic acid did interact with Ara h 2 and circular dichroism tests further showed that those compounds also induced changes in the Ara h 2 secondary structure upon binding. Previous studies using ATR-FTIR (Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy) had suggested, but did not confirm changes in secondary protein structure when peanut proteins were bound to polyphenols. Immunoblotting showed that procyanidin

C1, chlorogenic acid, and benzoic acid binding to Ara h 2 significantly inhibited IgE epitope binding capacity.

Collectively, these findings suggest that different classes of polyphenols can both non-covalently and covalently bind to peanut allergens and consequently inhibit epitope binding by epitope-specific IgE, presenting a potential strategy for alleviating peanut allergenicity symptoms in susceptible individuals.

Protein-Polyphenol Complexation to Reduce Food Protein Allergenicity

by
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BIOGRAPHY

Nathalie Plundrich was born on May 8th, 1985 in Weingarten i.A., Germany. Raised in southern Germany, she received an associate degree as a state-approved Biological Technical Assistant with a major in biology and biotechnology in 2007 and a B.Sc. degree in Bio- and Process Technology with a major in biotechnology in 2011. During her undergraduate program, she completed two internships in Dr. Lila's laboratory (Plants for Human Health Institute, North Carolina Research Campus, NCSU) working on collaborative projects developing functional food and skin applications utilizing edible protein-plant polyphenol aggregate ingredients. She went on to pursue her M.S. degree in Food Science at North Carolina State University under the direction of Dr. Mary Ann Lila in 2012, when she was introduced to this exciting and challenging project of reducing the allergenicity of food proteins by complexing them with plant bioactive compounds. In 2014 she graduated with her M.S. and continued to pursue her Ph.D. in Food Science at NC State under the direction of Dr. Mary Ann Lila and Dr. E. Allen Foegeding, continuing her work on hypoallergenic polyphenol-enriched food protein ingredients. Nathalie has to date already successfully published 10 refereed journal articles; 5 as first author, and she has presented her work in several professional venues (6^{th} International Workshop on Anthocyanins 2011, Berry Health Benefits Symposium 2013, NCSU Workshop on Allergy and Inflammation 2013, Experimental Biology 2014 and 2017, American Academy of Allergy, Asthma and Immunology 2017, and NCSU-Comparative Medicine Institute Translational Pharmacology and Physiology meeting 2017). She was awarded the NCSU Food Science Club Professional Development Scholarship (2015), the Dr. Gideon "Guy" Livingston Scholarship (2016) and

the Dr. Daryl B. Lund Student International Travel Scholarship (2017) of Phi Tau Sigma, and the IFT Nutraceutical and Functional Foods Division scholarship (2016).

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APPENDIX A

LIST OF TERMS

adaptive immune system	also called acquired immunity; includes response by highly specialized group of cells that respond specifically to eliminate or prevent reoccurrence of pathogens by immunological memory
alarmin	endogenous molecule secreted from cells undergoing non-programmed cell death that signal tissue and cell damage
allergen	type of antigen that although harmless can trigger an adverse immune response
allergic reaction	an allergen can cause an allergic reaction (inflammatory response which can range from itching to anaphylactic shock)
anaphylactic shock or anaphylaxis	severe (often life-threatening) allergic reaction including multiple symptoms
anergy	a lack of reaction by the body's defense mechanisms to foreign substances
antacid	a substance that neutralizes stomach acidity
antibody	large Y-shaped proteins that are produced by B cells and involved in immune responses to remove foreign substances (e.g. bacteria); also called immunoglobulin
antigen	an antigen is a molecule capable of inducing an immune response
antioxidant	a molecule that inhibits the oxidation of other molecules
apoptosis	programmed cell death
assay sensitivity	the smallest amount of substance in a sample that can accurately be measured by an assay

assay specificity	the ability of an assay to measure one particular organism or substance, rather than others, in a sample
asthma	chronic disease involving the airways in the lungs
atopy	a hereditary tendency to develop immediate allergic (hypersensitive) reactions
B cell	a type of white blood cell (lymphocyte) that produces antibodies
Bacteroidaceae	family of gram-negative bacteria
basophil	a type of white blood cell that plays a role in mediating hypersensitivity reactions of the immune system
bathochromic	shift to longer wavelength
Bifidobacterium lactis	a species of genus *Bifidobacterium* which can be found in the large intestines of most mammals, including humans
bioinformatics	interdisciplinary field that develops methods and software tools for understanding biological data
butyrate	a type of fatty acid
case-control study	type of observational study in which two existing groups differing in outcome are identified and compared on the basis of some supposed causal attribute
catalysis	the increase in the rate of a chemical reaction due to the participation of an additional substance called a catalyst
chemiluminescence	chemiluminescence is the emission of light, as the result of a chemical reaction
cholera toxin	protein complex secreted by the bacterium *Vibrio cholera*

circular dichroism	the difference in the absorption of left-handed circularly polarised light and right-handed circularly polarised light and occurs when a molecule contains one or more chiral chromophores (light-absorbing groups)
cohort study	an observational study design during which one or more subjects (called cohorts) are followed for a period of time. Subsequent status evaluations aid in evaluating associations between disease and exposure (i.e. habit of smoking)
conformation	structural arrangement or form (i.e. of molecule moieties)
conformational epitope	consists of discontinuous amino acids that can be recognized by the immune system through the protein's three-dimensional tertiary conformation
crystal structure	is composed of a unit cell, a set of atoms arranged in a particular way; which is periodically repeated in three dimensions on a lattice
C-section	Cesarean section; delivery of a baby through a surgical incision in the mother's abdomen and uterus
cutaneous	skin-related
cysteine	an amino acid
cytokine	cytokines belong to a group of small proteins that play an important role in cell signaling (mediators)
dermatitis	also known as eczema is a group of diseases that results in inflammation of the skin
desensitization	defined as temporary antigen hyporesponsiveness that depends on regular food ingestion

disulfide bond	covalent bond formed by two sulfur atoms derived from two sulfhydryl or thiol groups (R-S-S-R)
DNASTAR	sequence analysis software
eczema	see dermatitis
edema	medical term for swelling
ellipticity	deviation from perfect circular or spherical form toward elliptic or ellipsoidal form
endogenous	originating from inside an organism
Enterobacteriaceae	family of gram-negative bacteria
enzyme	enzymes are proteins and catalysts of many biological reactions. Enzymes are usually substrate-specific yielding certain enzymatic products
eosinophilic esophagitis	an allergic inflammatory condition of the esophagus that involves eosinophils, a type of white blood cell
epicutaneous	refers to the route of topical drug administration by which a drug or other substance is taken into the body via direct skin application
epidemiology	science that studies and monitors the patterns, causes, and effects of health and disease conditions in certain populations
epidermis	the outer layer of the two layers that make up the skin
epigenetics	the study of heritable changes in gene function that do not involve changes in the DNA sequence
epitope	surface portion of an allergen capable of eliciting an immune response

ex vivo	in science, experiments conducted with biological material but outside the organism without too much alteration of natural conditions (i.e. use of human blood, or animal tissue material)
exogenous	originating from outside an organism
fatal	deadly
fibrinogen	a blood clotting factor
flow cytometry	laser-based technology to analyze the characteristics of cells or particles
fluorescence	the property of a substance absorbing light (or other electromagnetic radiation) of short wavelength and the immediate emission of longer wavelength light/radiation
gastric	stomach-related
glycan	compound consisting of a large number of monosaccharides linked glycosidically
glycoprotein	proteins that contain sugars covalently bound to polypeptide side-chains
helminth	a parasitic worm
histamine	In this context: an inflammatory mediator part of allergic reactions
homotrimer	protein which is composed of three identical subunits
hydrogen bond	is a type of attractive interaction between polar molecules (a hydrogen atom can bind to a highly electronegative atom like nitrogen or oxygen)
hydrolysis	cleavage of a chemical bond in presence of water

hydrophobic interactions	interactions between hydrophobic molecules/or moieties (i.e. in an aqueous system hydrophobic molecules will closely come together and separate from the hydrophilic water, which is called hydrophobic effect)
hyperchromic	increase in UV absorption intensity
hypersensitivity	undesirable reactions produced by the normal immune system, including allergies
hypochromic	decrease in UV absorption intensity
hypsochromic	shift to shorter wavelength
immunoglobulin	see antibody
immunoreactive	in general: measure of immune response to an antigen; in context: intact and accessible IgE binding epitopes triggering allergic response
in silico	experiments conducted by means of computer modeling or computer simulation
in vitro	in science, experiments performed in test tubes, petri dishes, etc.
in vivo	in science, experiments conducted in the living organism
innate immune system	nonspecific defense mechanisms (first to respond)
intraperitoneally	into the body cavity
ionic interactions	chemical bond between two ions with opposite and attracting charges; typical for salts (i.e. Na+ Cl-)
isomer	molecules with same molecular formula but different structures/conformations
Lactobacillus casei	a species of genus *Lactobacillus* found in the human intestine and mouth

***Lactobacillus rhamnosus* GG**	bacterium that exists naturally in the body, primarily in the intestines, and has been used as a probiotic
Langerhans cell	a type of dendritic cell (antigen presenting cell)
leukemia	blood cancer
linear epitope	contains linear continuous amino acid sequence or primary structure
M cell	specialized epithelial cells (in upper gastrointestinal tract) that transport antigens from the lumen to cells of the immune system
macrophage	a type of white blood cell with several immune functions
Maillard reaction	non-enzymatic chemical reaction between reducing sugars and amino groups
mammal	warm-blooded vertebrate animals
mast cell	immune cell found in various tissues that is involved in regulating immune responses and plays an important role in allergic disease
meta-analysis	a study design that uses the compilation and analysis of different but similar studies (of a specific research scope) and evaluates the pooled data for statistical significance
monoclonal antibodies	antibodies produced by one B cell lineage
monocyte	type of white blood cell that can differentiate into macrophages and dendritic cells
mucosa	linings on body cavities that are exposed externally (environment) or internally (organs) such as nose, mouth or stomach
murine	relating to or affecting mice or related rodents
naïve T cell	T cell that has never been exposed to a certain antigen

N-terminus	refers to the start of an amino acid chain terminated by an amino acid with a free amine group (-NH2)
oligosaccharide	any carbohydrate of from three to six units of simple sugars (monosaccharides)
oral challenge	method for determining if a person has a specific food allergy (involves giving increasing amounts of a food and watching to see if an allergic reaction occurs)
oral tolerance	state of active inhibition of immune responses to an antigen through prior exposure to that antigen via the oral route
pathogenesis	the manner of development of a disease
pathophysiology	describes the functional (physiological) and conditional (pathological) changes associated with a disease or syndrome
pepsin	active proteolytic enzyme in the stomach (responsible for breakdown of proteins)
peptide	a chain of amino acids forms a peptide
Pi-Pi stacking	attractive, noncovalent interactions between aromatic rings, since they contain pi bonds
plasma	cell-free portion of blood
polyclonal antibodies	antibodies produced by different B cell lineage
post translational processing	also called post translational modifications, is a step in the biosynthesis of proteins. Proteins, translated from mRNA can be modified to increase their functional variety. Modifications include i.e. glycosylation, phosphorylation, or methylation
postnatal	after birth
prebiotic	a prebiotic is a food ingredient that induces the growth or activity of beneficial microorganisms
prenatal	before birth

primary antibody	an immunoglobulin that specifically binds to a particular protein or other biomolecule of research interest
probiotic	live micro-organism which, when administered in adequate amounts, confers a health benefit on the host
prostaglandin E_2	a type of prostaglandin (hormone-like substance) that participates in a wide range of body functions
proteolysis	breakdown of proteins into smaller peptides or amino acids
quaternary protein structure	the number and arrangement of multiple folded protein subunits in a multi-subunit complex
quinones	class or organic compounds, derived from aromatic compounds, such as benzene
randomized controlled trial	a study design that randomly assigns subjects into an experimental group or a control group (usually a placebo is used)
ribbon diagram	3D schematic representations of protein structure
salt bridge	see ionic interactions
SDS-PAGE	method for separating proteins by electrophoresis using a polyacrylamide gel matrix
secondary antibody	bind to the primary antibody to assist in detection, sorting and purification of target antigens
secondary protein structure	three dimensional form of *local segments* of proteins (e.g. α-helix)
sensitization	production of allergen-specific IgE after initial exposure to allergen
serum	plasma without clotting factors such as fibrinogen

skin barrier dysfunction	impairment of skin integrity which allows for irritants, allergens, and infectious organisms to penetrate
skin prick test	tests for immediate allergic reactions to different substances
small intestine	part of the gastrointestinal tract which includes the duodenum, jejunum, and ileum
spectrometry	the study of interactions between light and matter, and the reactions and measurements of radiation intensity and wavelength
splenocytes	a mixture of spleen cells such as lymphocytes, macrophages, and dendritic cells
sulfhydryl group	a functional group consisting of a sulfur bonded to a hydrogen atom
tannins	a class of phenolic compounds which includes proanthocyanidins and ellagitannins
tertiary protein structure	three dimensional shape of a protein
T-lymphocyte cell	type of white blood cell that plays a role in cell-mediated immune response (there are many different types of T cells with distinct functions)
trypsin	pancreatic protease acting in the upper part of the small intestine
uric acid	an AGE liver waste product metabolized from dietary fructose
X-ray crystallography	a technique used for determining the atomic and molecular structure of a crystal, in which the crystalline atoms cause a beam of incident X-rays to diffract into many specific directions
α-helix	a secondary protein structure form
β-hexosaminidase	an enzyme that is expressed in all cells of the body, in food allergy, it is a mediator of allergic reaction

LIST OF ABBREVIATIONS

AGE	advanced glycation end-products
ALAC	alpha-lactalbumin
Ana o 1	*Anacardium occidentale* (Cashew) allergen 1
ANC	anthocyanin
ANOVA	analysis of variance
APC	antigen presenting cell
Ara h	arachis hypogaea
B cell	B lymphocyte cell
BETALAC	beta-lactoglobulin
CBB	coomassie brilliant blue
CD	circular dichroism
CD	cluster of differentiation
DBPCFC	double blind placebo-controlled food challenge
DC	dendritic cell
DIT	data integration time
DNA	deoxyribonucleic acid
DP	degree of polymerization
DS	druggability score
ELISA	enzyme-linked immunosorbent assay
eM	eModel score
EPIT	epicutaneous immunotherapy

EWP	egg white protein
FcεRI	high affinity IgE receptor
FDA	food and drug administration
FRODOCK	fast rotational docking
GAE	gallic acid equivalents
HPLC	high performance liquid chromatography
HPP	high pressure processing
IES	InterEvScore
IFN-γ	interferon-γ
IgA	immunoglobulin A
IgE	immunoglobulin E (antibody)
IgE	immunoglobulin E
IgG	immunoglobulin G
IL	interleukin
LC	Langerhans cell
LDS	lithium dodecyl sulfate
M cell	Microfold cell
MBP	maltose binding protein
MDS	molecular dynamics simulations
MHC	major histocompatibility complex
MRE	mean residue ellipicity
NBT	nitroblue tetrazolium
OIT	oral immunotherapy

PAC	procyanidin
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PDB	protein data bank
PEF	pulsed electric field
pI	isoelectric point
PN	peanut
PN flour	light roasted 12% fat peanut flour
PVDF	polyvinylidene fluoride
RAGE	receptor for Advanced Glycation End-products
RBL-2H3	rat basophilic leukemia cell line
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SGF	simulated gastric fluid
SLIT	sublingual immunotherapy
SN	soluble nitrogen
SOAP_PP	statistically optimized atomic potentials protein-protein
SP	standard precision
T cell	T lymphocyte cell
Th1	T helper cell 1
Th2	T helper cell 2
TP	total phenolics

Treg cell	T regulatory cell
UV-Vis	ultraviolet–visible
XP	extra precision

CHAPTER 1

Literature review

Introduction to food allergy

The 2010 guidelines released by the US National Institute of Allergy and Infectious Diseases (NIAID) define the term food allergy as an "adverse health effect arising from a specific *immune response* that occurs reproducibly on exposure to a given food" (Boyce et al., 2011)**.** Adverse responses to food that do not involve an immune response, including metabolic disorders such as lactose intolerance or responses to pharmacologically-active food components such as caffeine, are not considered allergic disorders (Boyce et al., 2011; Sicherer & Sampson, 2010). The allergic reaction occurs in response to certain proteins within a food often called allergens or allergenic proteins. The terms "allergenic protein", "allergen", and "antigen" are interchangeably used in this text.

Oral tolerance

All dietary molecules (e.g. proteins) and biological agents (e.g. bacteria) are generally considered foreign antigens to the human immune system. Oral antigen administration can result in three different outcomes: 1) local secretory IgA antibody (plays crucial role in the immune function of mucous membranes) response in the gastrointestinal mucosa, 2) production of serum antibodies and cell-mediated immunity 3), or immunological tolerance development, preventing the induction of potentially damaging immune response pathways upon re-exposure to foreign antigen. The first two consequences occur to neutralize invasive, disease provoking pathogens, whereas dietary molecules such as proteins as well as commensal (non-harmful) bacteria predominantly induce immune tolerance (i.e. the immunologic state defined by a lack of reactivity to an antigen/allergen) (Faria & Weiner, 2005). More specifically, the state of immune tolerance is often referred to as "oral

tolerance" which refers to a state of active inhibition of immune responses to an antigen through *prior exposure* to that antigen *via the oral route* (Chase, 1946). It has been suggested that a breakdown in oral tolerance or failure to induce oral tolerance to antigens results in food allergy. The gastrointestinal tract is the biggest immunologic organ found in the human body and is continuously exposed to a variety of food components, including proteins, after consumption. Food proteins first are exposed to digestive conditions in the stomach where hydrochloric acid (low pH) and the protease pepsin begin to break down proteins into smaller fragments. The content of the stomach is then released into the small intestines, where pH, enzymes and digestive fluids contribute to further breakdown of proteins and peptides.

Many different immune cells are involved in oral tolerance induction, partially depending on the dose of antigen delivered to the cells. In the lower intestine a multitude of immune cells continuously sample protein antigens in proximity to the intestinal lumen, which results in different immune responses. For example, M cells (Microfold cells) are specialized to take up particulate (insoluble) antigens or antigens for which M cells express receptors (Sicińśki et al., 1990). The antigen is further delivered to and ingested by subepithelial dendritic cells (DCs), which are antigen-presenting cells (APCs) (Chehade & Mayer, 2005). It has been postulated that antigen tolerance is initiated by DCs. Lower doses or continuous consumption of antigen are proposed to favor the induction of T regulatory cells (T_{regs}) by DCs, whereas higher doses of antigen favor anergy (unresponsiveness)/deletion (apoptosis) of possibly reactive T cells as a mechanism of tolerance induction (Weiner, da Cunha, Quintana, & Wu, 2011). T_{regs} can produce cytokines (mediators) such as IL-10 that suppress other immune cells that are involved in allergy pathways overall shifting to tolerogenic type cells and cell responses (active suppression of

allergy pathways and establishment of antigen-unresponsive regulatory network) (Farrugia & Baron, 2016). In a healthy individual, oral tolerance is induced and sustained, preventing the immune system from reacting adversely to food proteins (or other components). Establishment of oral tolerance to dietary proteins is crucial to prevent the development of food allergy. Lack of oral tolerance induction or oral tolerance breakdown can occur due to several factors including antigen and host characteristics.

Pathogenesis of food allergy

In individuals that are susceptible to food allergy, the initial exposure to a food allergen results in a sensitization (towards the allergen) commonly involving an immune response cascade with production of certain immune cells and antibodies that eventually recognize the allergen after re-exposure. Upon re-exposure to the same allergen, antigen-specific immune responses can result in physical symptoms (allergic reaction). Generally, allergic immune responses can be immunoglobulin E (IgE) antibody-mediated, non-IgE mediated, or a combination of both. Non–IgE-mediated allergic reactions (cell-mediated) will not be further discussed here; most food allergies fall under the immediate type reaction category (IgE-mediated).

IgE-mediated reactions are generally classified as type I hypersensitivity reactions. When allergens are sampled from the intestine during the first encounter, as described earlier, they are captured and transferred across the mucosal barrier and processed (digested) by APCs such as DCs. Antigens are subsequently presented as protein peptides bound to Major Histocompatibility Complex class II (MHC II) molecules to naïve T lymphocyte cells (T cells). Naïve T cells, which have never been exposed to a certain antigen, are activated through the presentation of protein peptides by APCs to induce Th2 helper cells (T helper 2 cells), which in turn secrete allergy pathway-specific cytokines (i.e. IL-4 and IL-13). Those

cytokines further induce B lymphocytes (B cells) to differentiate into antigen-specific IgE antibody-producing plasma B cells (Figure. 1).

These antigen-specific IgE antibodies then bind to high affinity surface IgE receptors (FcεRI) on tissue mast cells and blood basophils. At this stage, an individual is sensitized to a given allergen and mast cells and basophils are weaponized to detect this allergen upon re-exposure. A second encounter results in binding and cross-linking of allergen IgE binding epitopes (surface portion of the allergen capable of eliciting an immune response) with the cell-bound allergen-specific IgE antibodies. There are two types of epitopes that exist on allergens: linear and conformational epitopes. Linear, or sequential, epitopes are recognizable by their linear continuous amino acid sequences or primary structures and conformational epitopes by their discontinuous amino acids through the protein's three-dimensional tertiary conformation. IgE cross-linking through the allergen leads to the degranulation of mast cells and basophils which involves the release of various inflammatory mediators, including histamine, responsible for the allergic immune response and associated physical symptoms (Burks et al., 2012). IgE-mediated reactions are characterized by an acute onset of symptoms, often just minutes after ingestion of or exposure to the triggering allergen (Figure 1). Those reactions to food can involve the skin, gastrointestinal tract, and respiratory tract (Burks et al., 2012). Subjects can have allergic sensitization (production of blood circulating IgE) to food allergens without having clinical symptoms of an allergic reaction on exposure. Thus, sensitization alone is not sufficient to define food allergy. An IgE-mediated food allergy requires both sensitization and the development of allergic symptoms upon food allergen exposure (Burks et al., 2012).

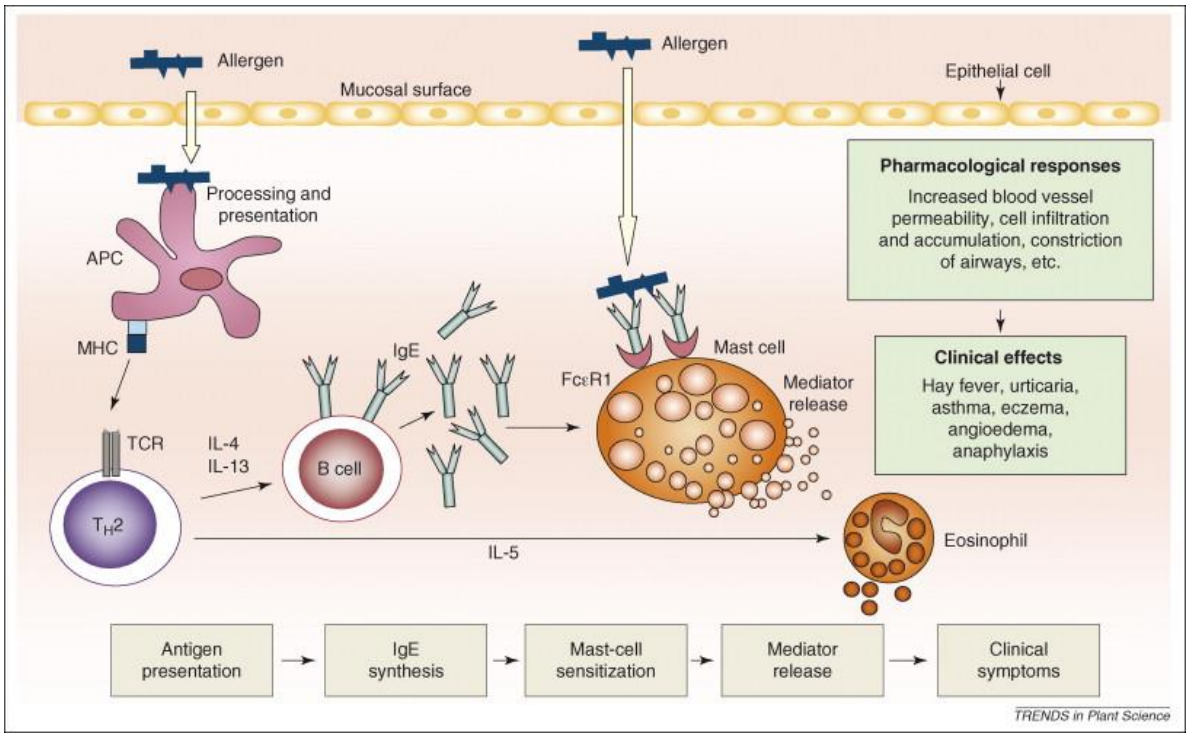

Figure 1. Schematic overview of allergy mechanisms
Sensitization: Allergens enter via mucosal surfaces in the gut and are taken up by local antigen-presenting cells (APCs; e.g. dendritic cells). The APCs process (digest) the allergens into peptides and present them to naïve T cells via major histocompatibility complex II (MHC II) which then commit to be T helper 2 (Th2) cells. Th2 cells then secrete cytokines which induce B lymphocyte cells (B cells) to differentiate into antigen-specific IgE antibody-producing plasma B cells. Th2 cells also mediate the stimulation of other proinflammatory cells, such as eosinophils. Allergen-specific IgE antibodies then circulate through the body and sensitize mast cells (which are resident in connective tissues within the body) and basophils (generally circulating in blood) by binding to high affinity Fcε receptors (FcεRI). Allergic reaction: Following subsequent exposure to the allergen, the allergen binds and cross-links IgE molecules located on mast cell and basophil surfaces, leading to cell degranulation and the secretion of mediators that are responsible for allergic inflammation and clinical symptoms of allergy (e.g. anaphylaxis) (reprinted with permission from *Genetic engineering for removing food allergens from plants*, Singh & Bhalla, 2008, copyright 2008 Elsevier Ltd).

Hypotheses for food allergy development

The reasons behind a worldwide increase in food allergies are not known, but, several potential causes have been suggested including the “hygiene hypothesis,” genetic factors, dietary trends and drug use. More recently, advanced-glycation end products (AGEs) and

protein structural features as allergy-promoting adjuvants have been discussed to play a role in the development of food allergy (Lack, 2012; Shreffler et al., 2006; Smith, Masilamani, Li, & Sampson, 2017).

Hygiene hypothesis

The "hygiene hypothesis" suggests that lifestyle changes (i.e. improved hygiene) in Westernized countries has led to a decrease of exposure and failure to build up tolerance to infectious agents, such as microbes and parasites which are associated with a rise in allergic diseases. The hypothesis was originally proposed by Strachan (1989). Following more than 17,000 British children born in the late 1950s, he found that with an increase of the number of older siblings, hay fever incidences decreased (Strachan, 1989). The study suggested that protection of younger children to develop allergy can be acquired through unhygienic interactions with older siblings (sources of diverse microbial and viral agents) or prenatally through a mother infected by contact with her older children.

The balance between Th1 (associated with bacterial and viral infections and autoimmune diseases) and Th2 (associated with helminth [parasitic worm] infections and allergic diseases) immune responses is crucial to maintain proper immune homeostasis (Matricardi & Bonini, 2000). It has been proposed that the lack or insufficient exposure to bacterial and viral agents during early childhood results in an inadequate stimulation of Th1 cells, which in turn cannot counterbalance the expansion of pro-allergic Th2 cells (Figure 2). Dendritic cells have closely been associated with immune activation or suppression, since those cells acquire signals from the microenvironment and deliver those to naïve T cells dictating them into development of polarized Th1, Th2, or T_{reg} cells (Jong et al., 2002;

McGuirk, McCann, & Mills, 2002). While helminths induce a Th2 type response, and bacteria and viruses induce a Th1 type response (Jong et al., 2002), it appears that high and diverse overall infection turnover plays a key role in the development of a balanced regulatory network (Figure 2). Bacteria, viruses, and helminths carry distinct recognition molecules or patterns that interact with DCs and orchestrate the path of immune response to the exogenous agent. The question remains, however, whether certain biological agents bear signature molecules that are better able to protect against allergy development than others (Matricardi & Bonini, 2000).

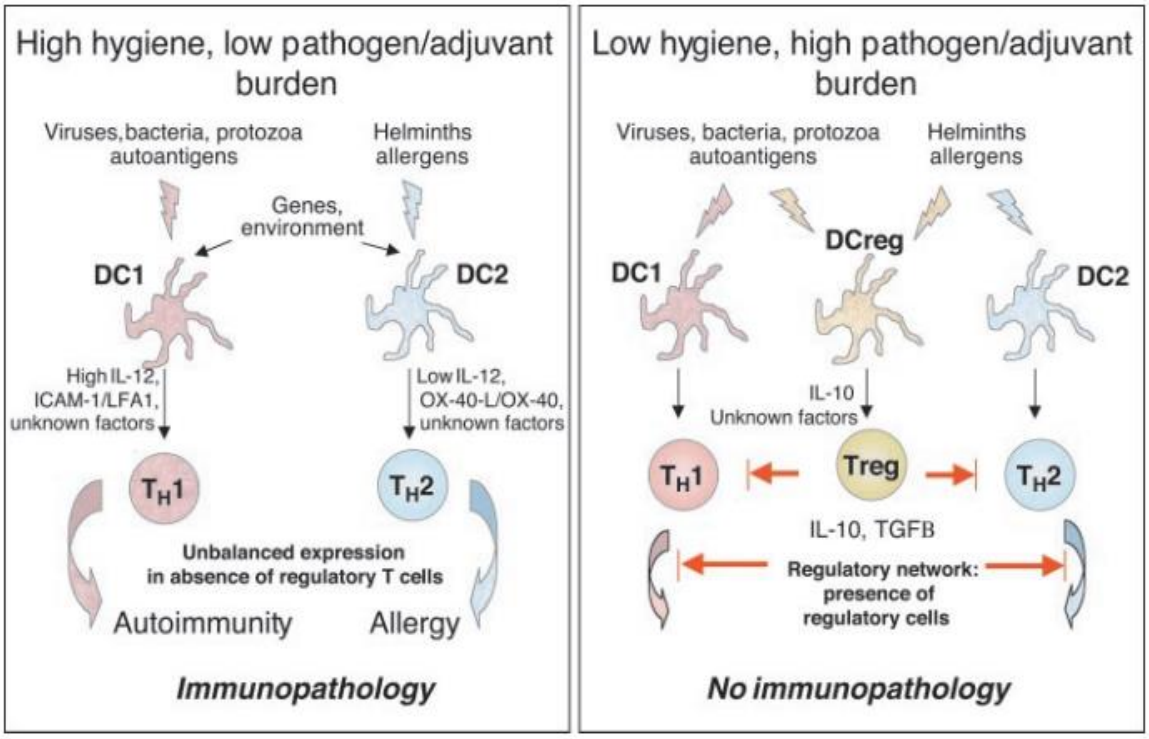

Figure 2. Hygiene hypothesis

The "hygiene hypothesis" focuses on the education of the immune system by environmental bacteria and viruses. Dendritic cells (DCs) can develop into (pro-inflammatory) DC1, (pro-allergic) DC2, or (pro-regulatory) DCreg cell subsets and further determine the fate of naïve T cells to differentiate into either (pro-inflammatory) Th1, (pro-allergic) Th2, or (pro-regulatory) Treg polarized subsets. A stable regulatory network between Th1, Th2, and Treg cells prevents atopy development, whereas a polarized strong Th1 response can result in autoimmunity and a Th2 response in allergy development (reprinted with permission from *Allergy, parasites, and the hygiene hypothesis*, Yazdanbakhsh, Kremsner, & Ree, 2002, copyright 2002 The American Association for the Advancement of Science).

The concept of early exposure to environmental microbes, parasites, and viruses has been supported by several epidemiological studies. A Swiss study found a link between farm life and a decreased risk of allergy development, proposedly due to high exposure to environmental bacterial and viral agents (Braun-Fahrländer et al., 1999). A survey conducted in traditional alpine farming areas in Germany, Austria, and Switzerland that analyzed about 1,000 children showed that maternal environmental exposure during pregnancy (i.e. actively

participating in farm life) or exposure of child during the first year of life was associated with a strong protection from allergic disorders (i.e. atopy, wheeze, and asthma) (Riedler et al., 2001).

However, limited evidence exists with respect to food allergy. Early presence of furry pets in an infant's environment can protect against food allergy as suggested by the Healthnuts Study which found significantly less egg, sesame, and peanut allergy amongst young children living with a dog during the first year of life (Koplin et al., 2012). This might provide indirect evidence for the role of microbial exposure in the prevention of food allergies.

Microbial exposure also naturally occurs during vaginal birth. Multiple studies have investigated the effect of infant delivery mode, vaginal or by cesarean section (C-section), on food allergy development. A 2003 Norwegian birth cohort study showed that birth through cesarean delivery was linked to a 7-fold increased risk of parental perceived allergic reactions of the child to some foods (Eggesbø, Botten, Stigum, Nafstad, & Magnus, 2003). A meta-analysis in 2008 on the relationship between cesarean delivery and allergy outcome found that 6 out of 26 studies confirmed association between cesarean delivery and increased risk of food allergy (20 studies reported other allergic outcomes such as asthma) (Bager, Wohlfahrt, & Westergaard, 2008). It is suggested that the exposure to maternal fecal and vaginal microbial agents during natural birth (limited with C-section) leads to early colonization and establishment of microbial populations in the infant, protecting against the development of allergic disease (Heavey, 1999). Adding to the limited exposure of an infant to maternal microbes during C-section delivery is the increased administration of antibiotics to the mother for proper recovery after surgery which further depresses infant microbial

colonization (Smaill & Grivell, 2014). A recent study by Dominguez-Bello *et al.* (2016) showed that a vaginal microbial transfer from the mother (i.e. obtaining vaginal fluids from the mother after birth and subsequently swabbing the infant's body with it) can partially restore the microbiota of cesarean-born infants.

Another theory for differences seen between delivery modes are that C-sections are generally associated with high maternal age, and this has been shown to be a possible risk factor for food allergy in a case-control study (Dioun, Harris, & Hibberd, 2003). Some studies describe elevated risk of food allergy with C-section, especially in conjunction with an atopic mother (Eggesbø, Botten, Stigum, Nafstad, & Magnus, 2003; Metsälä et al., 2010), but others found no evidence (Karpa et al., 2012; Koplin et al., 2012; Pyrhönen, Näyhä, Hiltunen, & Läärä, 2013). In general, there appears to be a common understanding that low diversity of infant gut microbiota and an increased Enterobacteriaceae/Bacteriodaceae ratio (linked to lack of or inadequate exposure to environmental microbes such as associated with C-sections) are connected with subsequent food sensitization and allergy development (Azad et al., 2015).

Since studies suggest that a lack of infant or child microbial diversity and/or high levels of certain microbial populations is related to development of atopy, some studies have investigated the effects of pro- and prebiotic supplementation (of pregnant or nursing mothers or infants) in an effort to change offspring commensal gut flora and prevent allergy. A 2008 meta-analysis evaluated 10 double-blind randomized controlled clinical trials (conducted between 1997 and 2007) on the efficacy of prenatal and postnatal probiotic administration for atopic dermatitis (itchy inflammation of the skin often associated with food allergy) and found a small protective effect (Lee, Seto, & Bielory, 2008).

Some report that there is no evidence that maternal or infant supplementation with probiotics prevent the development of food allergies (de Silva et al., 2014). In contrast, prenatal and postnatal maternal probiotic supplementation was reported to decrease subsequent infant sensitization to food allergens (Huurre, Laitinen, Rautava, Korkeamäki, & Isolauri, 2008). In contrast, a study with cow's milk-allergic infants (challenge proven) showed that the supplementation with two bacterial strains (*Lactobacillus casei* and *Bifidobacterium lactis*) with their extensively hydrolyzed infant formula for 12 months had no benefits on clinical tolerance development to cow's milk (Hol et al., 2008). Recent studies showed, however, that administration of an extensively hydrolyzed casein formula containing a different bacterial strain, *Lactobacillus rhamnosus* GG, accelerated the onset of oral tolerance in cow's milk-allergic infants and lowered events of other allergic manifestations (i.e. other IgE-mediated food allergies, asthma, eczema) (Berni Canani et al., 2013, 2017; Canani et al., 2012). When this group compared the fecal microbiota of infants that received probiotic supplement to that of infants that consumed formula alone they found an abundance of butyrate-producing bacteria and increased concentrations of fecal butyrate (Canani et al., 2016). Butyrate can act in many ways but it has been suggested that it ultimately promotes the production of T_{regs} (Tao et al., 2007).

Different microorganisms have been used in different studies, which could play a role in the possible preventive effect of certain probiotic strains on food sensitization or oral tolerance development in the infant. In fact, researchers have postulated that probiotic use against allergies is strain-specific, and results of studies for one selected strain cannot be adopted to others (Hill et al., 2014; Lomax & Calder, 2009; Maidens, Childs, Przemska, Dayel, & Yaqoob, 2013).

One systematic review did not find sufficient evidence to suggest that prebiotics (substances that induce the growth or activity of beneficial, probiotic, microorganisms) added into infant formula had any preventative effects on food allergy (Osborn & Sinn, 2013), and one randomized trial using a blend of oligosaccharides (prebiotics) found a benefit for eczema, but not for food sensitization (Grüber et al., 2010).

Genetic factors

Sex and ethnicity have been mentioned as important risk factors for the development of food allergies but will not be discussed here further. The role of genetics in risk and emergence of food allergy has been a hot research topic. Particularly, researchers want to understand the genetic mechanism and its interplay with environmental factors (Li, Maggadottir, & Hakonarson, 2016). A study has found that there is a 7-fold increase in risk for a child to develop peanut allergy if one of the parents or siblings suffers from peanut allergy (Hourihane, Dean, & Warner, 1996). Furthermore, depending on the study design, twin studies have been proposed to exhibit a 2-10 fold increased risk to develop food allergy (Crespo, James, Fernandez-Rodriguez, & Rodriguez, 2006; Hourihane et al., 1996; Koplin et al., 2013; Tsai et al., 2009). A study by Sicherer *et al.* (2000) investigated the heritability for peanut allergy of 58 twin pairs and showed it to be as high as 87%. While various genes involved in allergen presentation and/or a Th2-skewed immune system have been linked to food allergy or food sensitization, skin barrier dysfunction (e.g. through mutated genes) has attracted most interest, as this is known to be associated to susceptibility of various atopic diseases (Hong, Tsai, & Wang, 2009). Defenders of a hypothesis that involves genetic and environmental factors suggest that one or several environmental exposures, or the absence

thereof, can trigger epigenetic changes that result in the imbalance of the inherent immunologic state of tolerance (Sabounchi, Bollyky, & Nadeau, 2015).

Dietary trends

Another highly discussed theory has been that dietary choices influence allergy susceptibility. For example, an increased consumption of omega-6 polyunsaturated fatty acids and a decreased intake of omega-3 polyunsaturated fatty acids, high fat/low fiber diet, excess vitamin D intake or deficiency and low levels of vitamin A as well as marginal consumption of plant-derived antioxidants and obesity have been proposed to be involved in increased prevalence of allergies (Aitoro et al., 2017; Lack, 2012). Some studies suggest that a ***shift from diets high in animal to plant-based fatty acids*** has caused an increase in allergies. Researchers argue that a rise in intake of dietary omega-6 polyunsaturated fats (found in various seed oils) leads to the production of prostaglandin E_2 (proinflammatory mediator) which in turn reduces IFN-γ (cytokine involved in immune tolerance) production by B-cells resulting in increased IgE production by those cells. In contrast, omega-3 polyunsaturated fats (found in oily fish) inhibit the synthesis of prostaglandin E_2 (Black & Sharpe, 1997; Devereux & Seaton, 2005). Dietary fat, fiber, and vitamin A intakes have shown to be negatively correlated with allergies. Grimshaw *et al.* (2014) concluded from a birth cohort study in England that an infant diet consisting of high levels of fruits and vegetables, as well as home-prepared fresh foods (high fiber and low fat diet), is associated with decreased occurrence of food allergy by the age of 2 years. A mouse study showed that a high-fiber diet - which altered gut microbial ecology and expanded the release of short-chain fatty acids - supported oral tolerance and protected from peanut allergy by increasing

tolerogenic DC function, and this was vitamin A dependent (Tan et al., 2016). Similarly, ***excessive levels of vitamin D*** (in supplement form or in fortified foods) have been epidemiologically shown to increase allergy rates. Cohort studies in Northern Finnland and North America showed that infants who received vitamin D supplementation were more susceptible to food allergies (Hyppönen et al., 2004; Milner, Stein, McCarter, & Moon, 2004). In contrast, a study by Camargo *et al.* (2006) suggested that ***vitamin D deficiency*** related to low exposure to natural sunlight was correlated to increased prevalence of allergies. Further, two studies in Finland showed that maternal vitamin D intake may be related to a decreased risk of sensitization to food allergens, emergence of asthma or allergic rhinitis (Erkkola et al., 2009; Nwaru et al., 2010). Lack of adequate amounts of ***dietary antioxidants*** from of fruits and vegetables in association with allergy has been reported in a few studies from the United Kingdom (Allan, Kelly, & Devereux, 2010). Observational epidemiological studies of humans report potentially beneficial associations between dietary antioxidants and allergic outcomes (such as asthma), however, some report potentially adverse associations. No such data is currently available for food allergy.

Another theory is that a ***Western diet*** high in animal protein, sugar, and fat but low in fiber (especially processed foods) has led to the extinction of many species of gut microbiota and could explain the increase in food allergy rates (Filippo et al., 2010; Myles, 2014). Populations with a more wholesome diet (high fiber, low fat, vegetable heavy diet) and different sanitation and hygiene standards, as seen in developing countries such as Africa, have shown to have more diverse microbial variety in their gastrointestinal tract and a lower prevalence of food allergy (Filippo et al., 2010; Levin, Gray, & Marrugo, 2016). A comparative study in 2010 showed that children living in rural Africa have a healthier (more diverse) mix of microbes found in their feces than Italian children (Westernized country),

which may protect them from diseases that are common in modern developed countries such as food allergies (Filippo et al., 2010).

Drug use

The more common and frequent use of ***antacid medications*** (i.e. gastric acid neutralizers) has been hypothesized to lead to increased occurrence of food allergies. Recent studies in both murine models of food allergy and humans have yielded evidence that suggested that antacids can change the gastric acidity to an extent where increased IgE sensitization in both children and adults can be observed. The change of gastric pH can affect gastric enzyme (pepsin) activity and efficiency and may prevent or impair complete destruction of potential allergens during the digestive process, hence protein antigens or protein fragments may retain their immunoreactivity (Untersmayr & Jensen-Jarolim, 2008). More importantly, inadequate digestion of allergenic proteins can lead to greater exposure to intact proteins and potentially enhance the severity of food allergic reactions (Groschwitz & Hogan, 2009).

Advanced-glycation end products (AGEs)

A more recently emerged hypothesis for the dramatic increase of food allergy in developed countries suggests that the Western diet promotes innate danger signals and immune responses through production of so called "alarmins" (Smith et al., 2017). Alarmins are endogenous molecules secreted from cells undergoing non-programmed cell death that signal tissue and cell damage. Alarmins bind to the Receptors for Advanced Glycation End-products (RAGE) and those receptors can be found on DCs, macrophages, T cells, and B cells, as well as mast cells and basophils (Chen et al., 2008; Han et al., 2011;

Ramasamy, Yan, Herold, Clynes, & Schmidt, 2008; Sick et al., 2010; Ullah et al., 2014). Binding of alarmins to RAGE results in Th2 (allergic) immune cell responses (Dumitriu et al., 2005; Manfredi et al., 2008; Milutinovic, Alcorn, Englert, Crum, & Oury, 2012; Sick et al., 2010). The study proposed, based on numerous epidemiologic and experimental observations, that dietary AGEs (derived from cooked meats, oil, and cheese) and AGE-forming sugars (e.g. uric acid, an AGE liver waste product metabolized from dietary fructose) can "mimic" alarmins and cause misinterpretation by various immune cells, resulting in allergy-promoting immune responses.

Protein structural features as adjuvants for allergic response

Many studies on allergen immunogenicity have focused on physiochemical properties of the proteins (i.e. size, solubility, and stability) but not much on their potential inherent immunomodulatory properties. Interestingly, an *in vitro* study proposed that glycan structures found on plant proteins (such as peanut glycoprotein Ara h 1), similar to glycans from helminths (usually a trigger for a Th2-mediated immune response), can act as Th2 adjuvants and induce immune responses that contribute to the emergence of allergy in susceptible individuals (Shreffler et al., 2006). Glycans act as Pathogen-Associated Molecular Patterns (PAMPs) that can be recognized and responded to by immune cells. Peanuts contain several allergenic glycoproteins, which have shown to carry complex *N*-glycans (Burks, Cockrell, Stanley, Helm, & Bannon, 1995; Kolarich & Altmann, 2000). Experiments with Ara h 1 and human monocyte-derived DCs have shown enhanced uptake and activation by DCs and subsequent Th2 response priming by Ara h 1 - bearing glycan PAMPs and this effect was also seen with a pool of peanut allergens in the form of a soluble peanut extract.

Interestingly, Ara h 1 –activated DCs promoted Th2 polarization even when those DCs were co-stimulated with pro-inflammatory (Th1) cytokines (IL-1 and TNF-α). The contribution of glycan structures in food allergic sensitization by other non-mammalian proteins (e.g. shellfish) has been suggested. In addition, other exogeneous Th2 adjuvants have been suggested such as bioactive lipids or non-mammalian biopolymers (e.g. chitin) (Berin & Shreffler, 2008).

Food allergens

The "Big Eight"

The term "Big Eight" was born through the international Codex Alimentarius Commission in 1999 which recommended that member countries create a list of these eight common allergenic foods and their derivative ingredients to be listed on food labels. The "Big Eight" became a focus of the United States legislation in 2006, when the Food Allergen Labeling and Consumer Protection Act took effect. This law requires that manufacturers clearly label products containing any of the eight food allergens. Peanuts, tree nuts (e.g. cashews, Brazil nuts, and hazelnuts), soy, cow's milk, wheat, hen's egg, finned fish, and shellfish (crustaceans) are considered the "Big Eight" foods associated with food allergy both in the United States and internationally. Those foods are the cause for >90% of allergic reactions (Boyce et al., 2011). In particular, peanut and tree nuts are responsible for reported fatal and near-fatal reactions in the United States and Europe (Bock, Muñoz-Furlong, & Sampson, 2001; Sampson, Mendelson, & Rosen, 1992; Yunginger et al., 1988). It has been shown that peanuts and tree nuts account for 21%, shellfish for 19%, and fish for 10% of emergency department visits (Clark et al., 2004). Sesame has been considered an emerging food allergen

in Westernized countries (Dalal et al., 2002). Food allergens, compared to non-allergenic food proteins, are generally very resistant to heat and gastric digestion. They can withstand acidic stomach conditions and are relatively stable to proteolytic (enzyme) degradation in the gastrointestinal tract (Astwood, Leach, & Fuchs, 1996).

Peanut allergy and allergens

Reactions to peanut (*Arachis hypogaea* L.*)* are mostly IgE-mediated (immediate reactions). Peanut allergy can affect both children and adults as only about 20% of peanut-allergic children outgrow the immune disorder (Skolnick et al., 2001). In contrast, allergies to egg, milk, soy or wheat are more likely to be outgrown and food allergies are in fact common in the first three years of life (6% to 8% of children being affected) (Bock, 1987; Høst & Halken, 1990; Sicherer, 2003; Sicherer et al., 2004).

The WHO/IUIS Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies recognized 16 peanut allergens present in the edible seed (Table 1) (WHO/IUIS http://www.allergen.org/search.php?Allergensource=Arachis+hypogaea). Ara h 4 was renamed to an isomer of Ara h 3 and hence is not listed as a separate allergen by the WHO/IUIS.

Table 1. Allergen name, protein family, known isoelectric point, and molecular weight (MW) of peanut allergens

Allergen	Protein family	Isoelectric point (pH)	MW in kDa (reducing SDS-PAGE)
Ara h 1	Cupins (7S globulin)	4.55	64
Ara h 2	Conglutins (2S albumin)	5.2	17
Ara h 3	Cupins (11S globulin)	5.5	60, 37 (fragment)
Ara h 4	Cupins (11S globulin); Ara h 4 was identified as isomer of Ara h 3		
Ara h 5	Profilins	4.6	15
Ara h 6	Conglutins (2S albumin)	5.0	15
Ara h 7	Conglutins (2S albumin)	5.6	15
Ara h 8	Pathogenesis-related proteins	5.0	17
Ara h 9	Non-specific lipid transfer proteins	8.9	9.8
Ara h 10	Oleosins	9.6-9.8	16
Ara h 11	Oleosins		14
Ara h 12	Defensins		8
Ara h 13	Defensins		8
Ara h 14	Oleosins		17.5
Ara h 15	Oleosins		17
Ara h 16	Non-specific lipid transfer protein		8.5
Ara h 17	Non-specific lipid transfer protein		11

Of those 16 peanut allergens, Ara h 1, Ara h 2, Ara h 3, and Ara h 6 are the major allergens of concern and will be further discussed in the following sections (Sicherer, Muñoz-Furlong, Godbold, & Sampson, 2010; Skolnick et al., 2001).

Ara h 1 is a 63 kDa glycoprotein, belonging to the vicilin (7S) protein family, and occurs in peanuts as highly structured, stable homotrimers (quaternary structure consists of three identical subunits) (Koppelman, Bruijnzeel-Koomen, Hessing, & Jongh, 1999; Maleki, Kopper, et al., 2000; Shin et al., 1998). It comprises 12–16% of the total peanut protein (De Jong et al., 1998). The crystal structure of Ara h 1 has been elucidated (Figure 3) (Cabanos et al., 2011).

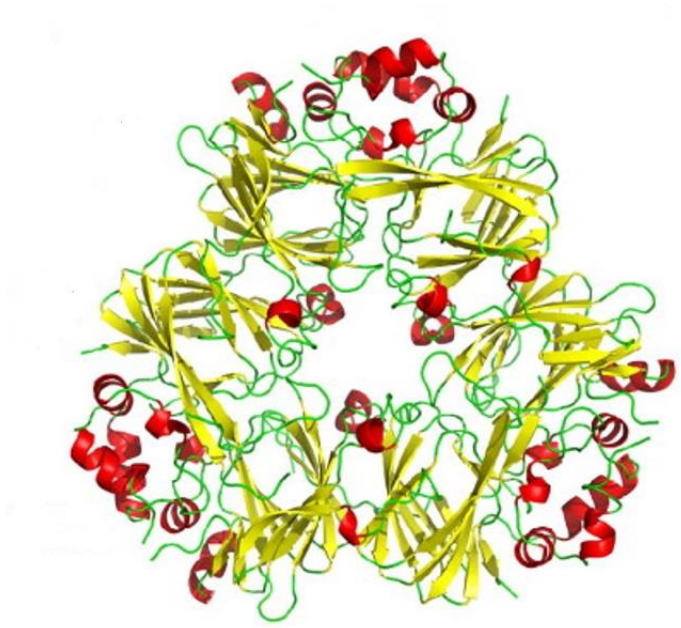

Figure 3. Crystal structure Ara h 1 (homotrimer form) (reprinted with permission from *Crystal structure of the major peanut allergen Ara h 1*, Cabanos et al., 2011, copyright 2011 Elsevier Ltd).

The trimeric form of Ara h 1 can form high molecular weight aggregates (600 -700 kDa) (van Boxtel, van Beers, Koppelman, van den Broek, & Gruppen, 2006; van Boxtel, van den Broek, Koppelman, Vincken, & Gruppen, 2007). Since the overall core structure of Ara h 1 is very similar to other known structures of 7S globulins, there is a potential for cross-reactivity between 7S globulins (Cabanos et al., 2011). However, *in vitro* IgE binding experiments did not find evidence of high-affinity cross-reactive IgE between vicillins from peanut and other foods (i.e. walnuts and cashews) (Astwood, Silvanovich, & Bannon, 2002; Teuber, Jarvis, Dandekar, Peterson, & Ansari, 1999; Wang et al., 2002). Studies with peanut allergen Ara h 1 and cashew allergen Ana o 1 (a vicillin protein like Ara h 1) showed that Ara 1 and Ana o 1 did not share any common linear epitopes. To date, 23 linear epitopes have been identified post-translational processing on Ara h 1 (Burks et al., 1997; Cong, Lou, Xue, Li, & Chena, 2008).

Ara h 2, also a glycoprotein, belongs to the 2S albumin protein family and accounts for around 6% - 9% of the total peanut protein (Koppelman et al., 2001). Ara h 2 exists as two isomers, Ara h 2.01 (17 kDa) and Ara h 2.02 (18 kDa), the former lacking 12 amino acids. This extra amino acid sequence contains one linear IgE binding epitope (Burks et al., 1992a; Chatel, Bernard, & Orson, 2003). Structurally, Ara h 2 contains five α-helices and four disulfide bonds. Along the polypeptide chain, 10 linear IgE binding epitopes have previously been identified for Ara h 2.02 (Stanley et al., 1997). The tertiary structure of Ara h 2 was derived from X-ray crystallography and is shown in Figure 4 (Barre, Borges, Culerrier, & Rougé, 2005).

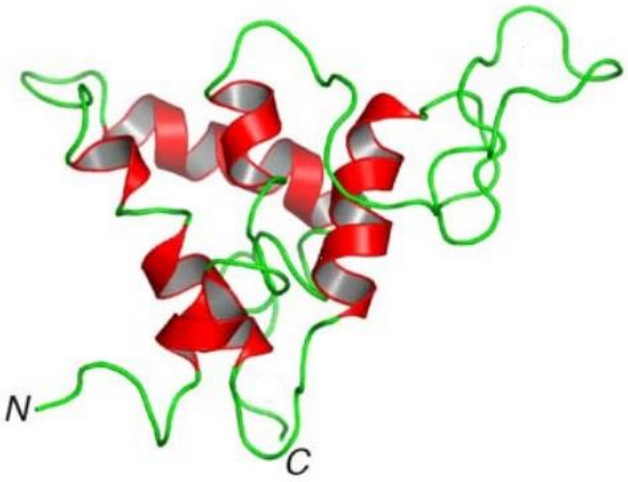

Figure 4. Ribbon diagram of Ara h 2 (reprinted with permission from *Homology modelling of the major peanut allergen Ara h 2 and surface mapping of IgE-binding epitopes*, Barre et al., 2005, copyright 2005 Elsevier B.V.).

Ara h 1 and Ara h 2 are recognized by >90% of peanut-allergic individuals (Burks et al., 1991, 1992a) and initial studies proposed that Ara h 1 was more important than Ara h 2 (Burks et al., 1991, 1992b; Buschmann, Petersen, Schlaak, & Becker, 1996). More recent experiments have revealed that Ara h 2 has a higher potential to trigger human basophil degranulation and has a higher reactivity in skin tests in peanut-sensitive patients compared to Ara h 1 (Palmer et al., 2005; Porterfield et al., 2009).

Ara h 3 (Figure 5) belongs to the legumin (11S) proteins (Koppelman et al., 2003). Originally it was found that Ara h 3 was a 14 kDa protein (Eigenmann, Burks, Bannon, & Sampson, 1996) but further investigations by cloning of the Ara h 3 gene, it was found that Ara h 3 is a 60 kDa protein (Rabjohn et al., 1999). A protein, that was previously identified and named as Ara h 4 (Kleber-Janke, Crameri, Appenzeller, Schlaak, & Becker, 1999) was later shown to be an isomer of Ara h 3. Today, Ara h 3 and Ara h 4 are considered as Ara h 3. Studies further revealed that Ara h 3 undergoes post-translational processing ultimately leading

to the formation of a hexamer (360 – 380 kDa) consisting of six basic and acidic subunits (Jin, Guo, Chen, Howard, & Zhang, 2009). The hexamer is formed by a head-to-head association of two trimers and each monomer was found to have 4 linear IgE binding epitopes (Jin et al., 2009; Rabjohn et al., 1999).

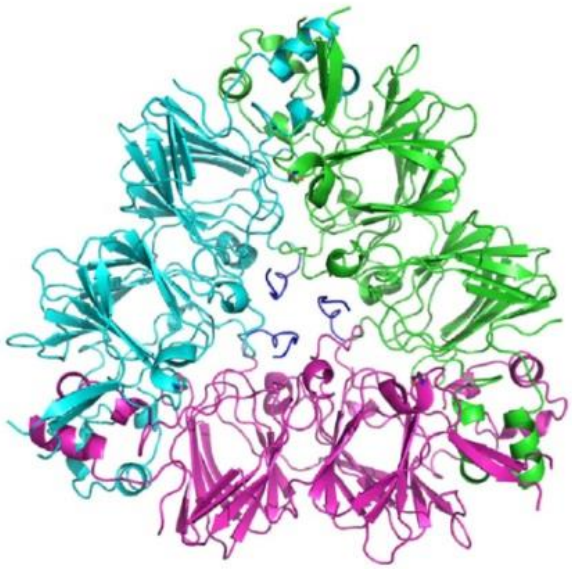

Figure 5. Ara h 3 homortimer (reprinted with permission from *Crystal structure of Ara h 3, a major allergen in peanut*, Jin et al., 2009, copyright 2009 Elsevier Ltd).

Under denaturing conditions, the native quaternary structure of the protein is separated into peptides with masses of 14, 16, 25, 28, 42, and 45 kDa (Koppelman et al., 2003). Ara h 3 was recognized from 44, 53, and 77% of peanut-sensitive individuals in three different studies (Kleber-Janke et al., 1999; Rabjohn et al., 1999; Shreffler, Beyer, Chu, Burks, & Sampson, 2004). *In vitro* and *in vivo* experiments suggest that it is less allergenic than Ara h 2 and Ara h 6 but about as allergenic as Ara h 1 based on its basophil degranulation capacity (Koppelman et al., 2003).

Ara h 6 is a 15 kDa protein and belongs to the albumin (2S) family (Figure 6) (Koppelman et al., 2005). Ara h 6 and Ara h 2 are 59% homologous in structure (Koppelman et al., 2005). Despite overlaps of amino acids sequence portions between Ara h 2 and Ara h 6 no linear IgE binding epitopes could be identified (Suhr, Wicklein, & Becker, 2002). Based on results, it was suggested that Ara h 6 contains conformational epitopes. An earlier study categorized Ara h 6 as a *minor* allergen (recognized by <50% of a population) (Kleber-Janke et al., 1999). However, a more recent study suggests that Ara h 6 has a similar allergenic potential as Ara h 2 (suggesting it to be a *major* allergen) and this was evaluated by IgE binding (immunoblotting) using human allergic serum and a basophil degranulation assay using human Peripheral Blood Mononuclear Cell (PBMC)-derived and sensitized basophils (Koppelman et al., 2005).

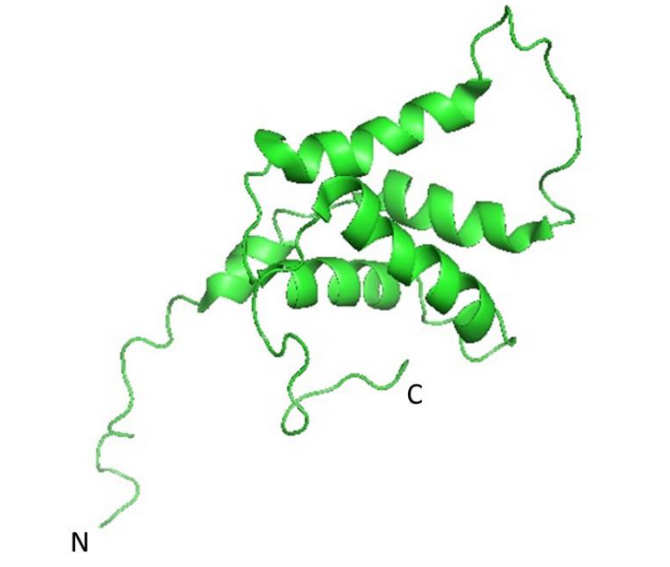

Figure 6. Ribbon diagram of Ara h 6, PDB entry: 1W2Q (reprinted with permission from *Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions*, Lehmann et al., 2006, copyright 2006 The Biochemical Society).

Prevalence and epidemiological overview

Many studies attempted to determine population-wide food allergy prevalence, but this has proved to be a difficult undertaking. Oral food challenge-based studies suggest that the prevalence of food allergy amongst young children in Westernized countries like the United Kingdom and Australia is currently between 5% and 10% (Prescott et al., 2013). The self-reported rate of food allergy in children in the United States lays between 4.8% and 8% (Dunlop & Keet, 2018). Food allergy in older children and adults has been reported to be present in about 2% to 4% (Eigenmann, 2001; Kanny et al., 2001; Mattila et al., 2003; Moneret-Vautrin et al., 2004; Roehr et al., 2004; Sicherer, 2003; Zuberbier et al., 2004). In respect to developing countries (e.g. Africa or Brazil) there continues to be lack of information regarding the prevalence and incidence of food allergy. Current assumptions are that prevalence rates are lower than in Westernized countries. A recent study reviewed the emerging body of evidence on the prevalence of various food allergies in developing countries (Boye, 2012). A study of 211 urban high school children in South Africa revealed a 5% prevalence rate of food allergy, using a skin prick test, and children were mostly allergic to egg white (3.3%), peanut (1.9%) and milk (1.9%) (Levin et al., 2011).

Cow's milk allergy has been suggested to have a prevalence of about 2% in infants (Teuber, Beyer, Comstock, & Wallowitz, 2006); however, rates vary based on population and prevalence as high as 7.5% has also been proposed (Bahna, 2002).

Shellfish allergy appears to be more prevalent in adults than adolescents or children. A digital dial population survey in the United States showed that 2% of a population (14,948 individuals) reported to be allergic to shellfish and over half of those affected were ≥ 41 years of age. Only 0.1% allergic to shellfish was younger than 5 years, and 0.7% were

between 6 and 17 years old (Sicherer, Muñoz-Furlong, & Sampson, 2004). The European Community Respiratory Health Survey found similar rates with 2.8% of individuals reporting that shrimp causes illness when consumed (Woods, Abramson, Bailey, & Walters, 2001).

The previously mentioned digital dial survey also revealed that the rate of ***fish allergy*** in the United States was between 0.2% (children) and 0.5% (adults) (Sicherer et al., 2004). ***Chicken (Gallus gallus domesticus) egg allergy*** mostly occurs during childhood and is outgrown by adulthood; however, some individuals suffer from persistent hen's egg allergy as adults. The prevalence of this food allergy is estimated to be around 1.3% in the United States (majority is children) and 0.2% in adults (Sampson, 2004).

As one of the most severe food allergies, ***peanut allergy*** is estimated to affect about 5% of young children and 3% to 4% of adults in Westernized countries (Sicherer & Sampson, 2010).

A survey of around 38,000 children has shown that about 0.4% of children suffered from ***soy allergy*** (Gupta et al., 2011).

There are 12 major types of ***tree nuts*** produced and consumed worldwide, namely almonds, Brazil nuts, cashews, chestnuts, coconuts, hazelnuts, macadamia nuts, pecans, pine nuts, pistachios, black walnuts, and English walnuts (Burket, 2000)**.** A random digit dial survey in the United States in 2002 reported that tree nut allergy prevalence was between 0.2% (children) and 0.5% (adults) (Sicherer, Muñoz-Furlong, & Sampson, 2003). A recent systematic review evaluated the outcomes of 36 studies (McWilliam et al., 2015). They found that food challenge-confirmed (IgE-mediated) tree nut allergy prevalence was < 2% while suspected tree nut allergy prevalence was between 0.05 to 4.9%.

Wheat allergy was found to affect about 0.4% to 1% of children worldwide (Poole et al., 2006; Venter et al., 2006). In the United States, a random dial survey by the United States Food and Drug Administration (FDA) reported the prevalence of the self-reported, doctor-diagnosed wheat allergy is 0.4% (Vierk, Koehler, Fein, & Street, 2007).

Methods to evaluate protein allergenicity

In vitro immunoassays

One of the first approaches to evaluate the immunogenicity (ability to induce an immune response) or allergenicity (ability to induce an allergic reaction) of a food protein is its capacity to bind to allergen-specific antibodies (usually IgG for immunogenicity and IgE for allergenicity).

One commonly used assay is ***immunoblotting*** (also called Western blotting). In this technique, a protein of interest (either isolated or in a protein mixture) is first separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE, which separates proteins by their molecular weight in a gel matrix) and subsequently transferred to either nitrocellulose (hydrophilic) or polyvinylidene difluoride (PVDF, hydrophobic) membranes. A simpler version of this is a so-called ***dot blot*** in which the protein of interest is directly applied onto the membrane. The membrane then undergoes several immunoblotting steps, which includes the incubation of the membrane with a primary antibody (which is specific to bind to the target protein) and subsequently with a secondary antibody (conjugated with a reporter enzyme or a fluorophore label) directed at the Fc region of the primary antibody (Burnette, 1981; Renart, Reiser, & Stark, 1979).

Primary antibodies used are generally either polyclonal (antibodies produced by *different* B cell lineages) or monoclonal (antibodies produced by *one* B cell lineage) antibodies derived from immunized animals such as mouse, rabbit, or goat. Plasma (cell-free portion of blood) or serum (plasma without clotting factors such a fibrinogen) are often also used as a source of primary antibodies. Depending on secondary antibody characteristics, a signal can be captured via colorimetric, chemiluminescent, or fluorescent imaging systems (Figure 7) (Nishi, Isobe, Zhu, & Kiyama, 2015; Roda & Guardigli, 2012). Immunoblots can provide qualitative and semi-quantitative results.

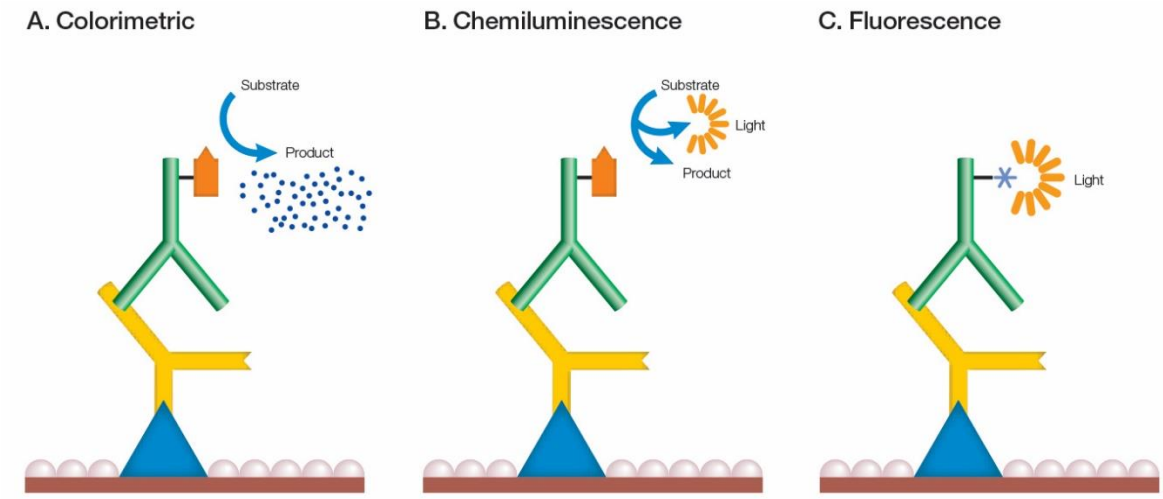

Figure 7. Different detection methods used during immunoblotting
In each detection method there is a reporter molecule translating primary antibody binding to a target protein as a measurable signal. Colorimteric detection (A) takes advantage of a reporter enzyme linked to the secondary antibody, which upon substrate addition catalyzes the reaction of the substrate to a visible (colored) chemical product. Detection via chemiluminescence similarly uses a suitable secondary antibody-conjugated enzyme which catalyzes the reaction of a substrate and with this emits a chemiluminescence light. In fluorescence detection, the secondary antibody is labled with a fluorophore which can emit light when excited (reprinted with permission from Bio-Rad Laboratories, Inc. and referenced from http://www.bio-rad.com/en-us/applications-technologies/detection-methods).

Another technique is an enzyme-linked ***immunosorbent assay (ELISA)***, which is a simpler, cheaper, quicker, and more quantifiable assay. It is very similar to immunoblotting, except ELISA starts with the manual adsorption (coating) of the isolated and purified target protein to a solid phase (microtiter plates made of plastic polymers). If the protein of interest is found in a protein mixture, then a ***sandwich ELISA*** is used. For this, a microtiter plate is coated with a target protein-specific antibody which can capture the target protein from the mixture. The rest of the procedure remains the same. Binding capacity of the target protein to primary antibody in this method is measured spectrophotometrically (visible color) (Crowther, 2000).

Besides investigating the ability of food allergens to bind to IgE antibodies, it is important to further evaluate the allergen's potential to induce activation of allergen-specific IgE-sensitized effector cells (mast cells and basophils) by crosslinking of cell-bound IgE. Different assays have been developed. For example, in the ***histamine release assay***, allergen-specific IgE-sensitized basophils derived from blood of allergic individuals or manually sensitized donor basophils (by incubation with allergen-specific IgE antibodies) are activated by the allergen and histamine (an allergic mediator involved in allergic response) release is fluorometrically detected.

The ***basophil activation test*** uses the same sources of basophils but activation of those cells is measured by flow cytometry (cell sorting and counting technique). Cells with cell-surface activation markers called clusters of differentiation (CD), specifically CD203c or CD63 with fluorochrome-tagged antibodies, are detected and counted with this method. Both assays are complex in nature and depend on frequent blood donations.

A simpler alternative is the ***humanized rat basophilic leukemia cell mediator release assay.*** A rat basophil cell line (RBL-2H3) modified to express the human high affinity IgE receptor is manually sensitized to the allergen and subsequently stimulated by the allergen. Degranulation is determined by detection of β-hexosaminidase or histamine release, respectively (Vogel, Lüttkopf, Hatahet, Haustein, & Vieths, 2005).

Further, evaluation of a protein's ability to induce T cell responses is often meaningful. A well-established method for that purpose is a ***T cell proliferation assay***. Peripheral mononuclear cells (PBMCs) are isolated from allergic patients or from splenocytes from an immunized animal and cultured in the presence of the target protein. After incubation, proliferation is determined by adding a radioactive nucleotide to the cells. This nucleotide is incorporated into the new chromosomal DNA during cell division and can be measured by scintillation counting using a scintillation counter (measures radiation). Proliferation assays can also be performed with allergen specific T cell lines and cytokines produced by those T cells in response to antigen exposure can be assessed (Jahn-Schmid et al., 2002).

Protein-uptake by bone marrow derived dendritic cells provides another way of assessing the immunogenicity of a protein. In this assay, DCs are incubated with the relevant protein conjugated with different fluorescent probes. The capture of the protein by the cell, protein internalization and processing (digestion) can then be evaluated by flow cytometry (Kitzmüller et al., 2015).

Animal models for food allergy

Several animal models have been used to provide insight into the complex immunologic mechanisms of human IgE-mediated (immediate-type) food allergy. Animal models are used to bypass naturally occurring tolerance to food proteins and induce sufficient allergen-specific IgE antibody production to cause sensitization and upon re-exposure to trigger an allergic response. Animals are also used to predict allergenicity of novel proteins and to test new immunotherapeutic approaches to treat food allergy. Any animal model aimed to simulate human allergic disease should meet the following criteria: 1) mimic the main pathological features of type I human allergic disease; 2) contain predisposed genetic background with high and low responders; 3) have innate or adaptive response characteristics; 4) permit oral sensitization; and 5) feature a variety of different well-known high, medium and weak food allergens as well as non-allergenic proteins (Helm, 2006). The following text highlights and discusses animal models that have been most widely used and that are of particular interest to scientists for various reasons discussed.

Rodent models

Several rodent (murine) food allergy models have been developed. Rodents like mice, rats, and guinea pigs are less expensive to obtain and keep under very specific environmental conditions compared to much bigger animals (e.g. pigs). There are a wide variety of immunological reagents commercially available that allow for mechanistic studies of food allergy response, and their genetic background has extensively been studied (McClain & Bannon, 2006). Different strains of mice have been investigated as suitable models for

human food allergy (Finkelman, 2007). Injected or aerosolized antigens results in IgE production in mice and mice have shown the ability to experience anaphylactic shocks.

Pig

Reports of naturally occurring allergic disease in the pig are rare and allergy in pigs is normally induced intentionally. A neonatal pig model of food allergy has been studied by a few groups (Helm et al., 2002; Helm, Ermel, & Frick, 2003; Schmied, Rupa, Garvie, & Wilkie, 2013). In this model, neonatal piglets are sensitized intraperitoneally (into the body cavity) with the antigen and cholera toxin (adjuvant to increase immune response) and then orally challenged repeatedly with the sensitizing allergen. IgE from pigs and its role in health and disease were reviewed by Rupa *et al*. (2009). Pigs, like humans, are big monogastric beings, with closely related gastrointestinal structure and function and can serve as a valuable translational model of food allergy. In particular, the gastrointestinal symptoms (functional and tissue morphology) experienced by pigs as a result of allergic reaction are very similar to those exhibited by humans. In addition, pigs can exhibit anaphylactic shock symptoms (Helm et al., 2003; McClain & Bannon, 2006). Pigs, as opposed to rodents, are able to vomit (Helm et al., 2002). While a certainly more expensive animal model with greater facilities requirements than rodents (i.e. availability and number of subjects possible, nursery space availability, and costs and labor involved), pigs are very useful for determining IgE-mediated immune mechanisms and conducting endoscopic histologic assessment of tissues (Helm et al., 2002).

Dog

In dogs, the occurrence of spontaneous allergy to foods is comparable to that in humans (10%) (Helm et al., 2003). Approximately 8% of dogs are affected by food allergy (Baker, 1974; Jeffers, Shanley, & Meyer, 1991; White, 1986). Clinical signs of food allergic reaction are very similar to human symptoms including vomiting, diarrhea and skin rashes (August, 1985; Baker, 1990; Halliwell, 1992; Jeffers et al., 1991; White, 1986, 1988). Several food allergies have been identified in dogs including reactions to milk, soybean, wheat, oats, beef, eggs, chicken, horsemeat, cornmeal, pork, yeast, and commercial foods with different protein ingredients (Helm et al., 2003). Ermel *et al*. (1997) established a dog model of food allergy in newborn pups. The animals were subcutaneously (under the skin) injected with cow's milk, beef, ragweed, and wheat extracts in alum and boosted several times over several weeks. Immunized pups responded with allergen-specific IgE and IgE titer could be maintained with injections of specific antigen in alum twice a month in addition to daily feeding of a maintenance diet containing minute amounts of the allergen. Oral challenge showed clinical manifestations of food allergy (e.g. nausea and vomiting, edema). Similarly, skin testing and oral food challenges with peanut, walnut, Brazil nut, wheat, cow's milk, soy, and barley in a dog model of food allergy showed identical allergic features to that in humans.

Table 2 shows a summary of possible methods that can be used to assess the immunogenicity of proteins.

Table 2. Methods to assess protein immunogenicity

Level	Method	Relevance/Comments
In silico	Computational predictions/bioinformatics methods	Fast method Computational prediction of allergenic proteins from sequence-derived protein structural and physicochemical properties (based on existing database/protein sequences) Can achieve good prediction accuracy, but less effective for novel proteins with no similarity to any known allergen
In vitro	Immunoblotting	Generally one of the first tests employed to assess protein immunogenicity/allergenicity Qualitative and semi-quantitative data can be obtained The availability of plasma/serum from well-characterized allergic patients with true food allergy symptoms (i.e., IgE-mediated anaphylaxis) is limited although there are commercial sources Non-functional immunochemical method that cannot measure the biological reactivity of proteins A 2 day process in most cases

Table 2 continued

Level	Method	Relevance/Comments
In vitro	ELISA	Mechanims similar to Western blotting Simpler, cheaper, quicker (1 day), and more quantifiable assay than immunoblotting Similar to immunoblotting it described binding potential of protein to antibodies and can provide no insight into the biological reactivity of a given protein/allergen
	Basophil activation	The ***histamine release assay*** was first developed, and requires great amounts of blood and has a low sensitivity and specificity A more recent adaption of this basophil activation test is based on flow cytometry, which uses *specific* antibodies to basophil markers and requires very little blood from the allergic patient Frequent blood donations required Basophil integrity declines rapidly over a period of 8 hours (robust validation of proposed protocol with controls needed)

Table 2 continued

Level	Method	Relevance/Comments
In vitro	Basophil activation	Both assays are complex in nature and depend on frequent blood donations Biological reactivity can be tested
	Humanized rat basophilic leukemia cell mediator release assay	No need for human donor blood Biological reactivity can be tested
	T cell proliferation assay	Biological reactivity can be tested Complex (a high number of T cells is needed)
	Protein-uptake by bone marrow derived dendritic cells	Biological reactivity can be tested The capture of the protein by the dendritic cell, protein internalization and processing (digestion) can then be evaluated
In vivo	Translational animal models	Validated animal models are the only method that can more completely evaluate the risk of a protein to be reactive/allergenic in humans (can closely mimic human digestion and allergic reactions)

Table 2 continued

Level	Method	Relevance/Comments
In vivo	Translational animal models	Human food allergy is very complex and includes multiple factors such as exposure and genetic predisposition, hence it is known that one animal model isn't able to fully mimic human food allergy Complex, time consuming, and expensive Can test for antigen specific IgE, total IgE, and skin prick test for antigen sensitization
Clinical	Human trials	Very complex, time consuming, and expensive Highest level of testing Can test for antigen specific IgE, total IgE, and skin prick test for antigen sensitization

Food allergy prevention and therapies

To date, there is no cure for food allergy and strict elimination of the allergenic food from the diet is the only proven preventative measure to avoid future allergic reactions. Despite that, 50% of affected patients accidentally ingest allergenic foods over a 2 year period (most of the "big eight" food allergens are present in many commercial food products or home cooked foods and cross-contamination is not uncommon) (Sicherer, Burks, & Sampson, 1998).

Approaches targeting host immune response

Immunotherapy (targeting the human immune response) for IgE-mediated food allergies has been under investigation for nearly a century (Noon & Cantab, 2005). Among the promising therapy approaches are peptide immunotherapy, traditional Chinese medicine, mutated protein immunotherapy, DNA immunization, immunization with immunostimulatory sequences, and anti-IgE therapy, some of which have been investigated for their potential *in vitro* and *in vivo* but not in humans (Gernez & Nowak-Węgrzyn, 2017). Current clinical research focuses on food allergen-specific immunotherapy through oral (OIT), sublingual (SLIT), or epicutaneous (EPIT) routes. Immunotherapy relies on the delivery of gradually increasing doses of specific allergens to induce desensitization (defined as temporary antigen hyporesponsiveness that depends on regular food ingestion) and, ultimately, tolerance (defined as the ability to ingest food without symptoms despite prolonged periods of avoidance or irregular intake). Although the majority of the patients treated with OIT achieve desensitization, only a minority achieves tolerance. OIT involves higher maintenance doses of food protein (300 mg-4g) compared with SLIT (2.5-7.5 mg) and EPIT (250-500 mcg). OIT efficacy is higher compared with SLIT, but OIT is associated with higher rate of

systemic adverse events compared with SLIT and EPIT. OIT is also associated with a minor risk of eosinophilic esophagitis. Combined treatment of OIT and anti-IgE monoclonal antibody has improved safety but not efficacy compared with OIT alone. Early initiation of peanut OIT in peanut-allergic infants and young children may afford superior efficacy and safety. In this review, we discuss the allergen-specific strategies currently explored for the treatment of food allergy, including immunotherapy with native and heat-modified food proteins. Additional research employs strategies to improve the safety and efficacy of allergen immunotherapy through modifications of allergen structure and addition of immunomodulatory adjuvants (Burks, Buchanan, & Pons, 2006; Gernez & Nowak-Węgrzyn, 2017). Therapies that have reached clinical trial stage are allergen-specific oral (OIT), sublingual (SLIT), and epicutaneous (EPIT) immunotherapy and will be further discussed.

Allergen-specific immunotherapy

Immunotherapy refers to the administration of gradually increasing amounts of specific allergens to allergic individuals to desensitize them (i.e. the ability to tolerate increased amounts of the allergen, for example, the amount that might be encountered in an accidental exposure) and build up tolerance against the food (the ability to ingest triggering food without allergic symptoms despite pronlonged periods of allergen avoidance or irregular intake). The focus of clinical research has been on immunotherapy through oral, sublingual, or epicutaneous routes. ***Oral immunotherapy*** utilizes the normal physiological pathways underlying oral tolerance induction to consumed food proteins (Figure 8). Generally, OIT protocols are set up in three phases starting with an initial dose escalation day (administration of several increasing doses of antigen in one day to identify the highest tolerated dose), which

is then followed by a gradual build-up of allergen dose every two weeks until a target dose is reached within 6-12 months. Once the target dose is reached individuals regularly consume one consistent dose of the allergenic food during a maintenance phase (300 mg - 4g) that can last for months or years. Desensitization is assessed then after a period of maintenance by a double blind placebo-controlled food challenge (DBPCFC) to the allergenic food. Tolerance, on the other hand, is assessed by discontinuing the daily maintenance allergen consumption for 4-12 weeks and subsequently re-introducing the triggering food during a DBPCFC. The state of no reaction after the oral challenge is defined as sustained unresponsiveness, which is also called permanent tolerance. Many studies with OIT established desensitization, but few studies have evaluated permanent tolerance. OIT studies, conducted, have focused on egg, milk and peanut allergy (Gernez & Nowak-Węgrzyn, 2017).

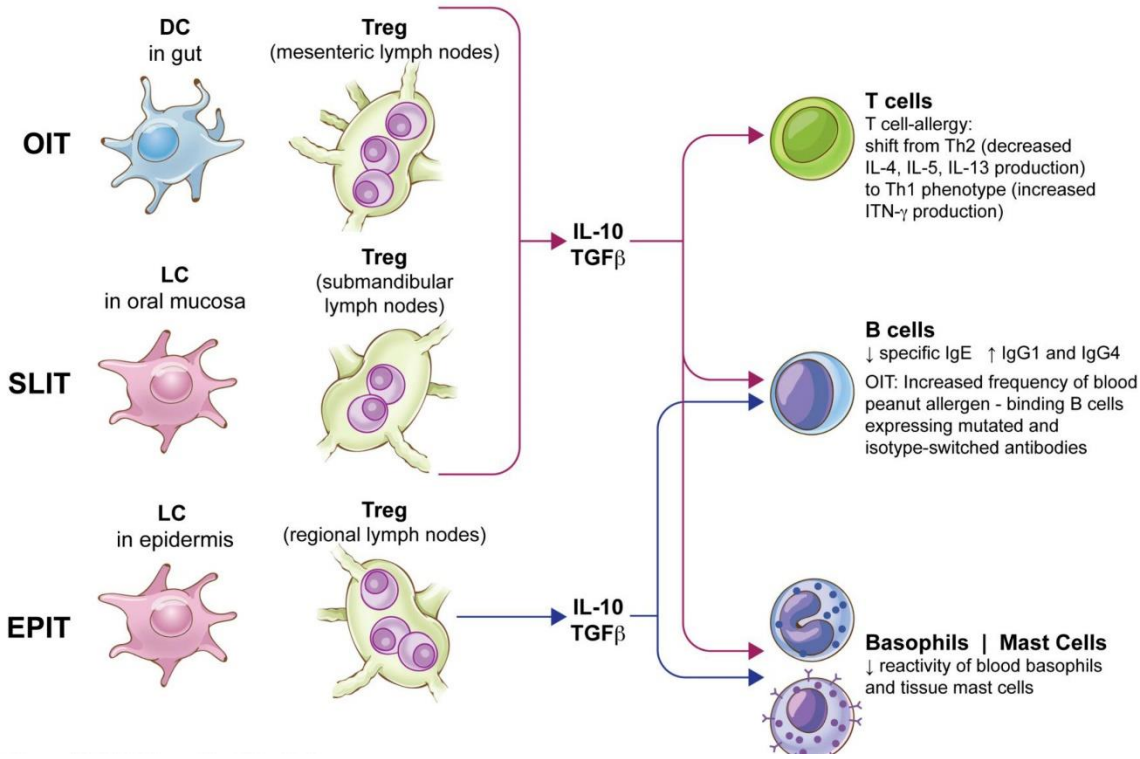

Figure 8. Suggested mechanisms of immunomodulation in different forms of food allergen-specific immunotherapy
Oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) enhance T regulatory cells (Tregs) via Langerhans cells (LC, a type of antigen presenting cell) in the gut and in the oral mucosa, respectively. The stimulation of Tregs decreases the production of allergy-associated cytokines by Th2 cells, decreases the production of IgE by B cells and induces antibody isotype-switching to IgA and IgG antibodies, and reduces the reactivity of basophils and mast cells. Epicutaneous immunotherapy (EPIT) has been shown to stimulate Tregs via Langerhans cells (LC), which decreases the production of food allergen-IgE antibodies and increases the production of IgG antibodies, and lowers the reactivity of basophils and mast cells (reprinted with permission from *Immunotherapy for food allergy: are we there yet?*, Gernez & Nowak-Węgrzyn, 2017, copyright 2017 American Academy of Allergy, Asthma & Immunology).

Sublingual immunotherapy very similarly works like OIT but significantly lower doses of allergen are used and this therapy approach is generally better tolerated than OIT (less side effects) (Gernez & Nowak-Węgrzyn, 2017). A small amount of the food allergen in liquid or solid form is administered sublingually and held for 2 min and then swallowed. Langerhans

cells (a form of dendritic cell residing in layers of the epidermis and in the epithelia of the respiratory, digestive and urogenital tracts) in the oral cavity tissues are activated which induces pathways that ultimately decrease the reactivity of effector cells (i.e. mast cells and basophils) and increase the emergence of T_{reg} cells (Gernez & Nowak-Węgrzyn, 2017). SLIT has been studied for hazelnut, peanut, milk, kiwi, and peach (Enrique et al., 2005; Fernández-Rivas et al., 2009; Keet et al., 2012; Kim et al., 2011; Mempel, Rakoski, Ring, & Ollert, 2003). SLIT efficacy is limited by allergen dose due to low concentration of available extracts and the volume of liquid that can be held sublingually. The maintenance dose for SLIT is between 2.5 – 7.5 mg protein (Gernez & Nowak-Węgrzyn, 2017).

The traditional ***subcutaneous immunotherapy*** involved the injection of small doses of allergen under the skin (epidermis was vascularized) and has shown to be useful in inhalant allergen sensitivity such as asthma but was unsafe in use for food allergens (systemic adverse reactions) (Nelson, Lahr, Rule, Bock, & Leung, 1997; Oppenheimer, Nelson, Bock, Christensen, & Leung, 1992; Till, Francis, Nouri-Aria, & Durham, 2004).

Now, ***epicutaneous immunotherapy*** provides a safer alternative (as adverse reactions are reduced by not injecting the allergen subcutaneously). EPIT takes advantage of a novel antigen delivery system using allergen-containing patches designed to activate skin Langerhans cells with subsequent systemic downregulation of the effector cell responses (Figure 8). Maintenance doses for EPIT are between 250 - 500 µg. Phase III multicenter international clinical trials of peanut and milk EPIT are currently ongoing (Gernez & Nowak-Węgrzyn, 2017).

Generally, OIT has been shown to have a higher efficacy compared with SLIT, however, OIT has been linked to a higher prevalence of systemic adverse reactions compared

with SLIT and EPIT. In addition, those immunotherapies have been evaluated in regards to the use of high and low allergen dosage and native and heat-modified food allergens. Ongoing research is being conducted to explore strategies to improve the safety and efficacy of allergen-specific immunotherapy (e.g. through alterations of allergen structure or the addition of immunomodulatory adjuvants). Interestingly, Australian researchers demonstrated that oral immunotherapy supplemented with a probiotic strain of *L. rhamnosus* led to sustained unresponsiveness to peanut in 82% of *allergic* children assessed 2-5 weeks after discontinuation of the immunotherapy treatment (probiotics used in allergy treatment not prevention) (Tang et al., 2015). None of the investigations to date investigated immunotherapeutic approaches have been shown to deliver a permanent cure of food allergy.

Nutritional intervention with plant polyphenols

Fruits, vegetables and other edible plant sources are rich in a variety of polyphenols which are secondary metabolites (usually not directly involved in plant growth and development) and generally produced in defense to various environmental stress factors (i.e. ultraviolet radiation, pathogens, and extreme temperatures) (Beckman, 2000). In food, polyphenols contribute to sensory properties including taste, color and flavor (Pandey & Rizvi, 2009). To date, more than 8,000 phenolic compounds have been identified and among them over 4,000 flavonoids. They commonly occur in conjugated forms, such as with monosaccharides (sugars), organic acids, lipids, or other phenols (Cheynier, 2005; Harborne & Williams, 2000; Kondratyuk & Pezzuto, 2004). Chemically, polyphenols are a group of compounds with phenolic structural features. Polyphenols can be categorized into different groups based on the number of phenolic rings and architecture of how those rings are linked with each

other. Main classes are phenolic acids, flavonoids, stilbenes, coumarins and tannins, which can be further divided into subclasses (Figure 9). The terms “polyphenols” and “phenolic compounds” are interchangeably used in this text.

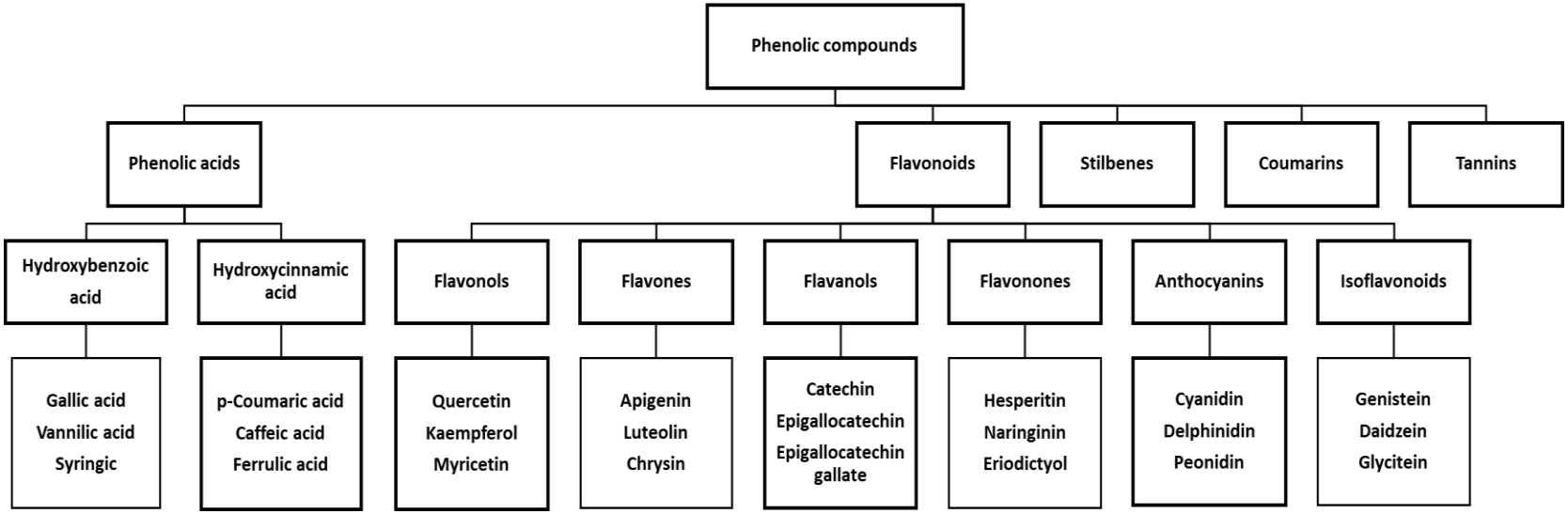

Figure 9. Classification of dietary phenolic compounds (reprinted and adapted with permission from *Potential synergy of phytochemicals in cancer prevention: mechanism of action,* Liu, 2004, copyright 2004 The American Society for Nutritional Sciences).

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Polyphenols are well known to provide health-promoting and disease preventative benefits against acute, chronic, and degenerative diseases mainly attributable to their antioxidant and anti-inflammatory properties (Calabrese et al., 2012; Nichenametla, Taruscio, Barney, & Exon, 2006; Rice-Evans, Miller, & Paganga, 1996). Polyphenols have also shown to have anti-allergic properties. Bellik *et al.* provides a comprehensive review of the anti-allergic mechanisms of polyphenols *in vitro* and their reported efficiency *in vivo* (2012). Polyphenols may interfere with the allergic response via mechanisms that include (a) inhibition of chemical mediator release (e.g. histamine) and cytokine production by mast, basophil or T cells and (b) inhibition of signal transduction and gene expression in mast, basophil or T cells.

Approaches targeting allergen immunoreactivity

Numerous processing and non-processing strategies to try to target protein allergenicity have been investigated, including 1) thermal and non-thermal food processing techniques (Verhoeckx et al., 2015), 2) enzymatic hydrolysis (Kasera, Singh, Lavasa, Prasad, & Arora, 2015), enzymatic protein cross-linking (Chung, Kato, & Champagne, 2005), fermentation, (Bu, Luo, Zhang, & Chen, 2010; El-Ghaish et al., 2011), 3) controlled Maillard modifications (Walter, 2014), and 4) genetic engineering/biotechnological methods (Ferreira et al., 2002). Since most of our food is processed prior to consumption, the following section will further discuss the effect of some food processing techniques on modulating the allergenic potential of food allergens.

Food processing

The types of food processing techniques that have been investigated in influencing protein allergenic properties include heat (thermal processing), enzymatic and acid hydrolysis, non-

thermal treatments (e.g. high pressure processing or pulsed electric field) (Galazka, Ledward, Dickinson, & Langley, 1995; Hinrichs & Rademacher, 2004; Johnson et al., 2010; Li, Chen, & Mo, 2007; Zhang, Cheng, Wang, & Guan, 2006), or combinations of these (Mills & Mackie, 2008; Thomas et al., 2007). Verhoeckx *et al.* (2015) provides a current review on the effect of different processing techniques on the allergenicity of some of the most prevalent food allergens namely, peanuts, tree nuts, cow's milk, hen's egg, soy, wheat, and mustard. The review revealed that most studies performed evaluate the capacity of processed food allergens to bind to IgE antibodies (i.e. is the processed allergen able to elicit an allergic response and to what extent?), while studies on the ability of processed food allergens to induce sensitization (i.e. is the processed allergen able to induce IgE antibody production and prime allergic pathways?) are scarce. Many investigations have shown that processing may influence but not eliminate the allergenic potential of proteins and in some cases allergenic potential is increased. For example, most commonly used food processing techniques involve one or multiple heat treatments (generally for food safety purposes) that can decrease protein allergenicity by destroying conformational epitopes but not linear epitopes (Figure 10). In addition, heating and other processing techniques, affecting native protein structure, can potentially alter the susceptibility of proteins to gastrointestinal digestion and their absorption through mucosa and thereby modulate their allergenic potential (Figure 11) (Rahaman, Vasiljevic, & Ramchandran, 2016). Heat has shown to reduce the allergenic potential in some allergenic foods including egg and milk. *In vitro* studies assessing IgE binding capacity of heated ovalbumin (major egg white protein) showed a reduction in the IgE binding capacity compared to unheated ovalbumin (Ma, Chen, Gao, Hu, & Li, 2013; Shin, Han, & Ahn, 2013). Studies with egg-allergic children have shown that the majority (50 – 85%) of

children could tolerate (no allergic reactions) extensively heated egg proteins but not dehydrated, freeze-dried, or raw egg proteins (Escudero et al., 2013; Urisu et al., 1997). The majority of milk-allergic children tolerate extensively heated milk (baked with wheat matrix) (Nowak-Wegrzyn et al., 2008). However, in peanut and shrimp, heat-induced Maillard reactions may increase allergenicity through creation of advanced glycation end products (AGEs) with increased allergenic potential (Nowak-Wegrzyn & Fiocchi, 2009). Several studies described that roasting of peanuts enhanced IgE binding capacity of peanut proteins *in vitro* (Beyer et al., 2001; Maleki, Chung, Champagne, & Raufman, 2000; Mondoulet et al., 2005). The high temperature during the roasting process increased AGE products, which could explain the observed enhanced IgE binding capacity (Chung & Champagne, 2001). Allergen interactions with other proteins, fat, and carbohydrates in the food matrix and their effects on allergenic potential are complex and poorly understood (Nowak-Wegrzyn & Fiocchi, 2009). ***Low pH*** is known to denature proteins and hence destroy conformational epitopes. Similarly, ***hydrolytic enzymes*** can hydrolyze peptide bonds within conformational epitopes and thereby decrease allergenicity.

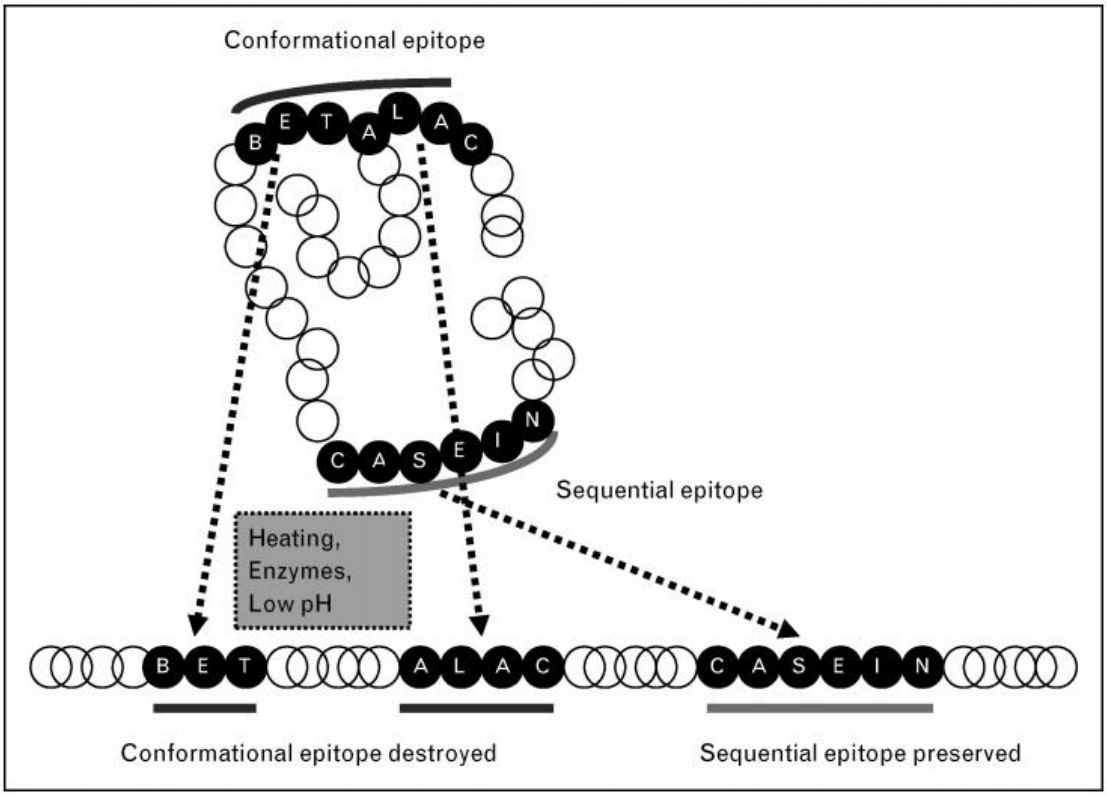

Figure 10. Schematic illustration of processing effects on conformational and sequential (linear) IgE binding epitopes

Processing can destroy conformational epitopes through the loss of protein tertiary structure but generally do not affect linear epitopes. ALAC, a-lactalbumin; BETALAC, b-lactoglobulin *(*reprinted with permission from *Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity*, Nowak-Wegrzyn & Fiocchi, 2009, copyright 2009 Wolters Kluwer Health, Inc).

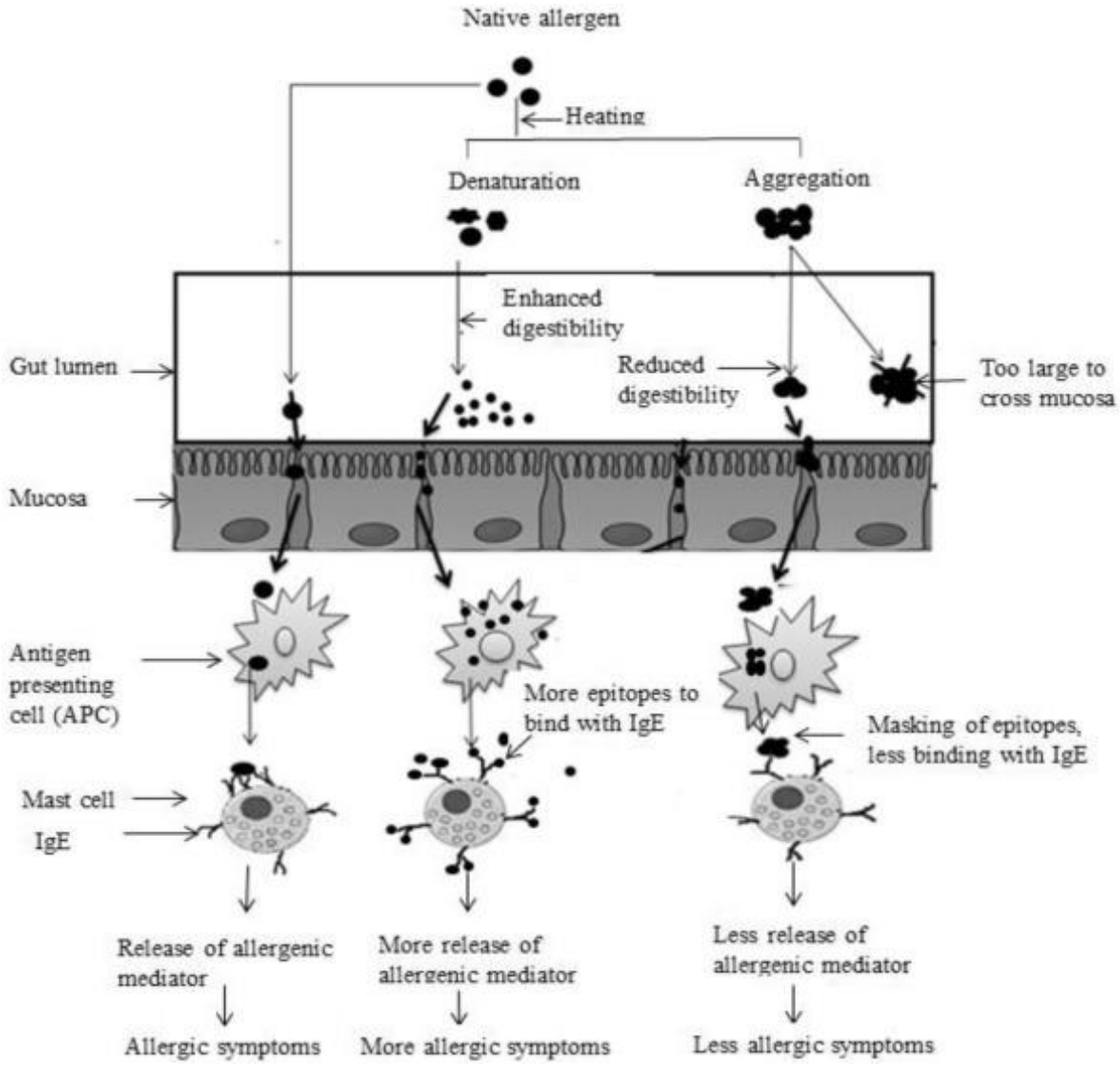

Figure 11. Processing-induced conformational changes and their consequences in digestion, absorption and proposed immune reactivity of food allergens (reprinted with permission from *Effect of processing on conformational changes of food proteins related to allergenicity*, Rahaman et al., 2016, copyright 2016 Elsevier Ltd).

Protein-polyphenol binding

Polyphenols have a natural affinity to bind to proteins. For example, polyphenols are responsible for the astringency of foods like tea or cranberries as they interact with basic salivary proline-rich proteins (Charlton et al., 2002). Similarly, tannins (a class of phenolic compounds which includes proanthocyanidins and ellagitannins) binding to proteins is the basis for leather making.

Polyphenols can bind reversibly via non-covalent forces (i.e. hydrogen bonds, van-der-Waals forces, charge-charge interactions and hydrophobic interactions) (Charlton et al., 2002) or irreversibly via covalent bonds (Haslam, 1996). The strength of non-covalent protein-polyphenol interactions depends on the size of polyphenols, polyphenol structure and amino-acid sequence of proteins (Frazier et al., 2010).

Phenolic compounds can readily oxidize either enzymatically or non-enzymatically (Cilliers & Singleton, 1991; Friedman, 1997). Enzymes responsible for the oxidation of polyphenols in damaged plant tissue, resulting in polymerization into brown pigments (e.g. browning of cut apple slices), can be inactivated or removed during food processing. Non-enzymatic oxidation, however, may still occur. Factors such as the presence of oxygen, elevated pH, and high temperatures can promote polyphenol oxidation (Hurrell & Finot, 1984; Kalt, 2005).

Oxidized polyphenols, or quinones, are highly reactive electrophilic intermediates (electron acceptors) that can react with nucleophiles (electron donors) such as certain amino acid functional groups. After an initial oxidation of a phenolic compound and the subsequent addition to a protein side chain, a second oxidation and binding to another protein chain is

possible. This results in protein-cross linked material with high molecular weight, a reaction essential to leather tanning (Kroll, Rawel, & Rohn, 2003; Rohn, 2014).

It is not unlikely that covalent bonds and non-covalent interactions take place simultaneously as shown for the binding of chlorogenic acid to protein (Prigent et al., 2003; Prigent, Voragen, Visser, van Koningsveld, & Gruppen, 2007). Further, polyphenols can form soluble (dispersible) and insoluble (precipitate) complexes with proteins depending on the extent of protein-complexation with phenolic compounds (i.e. concentration of protein vs. phenolic compound) (Figure 12) (Charlton et al., 2002).

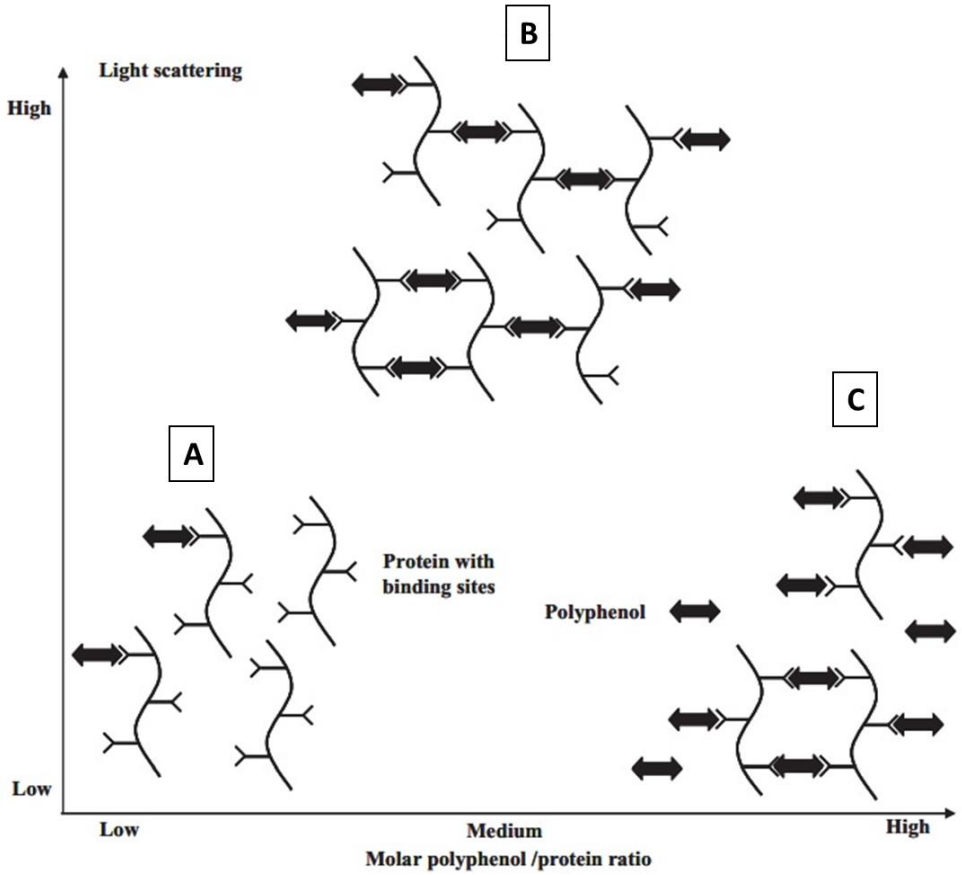

Figure 12. Protein-polyphenol binding model
A) At a low protein to polyphenol ratio it is thought that polyphenols interact with proteins through hydrophobic interactions and soluble complexes with low light scattering potential are formed. B) With an increase of polyphenol to protein ratio, polyphenols can act as "bridges" between two or more protein molecules which initially can stay dispersed (high light scattering) but can, if further complexed (at high levels of polyphenols) C) precipitate out of solution (phase separation) as particles of various sizes (reprinted with permission from *Interactions between polyphenols and macromolecules: quantification methods and mechanisms,* Le Bourvellec & Renard, 2012, copyright 2012 Taylor & Francis).

Furthermore, protein-polyphenol interactions are known to affect protein structure and digestibility, overall antioxidant capacity, and the bioavailability of both phenolic compounds and proteins (Bravo, 1998; Gonçalves, Mateus, & de Freitas, 2011; Grussu, Stewart, & McDougall, 2011). It has been shown that quinone–amino group reactions (covalent connections between proteins and polyphenols) decrease the digestibility and bioavailability

of protein-bound lysine and cysteine (if cysteine is located at *N*-terminus or with the sulfhydryl group of cysteine) (Damodaran, 1996).

Many studies have investigated protein structural changes upon polyphenol binding (Bourassa, Kanakis, Tarantilis, Pollissiou, & Tajmir-Riahi, 2010; Kanakis et al., 2011; Ognjenović et al., 2014; Vesic et al., 2015) and several studies have focused on the extent of (mostly *in vitro*) proteolytic polyphenol-bound protein breakdown by gastrointestinal enzymes (Bandyopadhyay, Ghosh, & Ghosh, 2012; Kroll et al., 2003; Kumar & Singh, 1984; Stojadinovic et al., 2013).

Polyphenol binding to proteins offers a novel approach to reducing the allergenicity of food proteins or food products containing allergens. One way is by making the allergenic proteins more susceptible to enzymatic hydrolysis into peptides that either cannot or have a lower ability to trigger an allergic response. For example, a study by Tantoush *et al.* (2012) showed that a catechin-enriched green tea extract facilitated the pepsin digestion of various food allergens *in vitro*. Polyphenols promoted pepsin activity resulting in a more rapid digestion of peanut allergens Ara h 1 and Ara h 2. One plausible mechanism is a direct effect of *free polyphenols* on pepsin activity, as proposed previously (Tagliazucchi, Verzelloni, & Conte, 2005). However, allergenicity of the resulting digesta was not evaluated. Similarly, we have shown that the *complexation* of peanut allergen Ara h 3 (basic subunit) in peanut flour with cranberry or green tea polyphenols resulted in a more rapid digestion of the allergen, and this was also true for Ara h 2 complexed with cranberry polyphenols (Plundrich et al., 2015). Overall, peptides from both complexes were substantially less allergenic (based on their capacity to bind to peanut-specific IgE from patient plasma) compared to peptides from uncomplexed peanut flour. Free polyphenols are able to bind to enzymes (proteins),

potentially changing their conformation and activity and rendering them either more active or impaired (He, Lv, & Yao, 2007; Tagliazucchi et al., 2005).

The mechanism of protein precipitation by phenolic compounds has also been taken advantage of, in order to remove allergenic food proteins from food products. A study by Chung & Champagne (2009) showed that the addition of phenolic compounds in peanut extract and liquid peanut butter lead to the irreversible precipitation of peanut allergens which rendered the remaining soluble material less allergenic (determined by IgE binding capacity in an ELISA test). They specifically showed that the majority of Ara h 1 and Ara h 2 allergens were irreversibly bound and precipitated by phenolic compounds. Chung & Reed (2012) further investigated the ability of tannic acid to precipitate peanut allergens from a peanut extract and considered the potential of peanut protein-tannic acid complexes to be disrupted by acidic gastric (stomach) or basic intestinal pH, pH 2 and 8, respectively. They found that tannic acid at the concentration used forms insoluble complexes with peanut allergens and the complexes, at pH 2 and 8, did not release major peanut allergens. IgE binding of the remaining soluble extract was significantly reduced (up to 100%; $p<0.05$) as was determined by ELISA and Western blotting. These approaches show that peanut allergens can be removed from food, based on forming a protein-polyphenol particle that is of sufficient size and density to be removed from the system.

The binding of phenolic compounds to allergenic proteins also has the potential to modulate or mask conformational and linear IgE binding epitopes. We recently described a novel approach to bind polyphenolic plant compounds to peanut protein that created protein-

polyphenol aggregate particles with reduced allergenicity as shown by complementary *in vitro*, *ex vivo* and *in vivo* experiments (Plundrich et al., 2014, 2015). IgE binding epitopes were masked or structurally modified by the complexation process that takes advantage of the natural affinity between proteins and medium polarity polyphenol molecules. These studies included commercially available fruit juice concentrates as sources of polyphenolic compounds. It was shown that particularly procyanidins (in the form of a purified fraction from cranberry) -compared to an anthocyanin-rich fraction from cranberry - were able to bind to peanut proteins and decrease IgE binding using allergic plasma from peanut-allergic individuals.

Conclusion and hypotheses for further investigation

Our previous studies demonstrated that the stable binding of plant compounds (polyphenols) from fruit and herbal extracts to milled roasted peanut flour (the active ingredient used in clinical peanut oral immunotherapy) lead to the formation of protein-polyphenol aggregate particles (peanut flour enriched with plant polyphenols) with reduced allergenic potential *in vitro* and *in vivo* (Plundrich et al., 2014). Proanthocyanidin-rich sources generally provided the most efficient reduction in allergenicity. Further investigations revealed that, when digested with pepsin, some peanut allergens were hydrolyzed more rapidly in protein-polyphenol aggregate particles compared to uncomplexed peanut flour. Moreover, digestive fragments from protein-polyphenol aggregate particles were considerably less immunoreactive compared to fragments from uncomplexed peanut flour based on their capacity to bind to peanut-specific IgE from a pool of peanut-sensitive patient plasma and peanut-specific polyclonal IgG *in vitro* (Plundrich et al., 2015). These results may partially explain the

attenuated allergenicity of protein-polyphenol aggregate particles observed in previous experiments. Solubility behavior of peanut protein-polyphenol aggregate particles in simulated gastric fluid (pH 2) used to mimic acidic harsh conditions *in vivo* showed that polyphenols from some fruit and herbal sources rendered peanut proteins significantly less soluble (more stable complexes). Further, results suggested that interactions between polyphenols and proteins are non-covalent in nature (hydrophobic interactions and hydrogen bonding).

In this work, we aimed to better understand the *molecular mechanisms* by which peanut protein-polyphenol aggregate particles are rendered less allergenic. We hypothesized that a) an increase in concentration of polyphenols complexed with peanut proteins, corresponds with a decrease in allergenic potential of the protein-polyphenol aggregate particles, b) certain classes of polyphenols non-covalently or covalently bind to peanut proteins responsible for the observed decreased allergenic potential of the particles, and c) certain types of polyphenols bind to peanut allergens and, more particularly, interact with amino acids that are part of IgE binding epitopes, hence preventing IgE binding epitope recognition by IgE antibodies.

Peanut protein-polyphenol aggregate particles were created containing 5, 10, 15, 30, and 40% (w/w) polyphenols and a) screened for their peanut-specific IgE binding potential by *in vitro* immunoblotting with peanut-allergic plasma, and b) tested for their inhibitory activity on ionomycin-induced degranulation in a RBL-2H3 mast cell allergen model system. Free and non-covalently protein-bound polyphenols were extracted from the aggregate particles using different extraction solvents, while covalently bound polyphenols were

extracted by alkaline hydrolysis. Polyphenols were subsequently identified and quantified by the Folin C assay and HPLC.

Further, different polyphenols were screened, using *in silico* molecular modeling techniques, to prioritize the ones predicted to have the strongest binding to Ara h 2 in general, and to specific IgE binding epitopes on the Ara h 2 allergen. All polyphenols were evaluated using spectroscopic and immunological methods (UV-Vis spectroscopy, circular dichroism, and immunoblotting, respectively) for their a) Ara h 2 binding potential, their b) capacity to induce protein secondary structural changes, and c) capacity to inhibit IgE binding epitopes.

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CHAPTER 2

Protein-bound *Vaccinium* fruit polyphenols decrease IgE binding to peanut allergens and RBL-2H3 mast cell degranulation in vitro

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Protein-bound *Vaccinium* fruit polyphenols decrease IgE binding to peanut allergens and RBL-2H3 mast cell degranulation *in vitro*†

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Peanut allergy is a worldwide health concern. In this study, the natural binding properties of plant-derived polyphenols to proteins was leveraged to produce stable protein–polyphenol complexes comprised of peanut proteins and cranberry (*Vaccinium macrocarpon* Ait.) or lowbush blueberry (*Vaccinium angustifolium* Ait.) pomace polyphenols. Protein-bound and free polyphenols were characterized and quantified by multistep extraction of polyphenols from protein–polyphenol complexes. Immunoblotting was performed with peanut-allergic plasma to determine peanut protein-specific IgE binding to unmodified peanut protein, or to peanut protein–polyphenol complexes. In an allergen model system, RBL-2H3 mast cells were exposed to peanut protein–polyphenol complexes and evaluated for their inhibitory activity on ionomycin-induced degranulation (β-hexosaminidase and histamine). Among the evaluated polyphenolic compounds from protein–polyphenol complex eluates, quercetin, – in aglycone or glycosidic form – was the main phytochemical identified to be covalently bound to peanut proteins. Peanut protein-bound cranberry and blueberry polyphenols significantly decreased IgE binding to peanut proteins at $p < 0.05$ (38% and 31% decrease, respectively). Sensitized RBL-2H3 cells challenged with antigen and ionomycin in the presence of protein–cranberry and blueberry polyphenol complexes showed a significant ($p < 0.05$) reduction in histamine and β-hexosaminidase release (histamine: 65.5% and 65.8% decrease; β-hexosaminidase: 60.7% and 45.4% decrease, respectively). The modification of peanut proteins with cranberry or blueberry polyphenols led to the formation of peanut protein–polyphenol complexes with significantly reduced allergenic potential. Future trials are warranted to investigate the immunomodulatory mechanisms of these protein–polyphenol complexes and the role of quercetin in their hypoallergenic potential.

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1. Introduction

Allergic reactions to peanuts, estimated to affect 1% of the American population, involve an immunoglobulin E (IgE)-mediated immunological type I disorder (immediate hypersensitivity) to various proteins (Ara h 1–17) within the edible seed.[1] A primary mechanism of allergenic food proteins is IgE binding to protein segments (epitopes) coupled with cross-linking on mast cell and basophil surfaces, ultimately resulting in downstream cascades responsible for the allergic reaction. There is no cure for peanut allergy, and the most effective management is the strict avoidance of the allergenic food. However, a lot of effort has been put forward to alleviate peanut allergy, either through attempted modulation of the human immune system (*e.g.* oral immunotherapy) or the allergenic food proteins (by processing techniques or genetic modification). Since most food allergies prevalent in North America are mediated by IgE, therapeutic intervention in allergic disease has commonly focused on suppressing IgE production and/or allergen cross-linking with high affinity IgE receptors (FcεRI) located on mast cell and basophil surfaces.

We recently described a novel, food grade, green chemistry approach to attenuate allergenicity of proteins.[2,3] The strategy takes advantage of the natural binding properties of plant-derived polyphenols to proteins. Binding of polyphenols from

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various plant and fruit extracts to peanut proteins in the form of milled roasted peanut flour resulted in the formation of protein–polyphenol complexes (dry phytochemical-enriched peanut matrices) with attenuated allergenic potential. Polyphenols are known for their many health promoting and disease preventative properties, such as innate anti-inflammatory and anti-allergic activities.[4,5] Polyphenols *alone* may interfere with the allergic response *via* mechanisms which include (a) inhibition of chemical mediator release (*e.g.* histamine, hexosaminidase or leukotrienes) and cytokine production by mast, basophil or T cells and (b) inhibition of signal transduction and gene expression in mast, basophil or T cells.[6] Polyphenols *bound to peanut proteins* may further interfere with allergenicity by masking and/or changing the conformation of allergenic epitopes.[2]

Both cranberry (*Vaccinium macrocarpon* Ait.) and lowbush blueberry (*Vaccinium angustifolium* Ait.) are rich sources of procyanidins, flavonols (mostly in glycosidic form) and anthocyanins; all polyphenols capable of binding to proteins. Polyphenols are able to bind to proteins non-covalently (reversible) or covalently (irreversible).[7,8] Non-covalent binding can involve hydrophobic, van der Waals, hydrogen and ionic forces.[9] For example, quercetin (a flavonol) has been shown to bind to soy glycinin covalently,[10] (−) epigallocatechin to bovine β-lactoglobulin non-covalently,[11] and pelargonidin to milk proteins non-covalently.[12] We hypothesized that different levels of polyphenols complexed to peanut proteins would result in modulation of their allergenic potential (*in vitro* IgE binding capacity) in a dose-dependent manner, and that certain polyphenolic compounds would be more tightly bound to proteins than others responsible for the blocking of IgE binding. We also hypothesized that the protein–polyphenol complexes would be able to attenuate degranulation of mast cells in a well-known model *in vitro*.

2. Materials and methods

2.1. Materials and reagents

Precast mini TGX 4–20% polyacrylamide gels, 10× tris/glycine/SDS running buffer, Precision Plus protein pre-stained standard, and 2× Laemmli sample buffer were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Chameleon duo pre-stained protein ladder, Odyssey PBS blocking buffer, 4× protein sample loading buffer, REVERT total protein stain, and REVERT total protein wash solution were purchased from LI-COR (Lincoln, NE, USA). Goat anti-human IgE DyLight 800 conjugated secondary antibody was obtained from Agrisera (Vännäs, Sweden). Polyvinylidene fluoride (PVDF) membranes and ionomycin were purchased from EMD Millipore (Billerica, MA, USA). Celite™ 545 Filter Aid, Tween20 and 10× PBS were purchased from Fisher Scientific (Hampton, NH, USA). Eagle's minimum essential medium (EMEM) and fetal bovine serum (FBS) were purchased from ATCC (Manassas, VA, USA) and DNP-BSA was obtained from Life Technologies (Grand Island, NY, USA). Anti-DNP IgE, nitroblue tetrazolium and Folin–Ciocalteu reagent were purchased from Sigma-Aldrich (St Louis, MO, USA). Reference standards with different degrees of polymerization (DP), namely epicatechin (DP1), procyanidin (PAC) dimer A2 (DP2) and PAC trimer C1 (DP3; epicatechin-(4β-8)-epicatechin-(4β-8)-epicatechin) were obtained from Chromadex (Irvine, CA, USA). PAC tetramer D (DP4; epicatechin-(4β-8′)-epicatechin-(4β′-8″)-epicatechin-(4β″-8‴)-epicatechin) was purchased from Planta Analytica (Danbury, CT, USA).

2.2. Protein–polyphenol complex formation

Cranberry (*Vaccinium macrocarpon* Ait.) pomace was provided by Ocean Spray (Lakeville-Middleboro, MA, USA) and lowbush blueberry (*Vaccinium angustifolium* Ait.) pomace was donated by Jasper Wyman & Son (Cherryfield, ME, USA). Light roasted 12% fat peanut (PN) flour (*Arachis hypogaea* L.) containing 50% ± 2% protein was obtained from Golden Peanut Co. LLC (Alpharetta, GA, USA). The pomaces were freeze-dried to remove residual moisture and were extracted and stored until further use as previously described.[2] Ethanolic extraction was performed for 2 h at 80 °C. The amount of extract (mL) and protein powder (g) required to generate dry, stable protein–polyphenol aggregate particles containing 5, 10, 15, 30, or 40% polyphenols (w/w) after complexation were added together and mixed under constant agitation for 15 min at room temperature. Mixtures were subsequently frozen and freeze-dried to form stable protein–polyphenol complexes.

2.3. HPLC-MS-IT-TOF profiling

LC-MS analysis of plant extracts prior to complexation with food proteins was performed according to Grace *et al.*[13] A Shimadzu LC-MS-IT-TOF instrument containing a Prominence HPLC system was used. The separation of polyphenolic compounds in the HPLC was accomplished using a Shim-pack XR-ODS column (50 mm × 3.0 mm × 2.2 μm) supplied with a constant column temperature of 40 °C. Extracts were filtered through 0.45 μm cellulose syringe filters (Fisher Scientific, Pittsburgh, PA, USA) and diluted in 100% methanol if needed prior to injection of 20 μL onto the column. The binary solvent system contained 0.1% formic acid in water (A) and methanol (B). Compounds were eluted into the ion source (electron spray ionization, ESI) at a flow rate of 0.35 mL min^{-1} with a step gradient of 5–60% B over 40 min, isocratic at 60% B over 2 min, and returned to 5% B over 2 min. Ionization was performed in the negative and positive mode but compounds of interest were identified in the negative mode. MS, MS/MS spectra, HPLC retention times and the literature were used to identify compounds. Polyphenolic compounds that were eluted from the protein–polyphenol complexes (eluates) were also identified by LC-MS as described above.

2.4. Total phenolics, procyanidins, flavonols and phenolic acids in extracts and eluates

The total phenolic, individual procyanidin, and select flavonols and phenolic acids content in fruit extracts (prior to complexation with proteins) and eluates (compounds extracted

from protein–polyphenol complexes) was determined. The concentration of total phenolic compounds was determined according to the 96-well microplate-adapted Folin–Ciocalteu method by Zhang *et al.*[14] with modifications described by Herald *et al.*[15] Results were expressed as mg mL^{-1} gallic acid equivalents (GAE) based on a gallic acid standard curve.

Procyanidins were determined according to the method of Wallace and Giusti[16] (which was adopted from Brownmiller[17]) by means of normal phase HPLC-fluorescence analysis. An Agilent 1200 HPLC system with a fluorescence detector (FLD) and photodiode array detector (DAD) was used (Agilent Technologies, Englewood, CO, USA). A Develosil Diol column (250 mm × 4.6 mm × 5 μm; Phenomenex, Torrance, CA, USA) was used and the mobile phase consisted of (A) acetonitrile : acetic acid (98 : 2, v/v) and (B) methanol : water : acetic acid (95 : 3 : 2, v/v/v). Compound separation was accomplished by means of a linear gradient with a flow rate of 0.8 mL min^{-1}: 0–35 min, 0–40% B; 35–40 min, 40–100% B; isocratic 100% B, 45 min; 100–0% B, 50 min; and 0% B to 55 min. The separation of procyanidins was surveilled by fluorescence detection (excitation at 230 nm and emission at 321 nm) as well as with the DAD (280 nm). Samples were filtered through 0.45 μm cellulose filters prior to injection of 10 or 20 μL onto the column. Peak areas and external calibration curves (monomer (DP1) through tetramer (DP4) commercial standards) were used to identify procyanidins. PAC with DP > 4 including polymers were expressed as DP4.

HPLC for phenolic acids and flavonols was performed according to Grace *et al.*[13] using an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA, USA). A Phenomenex Synergi 4 μm Hydro-RP 80A column (250 mm × 4.6 mm × 5 μm; Torrance, CA, USA) had a mobile phase containing 2% acetic acid in distilled water (v/v; solvent A) and 0.5% acetic acid in 50% acetonitrile in water (v/v; solvent B). A flow rate of 1 mL min^{-1} with a step gradient of 10, 15, 25, 55, 100, and 10% of solvent B at 0, 13, 20, 50, 54, and 60 min, respectively was applied. Compounds were quantified using peak areas recorded at 280 nm and by means of calibration curves obtained with reference standards.

2.5. Characterization of free and peanut protein-bound polyphenols

In order to characterize and quantify free and protein-bound polyphenols, 250 mg of protein–polyphenol complexes containing 40% cranberry or blueberry polyphenols, or 150 mg of unmodified PN flour (control; the amount of PN flour comparable to the amount in 250 mg of the 40% protein–polyphenol complex) were extracted and the eluates analyzed by the following procedure: first, 10 mL of 80% aqueous methanol were added to either the complexes or PN flour in a conical tube and gently inverted for 1 min. The dispersions were then centrifuged for 1 min at 3434*g* and the supernatant collected in a separate tube. These eluates were considered as the fractions of free (not bound by PN proteins) polyphenols present in the complex or PN flour (designated as A). Next, the remaining pellet was extracted three times each with 80% aqueous methanol containing 1% acetic acid (v/v) for five minutes by placing the conical tubes on an orbital shaker. The dispersions were centrifuged in between each extraction step for 10 min at 3434*g* and the supernatant collected. These eluates were considered as the fractions containing polyphenols that were non-covalently bound to PN proteins (designated as B). Lastly, 1 mL of 4 M NaOH was added to the remaining pellet, flushed with nitrogen (to prevent oxidation), and incubated for 1 h in a heated sonicator (~50 °C) similar to a method described by Collins *et al.*[18] which was adapted by Yang *et al.*[19] Subsequently after incubation, the pH of each sample was adjusted to ~pH 7 with HCl. Each sample was then added to a column packed with Celite (diatomaceous earth, a filter aid) and eluted with a total of 200 mL of 80% ethyl acetate in methanol. The collected eluates were categorized as fractions containing phenolic compounds that were covalently bound to PN proteins (designated as C). Methanol was removed from each collected eluate by evaporation (Rotavap system, Büchi, Switzerland) and eluates were then freeze-dried. Dried samples were then reconstituted with 50% aqueous methanol and analyzed by the Folin Ciocalteu assay for total phenolics, or by HPLC for procyanidins or phenolic acids and flavonols as described above. Data obtained from extracting PN flour alone (control) was subtracted from data obtained for complexes to eliminate the polyphenols eluted from the PN flour from consideration. Each full extraction procedure (A, B and C) for each protein–polyphenol complex and unmodified PN flour was performed three times.

2.6. Protein distribution and semi-quantitative immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to observe protein distribution in PN protein–polyphenol complexes and unmodified PN flour. The amount of PN protein–polyphenol complex or unmodified PN flour required to provide equivalent protein content (1 mg) was used. One milliliter dispersions in Laemmli SDS sample loading buffer (2×) were prepared and mixed thoroughly. Five microliter of each dispersion was subsequently added to 5 μL Laemmli SDS sample loading buffer (2×) containing 5% β-mercaptoethanol, resulting in 5 μg protein in 10 μL. Samples were mixed and incubated for 5 min at 95 °C and subsequently loaded onto a gel. The gel was run for 40 min at 200 V, stained with Coomassie Brilliant Blue (CBB) G-250 and de-stained overnight in deionized water.

For the detection of PN-specific IgE binding by PN proteins, semi-quantitative fluorescence Western blotting was performed with pooled plasma from seven PN-allergic individuals obtained from PlasmaLab International (Everett, WA, USA). PN-specific IgE levels ranged from 53 to >100 kU L^{-1} as determined *via* ImmunoCAP (Phadia, Uppsula, Sweden). Preparation of protein samples was as described above except a sample loading buffer lacking bromophenol blue was used (because this reagent interferes with fluorescence detection). SDS-PAGE resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using an iBlot blotting

system (ThermoFisher Scientific, MA, USA). The membrane was allowed to fully dry, was rehydrated in 100% methanol, and briefly washed with deionized water and 1× PBS. Normalization of protein load was performed by staining the proteins with REVERT total protein stain and capturing an image in the 700 nm channel of an Odyssey Classic (CLx) infrared imaging system (LI-COR, Lincoln, NE, USA). In order to account for possible autofluorescence of polyphenols at 700 nm, an image was taken of the rehydrated membrane prior to staining. The membrane was then blocked with PBS blocking buffer (containing non-mammalian proteins) for 1 h at room temperature. The membrane was subsequently incubated overnight with a 1 : 80 dilution (v/v) of pooled human plasma (primary antibody) in PBS blocking buffer containing 0.2% Tween20 (1× PBS-0.2%T). The following day, the primary antibody was poured off and the membrane was washed in 1× PBS containing 0.1% Tween20 (1× PBS-0.1%T) four times for 5 min. A goat anti-human IgE (epsilon chain) DyLight 800 conjugated secondary antibody was prepared in PBS blocking buffer containing 0.2% Tween20 and 0.01% SDS (1 : 4000; v/v), added to the membrane, and incubated for 1 h at room temperature in the dark. The membrane was then washed four times for 5 min with 1× PBS-0.1%T and briefly rinsed three times with 1× PBS to remove any residual Tween20. The membrane was visualized using an Odyssey CLx infrared imaging system. Semi-quantitative analysis was performed using Image Studio Lite software version 5.2.

2.7. Nitroblue tetrazolium (NBT) staining to reveal polyphenols

Following the transfer of separated proteins from unmodified PN flour and PN protein–polyphenol complexes (SDS-PAGE) to a PVDF membrane, the membrane was briefly hydrated in 100% methanol and PN protein–polyphenol complexes were detected with NBT and glycinate as described by Hagerman.[20] At alkaline pH, the catechol moiety of polyphenols catalyzes redox-cycling in the presence of glycinate, generating superoxide that reduces NBT to insoluble, visible formazan.[21]

2.8. Modulation of RBL-2H3 mast cell degranulation by peanut protein–polyphenol complexes

RBL-2H3 cells (ATCC # CRL-2256; ATCC, Manassas, VA, USA) were plated at 2.0×10^5 cell per well in 24-well plates in Eagle's minimum essential medium (EMEM) containing 15% fetal bovine serum (FBS) at 37 °C in 5% CO_2. Following 24 h after seeding, the cells were sensitized overnight with 1 μg mL^{-1} of anti-DNP IgE. The cells were then washed three times in Tyrode's buffer (pH 7.4) to remove excess IgE and challenged with 1 μg mL^{-1} DNP-BSA (bovine serum albumin conjugated with multiple dinitrophenyl molecules) and 10 μM ionomycin in Tyrode's buffer containing 1% BSA for 3 h along with PN flour (control) or PN protein–polyphenol complexes. Control with ionomycin treatment alone was also included. The exposure concentrations of complexes were normalized to 100 μg mL^{-1} of PN flour. Following DNP-BSA challenge, the supernatant in each well was collected and stored at −20 °C for subsequent histamine and β-hexosaminidase analyses. Data obtained for PN protein–polyphenol complexes was compared with data for ionomycin treatment only (control). The blank sample received no ionomycin but PBS 1× only and was set as value 1. All data was a fold difference of 1.

2.9. Histamine assay

Twenty μL of 1 mol L^{-1} NaOH was added to a 96-well fluorescent plate followed by 100 μL of supernatant collected from the above experiment. Then, 25 μL of *O*-phthalaldehyde (1% w/v in 99.9% HPLC grade methanol) was immediately added and incubated at room temperature for 4 min with constant agitation. The reaction was stopped by adding 20 μL of 3 mol L^{-1} HCl. Fluorescence was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm using a BioTek Synergy 2 multiplate reader (Winooski, VT, USA). The experiment was repeated six times.

2.10. Beta-hexosaminidase assay

Fifty μL of supernatant collected from the above experiment (section 2.8.) was plated into a 96-well plate to which 50 μL of *p*-nitrophenyl *N*-acteyl-β-glucosamide in citrate buffer (1.3 mg mL^{-1}; pH 4.5) was added and incubated for 1 h at 37 °C. The reaction was stopped by adding 150 μL of 0.05 mol L^{-1} sodium carbonate solution. The plate was read at 405 nm using a BioTek Synergy 2 multiplate reader (Winooski, VT, USA). The experiment was repeated six times.

2.11. Statistical analysis

All experiments were replicated three 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.

3. Results

3.1. Phytochemical analysis of pomace extracts

Analysis by LC-MS of the aqueous pomace extracts identified some major phenolic constituents as presented in Table 1. Some of these were subsequently quantified by HPLC using pure standard compounds (Table 2). The total phenolic content was also determined. Overall, blueberry pomace extract had a higher total phenolic content than cranberry pomace extract (4.80 mg mL^{-1} and 3.20 mg mL^{-1}, respectively) (Table 2). However, the cranberry pomace extract was richer in procyanidins, 1.50 mg mL^{-1} compared to 0.80 mg mL^{-1} in the blueberry pomace extract. Both extracts contained particularly high levels of polymerized procyanidins (oligomers and polymers). Cranberry pomace extract also contained about twice the levels of phenolic acids and flavonols (0.60 mg mL^{-1}) than blueberry pomace extract (0.31 mg mL^{-1}), which is attributable in part to higher quercetin levels in cranberry (0.48 mg mL^{-1}).

Table 1 Peak identification, retention times and mass spectral data of procyanidins, flavonols, and phenolic acids detected in pomace extracts using HPLC-MS-IT-TOF with ESI

Source	Phenolic compound	Rt (min)	MS $(M-H)^-$ *m/z*	MS-MS *m/z*	Tentative identification	Molecular formula
Cranberry pomace extract	Procyanidins	10.4, 18.6	289	245	(+) Catechin/(−) catechin	$C_{15}H_{14}O_6$
		25.2	575	423, 285	PAC dimer A-type	$C_{30}H_{24}O_{12}$
		9.2, 14.3	577	407, 289	PAC dimer B-type	$C_{30}H_{26}O_{12}$
		17.5	863	575, 423	PAC trimer A-type	$C_{45}H_{36}O_{18}$
		19.9	865	577, 407	PAC trimer B-type	$C_{45}H_{38}O_{18}$
		6.4	1151	711, 411	PAC tetramer A-type	$C_{60}H_{48}O_{24}$
		11.6	1439	575, 285	PAC pentamer A-type	$C_{75}H_{60}O_{30}$
	Flavonols	33.8	301	290, 271	Quercetin	$C_{15}H_{10}O_7$
		28.4	317	ND	Myricetin	$C_{15}H_{10}O_8$
		31.0	433	300, 271	Quercetin pentoside	$C_{20}H_{18}O_{11}$
		31.4	447	300	Quercetin derivative	$C_{14}H_{24}O_{16}$
		29.3	463	301	Quercetin 3-hexoside	$C_{21}H_{20}O_{12}$
		32.0	507	344	Syringetin-3-*O*-glucoside	$C_{23}H_{24}O_{13}$
		34.7	567	300, 271	Quercetin 3-benzoyl galactoside	$C_{28}H_{24}O_{13}$
		33.7	609	463, 301	Quercetin 3-coumaroyl galactoside	$C_{30}H_{26}O_{14}$
Blueberry pomace extract	Procyanidins	18.4	289	245	(−) Catechin	$C_{15}H_{14}O_6$
		25.2	575	423, 285	PAC dimer A-type	$C_{30}H_{24}O_{12}$
		9.1, 14.2	577	407, 289	PAC dimer B-type	$C_{30}H_{26}O_{12}$
		15.7	863	711, 411	PAC trimer A-type	$C_{45}H_{36}O_{18}$
		9.9	1153	865, 449	PAC tetramer B-type	$C_{60}H_{50}O_{24}$
	Flavonols	33.5	301	229	Quercetin	$C_{15}H_{10}O_7$
		30.7	317	316, 256	Myricetin	$C_{15}H_{10}O_8$
		30.9	433	300, 271	Quercetin 3-*O*-arabinoside	$C_{20}H_{18}O_{11}$
		31.2	447	301, 255	Quercetin derivative	$C_{21}H_{20}O_{11}$
		29.1	463	301	Quercetin-3-*O*-hexoside	$C_{21}H_{20}O_{12}$
		29.8	477	301	Quercetin 3-*O*-glucuronide	$C_{21}H_{18}O_{13}$
		26.3	479	479, 299	Myricetin 3-*O*-hexoside	$C_{21}H_{20}O_{13}$
		32.0	507	344, 273	Syringetin-3-*O*-glucoside	$C_{23}H_{24}O_{13}$
		29.6	609	301	Rutin	$C_{27}H_{30}O_{16}$
	Hydroxycinnamic acid derivatives	13.2	353	191	Chlorogenic acid	$C_{16}H_{18}O_9$
		23.7	355	193	Feruloyl hexose	$C_{23}H_{16}O_4$

ND, not detected.

3.2. Characterization of free and PN protein-bound polyphenols

The majority of polyphenols in the PN protein–polyphenol complexes were present in their free form (not bound to proteins). The same amount of phenolic compounds was theoretically present in each of the 250 mg of PN protein–blueberry or cranberry 40% polyphenol complexes prior to extraction. Of that amount more total phenolic compounds remained in protein-unbound (free) form in the PN protein–blueberry polyphenol complex than in the PN protein–cranberry polyphenol complex (52.9 mg and 10.6 mg, respectively, in 250 mg of protein–polyphenol complex) (Table 3). Interestingly, polyphenols appeared to be predominately non-covalently bound to PN proteins in the PN protein–blueberry polyphenol complex while polyphenols from cranberry seemed to primarily be covalently bound to proteins in the PN protein–cranberry polyphenol complex (Table 3, total phenolics column). Phenolic acids and flavonols were captured at higher levels in the PN protein–cranberry polyphenol matrix in both the free form (protein-unbound) (2272 μg) and bound forms (162 μg non-covalently bound and 83.1 μg covalently bound). PN protein–blueberry polyphenol complexes yielded lower values of 557, 50.4 and 20.1 μg for free, non-covalently bound, and covalently-bound polyphenols, respectively. Quercetin was found to be the compound, among phenolic acids and flavonols quantified, to be responsible for higher levels of total phenolic acids and flavonols observed to be covalently bound in the PN protein–cranberry polyphenol complex (Table 3). In fact, quercetin was the only compound found in hydrolysates (*i.e.* covalently bound polyphenol) from both PN protein–polyphenol complexes, whereas a small amount of caffeic acid was found in the PN protein–cranberry polyphenol hydrolysate.

More procyanidins remained unbound in the PN protein–cranberry complex (5671 μg) than in the PN protein–blueberry polyphenol complex (1116 μg), respectively (Table 3). Both blueberry and cranberry are particularly rich in oligomeric and polymeric procyanidins and those compounds non-covalently bound to PN proteins (Table 3). HPLC analysis of hydrolysates for procyanidins revealed several compounds showing strong fluorescence properties, however, none of the peaks could be identified as procyanidins and further analysis by LC-MS only showed a complex mixture of unidentified compounds (data not shown).

3.3. Protein distribution and semi-quantitative immunoblotting

The major PN allergens Ara h 1 (64 kDa),[22] Ara h 2 (16.7 and 20 kDa isomers)[23] and Ara h 3 (several peptides ranging from

Table 2 Concentration of total phenolics, select phenolic acids and flavonols, and individual procyanidins in plant extracts in mg mL^{-1}

	Phenolics	Phenolic acids & flavonols								Procyanidins						
Extract	Total[a]	Quercetin[a]	Myricetin[a]	Chlorogenic acid	Rutin	Hyperoside[a]	Caffeic acid[a]	*p*-Coumaric acid[a]	Total[a]	DP1	DP2	DP3[a]	DP4[a]	DP > 4	Polymers[a]	Total[a]
Blueberry	4.80	0.06	<0.01	0.09	0.02	0.07	0.06	<0.01	0.31	0.02	0.04	0.04	0.01	0.31	0.37	0.80
Cranberry	3.20	0.48	0.09	ND	ND	0.01	<0.01	0.01	0.60	0.01	0.06	0.11	0.04	0.35	0.94	1.50

[a] Values within each column for blueberry and cranberry extract are significantly different at $p < 0.05$. DP, degree of polymerization; DP1, monomers expressed as epicatechin; DP2, dimers expressed as PAC A2; DP3, trimers expressed as PAC C1; DP4, tetramers expressed as PAC D; DP > 4, oligomers expressed as PAC D; polymers, expressed as PAC D.

Table 3 Total phenolics, phenolic acids and flavonols, and individual procyanidins bound to peanut proteins in 250 mg of peanut protein–polyphenol complex

		Phenolics (mg)	Phenolic acids & flavonols (μg)								Procyanidins (μg)						
Complex		Total	Quercetin	Myricetin	Chlorogenic acid	Rutin	Hyperoside	Caffeic acid	*p*-Coumaric acid	Total	DP1	DP2	DP3	DP4	DP > 4	Polymers	Total
Blueberry	A	52.9	160[a]	5.57[a]	117	24.3	109[a]	141[a]	0.01[a]	557[a]	28.8	45.8[a]	52.8[a]	17.7[a]	266[a]	705[a]	1116[a]
	B	1.44	14.6[a]	ND	11.9	2.20	13.9[a]	7.80[a]	ND	50.4[a]	1.53	8.43	8.07	2.00	41.4[a]	98.3[a]	160[a]
	C	0.57	20.0	ND	ND	ND	ND	NA	NA	20.1	ND	ND	ND	ND	ND	ND	ND
Cranberry	A	10.6	1924[a]	244[a]	ND	ND	53.0[a]	13.0[a]	38.0[a]	2272[a]	28.9	150[a]	122[a]	82.8[a]	1954[a]	3333[a]	5671[a]
	B	0.67	144[a]	14.0	ND	ND	2.27[a]	0.57[a]	1.37	162[a]	1.29	16.0	10.7	4.99	109[a]	175[a]	317[a]
	C	1.35	82.0	ND	ND	ND	ND	1.05	NA	83.1	ND	ND	ND	ND	ND	ND	ND

[a] Values within each column for each polyphenol type and each extract (A, B or C) are significantly different at $p < 0.05$; A, 80% aqueous methanol extract (unbound polyphenol fraction); B, extract using 80% aqueous methanol containing 1% acetic acid (non-covalently bound polyphenol fraction); C, extract derived from alkaline hydrolysis (covalently bound polyphenol fraction). DP, degree of polymerization; DP1, monomers expressed as epicatechin; DP2, dimers expressed as PAC A2; DP3, trimers expressed as PAC C1; DP4, tetramers expressed as PAC D; DP > 4, oligomers expressed as PAC D; polymers, expressed as PAC D; ND, not detected; NA, not applicable (cancelled out after subtracting value for control, peanut flour alone).

45 to 14 kDa)[24] from both PN protein–polyphenol complexes and unmodified PN flour were separated by SDS-PAGE and identified by staining with CBB (Fig. 1A and B). An increase in molecular weight of Ara h 3 peptides (referred to Ara h 3 from here on) and Ara h 2 was observed and this was polyphenol concentration dependent (Fig. 1A and B). The band density of Ara h 3 and Ara h 2 appeared to decrease as polyphenol content increased in the samples. An increase in high molecular weight material above 250 kDa was observed which was stained by CBB, and this was also dependent on polyphenol concentration. Nitroblue tetrazolium staining indicated that the high molecular weight protein material in particular was modified by polyphenols and the degree of staining was dependent on the concentration of polyphenol (Fig. 1C and D). As expected, control PN proteins did not react with NBT (Fig. 1C and D).

Fluorescence Western blotting revealed that Ara h 1, Ara h 2 and Ara h 3 as well as unresolved protein material (smears in lanes) in unmodified PN flour and PN protein–polyphenol complexes were recognized by antigen-specific IgE antibodies from human plasma (Fig. 1E and F). However, replicates of those Western blots indicated that the overall IgE binding decreased with an increase in polyphenol content (decrease in fluorescence signal intensity). Semi-quantitative analysis of blots showed that the overall IgE binding capacity in fact decreased with an increase in polyphenol content in both cranberry and blueberry complexes compared to the control, unmodified PN flour (Fig. 1G and H). Cranberry polyphenols blocked IgE binding to PN proteins to the greatest extent (38% for the PN protein–40% polyphenol complex) and this was significant when compared to the control (unmodified PN proteins). Blueberry polyphenols decreased IgE binding up to 31% (for the PN protein–40% polyphenol complex) which was significantly different when compared to the control (Fig. 1G and H).

3.4. Modulation of RBL-2H3 mast cell degranulation by peanut protein–polyphenol complexes

Peanut protein–cranberry and blueberry polyphenol complexes decreased ionomycin-induced mast cell degranulation in a dose-dependent manner as shown in Fig. 2. Results showed that PN protein–cranberry and blueberry polyphenol complexes modulated histamine release from mast cells to a similar extent, a 65.5% and 65.8% decrease for the 30% polyphenol complexes, respectively (Fig. 2A and B). The PN protein–cranberry polyphenol complexes were observed to have a greater effect at reducing β-hexosaminidase release than the PN protein–blueberry polyphenol complexes (Fig. 2C and D). Cranberry polyphenols decreased ionomycin-induced β-hexosaminidase release by 60.7% (PN protein–30% polyphenol complex) and blueberry polyphenols by 45.4% (PN protein–40% polyphenol complex). The addition of PN flour alone to ionomycin-induced mast cells resulted in a slight decrease of histamine and β-hexosaminidase release (Fig. 2). Cranberry or blueberry extract alone was added to ionomycin-induced mast cells in a separate experiment as well (polyphenol concentrations tested were the same as present in the PN protein–cranberry or blueberry polyphenol complexes containing 5–40% polyphenols). Polyphenols had a greater effect on decreasing histamine release from mast cells than on β-hexosaminidase release (see ESI, Fig. 1S†). In fact, free unbound blueberry polyphenols showed no effect on β-hexosaminidase release. A cytotoxicity test (XTT assay) showed a concentration-dependent decrease in cell viability when the cells were exposed to cranberry or blueberry extract (see ESI, Fig. 2SA and B†). However, when cell viability was assessed using PN protein–polyphenol complexes this effect was abrogated and only PN protein–cranberry polyphenol complexes containing higher levels of polyphenols had a significant effect on cell viability (see ESI, Fig. 2SC and D†).

4. Discussion

Our previous studies included commercially available fruit juice concentrates as sources of polyphenolic compounds.[2,3] For the present study, A by-product from the fruit juice industry, pomace (*i.e.* skin and seeds residues leftover after juicing), was chosen as the source of polyphenolic compounds due to: (a) promising results using cranberry juice in previous studies[2,3] (b) low levels of sugar in the pomace extracts (data not shown) as opposed to fruit juice, which allowed for the creation of dry hypoallergenic polyphenol-fortified protein ingredients with low sugar content, and (c) ecological reasons (*i.e.* pomace is a waste product). Our previous work has shown that particularly procyanidins (in the form of a purified fraction from cranberry) -compared to an anthocyanin-rich fraction from cranberry- were able to bind to PN proteins and decrease IgE binding using allergic plasma from PN-allergic individuals. Many studies have investigated the binding and protein structure-modulating properties of procyanidins, especially monomers, such as epigallocatechin-3-gallate,[25] but also of flavonols and phenolic acids.[10] LC-MS analysis of the aqueous pomace extracts was performed to identify some major phenolic constituents belonging to those polyphenol classes which have also been reported by others[26–28] (Table 1). Some of these were quantified by HPLC using pure standard compounds (Table 2).

Phenolic compounds which remained protein-unbound (free) after complexation of PN proteins with either cranberry or blueberry extract or bound to PN proteins non-covalently or covalently were evaluated. Free polyphenols were extracted by a brief wash of the complexes in 80% aqueous methanol, whereas non-covalently bound phenolic compounds were released through multiple extractions with 80% aqueous methanol containing 1% acetic acid; solvent systems which have been described by others for extracting polyphenols.[29–31] Alkaline hydrolysis on the other hand was used to release covalent protein-bound phenolic compounds.[18,32] While this technique does not allow for an absolute distinction between protein-unbound or non-covalently and covalently protein-

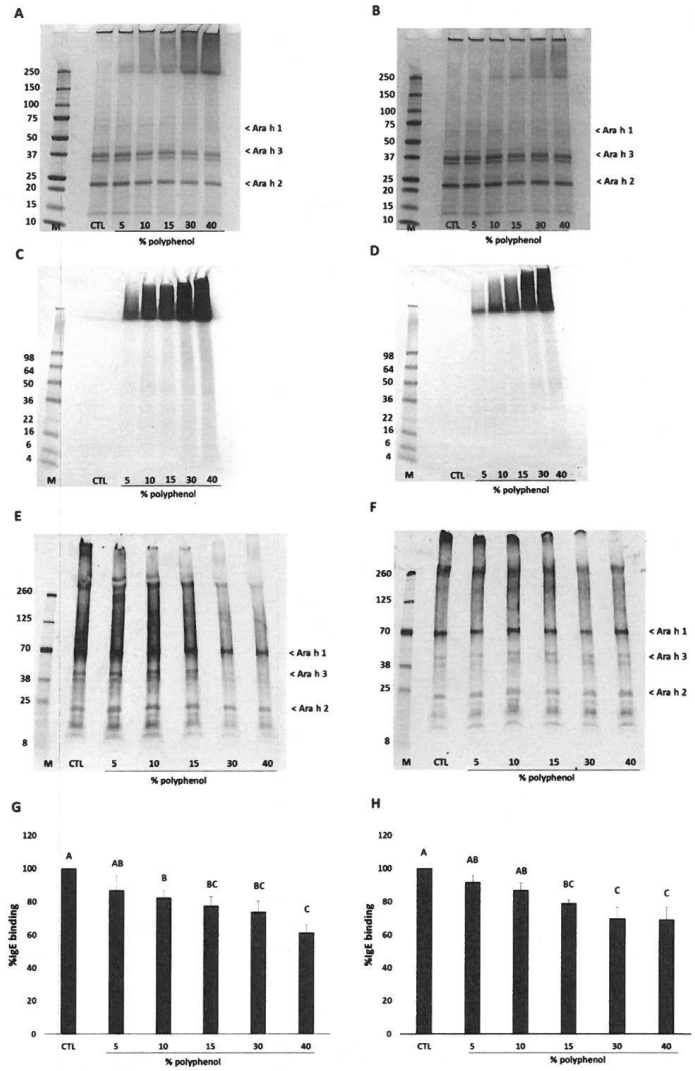

Fig. 1 SDS-PAGE of unmodified peanut protein or peanut protein–polyphenol complexes containing 5, 10, 15, 30, and 40% cranberry (A) or blueberry (B) polyphenols and stained with Coomassie Brilliant Blue; staining of cranberry (C) or blueberry (D) polyphenol-bound peanut proteins by NBT (nitroblue tetrazolium) following SDS-PAGE and subsequent electrophoretic transfer to a PVDF membrane; representative corresponding Western blot for cranberry (E) and blueberry (F) complexes; semi-quantitative analysis of IgE binding capacity (based on Western blots) of cranberry (G) and blueberry (H) peanut protein–polyphenol complexes (mean of four replicates with SE; values with different letters are significantly different at $p < 0.05$). M, molecular weight marker (kDa); CTL, control (unmodified peanut proteins). Tentative approximate locations for major peanut allergens are indicated. Gray scale was used for gels and membranes and contrast was optimized to improve visualization.

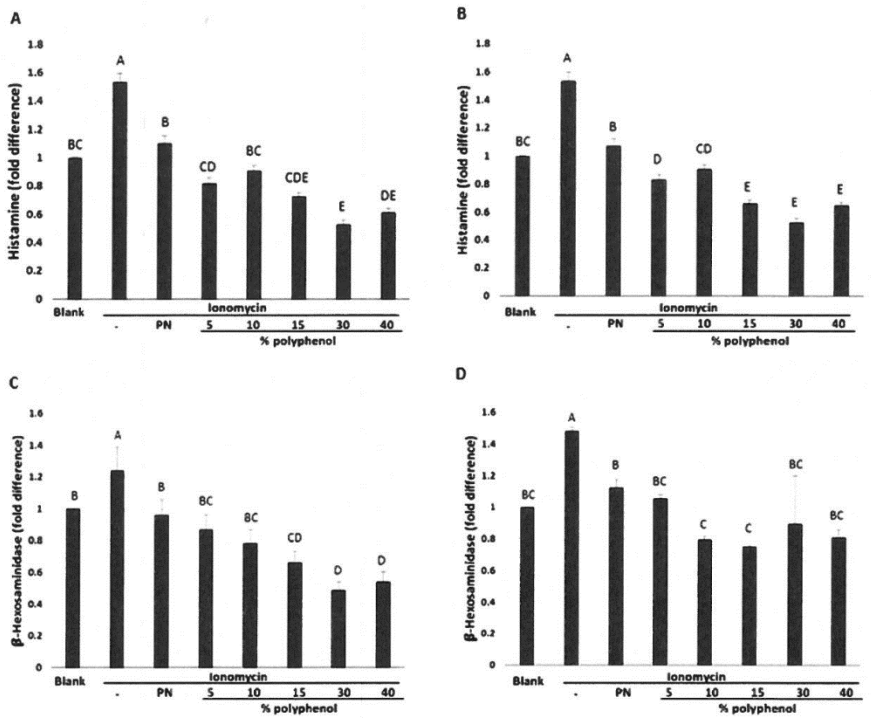

Fig. 2 Histamine (A and B) and β-hexosaminidase (C and D) release following peanut protein–cranberry (A and C) or blueberry (B and D) polyphenol complex (5–40% polyphenols) or unmodified peanut protein (PN) co-exposure with DNP-BSA in RBL-2H3 cells sensitized with anti-DNP IgE and ionomycin. Controls were cells which only received ionomycin or PBS 1× (blank). Data shown are means of six replicates with SE; values with different letters are significantly different at $p < 0.05$. The data was normalized against cell death (for cell viability data please refer to Fig. 2S†). DNP, dinitrophenyl; BSA, bovine serum albumin, PBS; phosphate buffered saline.

bound polyphenols, this approach was able to provide information about estimated amounts and types of polyphenols in each of those fractions (Table 3).

To understand the potential for IgE binding modification through the binding of polyphenols to PN proteins, PN protein–polyphenol complexes were further screened using SDS-PAGE, NBT staining and subsequent fluorescent Western blotting using PN-specific IgE (Fig. 1). Since the addition of polyphenols to PN flour resulted in a dilution of protein content per unit weight (data not shown) experiments were normalized to equal protein content. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that polyphenol-binding to PN proteins resulted in PN protein–polyphenol complexes with increased molecular weight (decreased electrophoretic mobility as can be seen with an upward move of protein bands on the gel) as well as unresolved high molecular weight material (see top of gel). The fact that these complexes were not broken down by SDS (disrupts non-covalent interactions) or β-mercaptoethanol (cleaves covalent disulfide bonds) indicates that the bonds formed between the molecules in those complexes must be of covalent nature. This has also been observed in other studies focusing on polyphenol binding with food proteins and enzymes.[33,34] Further indication for at least partial covalent polyphenol modification of PN proteins was seen when SDS-PAGE resolved PN proteins were electroblotted onto a PVDF membrane and stained with NBT. Nitroblue tetrazolium in glycinate buffer (pH 10) allows for a specific reaction with quinones (oxidized polyphenols). The stain specifically stained PN protein–quinone complexes because unmodified PN proteins did not react with NBT. The high molecular weight protein material, which was also stained by CBB on a gel, was also detected by NBT and this was polyphenol concentration dependent. Poorly resolved proteins (lane smears) were also stained by NBT.

Polyphenols can readily undergo oxidation either enzymatically or non-enzymatically.[35,36] The oxidoreductases peroxidase and polyphenol oxidase can, in the presence of oxygen and upon tissue damage, catalyze the oxidation of mono- and diphenols to quinones which ultimately can polymerize to brown pigments in plant tissue. During food processing, those enzymes can be removed or inactivated, however, non-enzymatic oxidation of polyphenolic compounds can still occur. Environmental conditions such as the presence of oxygen, pH, and temperature play a role in non-enzymatic polyphenol oxidation. It has been shown that elevated temperatures, such as during thermal treatment, and alkaline pH (>7) can cause polyphenols to oxidize.[37,38] However, while a higher pH generally promotes formation of quinones it has been shown that oxidation of polyphenols can also occur in acidic medium (pH 4).[39,40] Quinones are highly reactive electrophilic intermediates which can readily undergo attack by nucleophiles which can be found in many amino acid side chains in a protein polypeptide chain. The amino acid residues of cysteine, methionine and tryptophan represent a few of the possible nucleophilic sites. The reaction between electrophiles and nucleophiles results in nucleophilic (Michael-type) addition and ultimately in the formation of covalent bonds between the two molecules involved. After the addition of phenolic compounds to a protein side chain, a second oxidation is possible and subsequent binding to another protein chain. This results in protein-crosslinked material with high molecular weight.[34,41]

Fluorescence Western blotting showed that the high molecular weight protein material detected by CBB and NBT was modified by bound polyphenolic compounds to the extent that IgE epitopes (*i.e.* linear epitopes if conformational IgE epitopes were disrupted) could not be recognized by PN-specific IgE antibodies (Fig. 1). In other words, protein-bound polyphenolic compounds (possibly in the form of protein-bound quinones as discussed above) blocked PN-specific IgE antibody binding. Lack of antibody binding due to absence of antigen was ruled out since a total protein stain prior to immunoblotting verified the presence of proteins (data not shown). This was also seen for poorly resolved proteins (smears in lanes) and protein bands to various degrees. Overall, total IgE binding to PN proteins was decreased substantially once they were bound to cranberry and blueberry polyphenols. Based on results obtained from PN protein–polyphenol complex extraction experiments (Table 3) it appears that of the compounds evaluated, the flavonol quercetin, in aglycone or glycosidic form (*i.e.* with or without a sugar attached, respectively), was covalently bound to PN proteins and partially responsible for PN-specific IgE antibody blocking. Other polyphenolic compounds such as procyanidins (which were not identifiable in hydrolyzed form by analytical methods employed) also seemed to have been involved in this blocking. The presence of covalent protein-bound polyphenols was also shown by an estimation of total phenolic compounds (Table 3, extract C). Additional experiments are warranted to better understand the mechanisms of binding between PN protein and cranberry or blueberry polyphenols. Studies have shown that both non-covalent and covalent forces between food proteins and polyphenols are possible.[42] Our previous study as well as the present study indicate that both occur during protein–polyphenol complex formation.[3]

In order to investigate the ability of the PN protein–polyphenol complexes to modulate the typical anaphylaxis process, a well-known mast cell model system consisting of DNP-BSA and RBL-2H3 cells armed with anti-DNP IgE, was used. Mast cell degranulation experiments are commonly performed *in vitro* to study allergic response of the human body upon allergen stimulation.[43,44] The process of anaphylactic reaction involves release of cytokines, eicosanoids, and leukotrienes. Mast cells also release granule-associated mediators, mainly histamine, β-hexosaminidase, proteoglycans, proteases and cathepsin D.[45] These granules fuse with the cell plasma membrane and trigger exocytosis thereby leading to calcium (Ca^{2+}) accumulation.[46] We tested the involvement of PN protein–polyphenol complexes in inhibiting mast cell exocytosis caused due to elevated Ca^{2+} concentration by treating the cells with ionomycin, a known Ca^{2+} ionophore which causes intracellular Ca^{2+} increase. DNP-BSA was co-exposed with either PN flour alone, PN protein–polyphenol complexes or polyphenols alone (at the same concentration as present in the complexes) to the cells and the supernatant was screened for markers of degranulation (histamine and β-hexosaminidase). RBL-2H3 cells (containing anti-DNP IgE on the cell surface) treated with PN protein–cranberry and blueberry polyphenol complexes, challenged with DNP-BSA and stimulated with ionomycin showed decreased β-hexosaminidase and histamine release suggesting a Ca^{2+}-mediated inhibitory mechanism (Fig. 2). Peanut protein–cranberry complexes showed a stronger inhibitory effect on degranulation of RBL-2H3 cells than PN protein–blueberry complexes. Our results show that PN protein–polyphenol complexes exert potential anti-allergic affects, at least in part, through inhibition of degranulation triggered by Ca^{2+} influx upon onset of anaphylactic reaction in mast cells.

5. Conclusion

In the present study, peanut protein–polyphenol complexes created with polyphenol-rich extracts from two *Vaccinium* species were evaluated for their hypoallergenic potential *in vitro*. We demonstrated that polyphenolic compounds bound to peanut proteins reversibly and irreversibly. The peanut protein–polyphenol complexes were shown to be hypoallergenic in nature based on decreased peanut protein-specific IgE binding to IgE epitopes on peanut proteins (allergen-specific, immunoblotting) and based on non-allergen-specific anti-allergic effects observed using a well-known *in vitro* allergen model system. Future studies are warranted to investigate the molecular mechanisms involved and the role of quercetin and procyanidins in the hypoallergenicity and anti-allergic potential of peanut protein–polyphenol complexes.

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CHAPTER 3

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

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

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ABSTRACT

Peanut allergy is a worldwide health concern and among the identified peanut allergens, Ara h 2 is one of the most potent. Plant polyphenolic compounds have a natural affinity to bind with proteins, including edible peanut proteins which contain the allergenic epitopes. In this study, we investigated a range of polyphenolic compounds found in cranberry (*Vaccinium macrocarpon* Ait.) and/or lowbush blueberry (*Vaccinium angustifolium* Ait.) for their potential to bind to Ara h 2 and inhibit binding to IgE binding epitopes (crucial components for eliciting an allergic response). A total of 42 polyphenols were screened, using *in silico* molecular modeling techniques, to prioritize the ones predicted to have the strongest binding to Ara h 2 in general, and to specific IgE binding epitopes on the Ara h 2 allergen. Based on *in silico* results, the four polyphenols tested *in vitro* were: delphinidin-3-glucoside, cyanidin-3-glucoside (anthocyanins), procyanidin C1, and chlorogenic acid (phenolic acid). Also, benzoic acid was tested as a control because it showed minimal *in silico* binding. All polyphenols were evaluated using spectroscopic and immunological methods (UV-Vis spectroscopy, circular dichroism, and immunoblotting, respectively) for their a) Ara h 2 binding potential, their b) capacity to induce protein secondary structural changes, and c) capacity to inhibit IgE binding epitopes. Various Ara h 2:polyphenol molar ratios (i.e. 1:5, 1:10, and 1:20) were examined. UV-Vis spectroscopy suggested that procyanidin C1 and chlorogenic acid did interact with Ara h 2 and circular dichroism tests further showed that those compounds also induced changes in the Ara h 2 secondary structure upon binding. Binding of procyanidin C1 to Ara h 2 resulted in a 6% *increase* in α-helical content, whereas chlorogenic acid binding *decreased* the α-helical content by 3%. Computational studies, focusing on non-covalent interactions, predicted that all selected phenolic compounds bind in

a binding pocket located between α-helix 2 and α-helix 3 (extended loop region) interacting with amino acids that are part of one or more IgE binding epitopes. Immunoblotting using pooled human peanut-allergic plasma (containing Ara h 2-specific IgE antibodies) showed that procyanidin C1 binding to Ara h 2 significantly decreased ($p<0.05$) the IgE binding capacity by up to 37%, chlorogenic acid by up to 50%, and benzoic acid up by to 37%. Interestingly, benzoic acid was predicted based on docking studies to likely not bind to Ara h 2 and was used as a control in this study, but these immunoblotting results show that benzoic acid *did* bind to Ara h 2 and inhibited IgE binding to IgE epitopes on Ara h 2. *In silico* predictions simulate binding of one polyphenol to the surface of Ara h 2 at a time. It is possible that benzoic acid non-specifically bound to multiple surface sites on Ara h 2, and specifically to specific IgE binding epitopes. Another possibility is that benzoic acid binding to the surface of Ara h 2 resulted in the loss of tertiary protein structure (protein unfolding) which increased the availability of protein core-hidden IgE binding epitopes and resulted in their subsequent binding by benzoic acid. Observed changes in α-helical content imply that local changes in native Ara h 2 secondary structure occurred upon polyphenol binding. A decrease in IgE binding capacity suggests that procyanidin C1, chlorogenic acid, and benzoic acid bound with amino acid residues on Ara h 2 that are part of IgE binding epitopes, and those polyphenols were bound tightly enough or covalently to survive SDS-PAGE conditions. Collectively, these findings suggest that hypoallergenic Ara h 2-procyanidin C1, -chlorogenic acid, and -benzoic acid complexes were formed.

1. Introduction

Peanut allergy is a worldwide health concern. 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) (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 (FcεR receptors) on mast cell and basophil surfaces. Upon re-encounter with peanut allergens, certain protein epitopes bind and cross-link peanut-specific IgE antibodies located on mast cells and basophils. Downstream cascade reactions trigger the cells to release preformed granules containing inflammatory mediators like histamine or prostaglandins, which are ultimately responsible for allergic symptoms (Burks, 2008). Out of all the known peanut allergens, Ara h 2 is one of the most potent allergens, as it is recognized by > 90% of peanut-allergic individuals (Koppelman, Wensing, Ertmann, Knulst, & Knol, 2004; Palmer et al., 2005; Stanley et al., 1997). 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 amino acid sequence contains a linear IgE binding epitope 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 10 linear IgE binding epitopes have been identified for Ara h 2.01 (Stanley et al., 1997).

In a recent study, we have shown that complexing peanut flour with polyphenols derived from cranberry (*Vaccinium macrocarpon* Ait.) and lowbush blueberry (*Vaccinium angustifolium* Ait.) decreased allergenicity based on complementary *in vitro* experiments (Plundrich, Bansode, Foegeding, Williams, & Lila, 2017). It was hypothesized that binding of polyphenols might either mask or alter the structure of allergenic epitopes, attenuating the allergenicity of the aggregate complexes. Among the polyphenolic compounds analyzed (*e.g.*, phenolic acids, quercetin, quercetin derivatives, procyanidins), we demonstrated that binding to peanut proteins was achieved by non-covalent and/or covalent interactions (Plundrich et al., 2017). One of the antigens (peanut allergens) bound by blueberry and cranberry polyphenols was Ara h 2.

While previous investigations have shown the potential of using polyphenol binding as a way to mediate peanut allergenicity (Plundrich et al., 2014, 2015, 2017), the activity of individual polyphenols was not investigated. One way to address a large number of possible compounds involved in allergy attenuation is to start by virtually screening a library of polyphenols using *in silico* molecular modeling techniques, prioritizing the ones predicted to be the strongest Ara h 2 binders, and experimentally confirming those *in silico* hits.

In this study, we investigated a total of 42 diverse polyphenolic compounds found in cranberry and/or lowbush blueberry (Gavrilova, Kajdžanoska, Gjamovski, & Stefova, 2011; Grace, Esposito, Dunlap, & Lila, 2014; Khanal, Howard, Brownmiller, & Prior, 2009; Lee & Wrolstad, 2004; White, Howard, & Prior, 2010). We used structure-based computational docking to assess their binding potential to Ara h 2. Four polyphenols predicted to be Ara h 2 and IgE binding epitope (on Ara h 2) binders, belonging to the anthocyanin (delphinidin-3-glucoside and cyanidin-3-glucoside), procyanidin (procyanidin C1), and phenolic acid

(chlorogenic acid) polyphenol classes were selected for experimental validation. Also, benzoic acid was tested as a control because it showed minimal *in silico* binding. The compounds were evaluated for their Ara h 2 binding potential and their capacity to induce protein structural changes through spectroscopic methods using peanut-isolated Ara h 2 (containing both isoforms) *in vitro*. An X-ray crystal structure available for Ara h 2.01 (but not for Ara h 2.02) was used for computational studies (Mueller et al., 2011). The term "Ara h 2" refers to both isoforms in this text, unless specified as Ara 2.01 or Ara h 2.02. Finally, Ara h 2-polyphenol complexes were further tested for their hypoallergenic potential through IgE immunoblotting. We hypothesized that all proposed polyphenols bind to Ara h 2 as predicted *in silico* and that some of them may be able to induce protein secondary structure changes. We also hypothesized that some of the formed protein-polyphenol complexes are hypoallergenic *in vitro* based on the polyphenols' predicted potential to bind with amino acids that are part of linear IgE binding epitopes.

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). Peanut protein-40% cranberry polyphenol aggregate particles were prepared by complexing the amount of aqueous cranberry pomace extract (mL) with light roast peanut flour (g) required to generate dry particles containing 40% cranberry polyphenols (w/w) after complexation and subsequent freeze-drying (Plundrich et al., 2017).

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). Egg white protein (EWP), nitroblue tetrazolium, chlorogenic acid, and benzoic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Precast mini TGX 4–20% polyacrylamide gels, 10× tris/glycine/ SDS running buffer, Precision Plus protein pre-stained standard, and 2× Laemmli sample buffer were purchased from Bio-Rad Laboratories (Hercules, CA, USA). NativePAG Novex 4–16% Bis-Tris Gels, NativePAGE Sample Buffer (4X), NativeMark Unstained Protein Standard, NativePAGE 20X Running Buffer, NativePAGE 20X Cathode Buffer Additive, and SimplyBlue SafeStain G-250 were obtained from Invitrogen (Carlsbad, CA, USA). Chameleon duo prestained protein ladder, Odyssey PBS blocking buffer, 4×protein sample loading buffer, 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, Uppsula, 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 10× PBS 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

The X-ray crystal structure (resolution of 2.71 Å, PDB code: 3OB4) of Ara h 2.01 bound with Maltose Binding Protein (MBP) was extracted from the Protein Data Bank (Mueller et al., 2011). 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 our docking studies, the MBP protein was removed and Ara h 2.01 was curated using the Schrödinger Suite's Protein Preparation Wizard (Sastry, Adzhigirey, Day, Annabhimoju, & Sherman, 2013; Schrödinger Release 2017-2: Schrödinger Suite 2017-2 Protein Preparation Wizard). 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 Prime (software that helps refine ligand geometries when docking to a protein). All water molecules were removed (Jacobson et al., 2004; Jacobson, Friesner, Xiang, & Honig, 2002; Schrödinger Release 2017-2: Prime).

The EPIK program was used to determine the protonation state of Ara h 2.01 at pH 7 (Greenwood, Calkins, Sullivan, & Shelley, 2010; Shelley et al., 2007; Schrödinger Release 2017-2: Epik). A restrained minimization was performed with the OPLS3 force field (Harder et al., 2016; Schrödinger Release 2017-2). The refined structure is available in the supplementary material (supplementary Figure 1) to facilitate the reproducibility of this study. The amino acid sequence of the refined structure is as follows: RRCQSQLERA$_{10}$NLRPCEQHLM$_{20}$QKIQRDEDSY$_{30}$ERDPYSPSQD$_{40}$PYSPSPYDRR$_{50}$GAGSSQHQER$_{60}$CCNELNEFEN$_{70}$NQRCMCEALQ$_{80}$QIMENQSDRL$_{90}$QGRQQEQQFK$_{100}$RELRNLPQQC$_{110}$GLRAPQRCDL$_{120}$D. IgE binding epitopes distributed along the linear sequence of Ara h 2.01 are shown in Table 1.

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 (Friesner et al., 2004, 2006, Halgren, 2007, 2009; Shelley et al., 2007; Schrödinger Release 2017-2: Glide, Sitemap; Schrödinger 2016: Knowledge Base). First, we utilized the *SiteMap* program, which identifies the possible binding sites and calculates their physicochemical characteristics (*e.g.*, exposure to solvent, volume, and hydrophobic and hydrophilic spaces) (Halgren, 2007, 2009; Schrödinger Release 2017-2: Sitemap). Using these quantitative parameters, a Druggability Score (Dscore) was calculated (Halgren, 2009). A Dscore>0.8 associated to a binding site indicates a great likelihood 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 (Friesner et al., 2004, 2006, Halgren, 2007, 2009; Shelley et al., 2007; Schrödinger Release 2017-2: Glide, Sitemap). At last, 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 = 0.95 binding pocket. Second, we used a *BlindDocking* protocol. Briefly, it leads to the creation of a receptor grid without specifying an actual binding pocket. Here, the entire protein was included in the grid in order to calculate all optimal and 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 (Schrödinger 2016: Knowledge Base).

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 (Gavrilova et al., 2011; Grace et al., 2014; Khanal et al., 2009; Lee & Wrolstad, 2004; White et al., 2010)(supplementary Table 1). All these compounds were processed using LigPrep from the Schrödinger Suite with an OPLS3 force field (Harder et al., 2016; Schrödinger Release 2017-2; Schrödinger Release 2017-2: LigPrep). Tautomeric states of each compound were generated at pH 7 using EPIK, while retaining their specified chiralities (Greenwood et al., 2010; Shelley et al., 2007; Schrödinger Release 2017-2: Epik).

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 (Friesner et al., 2004, 2006; Shelley et al., 2007; Schrödinger Release 2017-2: Glide). Overall, this 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 (Friesner et al., 2004, 2006, Halgren,

2007, 2009; Shelley et al., 2007; Schrödinger Release 2017-2: Glide). To consider a compound as "active" in the binding site, DS ≤ -7 kcal mol^{-1} and eM ≤ -50 kcal mol^{-1} are required. The DS and eM thresholds were previously discovered through virtual screening protocols of micromolar binders. These thresholds can vary based on the protein and the scoring function and are considered guidelines for what is predicted to bind (Fourches, Muratov, Ding, Dokholyan, & Tropsha, 2013; S. Shityakov & Förster, 2014; Sergey Shityakov & Förster, 2014).

2.2.5 Molecular dynamics and dimer binding potential

The dynamic molecular interactions between polyphenols and Ara h 2.01 were studied using Molecular Dynamics Simulations (MDS). Molecular docking produced DS and eM scores close to the -7 kcal mol^{-1} and -50 kcal mol^{-1} thresholds, resulting in further testing. The stability of the molecular docking poses was analyzed using Desmond. Each model was built using the docked pose which was explicitly solvated with water molecules and a 50 ns MD simulation was conducted. All MDS ran for 50 ns with TIP3P solvation model, an OPLS3 force field energy minimization, and an orthorhombic volume. The calculation interval ran at 1.0 ps with an integration output of 1.0 fs (Bowers et al., 2006; Guo, 2012; Shivakumar et al., 2010; Schrödinger Release 2017-2: Desmond Molecular Dynamics System). The MDS can help determine the nature and persistence of dynamic molecular interactions between the Ara h 2.01 protein and a small molecule ligand.

Moreover, dimers of Ara h 2.01 were computationally created to analyze the effects of a larger binding pocket on protein-ligand interaction potential. An Ara h 2.01 dimer was generated using the protein-protein interaction software InterEvDock, which resulted in a

larger binding pocket that could cause more variability in ligand DS (Yu et al., 2016). The potential increase in DS can assist in identifying stronger binders. InterEvDock calculated 150 dimer combinations using three different criteria's: 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, and 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 cell 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 ($A= l*c*\varepsilon$) 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 $\varepsilon_{280\ nm}$ of 13,950.5 $1* mol^{-1} * cm^{-1}$ 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 mM. The protein concentration was kept constant (~ 0.1 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 and a data pitch of 0.5 nm, and 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 deg cm^2 $dmol^{-1}$ based on the following equation (Eq. 1) (Pan, Qin, Liu, & Wang, 2011):

$$\text{MRE (deg cm}^2\text{ dmol}^{-1}) = \frac{\theta}{Cp \times n \times l} \qquad \text{(Eq. 1)}$$

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 as a function 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):

$$\alpha - helix\ (\%) = \frac{-MRE208-4{,}000}{33{,}000-4{,}000} \times 100 \qquad \text{(Eq. 2)}$$

Where MRE_{208} is the observed MRE value at 208 nm, 4,000 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, & Badhei, 2015).

2.4. UV-Vis absorption spectroscopy

The interaction between Ara h 2 and polyphenols was further investigated using UV-Vis absorption spectroscopy. Ara h 2 (in MilliQ water) spectra were subtracted from sample spectra. 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 900 nm – 200 nm at room temperature.

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

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or native PAGE was used to observe protein distribution in 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 μg) was mixed with the respective sample loading buffer containing β-mercaptoethanol.

For native PAGE, 0.7 μg protein were also loaded per well. Samples were prepared in NativePAGE sample buffer (4X) and loaded onto NativePAGE 4-16% Bis-Tris protein gels. Gels were run for 100 min at 150 V, stained with SimplyBlue SafeStain and destained with distilled water for several hours. Gel images were taken with a Bio-Rad Gel Doc XR$^+$ imaging system (Hercules, CA, USA).

2.6. Nitroblue tetrazolium (NBT) staining

The presence of polyphenols with electrophoretically separated proteins can be determined by a redox-cycling mechanism involving NBT as described elsewhere (Hagerman, 2002)**.** Proteins were separated by SDS-or native PAGE and electro-blotted onto a polyvinylidene fluoride (PVDF) membrane prior to NBT staining.

2.7. Statistical analysis

Immunoblotting was replicated four times 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.

3. Results and discussion

3.1. Molecular docking

In this study, we computationally examined the potential protein-ligand interactions between Ara h 2.01 and 42 naturally occurring polyphenols found in lowbush blueberries (*Vaccinium angustifolium* Ait.) and cranberries (*Vaccinium macrocarpon* Ait.). Using *SiteMap*, we identified a binding pocket with a computed volume of 381.5 $Å^3$ which is located between α-helix 2 and α-helix 3 in the extended loop region (Figure 1). All 42 compounds were docked according to that receptor grid and both SP and XP scoring functions were used.

Moreover, we used a *BlindDocking* receptor grid to dock these compounds in all other binding pockets detected for Ara h 2.01. Interestingly, when examining the positioning of the binding sites and binding modes of the different compounds, it appeared that the optimal docking location determined by both, *SiteMap* and *BlindDocking*, was towards the

center of the protein. Therefore it is not surprising that we observed that the DS and eM scores for both grids were close to identical.

The docking results for all 42 compounds at pH 7 are given in supplementary Table 2. Out of the 42 compounds, 13 compounds received an average docking score lower than the -7 kcal mol^{-1} activity threshold. But only 12 compounds were considered to be likely binders (docking score > -7 kcal mol^{-1}) since tannic acid afforded an eM score higher than -50 kcal mol^{-1}, disqualifying it from the list of possible compounds. The compound affording the best docking score was the anthocyanin delphinidin-3-glucoside (Figure 2): the compound was characterized by a docking score of -8.70 kcal mol^{-1}. As shown in Figure 2, delphinidin-3-glucoside interacts with five different amino acids Lys, Arg, Glu, Tyr, and Gln throughout the Ara h 2.01 protein at locations 22, 25, 31, 47, and 58 respectively between alpha helix 2 and 3 via hydrogen bonds. One linear epitope was involved in the interactions (epitope 5). Epitope and helix data are illustrated in Figure 3. The compound that has the lowest *overall (average)* DS and eM score is also delphinidin-3-glucoside, with a DS of -8.26 kcal mol^{-1} and eM score of -68.9 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 2). Those docking scores were between -7 and -9 kcal mol^{-1}, which typically correspond to micromolar binders (Fourches et al., 2013; S. Shityakov & Förster, 2014; Sergey Shityakov & Förster, 2014).

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. A representation of an Ara h 2.01 dimer is shown in Figure 4, with the two Ara h 2.01 polypeptide chains colored teal and green and the

polyphenol ligand (PAC C1) colored purple. Once dimers were obtained using the InterEvDock, each of the top 10 compounds, listed in Table 2, were docked between the two protein molecules. Ten different dimer positions were obtained from the 150 dimers calculated. These ten dimers were the likeliest conformations to form (data not shown). The top 10 polyphenolic compounds were docked to determine if polyphenols could potentially stably connect two Ara h 2.01 molecules and to evaluate if protein-polyphenol interactions increase (i.e. lower DS). Each of the DS for the compounds slightly increased when docked in the binding pocket of the dimer (data not shown), resulting in no changes in the overall order of most or least likely binders.

Overall, *in silico* docking results were evaluated for the compounds with the highest predicted potential to bind to Ara h 2 in general, and to IgE binding epitopes on Ara h 2, and the occurrence of compounds in lowbush blueberries and cranberries taken into consideration to determine the top four compounds to be tested in *in vitro* 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 (Figure 5). Specific interactions between delphinidin-3-glucoside and specific amino acids on the Ara h 2.01 polypeptide chain were discussed above. Cyanidin-3-glucoside docking to Ara h 2.01 showed that the polyphenol interacts with two amino acids, Lys and Arg at locations 22 and 25, respectively, via hydrogen bonds, on α-helix 2. Both amino acids taking part in the interactions belong to a linear epitope (epitope 5). Chlorogenic acid was predicted to interact with four amino acids, Arg, Arg, Ser, and Gln at locations 25, 49, 54, and 56, respectively, between α-helix 2 and 3

via hydrogen bonds which involved one linear epitope (Arg, epitope 5). PAC C1 showed a total of 12 interactions with amino acids of the polypeptide chain. The amino acids Lys, Arg, Asp, Ser, Tyr, Gly, and Gln at locations 22, 25, 26, 45, 47, 53, 54, and 56, respectively, via hydrogen bonds, salt bridges, and Pi-Pi stacking interactions between α-helix 2 and 3. Two linear epitopes on the Ara h 2.01 sequence were directly involved in PAC C1 binding, epitope 5 (Lys, Arg, and Asp) and 7 (Ser).

A low-predicted binder, benzoic acid (a phenolic acid), was also further evaluated (Figure 5). Benzoic acid, which had an average DS of -4.58 kcal mol^{-1} and eM score of -20.3 kcal mol^{-1}, was above the DS and eM thresholds and was predicted to have limited to no binding capabilities. It was predicted to interact with three amino acids, Gln, Ser, and Arg at positions 49, 54, and 58, respectively, between α-helix 2 and 3 via salt bridges and hydrogen bonds. No linear epitopes were involved in the interactions. A summary of amino acids and IgE binding epitopes involved in binding with polyphenols is shown in Table 3. Amino acids that are within 4 Å to the docked polyphenol have a chance of altering predicted interactions and are described in Table 4. Since our predicted interactions are based on Ara h 2.01 which lacks an additional 12 amino acids (containing IgE binding epitope peptide "DPYSPS") compared to Ara h 2.02 (Figure 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 an α-helical content between 30% - 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 structure (Figure 6). 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 (Figure 6 A 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 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 (Figure 6C) 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, either (supplementary Figure 2A and B). 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 a one way to confirm 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 (Figure 6D). 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 (Figure 5E). 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 a recent 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

(Figure 5E), since it is well known that small impurities can affect absorption spectra in the UV region of the UV spectrum (Litjens, Quickenden, & Freeman, 1999). UV spectra of both, cyanidin-3-glucoside and delphinidin-3-glucoside, after addition to Ara h 2 were distinct to UV spectra of the anthocyanins alone (supplementary Figure 2C and D). Anthocyanins have an absorption maximum at around 520 nm. After reaction with Ara h 2, two new maxima appeared and the absorption intensity greatly increased (hyperchromic shift). The appearance of the two new maxima may have been an effect of change in pH as anthocyanin color and hence UV absorption are dependent on pH. This was also shown in a recent study which investigated the UV-Vis spectrum of cyanidin-3-glucoside at various pH (Skaar et al., 2014). Based on those results it is not possible to determine if either of the pigments interacted with Ara h 2.

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

Ara h 2 was stained by CBB after native PAGE as a single band close to 20 kDa (Supplementary Figure 3). In addition, small molecular weight protein fragments, likely peptides from Ara h 2 breakdown, were also observed at the bottom of the gel. Native PAGE indicated that some polyphenols may have formed higher molecular weight complexes with Ara h 2 (smears in lanes above Ara h 2 bands) and this was polyphenol concentration dependent for both, cyanidin-3-glucoside and delphinidin-3-glucoside (Supplementary Figure 3). A stain with NBT also showed that cyanidin-3-glucoside and delphinidin-3-glucoside tightly bound to Ara h 2 but not PAC C1, chlorogenic acid, or benzoic acid (Supplementary Figure 4). Staining observed by NBT for the negative controls used, Ara h 2 and egg white

protein (EWP), are artifacts from the Coomassie dye used during native PAGE that were transferred over to the PVDF membrane prior to NBT treatment.

After SDS-PAGE, two distinct bands (doublet) were observed at approximately 20 kDa as well as smaller molecular weight fragments described above (Figure 7). NBT staining of proteins separated by SDS-PAGE and subsequent transfer onto a membrane was inconclusive for Ara h 2-poylphenol complexes. An Ara h 2 negative control (no polyphenols present) faintly stained, although not to the same extent as Ara h 2-polyphenol samples (equal amount of protein was loaded onto gel) (Supplementary Figure 5). It was shown that NBT can cause staining artifacts with proteins under certain conditions (Sri Venugopal & Adiga, 1980). Peanut proteins from peanut flour were stained by NBT to a small degree as well. Peanut flour is known to contain phenolic compounds, mainly from peanut skins which could have reacted with peanut proteins (Bansode et al., 2014). As expected, a peanut protein-40% cranberry polyphenol complex sample (positive control) was stained by NBT which was previously reported (Plundrich et al., 2017).

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 (Shreffler, Beyer, Chu, Burks, & Sampson, 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 (Figure 8).

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 µM and 106 µM, 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 µM chlorogenic acid; 50% decrease in IgE binding), and for benzoic acid for the 1:10 and 1:20 ratio, 55 µM benzoic acid (37% decrease) and 106 µM 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 molecule polyphenol binding to one molecule Ara h 2 (1:1 molar ratio) has the same effect on 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.

Results from this study suggest that proteins did *not* form aggregates through binding with polyphenols and therefore impaired IgE antibody binding to epitopes. This is because Ara h 2 bands migrated in the same manner with or without bound polyphenols on the SDS-PAGE gel (Figure 7). This suggests that IgE binding epitopes on *un-aggregated* proteins were inhibited by bound polyphenols. 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. Blocking of one amino acid in an IgE binding epitope sequence is enough to impair sequence-specific binding of IgE antibodies. 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 applies for conformational epitopes.

IgE binding capacity to Ara h 2 complexed with delphinidin-3-glucoside and cyanidin-3-glucoside was not significantly different to the control, Ara h 2 alone (supplementary Figure 6). However, NBT stain results from both native PAGE and SDS-PAGE indicate that both anthocyanins covalently or tightly bound to Ara h 2 (supplementary Figure 4 and 5). This could mean that delphinidin-3-glucoside and cyanidin-3-glucoside molecules were either 1) bound to Ara h 2 molecules but did not mask linear IgE binding epitopes, or 2) bound to *some* epitope/s on Ara h 2 molecules, yet did not significantly reduce their overall IgE binding potential.

Interestingly, benzoic acid was previously predicted to likely not bind to Ara h 2 (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 of a linear IgE binding epitope. It was hence expected that benzoic acid would have no capacity at blocking 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 (Figure 6F). Immunoblotting confirmed that benzoic acid *did* bind to Ara h 2 and prevented Ara h 2-specific IgE antibodies from binding to one or more IgE binding epitopes on Ara h 2 (Figure 8).

Evaluation of amino acids within 4 Å distance to a predicted bound benzoic acid molecule indicated potential interactions with amino acids that are part of two linear epitopes (Table 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, Pi-Pi stacking, and salt bridges (supplementary Figure 6).

Recently, two studies proposed a role of conformational Ara h 2 IgE binding epitopes (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 we predicted the tested polyphenols to bind. Since the CBB dye that was used during native PAGE interfered with the total protein stain prior to immunoblotting, IgE binding capacity to native Ara h 2-polyphenol complexes could not be evaluated this way (*i.e.* no evaluation of conformational IgE binding epitopes was possible using immunoblotting). 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 IgE binding 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. As mentioned above, polyphenol interactions with protein portions adjacent to IgE binding epitopes could also impair epitope accessibility for epitope-specific IgE antibodies. Table 5 shows a summary of these potential mechanisms and analytical techniques that can aid in mechanism assessment.

Collectively, our results show that the binding of procyanidin C1, chlorogenic acid, and benzoic acid to Ara h 2 lead to the formation of protein-polyphenol complexes with reduced allergenic potential *in vitro*.

4. Conclusion

Forty two polyphenols found in cranberry (*Vaccinium macrocarpon* Ait.) and lowbush blueberry (*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 micromolar 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, their b) capacity to induce protein secondary structural changes, and c) capacity to inhibit IgE binding epitopes. Ara h 2-polyphenol complexes were further tested for their hypoallergenic potential through *in vitro* immunoblotting. We demonstrated that three out of five polyphenols, 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 electrophoretic and immunoblotting *in vitro* methods. Further, the resulting Ara h 2-polyphenol complexes were proven to be hypoallergenic in nature based on decreased Ara h 2-specific IgE binding to IgE binding epitopes on peanut proteins (*in vitro* immunoblotting). Additional *in vitro* experiments are warranted to investigate the immunomodulatory mechanisms of these Ara h 2-polyphenol complexes, in particular for Ara h 2-benzoic acid complexes, and to determine the number of Ara h 2-bound polyphenols and the precise polyphenol binding sites on Ara h 2.

Acknowledgements

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

Peptide	Amino acid sequence	Epitope position on Ara h 2 sequence
1	HASARQQWEL	NA
2	QWELQGDRRC	NA
3	$DR_1RCQSQLERA_{10}$	1-8
4	$NLRPCEQHLM_{20}Q$	12-21
5	$KIQRDEDSY_{30}E$	22-29
6	RDPYSP	32-37
7	$SQD_{40}PYSPS$	38-45
8	$DRL_{90}QGRQQEQ$	90-95
9	$QFK_{100}RELRNLPQQ$	100-109
10	$QRCDL_{120}DVESG$	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.

Table 2. XP Docking and eModel scores for top 10 polyphenolic compounds at pH 7

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

Table 3. 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

Table 4. 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

Table 5. Potential mechanisms by which polyphenol-binding to a food allergen can result in inhibition of IgE antibodies to bind linear and/or conformational IgE binding epitopes

Mechanism	Techniques that contribute to assessing mechanism
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)	**NMR** (provides information about location and number of polyphenol binding sites, mechanism of interaction, dissociation constants, can be used for structure/properties relationship) **CD** (evaluation of polyphenol binding-induced tertiary structural changes) **Native PAGE** (evaluation of native vs. denatured proteins, qualitative analysis of protein-polyphenol complexes/aggregates) **FTIR** (protein secondary structural changes upon polyphenol binding) **Surface plasmon resonance** (evaluation of protein denaturation) **X-ray crystallography** (formation of a protein-polyphenol complex crystal can help determine where polyphenols are binding and if protein structure is different compared with unmodified protein)
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	**FTIR** (protein secondary structural changes upon polyphenol binding) **MS** (enzymatic digestion of protein-polyphenol complex and analysis of peptides by MS compared to peptides from unmodified protein; may give insight into which portions of the polypeptide chain were modified by polyphenol binding, usually applicable for covalent protein modifications) **X-ray crystallography** (formation of a protein-polyphenol complex crystal can help determine where polyphenols are binding and if protein structure is different compared with unmodified protein)

Table 5 Continued

Mechanism	Techniques that contribute to assessing mechanism
Binding of polyphenols to the protein and subsequent aggregation (polyphenols cross-link protein molecules) masking IgE binding epitopes	**SDS-PAGE/ native PAGE** (change in protein migration pattern compared to unmodified protein; high molecular weight protein material) **Size exclusion chromatography** (for soluble complexes; informs on molecular size and behavior of the soluble complexes, structure/properties relationship, gives some indication on mechanism) **Single molecule force microscopy** (interactions between proteins and polyphenols at a molecular level, can determine whether several protein molecules interact cooperatively with one polyphenol molecule [cross-linking] or whether a polyphenol interacts cooperatively at several sites within the same protein) **FTIR** (protein secondary structural changes upon polyphenol binding) **Dynamic light scattering** (informs on particle size distribution, under favorable conditions gives the shape of the aggregates, interaction between the aggregates) **Turbidimetry** (gives information on size of the aggregates, rate of aggregation in solution)
A combination of the three above proposed scenarios	A combination of techniques

NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CD, circular dichroism; MS, mass spectrometry; FTIR, Fourier transform infrared spectroscopy

Figure 1. 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.

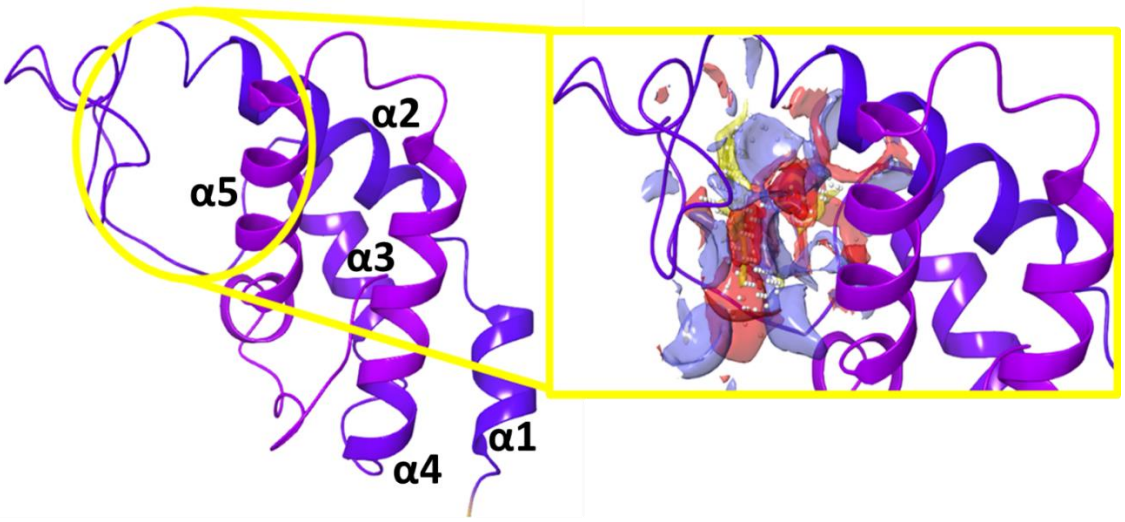
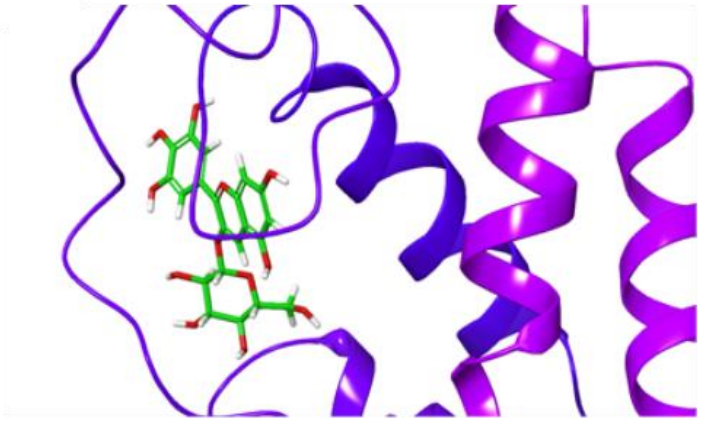

α2
α5
α3
α1
α4

Figure 2. Docking of delphinidin-3-glucoside to identified binding pocket on Ara h 2.01; Dscore = -8.6, with *SiteMap* function at a pH of 7. Figure 2A is a 3D representation of the protein-ligand interaction. Figure 2B is a 2D representation with the functional groups interacting with the respective amino acid.

A.



B.

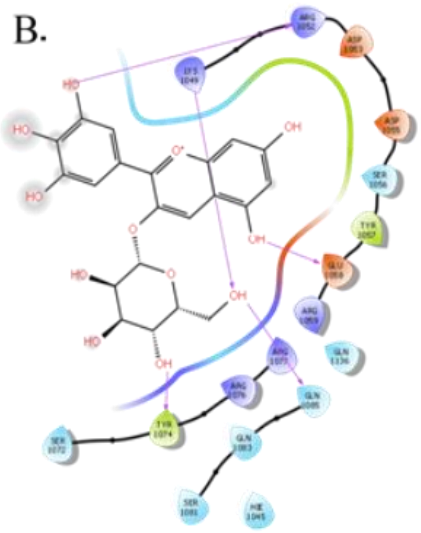


Figure 3. Illustration of the Ara h 2.01 sequence with indication of the five α-helices (blue) and 10 linear IgE binding epitopes (orange). Cysteines involved in a total of four disulfide bonds are marked in red (adapted from Mueller et al., 2011). Ara h 2.02 has an insertion of 12 amino acids containing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

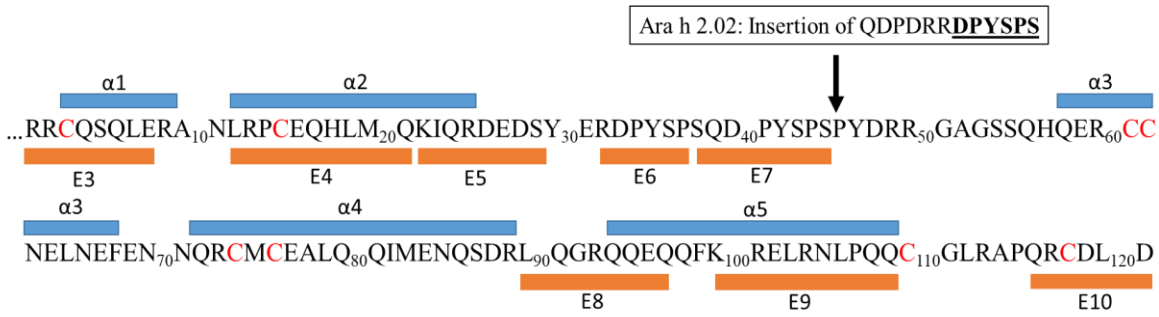

Ara h 2.02: Insertion of QDPDRRDPYSPS
α1
α2
α3
...RRCQSQLERA10NLRPCEQHLM20QKIQRDEDSY30ERDPYSPSQD40PYSPSPYDRR50GAGSSQHQER60CC
E3
E4
E5
E6
E7
α3
α4
α5
NELNEFEN70NQRCMCEALQ80QIMENQSDRL90QGRQQEQQFK100RELRNLPQQC110GLRAPQRCDL120D
E8
E9
E10

Figure 4. Dimer representation of Ara h 2.01-Ara h 2.01 (green and teal 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 kcal moL^{-1} at pH 7 on dimer FRODOCK3.

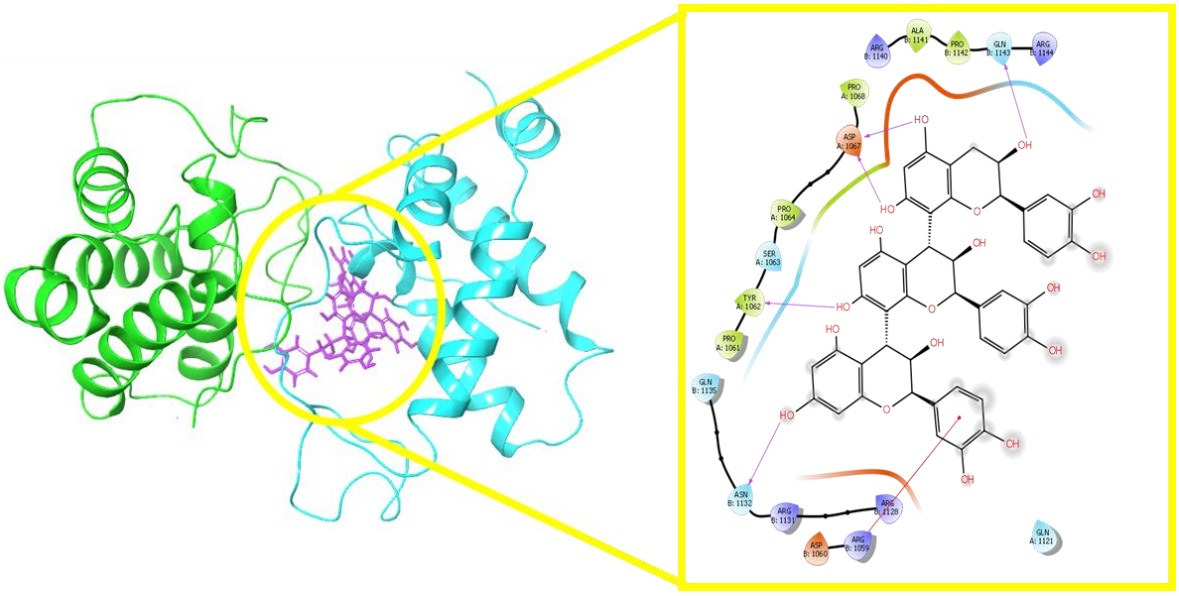

ARG B: 1140
ALA B: 1141
PRO B: 1142
GLN B: 1143
ARG B: 1144
PRO A: 1068
ASP A: 1067
PRO A: 1064
SER A: 1063
TYR A: 1062
PRO A: 1061
GLN B: 1135
ASN B: 1132
ARG B: 1131
ARG B: 1128
ASP B: 1060
ARG B: 1059
GLN A: 1121
HO
OH
O

Figure 5. Chemical structures of delphinidin-3-glucoside (A), cyanidin-3-glucoside (B), procyanidin C1 (C), chlorogenic acid (D), and benzoic acid (E) at pH 7.

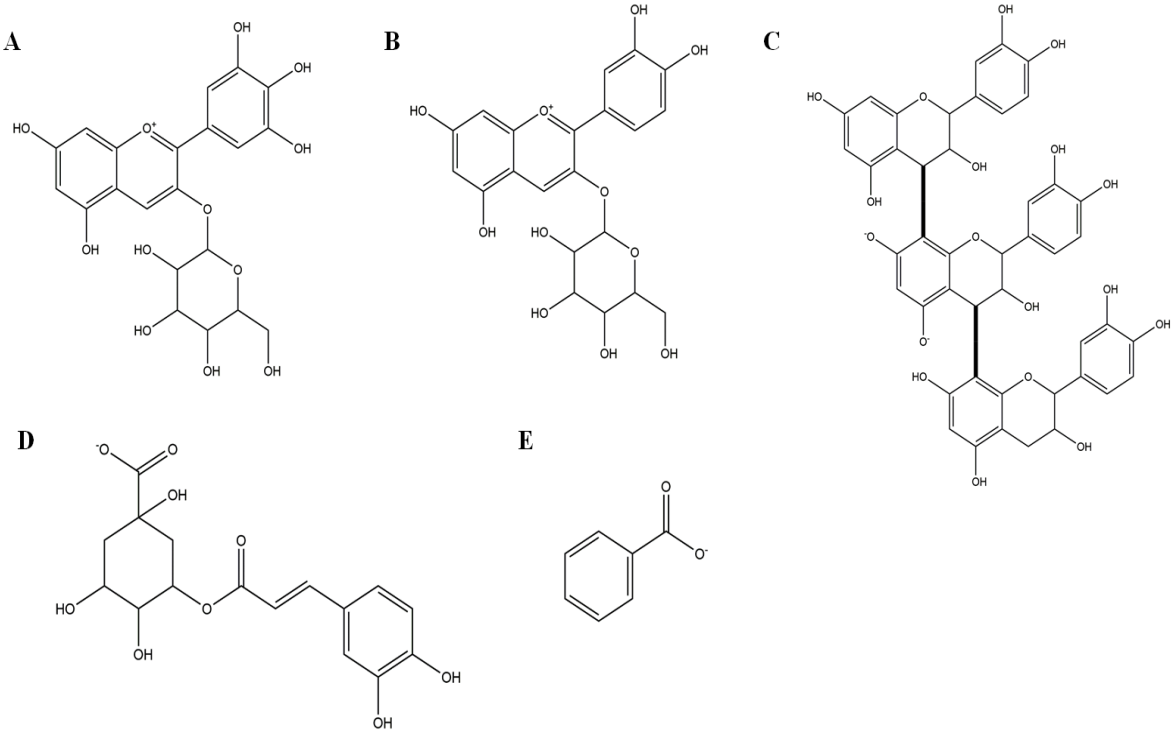

A
B
C
D
E

Figure 6. (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 molar ratio); (B) spectra of Ara h 2 and Ara h 2-chlorogenic acid (1:10 molar ratio); (C) spectra of Ara h 2 and Ara h 2-benzoic acid (1:19 molar ratio). (D-F) UV-Vis absorption spectra of polyphenols before and after addition of Ara h 2. (D) spectra of procyanidin C1 and procyanidin C1 after Ara h 2 addition (molar ratio Ara h 2:procyanidin C1; 1:9); (E) spectra of chlorogenic acid and chlorogenic acid after Ara h 2 addition (molar ratio Ara h 2:chlorogenic acid; 1:13); (F) spectra of benzoic acid and benzoic acid after Ara h 2 addition (molar ratio Ara h 2:benzoic acid; 1:20). PAC, procyanidin.

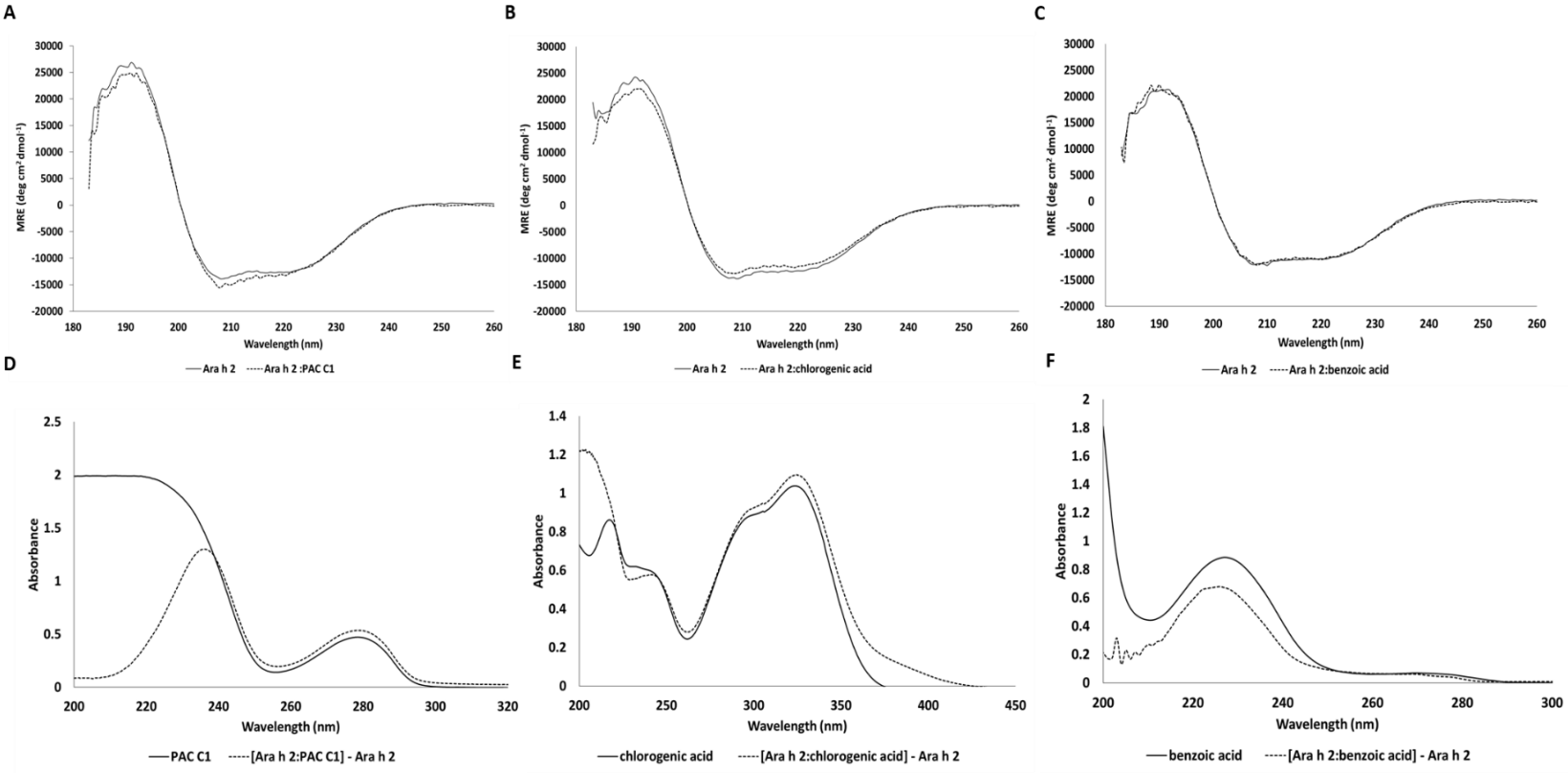

A
B
C
MRE (deg cm² dmol⁻¹)
Wavelength (nm)
Ara h 2
Ara h 2 :PAC C1
Ara h 2:chlorogenic acid
Ara h 2:benzoic acid
D
E
F
Absorbance
PAC C1
[Ara h 2:PAC C1] - Ara h 2
chlorogenic acid
[Ara h 2:chlorogenic acid] - Ara h 2
benzoic acid
[Ara h 2:benzoic acid] - Ara h 2

Figure 7. 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.

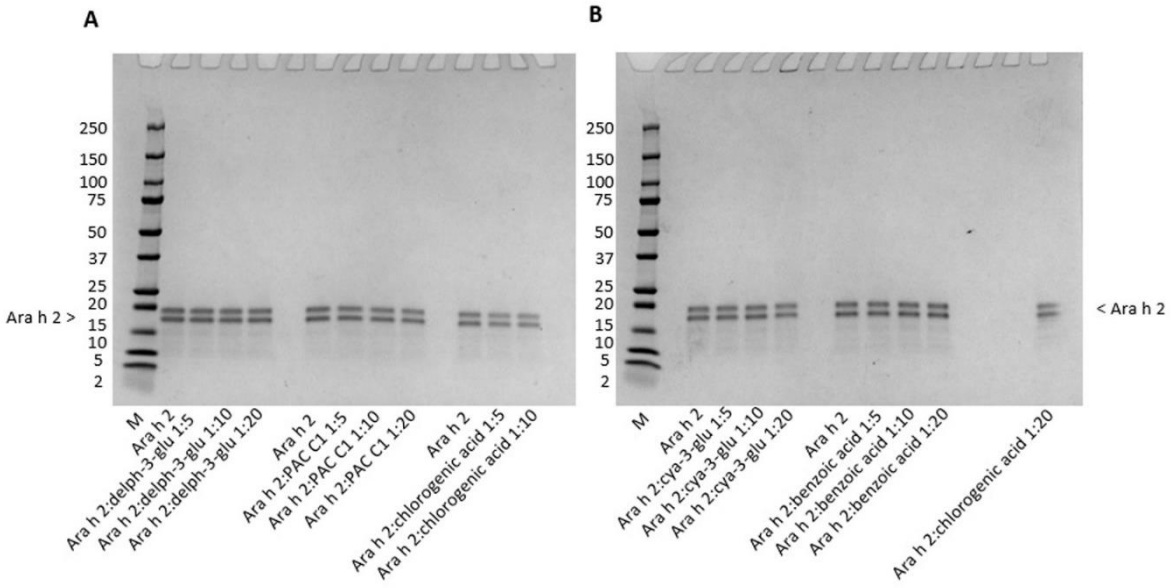
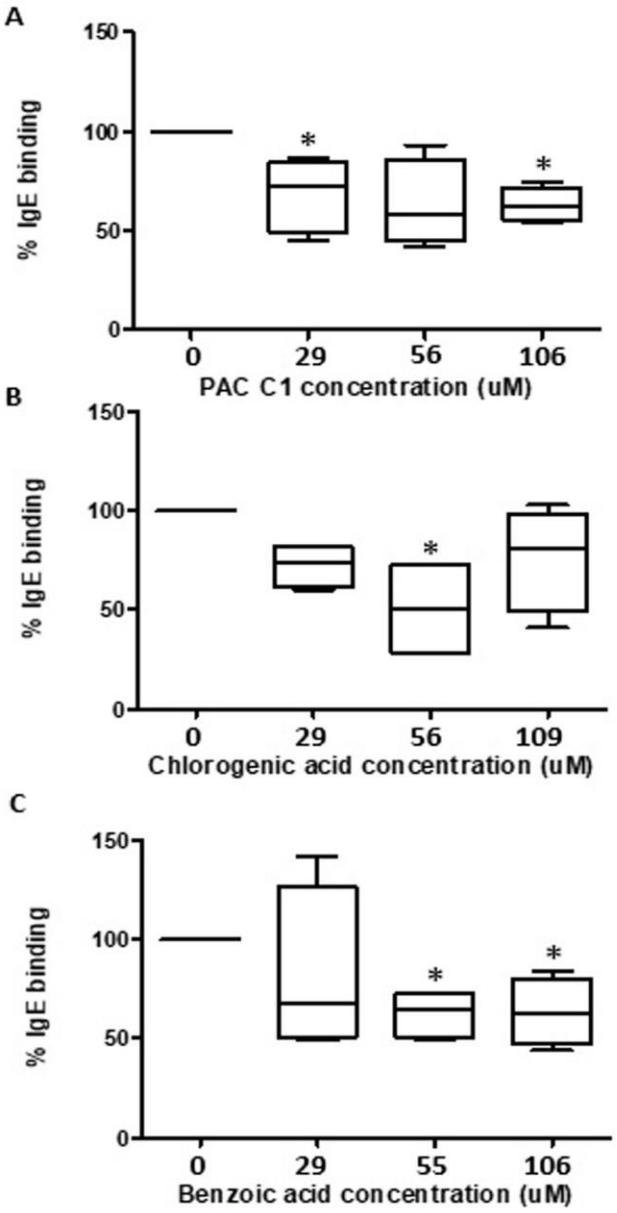

A
B
250
150
100
75
50
37
25
20
15
10
5
2
Ara h 2 >
< Ara h 2
M
Ara h 2
Ara h 2:delph-3-glu 1:5
Ara h 2:delph-3-glu 1:10
Ara h 2:delph-3-glu 1:20
Ara h 2
Ara h 2:PAC C1 1:5
Ara h 2:PAC C1 1:10
Ara h 2:PAC C1 1:20
Ara h 2
Ara h 2:chlorogenic acid 1:5
Ara h 2:chlorogenic acid 1:10
M
Ara h 2
Ara h 2:cya-3-glu 1:5
Ara h 2:cya-3-glu 1:10
Ara h 2:cya-3-glu 1:20
Ara h 2
Ara h 2:benzoic acid 1:5
Ara h 2:benzoic acid 1:10
Ara h 2:benzoic acid 1:20
Ara h 2:chlorogenic acid 1:20

Figure 8. Semi-quantitative analysis of IgE binding capacity (based on Western blots) of Ara h 2 (0 µM polyphenol, set as 100% IgE binding) and Ara h 2–polyphenol complexes containing different concentrations of polyphenol (µM) (mean of four replicates with SE; asterisk indicates significant difference compared to the Ara h 2 control at $p < 0.05$).


A
150
100
50
0
% IgE binding
*
*
0
29
56
106
PAC C1 concentration (uM)
B
150
100
50
0
% IgE binding
*
0
29
56
109
Chlorogenic acid concentration (uM)
C
150
100
50
0
% IgE binding
*
*
0
29
55
106
Benzoic acid concentration (uM)

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Supplemental material

Supplementary Table 1. 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

Supplementary Table 1 Continued

Compound	Molecular weight (g mol^{-1})	HBA score	HBD score
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

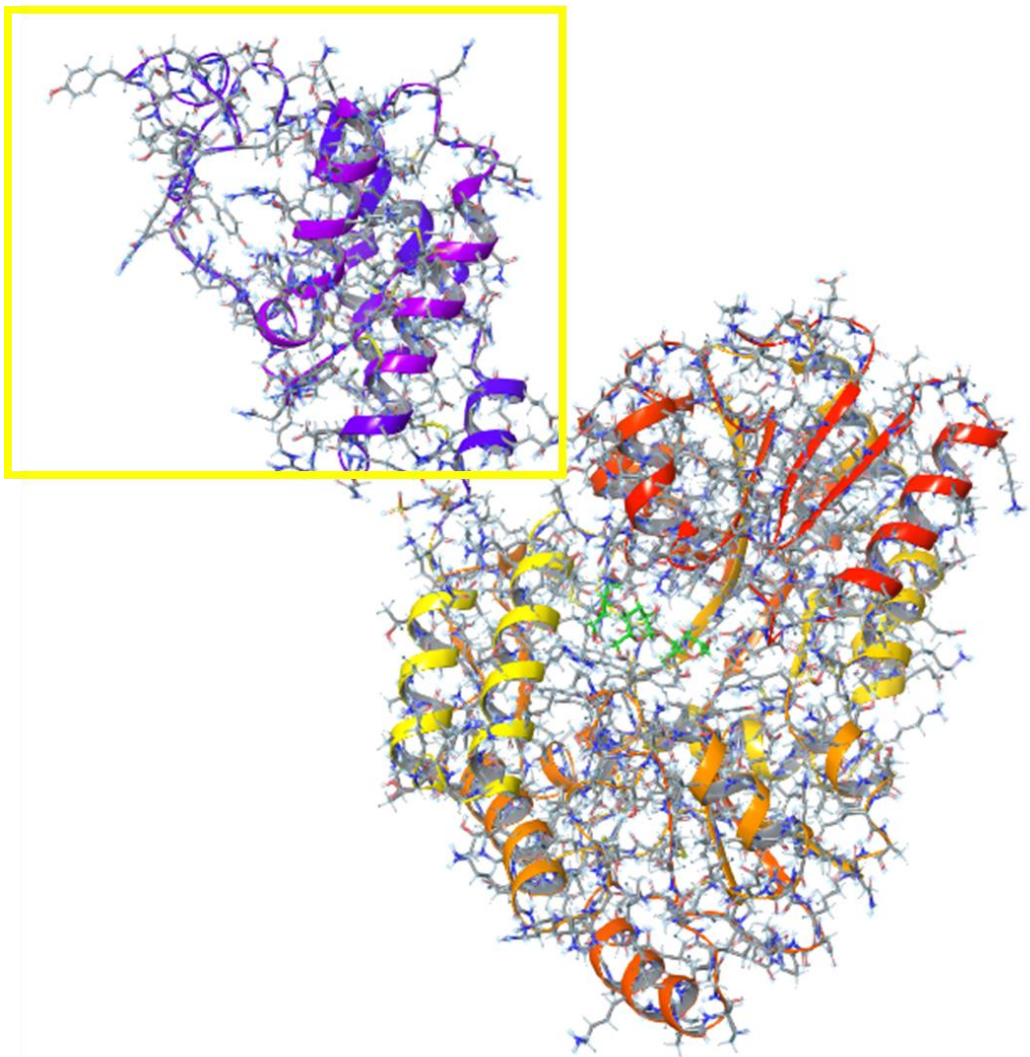
Supplementary Table 2. Average of XP Docking and eModel Scores for *SiteMap* and *BlindDocking* at pH 7

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

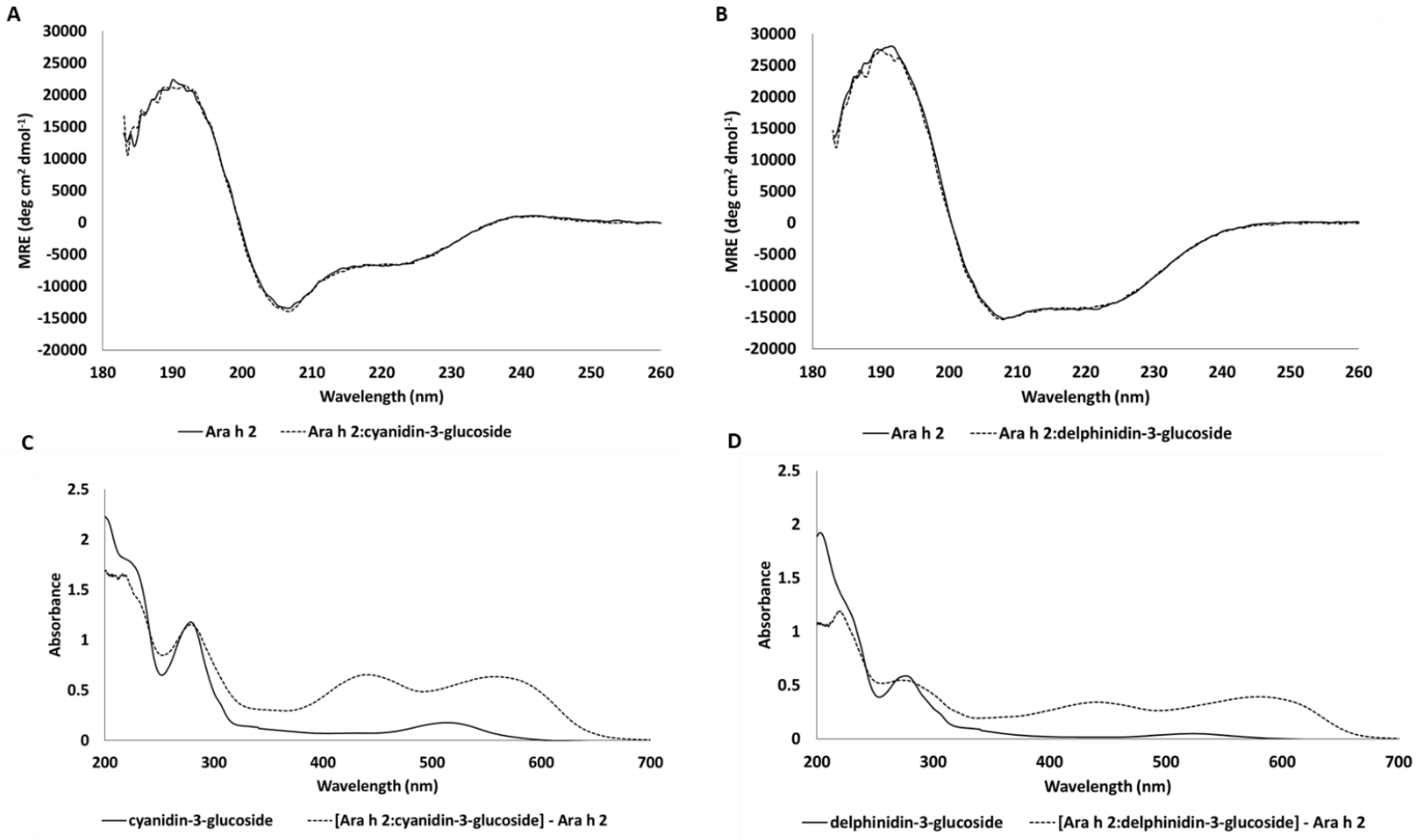
Supplementary Table 2 Continued

Compound	Docking Score	eModel Score
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

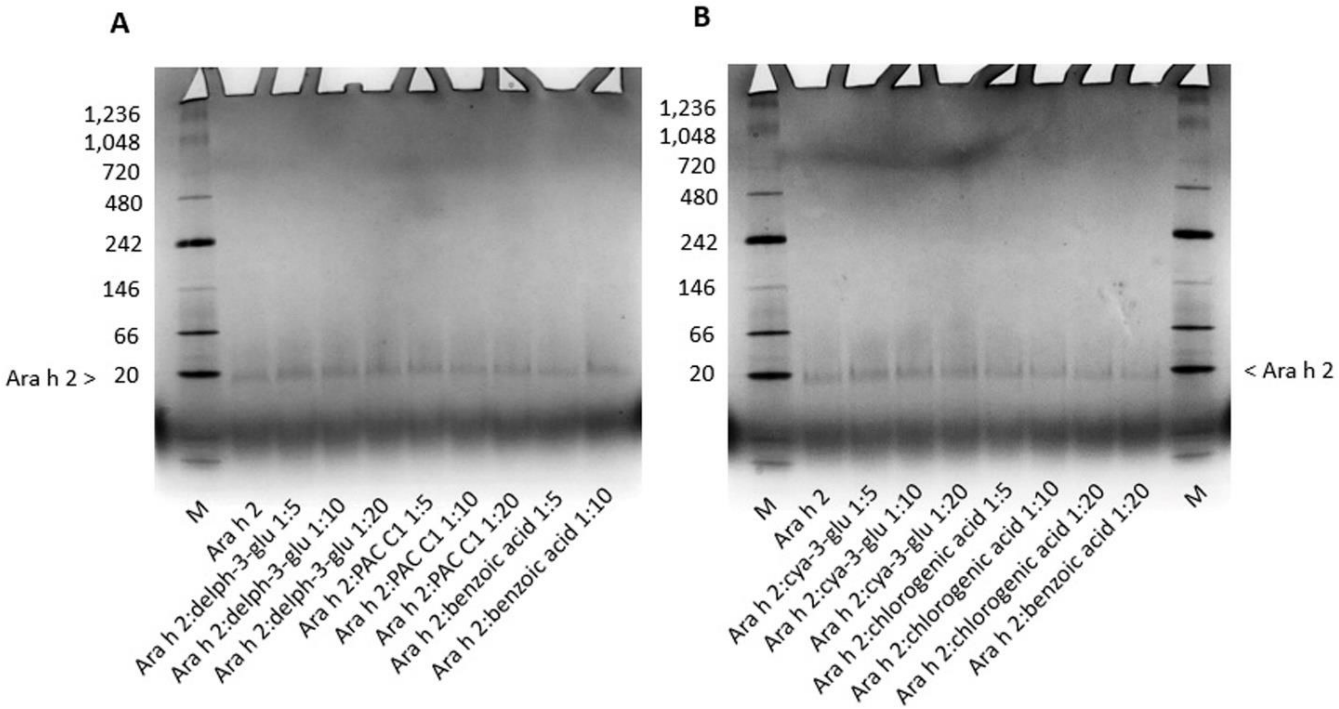
Supplementary Figure 1. PDB entry 3OB4: Maltose binding protein (MBP)-fusion protein of Ara h 2. Highlighted with a yellow square is Ara h 2.



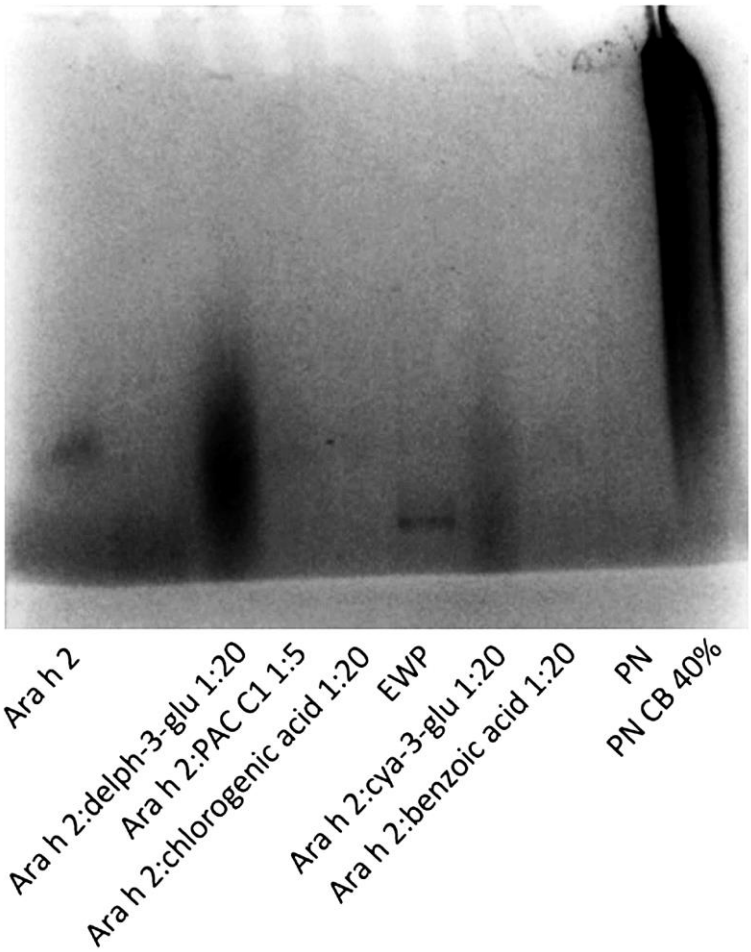
Supplementary Figure 2. (A and B) Circular dichroism spectra of unmodified Ara h 2 and Ara h 2-polyphenol mixtures. (A) Spectra of Ara h 2 and Ara h 2-cyanidin-3-glucoside (1:15 molar ratio); (B) spectra of Ara h 2 and Ara h 2-delphindin-3-glucoside (1:11 molar ratio); (C and D) UV-Vis absorption spectra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


A
MRE (deg cm² dmol⁻¹)
Wavelength (nm)
Ara h 2
Ara h 2:cyanidin-3-glucoside
B
MRE (deg cm² dmol⁻¹)
Wavelength (nm)
Ara h 2
Ara h 2:delphinidin-3-glucoside
C
Absorbance
Wavelength (nm)
cyanidin-3-glucoside
[Ara h 2:cyanidin-3-glucoside] - Ara h 2
D
Absorbance
Wavelength (nm)
delphinidin-3-glucoside
[Ara h 2:delphinidin-3-glucoside] - Ara h 2

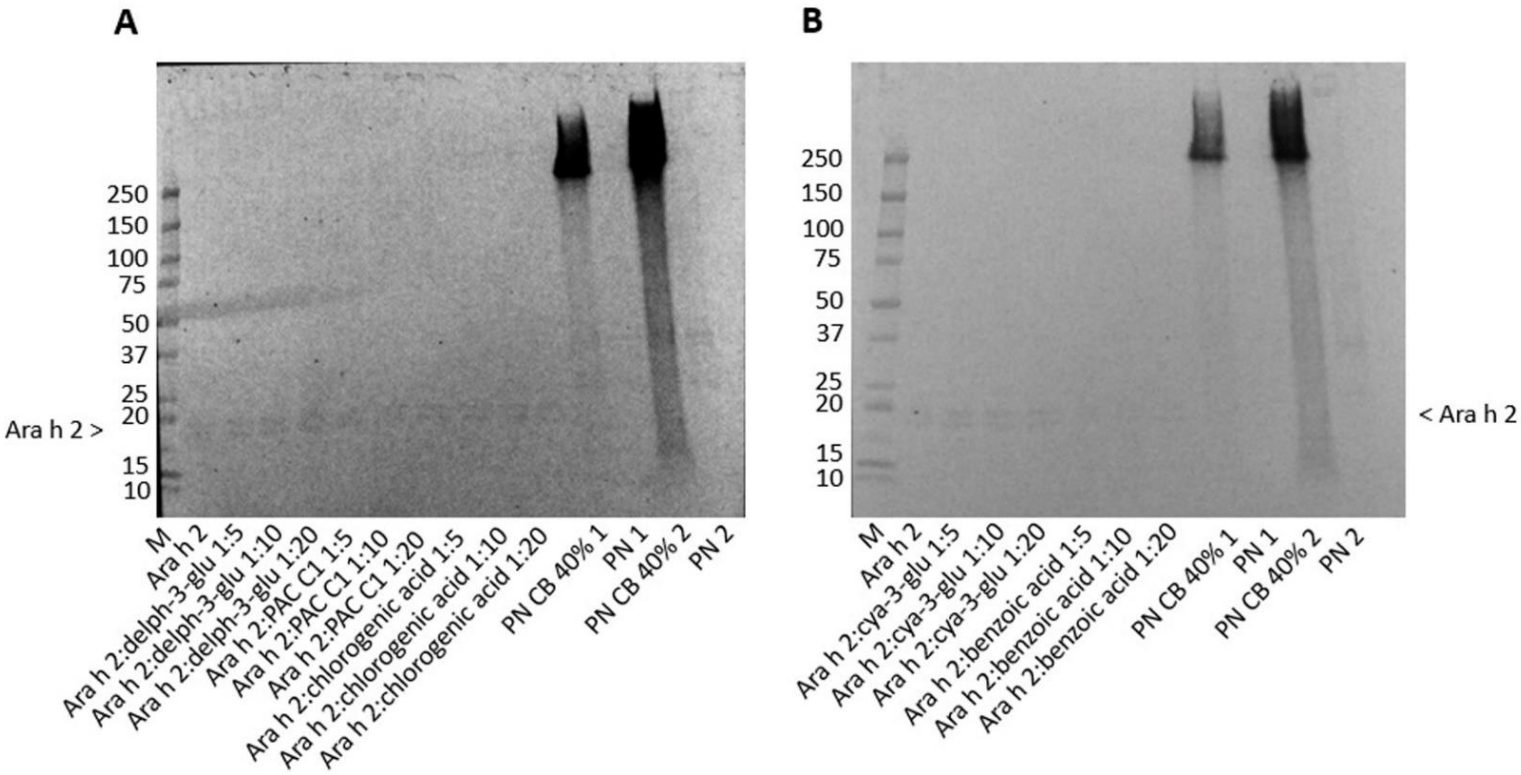
Supplementary Figure 3. Representative Native PAGE of Ara h 2 and Ara h 2:polyphenol complexes at 1:5, 1:10, and 1:20 molar ratios stained with Coomassie Brilliant Blue. M, molecular weight marker.


A
1,236
1,048
720
480
242
146
66
Ara h 2 > 20
M
Ara h 2
Ara h 2:delph-3-glu 1:5
Ara h 2:delph-3-glu 1:10
Ara h 2:delph-3-glu 1:20
Ara h 2:PAC C1 1:5
Ara h 2:PAC C1 1:10
Ara h 2:PAC C1 1:20
Ara h 2:benzoic acid 1:5
Ara h 2:benzoic acid 1:10
B
1,236
1,048
720
480
242
146
66
20
< Ara h 2
M
Ara h 2
Ara h 2:cya-3-glu 1:5
Ara h 2:cya-3-glu 1:10
Ara h 2:cya-3-glu 1:20
Ara h 2:chlorogenic acid 1:5
Ara h 2:chlorogenic acid 1:10
Ara h 2:chlorogenic acid 1:20
Ara h 2:benzoic acid 1:20
M

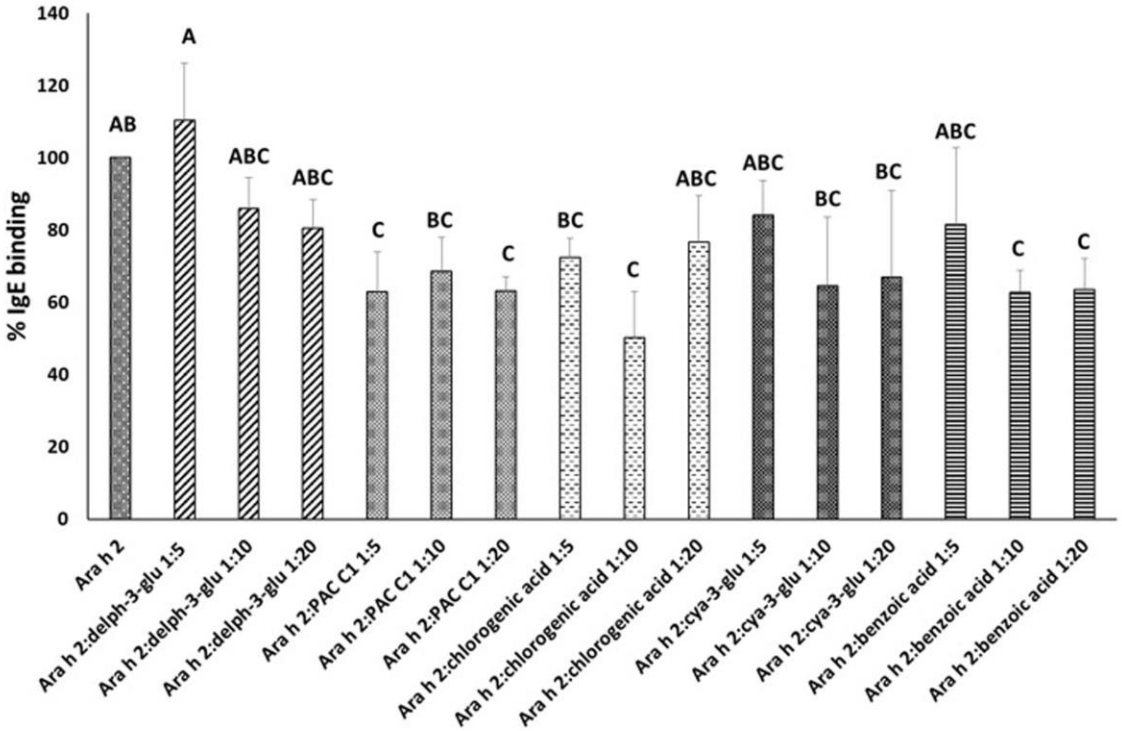
Supplementary Figure 4. Staining of some polyphenol-bound peanut proteins by NBT (nitroblue tetrazolium) following native PAGE and subsequent electrophoretic transfer to a PVDF membrane. Egg white protein (EWP) and peanut flour (PN) were used as negative controls and peanut flour complexed with 40% cranberry polyphenols (PN CB 40%) was used as a positive control.


Ara h 2
Ara h 2:delph-3-glu 1:20
Ara h 2:PAC C1 1:5
Ara h 2:chlorogenic acid 1:20
EWP
Ara h 2:cya-3-glu 1:20
Ara h 2:benzoic acid 1:20
PN
PN CB 40%

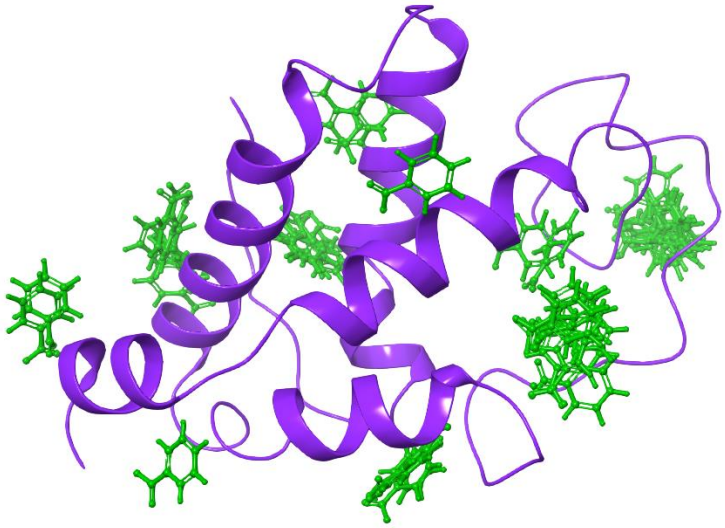
Supplementary Figure 5. Staining of polyphenol-bound peanut proteins by NBT (nitroblue tetrazolium) following SDS-PAGE and subsequent electrophoretic transfer to a PVDF membrane. Peanut flour containing 0.7 µg protein (PN 1) or 5 µg protein (PN 2) were used as negative controls and peanut flour complexed 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.


A
B
250
150
100
75
50
37
25
20
15
10
Ara h 2 >
< Ara h 2
M
Ara h 2
Ara h 2:delph-3-glu 1:5
Ara h 2:delph-3-glu 1:10
Ara h 2:delph-3-glu 1:20
Ara h 2:PAC C1 1:5
Ara h 2:PAC C1 1:10
Ara h 2:PAC C1 1:20
Ara h 2:chlorogenic acid 1:5
Ara h 2:chlorogenic acid 1:10
Ara h 2:chlorogenic acid 1:20
PN CB 40% 1
PN 1
PN CB 40% 2
PN 2
Ara h 2:cya-3-glu 1:5
Ara h 2:cya-3-glu 1:10
Ara h 2:cya-3-glu 1:20
Ara h 2:benzoic acid 1:5
Ara h 2:benzoic acid 1:10
Ara h 2:benzoic acid 1:20

Supplementary Figure 6. 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 $p < 0.05$).


% IgE binding
140
120
100
80
60
40
20
0
AB
A
ABC
ABC
C
BC
C
BC
C
ABC
ABC
BC
BC
ABC
C
C
Ara h 2
Ara h 2:delph-3-glu 1:5
Ara h 2:delph-3-glu 1:10
Ara h 2:delph-3-glu 1:20
Ara h 2:PAC C1 1:5
Ara h 2:PAC C1 1:10
Ara h 2:PAC C1 1:20
Ara h 2:chlorogenic acid 1:5
Ara h 2:chlorogenic acid 1:10
Ara h 2:chlorogenic acid 1:20
Ara h 2:cya-3-glu 1:5
Ara h 2:cya-3-glu 1:10
Ara h 2:cya-3-glu 1:20
Ara h 2:benzoic acid 1:5
Ara h 2:benzoic acid 1:10
Ara h 2:benzoic acid 1:20

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



CHAPTER 4

Conclusions and future perspectives

With this work, we successfully demonstrated that a mixture of different plant polyphenols as well as individual polyphenols can modulate the allergenicity of peanut allergens by binding to the allergenic proteins and inhibiting IgE binding epitopes from binding allergen-specific IgE. Our studies using polyphenol mixtures (extracts) derived from cranberry (*Vaccinium macrocarpon* Ait.) or lowbush blueberry (*Vaccinium angustifolium* Ait.) showed that peanut protein-polyphenol aggregate particles comprising higher concentrations of polyphenols correspond with a greater reduction of IgE binding capacity *in vitro.* Results suggested that the creation of high molecular weight protein-polyphenol covalently cross-linked particles (*aggregated* allergens) rendered IgE binding epitopes inaccessible for binding by peanut allergen-specific IgE. Results from experiments with the major peanut allergen Ara h 2 and polyphenols procyanidin C1, chlorogenic acid, and benzoic acid showed that an increase in (molar) polyphenol to protein ratio did not result in greater IgE binding epitope inhibition at the concentrations tested. This suggests that IgE binding epitopes on *un-aggregated* proteins were inhibited by bound polyphenols. It is possible that a few polyphenols bound to Ara h 2 molecules but didn't cross-link them to form bigger particles. We also observed changes in α-helical content by circular dichroism (CD) which implies that local changes in native Ara h 2 secondary structure occurred upon polyphenol binding.

Besides the protein:polyphenol ratios tested (1:5, 1:10, and 1:20), it needs to be evaluated if one molecule polyphenol binding to one molecule Ara h 2 (1:1 molar ratio) also results in IgE binding epitope inhibition. Higher concentrations should also be tested to evaluate if the addition of more polyphenol molecules results in a greater decrease of IgE binding. Stoichiometry required for peanut allergen binding and subsequent IgE binding

epitope inhibition can be evaluated by mass spectrometry (MS). It has been shown that ESI (electron spray ionization)-MS can be used to investigate non-covalent stable protein-polyphenol complexes. Binding stoichiometry (number of protein-bound polyphenols) and affinity (binding strength between polyphenols and protein) can be determined this way. Such experiments may shed light onto possible common polyphenol structural and/or chemical characteristics required for allergen binding.

While our findings suggest that different classes of polyphenols both non-covalently and covalently bound to peanut allergens, future studies can provide more insight into polyphenol binding mechanisms. This particularly would facilitate elucidation of binding mechanisms of benzoic acid to Ara h 2. Non-covalent interactions between proteins and polyphenols can be evaluated by several methods including equilibrium dialysis. This technique allows a researcher to determine the structure/properties relationship of both polyphenols and proteins and to calculate the number of polyphenols bound per protein. Implications for covalent protein modifications by polyphenols can be obtained by performing a full amino acid analysis of the protein-polyphenol complex and subsequent comparison of results with those obtained for the unmodified protein. Modification of amino acids side chains can be evaluated this way. Protein denaturation and fluorescence measurement can also be used for the estimation of a tryptophan modification (e.g. was the amino acid residue tryptophan covalently bound with oxidized polyphenol?). This method takes advantage of tryptophan's natural strong UV light absorption at 295 nm and subsequent emission of fluorescent light (320-390 nm). If a polyphenol covalently bound to a tryptophan residue the fluorescence of tryptophan is quenched. To confirm that binding occurred

covalently, proteins are generally denatured using high concentrations of urea (breaks non-covalent interactions).

In addition, more studies on polyphenol binding to native Ara h 2 would give valuable information on tertiary structural changes upon polyphenol binding. This can be done by CD spectroscopy in the near UV range (250 – 320 nm). The CD spectrum in that wavelength range reflects the environments of the aromatic amino acid side chains tryptophan, tyrosine, and phenylalanine, and thus gives information about the tertiary structure of the protein. Further, conformational IgE binding epitope inhibition can be investigated by ELISA (enzyme-linked immunosorbent assay) or by cell culture techniques (e.g. using RBL-2H3 cell line).

Additional investigations also need to address where polyphenols bind on peanut allergens (i.e. which IgE binding epitopes are inhibited from binding to allergen-specific IgE?). Nuclear magnetic resonance (NMR) spectroscopy could be employed to gather precise identification of polyphenol binding sites, mechanisms of interaction, number of binding sites, and dissociation constants.

Further, comprehensive simulated *in vitro* digestion models including physically relevant proteolytic enzymes such as pepsin, trypsin, or chymotrypsin can be a means to mimic digestion and can be used to get insight into the degradation behavior of Ara h 2-procyanidin C1, chlorogenic acid, or benzoic acid complexes and the allergenic potential of digested peptides.

Finally, future experiments should further investigate the immunomodulatory mechanisms of these protein–polyphenol aggregate particles *in vitro*, *in vivo*, and ultimately in humans in clinical trials. For example, exposing antigen presenting cells (e.g. dendritic

cells) to Ara h 2-polyphenol complexes can give insight into cellular uptake of the complexes, cell internal degradation of the complexes, presentation of the resulting digested peptides, and respective T cell responses. *In vivo* work using animal models of food allergy (e.g. peanut-allergic mice) will be required to evaluate if the reduced IgE binding capacity of protein-polyphenol aggregate particles containing different concentrations of polyphenols observed *in vitro* exclusively results in polyphenol concentration-dependent decreased cross-linking with mast or basophil cells, their degranulation and ultimately reduced allergic reactions *in vivo*. Animal models could also shed light onto possible mechanisms of hypoallergenic particles *in vivo* (e.g. are particles less allergenic because the majority of particles bypass enzymatic digestion and sampling in the gastrointestinal tract, and leave the body as high molecular weight aggregated protein-polyphenol particles?). For this, feces could be collected after oral feeding challenges and analyzed for protein content and/or presence of polyphenols.

Ultimately, trials in humans will be needed to assess whether protein-polyphenol aggregate particles result in reduced or alleviated allergic reactions, and potentially promote allergen desensitization in sensitive individuals (possible application in oral immunotherapy).

APPENDIX

APPENDIX A

Protein-bound polyphenols create “ghost” band artifacts during chemiluminescence-based antigen detection

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RESEARCH NOTE

REVISED Protein-bound polyphenols create "ghost" band artifacts during chemiluminescence-based antigen detection [version 2; referees: 2 approved, 1 approved with reservations]

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Abstract

Antigen detection during Western blotting commonly utilizes a horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescent substrate. We utilized this technique to examine the impact of green tea-derived polyphenols on the binding of egg white protein-specific IgE antibodies from allergic human plasma to their cognate antigens. Our experiments unexpectedly showed that green tea-derived polyphenols, when stably complexed with egg white proteins, caused "ghost" band formation in the presence of horseradish peroxide. This study suggests that caution should be taken when evaluating polyphenol-bound proteins by enhanced chemiluminescence Western blotting using horseradish peroxidase and demonstrates that protein-bound polyphenols can be a source of "ghost" band artifacts on Western blots.

Open Peer Review

Referee Status: ✔ ? ✔

	Invited Referees 1	2	3
REVISED **version 2** published 26 May 2017	✔ report		✔ report
version 1 published 13 Mar 2017	? report	? report	

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REVISED **Amendments from Version 1**

The authors have revised the manuscript based on referees' suggestions to create a new version that is more clear and streamlined. The following revisions were made:

1. Part of the introduction was revised to emphasize focus on HRP artifacts to coincide with the manuscript title
2. The term "hyperactivate/d" for HRP was removed and reworded, since there is no evidence for HRP hyperactivation
3. Authors' previous work containing "ghost" band artifacts was discussed
4. The NBT method was slightly reworded to increase clarity
5. The last paragraph of the immunoblotting method was slightly reworded to increase clarity
6. Additional proteins stained by NBT (Figure 1B) that were poorly stained by CBB were indicated with stars in the figure and text
7. The term "secondary HRP conjugated antibody" was rephrased to "biotinylated goat IgG anti-human IgE secondary antibody-NeutrAvidin HRP conjugate" for clarity
8. CBB staining of proteins on gels is now mentioned in Supplementary Figure S1

See referee reports

Introduction

Western blotting has been used extensively to identify and quantify relative amounts of specific proteins in complex mixtures. Proteins are identified using antigen-specific primary antibodies followed by various enzyme-coupled secondary antibodies. Commonly used conjugated enzymes are alkaline phosphatase and horseradish peroxidase (HRP)[1]. HRP is more popular due to its stability and smaller size, which allows for conjugation of multiple HRP moieties per secondary antibody and increased sensitivity[2]. Avidin-biotin systems can also be used to amplify reactivity and luminol-based enzyme substrates are commonly used to create a visible chemiluminescent signal.

We recently evaluated peanut protein-polyphenol aggregate particles for their binding capacity to peanut-specific plasma IgE from allergic patients using complementary assays, including chemiluminescence-based Western blotting[3]. Previous studies have shown "ghost" bands on some blots. In the present study, we demonstrate that protein-bound polyphenols can cause "ghost" band artifacts during chemiluminescence-based antigen detection. We investigated the binding of IgE antibodies to hen egg white proteins complexed with green tea-derived polyphenols. For detection on the blots, we used primary antibodies from allergic human plasma, secondary biotin-coupled goat anti-human IgE, avidin-HRP, and an enhanced luminol substrate. Results showed that HRP is required for "ghost" band formation. Caution should be taken when evaluating polyphenol-bound proteins by enhanced chemiluminescence Western blotting.

Methods

Materials

Precast mini TGX 4–20% polyacrylamide gels were purchased from BioRad (Hercules, CA, USA). Nitroblue tetrazolium and glycine were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). All other SDS-PAGE and immunoblotting reagents used are listed elsewhere[3]. Egg white protein (EWP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercially available organic dry green tea leaves (*Camellia sinensis* [L.] Kuntze) were provided by QTrade Teas & Herbs (Cerritos, CA, USA). Ground leaves were extracted and stored until further use as previously described[1]. Extraction was performed for 2 h at 80°C.

Preparation of egg white protein-green tea polyphenol aggregate particles

The total phenolic content in the green tea extract was determined (36.8 mg mL^{-1} ± 0.26 mg mL^{-1}, see Table S1) according to the 96-well microplate-adapted Folin-Ciocalteu method by Zhang *et al.*[4] with modifications described by Herald *et al.*[5]. The amount of extract (mL) and protein powder (g) required to generate dry, stable protein-polyphenol aggregate particles containing 5, 10, 15, 30, or 40% polyphenols after complexation was added together and mixed under constant agitation for 15 min at room temperature. Mixtures were subsequently frozen at -20°C and freeze-dried (FreeZone12, Labconco, Kansas City, MO, USA) to form stable protein-polyphenol aggregate particles.

Nitroblue tetrazolium (NBT) staining to reveal polyphenols

Following transfer of proteins by electroblotting from unmodified EWP and aggregate particles to a polyvinylidene difluoride (PVDF) membrane, the membrane was briefly hydrated in 100% methanol. Subsequently, polyphenol-modified proteins were detected with NBT and glycinate as described by Hagerman [6; www.users.muohio.edu/hagermae/]. At alkaline pH, the catechol moiety of polyphenols catalyzes redox-cycling in the presence of glycinate, generating superoxide that reduces NBT to insoluble, visible formazan[7].

SDS-PAGE and immunoblotting

Amounts of protein-polyphenol aggregate particles or unmodified EWP were normalized to provide 2 mg protein for SDS-PAGE. Samples were prepared in sample loading buffer containing 5% β-mercaptoethanol, resulting in 10 µg protein in 10 µL. Samples (10 µg protein/10 µL) were incubated for 5 min at 95°C, loaded onto a gel, run (40 min at 200 V), and then stained with Coomassie Brilliant Blue (CBB). The immunoblotting method used, including reagent sources, is described elsewhere[3]. The following minor modifications were made: Pooled human plasma (containing polyclonal antibodies, among them egg white-specific IgE) from 7 egg white-allergic individuals (PlasmaLab International, Everett, WA, USA; 1:80; v/v) was used to bind antigens on the membrane. EWP-specific IgE levels ranged from 15.4 to 100 kU L^{-1} as determined via ImmunoCAP (Phadia, Uppsula, Sweden). Biotinylated polyclonal goat IgG anti-human IgE (Kirkegaard & Perry Laboratory, Inc., reference no. 01-10-04, Gaithersburg, MD, USA; 1:8,000; v/v)

and NeutrAvidin HRP conjugate (Thermo Scientific, Rockford, IL, USA; 1:20,000; v/v) were used to bind plasma antibodies.

In separate experiments, proteins in aggregate particles containing 15% polyphenols were blotted onto a PVDF membrane. The membrane was subsequently cut into strips and subjected to various combinations of immunoblotting reagents. Transferred proteins from unmodified EWP served as controls. The proteins from unmodified EWP were subjected to the full immunoblotting procedure.

Results and discussion

Protein distribution, NBT staining, and IgE binding capacity

The major EWPs ovotransferrin (76.6 kDa), ovalbumin (45 kDa) and lysozyme (14.3 kDa)[8] from both aggregate particles and unmodified EWP were separated by SDS-PAGE and identified by staining with CBB (Figure 1A). An increase in molecular weight of ovotransferrin and ovalbumin, but not of lysozyme, was observed and this was polyphenol concentration dependent (Figure 1A). In fact, NBT staining indicated that ovalbumin and ovotransferrin, but not lysozyme were modified by polyphenols and the degree of staining was dependent on the concentration of polyphenol (Figure 1B). The staining also revealed several additional proteins poorly stained with CBB (indicated with stars), suggesting that the NBT staining of polyphenols more sensitively reveals the presence of protein than does CBB staining. As expected, control EWP did not react with NBT (Figure 1B). The finding that polyphenols remain bound to proteins following SDS-PAGE and membrane transfer suggests a strong, perhaps covalent association between the molecules.

As shown in Figure 1C, ovotransferrin, ovalbumin and lysozyme in unmodified EWP were recognized by antigen-specific IgE antibodies from human plasma. However, for protein samples that contained polyphenols, ovotransferrin and ovalbumin as well as several of the proteins revealed by NBT but not CBB staining, appeared as white "ghost" bands (Figure 1C). Generally, "ghost" bands occur when the substrate is depleted quickly by the enzyme at that location and ceases to produce light[2]. Commonly, this is a result of a high concentration of one or more of the components of the enzymatic reaction. However, in this case, the phenomenon was not observed for the EWP control sample (which did not contain polyphenols) and increased with increasing amount of polyphenols, suggesting that the polyphenols are triggering the excessive consumption of substrate and appearance of the "ghost" bands. The phenomenon was also observed with other aggregate particles including whey protein isolate-green tea polyphenol and whey protein isolate-blueberry polyphenol aggregate particles (see Figure S1) indicating that "ghosting" was not dependent on specific EWPs. "Ghost" bands also occurred on a few blots in our previously published work, however, this did not affect data interpretation[3,9]. The same treatments were re-tested by fluorescence-based Western blotting and the data was consistent with that previously reported.

To further investigate the mechanism underlying "ghost" band formation on those blots, PVDF membrane-transferred unmodified and polyphenol-modified EWPs underwent treatment with a combination of different immunoblotting reagents. Results revealed that polyphenols promoted "ghost" band formation by interacting with HRP during HRP-substrate reactions (Figure 2). "Ghost" bands were only observed on membrane strips containing green tea polyphenols and HRP (Figure 2B, D, and G) and only HRP was required to produce "ghost" bands with polyphenol-modified EWPs (Figure 2G). No "ghost" bands were observed

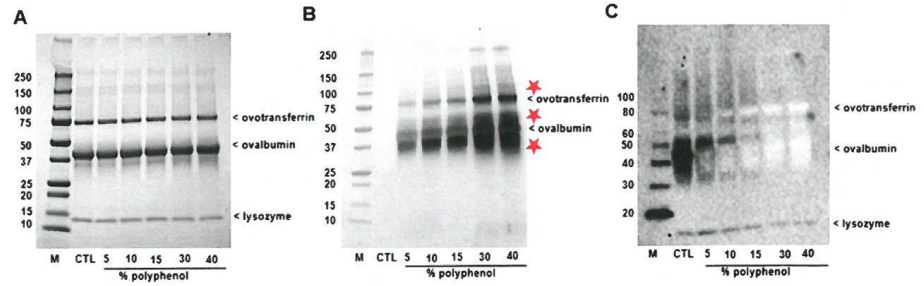

Figure 1. Protein distribution visualized by Coomassie Brilliant Blue staining (CBB), nitroblue tetrazolium (NBT) staining, and IgE binding capacity. (**A**) SDS-PAGE of unmodified egg white protein (CTL) or egg white protein-polyphenol aggregate particles containing 5, 10, 15, 30, and 40% polyphenols and stained with CBB; (**B**) Staining of green tea polyphenol-bound egg white proteins by NBT, following SDS-PAGE and subsequent electrophoretic transfer to a PVDF membrane; (**C**) corresponding Western blot. Pooled human plasma from 7 egg white-allergic individuals was used to bind antigens on the membrane. Egg white-specific IgE levels ranged from 15.4 to 100 kU L^{-1} as determined via ImmunoCAP (Phadia, Uppsula, Sweden). Biotinylated goat IgG anti-human IgE was used as the secondary antibody and NeutrAvidin HRP conjugate and substrate were used for signal production. M: molecular weight marker (kDa); CTL: control (unmodified egg white protein). Approximate locations for egg white allergens are indicated. Gray scale was used for gels and membranes and contrast was optimized to improve visualization.

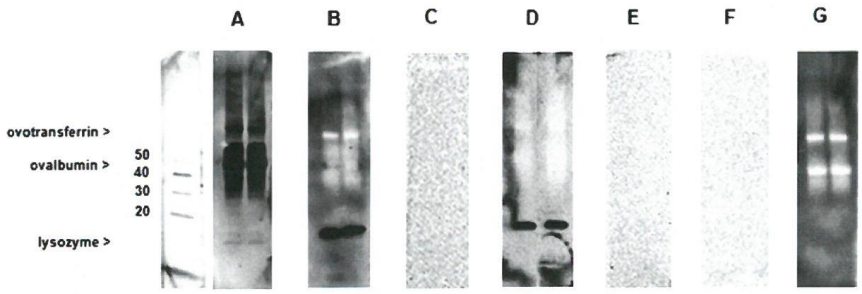

Figure 2. Evaluation of horseradish peroxidase hyperactivation by polyphenols. Western blot strips of (**A**) unmodified egg white proteins and (**B**–**G**) egg white protein-green tea polyphenol aggregate particles containing 15% total polyphenol content, after various immunoblotting treatments. (**B**) received all immunoblotting reagents after membrane blocking - primary antibody (pooled human plasma from 7 egg white allergic individuals with egg white-specific IgE levels ranging from 15.4 to 100 kU L^{-1}), biotinylated goat IgG anti-human IgE secondary antibody, NeutrAvidin HRP conjugate, and substrate; (**C**) the secondary antibody and NeutrAvidin HRP conjugate were omitted; (**D**) the primary antibody was omitted and (**E**) the primary and secondary antibody and NeutrAvidin HRP conjugate were omitted; (**F**) the primary antibody and NeutrAvidin HRP conjugate were omitted and (**G**) the primary antibody and secondary antibody were omitted. A molecular weight marker (kDa) is shown on the far left. Approximate locations for egg white allergens are indicated. Gray scale was used and contrast was optimized to improve visualization.

when substrate alone was added to a membrane containing polyphenol-bound proteins (Figure 2E). It should be noted that the light background in Figure 2C, E, and F is caused through a different mechanism than white "ghost" bands seen in B, D, and G. Since HRP is required for signal production, antibody-bound proteins on membranes not exposed to HRP (Figure 2C, E, and F) were not detected, hence, the membrane appeared blank when imaged (grey spotting is an imaging artifact). In contrast, on membranes that were treated with HRP and contained polyphenols (Figure 2B, D, and G), polyphenol-bound proteins appeared as white "ghost" bands due to depletion of locally available substrate and subsequent cessation of local light production. Interestingly, the lysozyme band was unaffected and apparently represents another artifact. This band did not require the presence of the primary antibody (Figure 2D), indicating it occurs due to a non-specific reaction between the biotinylated goat IgG anti-human IgE secondary antibody-NeutrAvidin HRP conjugate and the substrate. Further, the intensity of this band increased in the presence of polyphenols (Figure 2A, B and D), which seems contradictory since the NBT stain did not indicate polyphenols bound to lysozyme (Figure 1B). It is possible that in the presence of polyphenols, specific binding of primary and therefore secondary antibodies to proteins may be reduced resulting in excess free secondary antibodies to bind lysozyme (which did not contain bound polyphenols).

Based on this experiment, exact mechanisms of HRP promotion by polyphenols cannot be determined. It is possible, based on the fact that polyphenols are able to act as "bridges" between proteins[10], that HRP non-specifically binds to protein-bound polyphenols at high concentrations, therefore rapidly depleting substrate (luminol) in close proximity to the enzyme. Further, it is possible that protein-bound polyphenols are able to promote HRP activity, as has been observed similarly with digestive enzymes[11]. In both cases, this could result in the cessation of light emittance (depletion of locally available luminol).

It is important to note that the observations made in this study applied to a specific set of protein samples, secondary antibody, enzyme and chemiluminescence substrate. Other types of conjugated or unconjugated secondary antibodies, enzymes (e.g. alkaline phosphatase), or substrates have not been evaluated. However, while proper Western blot experimental designs include appropriate controls such as evaluation of unmodified proteins or antibody-antigen specificity, no control for protein-bound polyphenols as shown above has been described to date. The present study highlights the importance of evaluating polyphenol effects on chemiluminescence-based antigen detection in order to prevent false interpretation of data and reveals a new source of "ghost" band artifacts.

Conclusion

We demonstrated that when attempting to evaluate IgE binding capacity of EWP-green tea polyphenol aggregate particles by enhanced chemiluminescence-based Western blotting, polyphenols which remained bound to egg white proteins after electrophoretic transfer to PVDF membrane created "ghost" bands in the presence of HRP. This study reveals protein-bound ligands as an unintended source of "ghost" band artifacts, and suggests that caution should be taken when evaluating polyphenol-bound proteins by enhanced chemiluminescence Western blotting.

Dataset 1. Raw data for Figure 1. Protein distribution visualized by Coomassie Brilliant Blue staining (CBB), nitroblue tetrazolium (NBT) staining, and IgE binding capacity

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(Full legend and table are in the file).

Dataset 2. Raw data for Figure 2. Evaluation of horseradish peroxidase hyperactivation by polyphenols

http://dx.doi.org/10.5256/f1000research.10622.d152367

(Full legend and table are in the file).

Dataset 3. Raw data for Supplementary figure S1. Protein distribution, nitroblue tetrazolium (NBT) staining, and IgE binding capacity

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(Full legend and table are in the file).

Data availability

Dataset 1: Raw data for Figure 1. Protein distribution visualized by Coomassie Brilliant Blue staining (CBB), nitroblue tetrazolium (NBT) staining, and IgE binding capacity. (Full legend and table are in the file).

DOI, 10.5256/f1000research.10622.d152366[12]

Dataset 2: Raw data for Figure 2. Evaluation of horseradish peroxidase hyperactivation by polyphenols. (Full legend and table are in the file).

DOI, 10.5256/f1000research.10622.d152367[13]

Dataset 3: Raw data for Supplementary Figure S1. Protein distribution, nitroblue tetrazolium (NBT) staining, and IgE binding capacity. (Full legend and table are in the file).

DOI, 10.5256/f1000research.10622.d162634[14]

Author contributions

NJP carried out the research, contributed to experimental design and wrote a first draft of the paper. MAL served as corresponding author and contributed to the preparation of the manuscript. EAF contributed to the design of experiments and provided expertise in protein chemistry. SML helped design experiments, shared expertise in immunology and was involved in manuscript preparation. All authors were involved in manuscript revision and have agreed to the final content.

Competing interests

No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supplementary material

Figure S1: Protein distribution, nitroblue tetrazolium (NBT) staining, and IgE binding capacity. (Full legend and table are in the file).

Click here to access the data.

Table S1: Replicate measurements of green tea extract for total phenolic content. SD: standard deviation.

Click here to access the data.

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Data Source

13. Lila MA, Plundrich N, Foegeding E, *et al.*: **Dataset 2 in: Protein-bound polyphenols create "ghost" band artifacts during chemiluminescence-based antigen detection.** *F1000Research.* 2017.
Data Source

14. Lila MA, Plundrich N, Foegeding E, *et al.*: **Dataset 3 in: Protein-bound polyphenols create "ghost" band artifacts during chemiluminescence-based antigen detection.** *F1000Research.* 2017.
Data Source