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Development of novel therapeutic approaches: (I) Targeting allergic responses to peanuts and (II) Inhibition of DPP-IV enzyme involved in diabetes

Savan V. Patel
Rowan University

DEVELOPMENT OF NOVEL THERAPEUTIC APPROACHES: (I) TARGETING ALLERGIC RESPONSES TO PEANUTS AND (II) INHIBITION OF DPP-IV ENZYME INVOLVED IN DIABETES

by

Savan V. Patel

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Dedications

I dedicate this work to the love of my life and my family. Thank you for being there for me during the ups and down.

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I would like to acknowledge Dr. Catherine F. Yang for accepting me as her student and bestowing upon me her teachings, insights and experiences that guided me through this project. I would also like to acknowledge Dr. Gregory A. Caputo and Dr. Gustavo Moura-Letts for their knowledge and guidance during pivotal times. A big thank you to all the lab technicians and staff of Rowan University who I have had the pleasure of asking for help from the smallest to the biggest tasks.

Abstract

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Catherine F. Yang, Ph.D.
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There is an increase in prevalence of peanut allergy, especially in the western world. The only current treatment for peanut allergy is avoidance of peanut from diet and contact. Additionally, there is currently no specific vaccine that can be taken to decrease peanut allergies. The method of desensitizing a person allergic to peanut allergy is deemed unsafe as the smallest amount of peanut can trigger an anaphylaxis reaction. Thus, various allergoids were created by modifying major peanut protein allergen Ara h2 using various cross-linkers and modification agents in order to disrupt the binding surface epitopes of the antigen to antibody.

Diabetes is also a growing problem that affects millions of people globally. Having diabetes is known to lead to various morbidity as well which can be detrimental to the patient and a burden to the economy. Various drugs are available as monotherapy for diabetes; however, we aimed to develop dual acting compounds that would not only control type 2 diabetes but also commonly associated diagnosis of hypertension as well. Thus, we have successfully synthesized and tested compounds *in vitro* as effective dipeptidyl peptidase-IV (DPP-IV) inhibitors and potentially effective in treating hypertension (future study) associate with diabetes by utilizing cyanopyrrolidines and β-amino alcohols scaffolds in the structure, respectively.

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Chapter 1

Understanding Allergens in Peanuts

Introduction

Species of peanut. Peanuts are widely used around the world for their source of high quality protein and oil content. World production of peanut is about 30 million metric tons per year with China, India and U.S. producing 13.4, 7.7 and 1.8 million metric tons, respectively. Just for comparison to see the utilization and importance of peanuts, the global corn production in year 2004/2005 was 706 thousand metric tons out of which U.S. produced about 300 thousand metric tons of corn. Seven states in the U.S. account for 99 percent of all peanuts grown. Georgia produces the largest (41%), followed by Texas (24%), Alabama (10%), North Carolina (9%), Florida (6%), Virginia (5%) and Oklahoma (5%). Peanuts are used as value-added product in numerous applications which include but not limited to peanut flour, peanut oil, roasted peanuts and peanut butter.[1] Despite such high usage of peanuts, the prevalence of peanut allergy is becoming a growing concern in the westernized world thus making them an important plant seed of study.[2-7]

Peanuts are different from other tree nuts like almonds and walnuts because they produce their seeds just below the ground. Peanuts are of the genus *Arachia,* species *Arachis hypogaea,* which is a member of the legume family (Fabaceae) dating back to 2000-3000 B.C. in the Peruvian excavations.[8] Peanut species *A. hypogaea* is divided into two subspecies, *hypogaea (hypogaea hypogaea) and fastigiata (fastigiata vulgaris* and *fastigiata fastigiata)*, commonly called Virginia, Spanish, and Valencia, respectively. There is a hybrid between subspecies of Virginia and Spanish called Runner.[9] Runners are

the dominant peanut type grown in U.S. (80% of total U.S. production) since they provide the most yield and are used to make the most eaten style of peanut in U.S., peanut butter. Virginia peanuts (account for about 15% of total U.S. production) have the largest kernels and sold predominantly as roasted peanuts in their shells. Spanish peanuts (account for 4% of U.S. production) have a smaller kernel with reddish-brown skin with highest oil content than other types of peanuts. Valencia peanuts (account for less than one percent U.S. production) have three or more small kernels per pod and are very sweet sold in shell, or eaten boiled.[1]

Although there were numerous different types of species, subspecies and varieties of peanut in the beginning and middle of the 20th century, the only cultivars used for breeding now are Virginia, Spanish, and Valencia. Runner, although it is a hybrid, is also considered a cultivar since it has been in use for a long time.[9] Thus, it is important to focus on these different varieties as they are the most used around the world. It has been shown by Sheppard and Rudolf[10] that the four different peanut types have no significant differences in fat, ash or protein content. Approximately 90% of total fatty acid content of peanut oil is composed of 10% palmitic acid and 80% oleic and linoleic acid. Runner and Virginia type showed similar oleic and linoleic acid content than Valencia and Spanish subspecies.[10] Peanut seeds in general contain about 44-56% oil content and about 24-29% protein content across various types of peanuts.[9,11] Along with same protein content, the amount of protein that is extractable is about the same among different peanut varieties as well.[9]

Proteins that elicit allergic reaction from peanuts, specifically Ara h2. Since mostly proteins are responsible for mediating allergic reaction, it is important to focus on

the different types of allergens in the peanut seed. Currently up to date, World Health Organization (WHO) and International Union of Immunological Societies (IUIS) Allergen Nomenclature Subcommittee have identified 16 different peanut allergens that are capable of eliciting allergic reactions with the exception of Ara h4 which is now classified and termed as Ara h3.02 under Ara h3 family. The allergens are classified from Ara h1 to Ara h17 with their biochemical name as shown in Table 1 below.[12]

Table 1

List of major allergens of peanut and its corresponding protein family name and molecular weight as resolved by SDS-PAGE.

Allergen	Biochemical/Protein family name	MW (kDa)
Ara h1	Cupin (Vicillin-type, 7S globulin)	64
Ara h2	Conglutin (2S albumin)	17
Ara h3	Cupin (Legumin-type, 11S globulin, Glycinin)	60, 37 (fragment)
Ara h4	Renamed to Ara h3.02	
Ara h5	Profilin	15
Ara h6	Conglutin (2S albumin)	15
Ara h7	Conglutin (2S albumin)	15
Ara h8	Pathogenesis-related protein, PR-10, Bet v 1 family member	17
Ara h9	Non-specific lipid-transfer protein type 1	9.8
Ara h10	16 kDa oleosin	16
Ara h11	14 kDa oleosin	14
Ara h12	Defensin	8 (reducing), 12 (non-reducing), 5.184
Ara h13	Defensin	8 (reducing), 11 (non-reducing), 5.472
Ara h14	Oleosin	17.5
Ara h15	Oleosin	17
Ara h16	Non-specific Lipid Transfer Protein 2	8.5 (reducing)
Ara h17	Non-specific Lipid Transfer Protein 1	11 (reducing)

Note: Obtained from allergen.org which is managed by IUIS Allergen Nomenclature Subcommittee.
(http://allergen.org/search.php?allergensource=peanut&searchsource=Search)

The protein super families are cupin (Ara h1, Ara h3, Ara h3.02), the prolamin (Ara h2, Ara h6, Ara h7, Ara h9, Ara h16, Ara h17), the profilin (Ara h5), the Bet v 1 (Ara h8), the glycosyl transferase GT-C (Ara h10, Ara h11, Ara h14, Ara h15), and the scorpion toxin-like knottin (Ara h12, Ara h13).[11] Proteins are classified as such due to their similarity in structure and sequence to the superfamilies. The cupin allergen Ara h1 is a member of vicilins or 7Sglobulins which are characterized as lacking cysteine residues and by disk-shaped trimeric proteins whose subunit vary due to posttranslational proteolytic processing and glycosylation. The other cupin protein Ara h3 is from legumin or 11S globulins family which are hexameric proteins that are linked by a single disulfide bond. The prolamin superfamily are characterized by containing eight cysteine residues but have very little to none sequence similarity. The 3-dimensional structure consists of bundles of four α-helices stabilized by disulfide bonds.[11] Other protein superfamilies are important; however, the most allergenic groups are cupin and prolamin, specifically allergens Ara h1 and Ara h2 as described below.

Out of the 17 different peanut allergens, Ara h1 and Ara h2 have been found to be the most potent in eliciting allergic reaction.[13,14] The reason they are categorized as a major peanut allergen is because they are recognized by greater than ninety percent IgE of peanut allergic patients.[9,13-15] IgE is short for immunoglobulin E which are responsible for protecting the body from foreign substances (talked below in section "peanut as an allergen"). Ara h3 is sometimes identified as a major or minor allergen depending on the population sample with a range of 44-77% of IgE mediated reaction in peanut allergic population.[16] Another definition of major peanut allergen is proteins that are responsible for a majority of effector activity of food rather than just IgE recognition. Effector activity

is referred to the ability of an allergen to effectively cross-link IgE and activate mast cells and basophils since this is clinically relevant, and according to this definition, Ara h2 and Ara h6 have been found as major peanut allergens in crude peanut extracts.[17,18]

Most protein from the peanut seed is stored in the protein superfamilies' of cupin or prolamin of peanut. The cupin Ara h1 was determined by Koppelman et al.[9] to be 12-16% of total protein content and the 2S albumin Ara h2 to be 5.9-9.3% of total protein content in peanut. All of the classes combined account for 85% of total peanut protein while Ara h1, Ara h2 and Ara h3 together account for 75% of total protein content.[11] Study by Koppelman et al.[9] concluded that differences in serology between peanut allergic patients from different parts of the world are not explained by the allergen composition of different peanut types. Thus, a focus on the different protein allergens from peanut seed are of importance, particularly Ara h2.

It has been found that Ara h2 has peptide sequences that are similar to peptides in Ara h1 and Ara h3, which helps to explain co-sensitization to the three major allergens even though the structure, sequence, and protein groups are not similar.[19] It has been shown that Ara h2 is resistant to further enzymatic fragmentation after treatment with trypsin, chymotrypsin, or pepsin. The remaining large fragments are shown to contain intact IgE-binding epitopes which makes Ara h2 a very potent allergen.[20]

This research is specifically on the protein Ara h2 since it is found to be the most potent allergen via IgE recognition and as a major effector activity of peanuts.[17,21] Ara h2 is found to have two classes of protein isoforms (2.01 with 18.0 kDa and 2.02 with 17.7 kDa) which vary with addition of a 12-residue sequence in the flexible loop region of the

protein structure.[22,23] Native Ara h2 consists of 31% α-helix, 10% β-strand, and 59% random coil and/or loops; roasted Ara h2 consists of 29% α-helix, 12% β-strand, and 59% random coil and/or loops.[24] Ara h2 is a monomeric protein with 5 helices and 8 cysteine residues that form 4 disulfide bonds with at least one disulfide bond connecting each helix to another.[25] The disulfide bonds are important in stability of the secondary structure and maintaining allergenicity. A 3D molecular model of Ara h2 can be seen in Figure 1 that was created using VMD software.[26] Ara h2 is found to be a weak trypsin inhibitor (trypsin binding residues 76 to 85) whose activity is enhanced by 3.5-fold due to roasting. Ara h1 is known to under go degradation by trypsin, however, Ara h2 being a weak trypsin inhibitor, it protects Ara h1 and increases potency of Ara h1 in roasted peanuts.[24]

It is important to be able to recognize important IgE-binding epitopes of Ara h2 since they can further help shed light on the therapeutic approaches to peanut hypersensitivity to prevent anaphylaxis and potentially desensitize patients. Ara h2 contains 157-amino acids and within the protein there are 10 IgE-binding epitopes. Three epitopes are found between aa17-39, four epitopes between aa41-80 and three epitopes between aa114-157. These 10 IgE-binding epitopes include 63% amino acids that are either polar uncharged or apolar residues. Out of the 10 IgE, there are 3 immunodominant IgE at aa27-36, aa57-66, and aa65-74. It was shown that even one amino acid modification from the immunodominant IgE epitopes significantly decreased IgE binding.[15]

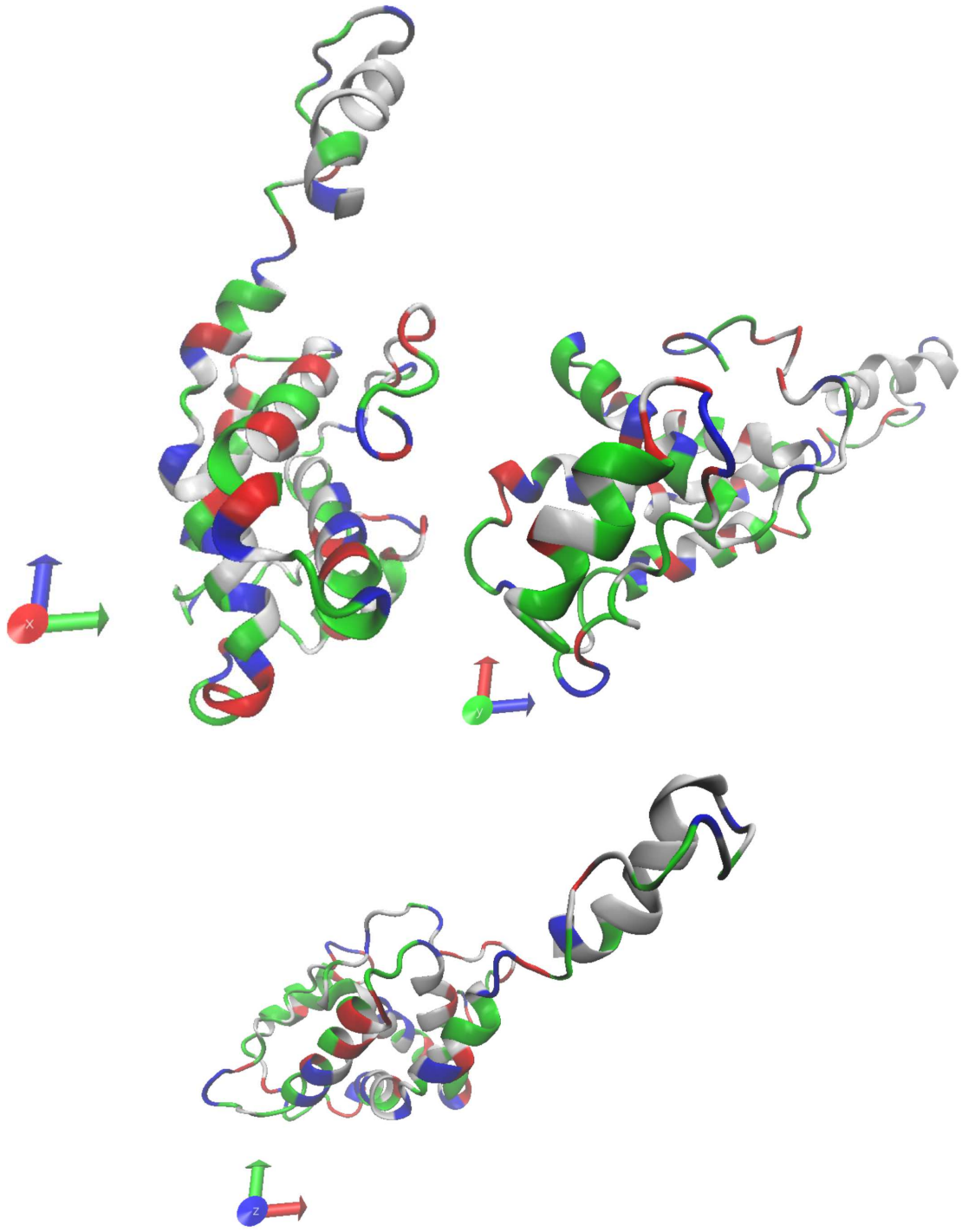


Figure 1. Molecular modeling of Ara h2 protein using VMD software. Note: non-polar residues (white), basic residues (blue), acidic residues (red) and polar residues (green).[26]

Peanut as an allergen. Despite such heavy use of peanuts by the world and a long history of cultivation, peanut allergies are on the rise. Research on food allergy has been discussed in scientific literature as early as 1912 with the first major peanut allergen to be documented and named (Ara h1) in 1991.[14,27] Not just peanut allergies but allergies to food have been increasing in the westernized countries. Although any food can cause an allergic response, the most common are milk, eggs, fish (e.g., bass, flounder, cod), crustacean shellfish (e.g., crab, lobster, shrimp), tree nuts (e.g., almonds, walnuts, pecans), peanuts, wheat and soybeans. According to the Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004, it is mandated that these eight major allergens be labelled in foods that contain these declared major food allergens. These common allergies account for 90% of all food allergies.[28,29]

According to guidelines for the diagnosis and management of food allergy in the U.S., food allergy is defined as "an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food." Immune related food allergy can be IgE mediated, non-IgE mediatd, Mixed IgE and non-IgE mediated, or cell mediated. Non-immune related adverse reaction to food can be metabolic, pharmacologic, toxic or idiopathic/undefined in relation so it is important to properly identify the allergen according to guidelines for food allergy management. Foods that do elicit reproducible adverse reactions but not linked immunologically are not considered food allergens and instead are called food intolerances (e.g. lactose intolerance).[2]

Peanut allergy is IgE-mediated and there are two stages in the development of an IgE-mediated allergy. Upon first exposure to the allergen, sensitization stage occurs. When an allergen is consumed, gastric acid and digestive enzymes break down the protein that

are then exposed to mucosal immune system in gastrointestinal tract. This exposure causes plasma cells to produce allergen-specific IgE (a-sIgE) to the peptide fragment that was exposed on the allergen. This a-sIgE then attaches to mast cells and basophils via FcεRI receptors, concluding the first stage.[27]

The second stage, allergic reaction, is initiated upon second exposure to peanuts, specifically peanut protein and their peptide fragment containing epitopes. These allergens then bind to and cross-link two or more IgE-FcεRI complexes that are on the surface of the mast cells or basophils resulting in basophil or mast cell degranulation and subsequent release of various inflammatory mediators, such as cytokines, chemokines, lipid mediators, and histamines. These chemicals cause a series of local symptoms, such as itching, swelling, nausea/vomiting, and diarrhea, in addition to various systemic symptoms, such as airway obstruction, hives, low blood pressure, and even fatal anaphylaxis.[27,30]

The most common types of allergies among children are food or digestive allergies, skin allergies such as eczema, and respiratory allergies such as hay fever. There has also been an increase in prevalence of skin allergies among children as well. Children with skin allergies went from 7.4% in 1997-1999 to 12.5% in 2009-2011. There is no significant trend in respiratory allergies from 1997-1999 but from 2009-2011, the prevalence rate has been 17%.[31]

Although peanuts contain approximately the same protein content across different varieties, there is a difference in prevalence of peanut allergies across different parts of the world.[9] There have been many theories postulating why the prevalence of peanut allergy is increasing in the western world over the last decade. The theories include; imbalance

between the T-helper 1 (Th1)/T-helper 2 (H2)-biased cellular responses in early life, exposure of peanut allergens in breast milk of lactating women sensitizing the infant,[32] preparation of peanut (dry roasting in U.S. and other western countries), family history of peanut allergy, consumption of soy during infancy along with onset of skin allergies and exposure to topical preparations (e.g. treatment for anti-rash, eczema, dry skin, etc.) containing peanut oil.[2,24,29] On the contrary, it has been shown by Du Toit et al.[6] through their Learning Early about Peanut Allergy (LEAP) trial that early introduction of peanuts significantly decreased the frequency of the development of peanut allergy among children at high risk for this allergy and modulated immune responses to peanuts.

One study by Du Toit et al.[33] was able to show that early introduction of peanut in infants' diet relates to a decrease and ultimately prevention of development of peanut allergy. They studied prevalence of Jewish children living in UK and living in Israel. The reason for this group of children that were studied was that children in Israel were fed peanut within the first year of their life and children in UK had avoidance of peanut till later age. The results showed a 10-fold higher prevalence of peanut allergies in Jewish children living in UK versus Jewish children in Israel.[33] This goes to show that the guidelines recommending avoidance of peanuts during infancy is rather incorrect and that actually an early exposure is better to preventing peanut allergies.[6]

Unfortunately, the current treatment for food allergy is avoidance of that food allergen. In an incidence of accidental ingestion of allergen, prompt administration of epinephrine is recommended to treat anaphylactic reaction.[2] Peanuts and other tree nuts have shown to be persistent allergen with lowest amount of resolution as the children ages among the common allergens. Study by Skolnick et al.[34] demonstrates that about 21.5% of

children outgrown their peanut allergy and that children with low peanut IgE levels should be offered a peanut challenge in a medical setting to demonstrate if they can now tolerate peanuts. For comparison, children with allergies to egg and milk is resolved by 11% and 19%, respectively, by age 4 years and that about 80% of these allergies are resolved by age 16 years.[5] Due to consistency of peanut allergies in children and as adults, it is of the most important allergy to be studied and focused on.

The average number of food related incidence per year in the U.S. shows 125,000 emergency department visits and 53,700 episodes of anaphylaxis.[5] Food-induced anaphylaxis occurred every 5 minutes in the U.S. between years 2001 and 2005 with nearly one-half million emergency department visits.[35] The annual incidence rate of children with food allergy is at 14.3% and surprisingly 85% of these children attended schools prohibiting peanuts.[36] Food allergies are an important concern for U.S. as they impact the economy. The overall economic cost of food allergy was estimated at $24.8 billion annually, which is about $4184 per year per child. Direct medical costs are estimated to be $4.3 billion annually, costs borne by the family totaled $20.5 billion annually, lost labor productivity costs totaled $0.77 billion annually, out-of-pocket costs totaled $5.5 billion annually and opportunity costs totaled $14.2 billion annually. The caregivers reported a willingness to pay $20.8 billion annually which is $3504 per year per child for food allergy treatment.[37] Clearly, there is a significant medical cost to having food allergy that is bared by the U.S. health care system and even more so by families with a food allergic child.

The adverse immune responses to foods affect approximately 15 million people.[4,38] Out of the 15 million people, an estimated 9 million or 3-4% are adults who have food allergies.[5] A 2008 CDC prevention report indicated an 18% increase in children food

allergy from year 1997 to 2007, with an estimated 3.9% of children currently affected. Specifically, for peanut allergy in the U.K. and U.S., the prevalence rates have essentially doubled for children exceeding 1%.[5] Looking at 11-year follow-up study by Sicherer et al.[7] showed prevalence of peanut allergy in children in 2008 to be 1.4% compared with 0.8% in 2002 and 0.4% in 1997.

However, a more recent study completed by Gupta et al.[38] suggests that the prevalence and severity of childhood food allergy is greater than in previously reported studies. Gupta et al.[38] suggests that food allergy prevalence is actually 8.0% rather than 3.9% in children, which is nearly 5.9 million affected children in U.S. With no surprise, the prevalence among all children (without food allergy) surveyed for all ages showed peanuts (2.0%) to be the highest prevalence frequency followed by milk (1.7%), shellfish (1.4%), tree nut (1.0%), egg (0.8%), fin fish (0.5%), wheat (0.4%) and soy (0.4%). Prevalence among children (with food allergy) surveyed showed peanuts (25.2%) to be the highest followed by milk (21.1%), shellfish (17.2%), tree nut (13.1%), egg (9.8%), fin fish (6.8%), wheat (5.0%) and soy (4.6%).[38]

Gupta et al.[38] also conducted a study to evaluate the history of severity of common food allergies among affected children. Severe reaction was seen in 38.7% of affected children and mild-to-moderate reaction in 61.3% of affected children. Children who had multiple food allergies accounted for 2.4% of all children corresponding to 30.4% of children with food allergy. Needing further studies, Hispanic children have lower rates for food allergies than non-Hispanic white and black.[31,38] There are socially constructed disparities that suggest difference in prevalence of food allergy by race and income.[38]

Modification of Ara h2 as a potential immunotherapy stimulant. Proteins of peanut allergens are generally stable under high pressures, varying temperatures and digestive conditions.[20,39] One of the reasons for this is roasting the peanut which causes modification of lysine and possibly arginine residues by the Maillard reaction.[40] The Maillard reaction is a nonenzymatic chemical modification between amino acids and reducing sugars when cooked at high temperature to form advanced glycation end-products (AGEs).[41] Roasting induces protein structural changes that enhance allergenic properties of Ara h1 and Ara h2. The Maillard reaction explains stability of other food allergens as well in that it preserves protein structure and IgE-binding epitopes rendering them from digestion by specific peptides in the digestive tract.[40,42] Thermal treatment also results in formation of variety of advanced glycation end products which increases the weight of the allergen. The increased weight of an allergen can potentially make the allergen more potent since the digestive enzymes are not able to access the cleavable sites.[22,43]

It has been shown that boiling or frying of peanut matrix, as practiced in China, reduces the allergenicity of peanuts.[44] This could explain why the prevalence of peanut allergy is increasing in the western world where roasting peanuts is more common compared to countries where peanuts are commonly prepared by boiling or frying such as China. Since most of the peanuts eaten in U.S. are in the form of roasted peanuts, it was important for us to use roasted peanuts in the development of an allergoid as potential therapeutic.

There has been evidence of modification of IgE-binding epitope of peanut allergen to decrease serum IgE binding from peanut-hypersensitive patients and stimulate T-cell proliferation and activation.[45-47] It has been studied that no specific epitopes were

associated with severe reactions to peanut and that clinical sensitivity is positively related to a more polyclonal IgE response.[48] King et al.[45] were able to engineer a mutant Ara h2 that showed less binding to allergen-specific IgE which was able to interact with T-cells and release significantly lower amounts of mediators from passively sensitized mast cells thus lowering allergenicity. Another option to reduce allergen-specific IgE is to mask allergen epitopes by crosslinking protein via specific amino acid. Wu et al.[47] were able to crosslink Ara h2 (either inter- or intramolecular) at tyrosine using polyphenol oxidase as catalyst. This allowed crosslinked Ara h2 to be more digestible by gastric fluid (however more difficult to digest by intestinal fluid) and a decrease in allergenicity was noted since three of the five tyrosine that were crosslinked were located in two of three main epitopes of Ara h2.[47] Another study showed the combined effects of polyphenol oxidase and caffeic acid, a phenolic compound, leading to decreased IgE binding of Ara h1 and Ara h2 by cross-linking via tyrosine residues of protein 1 and protein 2.[49-51]

There are various immunotherapies in the market for various pollen like ragweed or venom anaphylaxis such as wasp allergy but there is no FDA approved immunotherapy for food allergies.[27] There has been a study done by Nelson et al.[52] where they injected peanut extract into patients with peanut allergy using subcutaneous immunotherapy (SCIT). Their study concluded that due to over reaction in patients upon injection of peanut allergen that a modified peanut extract is needed instead of unmodified. This has opened doors to antigen-specific approaches to decreasing peanut allergenicity which include oral, sublingual, and epicutaneous immunotherapy.[53]

Oral immunotherapy (OIT) delivers antigens to the mucosal immune system via ingestion with absorption through the small intestine. OIT has been recently recognized

clinically to desensitize patients to peanut with suppression of mast cell and basophile activation along with increase in blocking peanut-specific IgG and IgG4 antibodies which compete with IgE for the binding of peanut proteins.[54] These studies have been demonstrated in European countries as well such as Germany and England, however, OIT is still deemed as an experimental treatment and not ready for widespread implementation.[27]

Sublingual immunotherapy (SLIT) delivers antigens to the oral mucosa by placing protein under the tongue. A recent study by Burks et al.[55] was able to conclude that SLIT was able to desensitize peanut allergic patients with decreased immunologic activity over 3 years along with excellent long-term safety profile. By the end of the study, 10.8% of patients were fully desensitized to 10 g of peanut powder, however, more than 50% discontinued therapy by the end of year 3.[55]

Another approach is epicutaneous immunotherapy (EPIT) which uses spray-dried allergens on a membrane applied to patient's skin. The superiority of this therapy over OIT and SLIT is that the effect of peanut allergy is localized to the area of the patch and not systematic. A current treatment in clinical trials is Viaskin Peanut, which showed peanut EPIT administration was safe with reduced sensitivity to peanut and increased IgG4 levels and IgG4/IgE ratio along with reduced basophil activation and peanut-specific T_H2 cytokines. This study was after 52 weeks of treatment which showed higher success among younger children of age 4-11.[56]

There are also non-antigen-specific approaches which involve administration other than the allergen to decrease allergenicity. There has been many anti-IgE therapy like

monoclonal antibody against human IgE, TNX-901 and antihuman IgE monoclonal Xolair (FDA approved but later discontinued) which have shown effectiveness by decreasing circulating IgE towards allergens and treating asthma, respectively. These anti-IgE therapies maybe impractical as they are expensive with lifelong injections to decrease continuous IgE proliferation in the body. There is an immunomodulatory phytochemicals category as well which are small molecules extracted from plants. This type of therapy has shown promise as a Chinese medicine called food allergy herbal formula-1 (FAHF-1), which contains 11 herbal extracts and FAHF-2 containing 9 herbal extracts in treatment of food allergy. FAHF-2 has been shown to decrease peanut, egg and fish allergy in murine study with no human trials. Recent study of FAHF-2 in human showed favorable in vitro immunomodulatory effects however with no improvement of tolerance to food allergens.[57]

The future goal of this study in creating a modified Ara h2 protein is so that it can be utilized in OIT or for some patients allergic to peanut, SIT since it offers a long lasting clinical efficacy as well.[58] The problem with using OIT or SIT to desensitize patient with peanut allergy is that the peanut proteins (especially Ara h2) are very potent leading to high risk of systemic adverse events such as anaphylaxis as show back in a study back in 1997.[52] Thus, if Ara h2 can be modified in a way to either mask or disrupt epitope binding amino acids, it can be used in a controlled dosage form to desensitize patient with peanut allergy, which is the goal of this part of the thesis.

Chapter 2

Purification of Ara h2 and Modification with Cross-linkers

Introduction to Cross-linkers

Cross-linkers are chemical reagents used to conjugate molecules together by the formation of covalent bond. Different cross-linkers have different functional groups that allow differential reactivity to specific target moiety. The cross-linkers used in this study were BS^3 (bis(sulfosuccinimidyl) suberate, MW: 572.43, MF: $C_{16}H_{18}N_2Na_2O_{14}S_2$), DST (disuccinimidyl tartrate, MW: 344.24, MF: $C_{10}H_{12}N_2O_{10}$), Sulfo-EGS (ethylene glycolbis(sulfosuccinimidylsuccinate), MW: 660.45, MF: $C_{18}H_{18}N_2Na_2O_{18}S_2$), $BM(PEG)_3$ (1,11-bis(maleimido)triethylene glycol, MW: 352.34, MF: $C_{16}H_{20}N_2O_7$) and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, MW: 191.7, MF: $C_8H_{17}N_3 \cdot HCl$) (Figure 2).

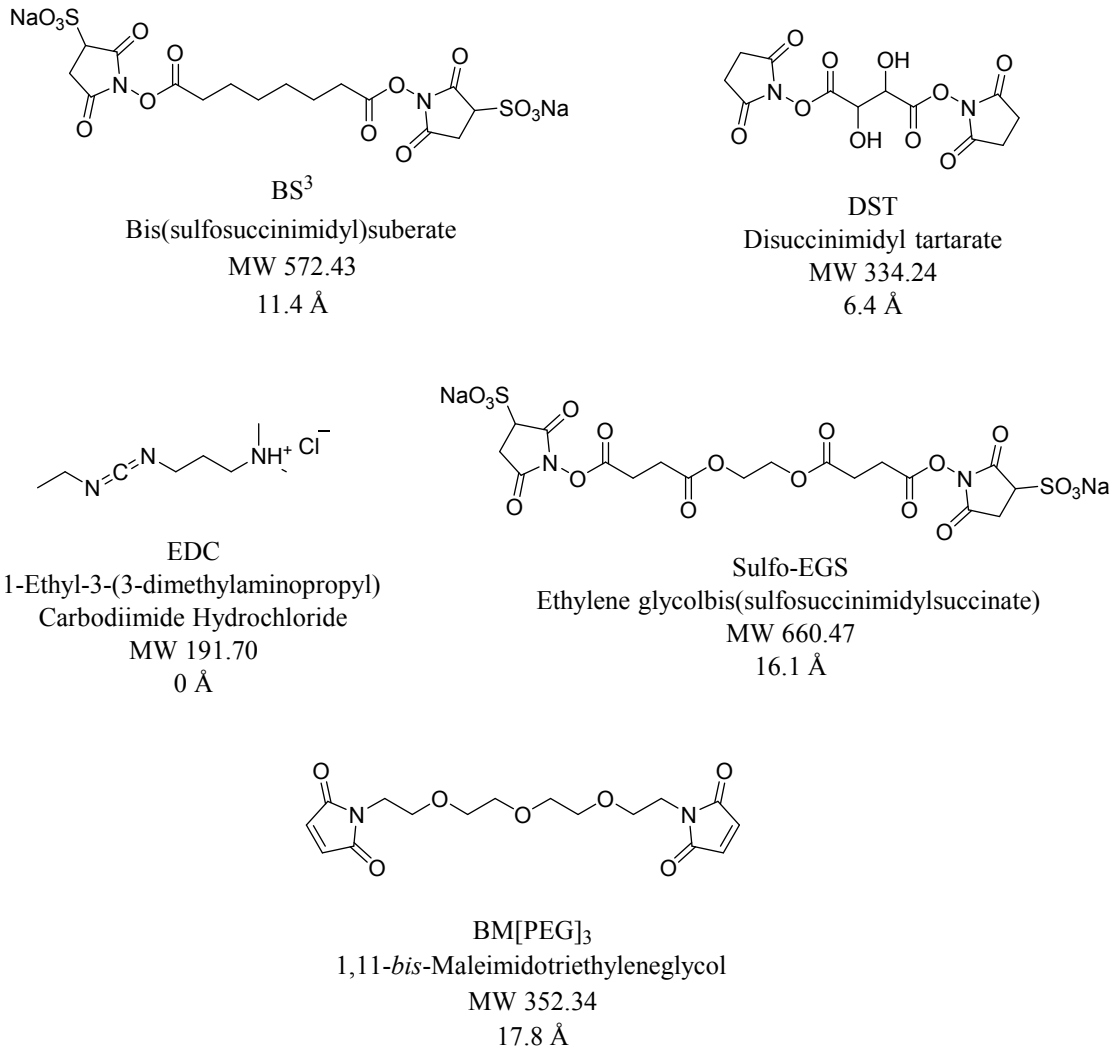


Figure 2. Chemical structures of cross-linkers used to modify Ara h2.

BS^3, DST, and sulfo-EGS all contain N-hydroxysccinimide ester (NHS ester) that react with nucleophiles to release the NHS or sulfo-NHS leaving group and form an acylated product with varying spacer arm length (reaction schematic described below).[59] NHS ester can react with sulfhydryl or hydroxyl group but the conjugation is not stable and it can be hydrolyzed in aqueous solutions. Reaction with primary and secondary amines is however more stable. A 3D molecular modeling of Ara h2 showing lysine residues which

contain primary amines for reaction can be seen in Figure 3 which was created using VMD software.[26] $BM(PEG)_3$ cross-linker has maleimide group on each side which helps conjugate between sulfhydryl groups. $BM(PEG)_3$ contains a poly(ethylene glycol) (PEG) as spacer arms which helps make PEG cross-linker hydrophilic compared to hydrocarbon spacers. EDC is a carboxyl and amine-reactive zero-length cross-linker that forms amide bonds. It is named zero-length cross-linker since the bond formed between the conjugates contain no additional atoms, it is simply a covalent bond between two molecules.[60]

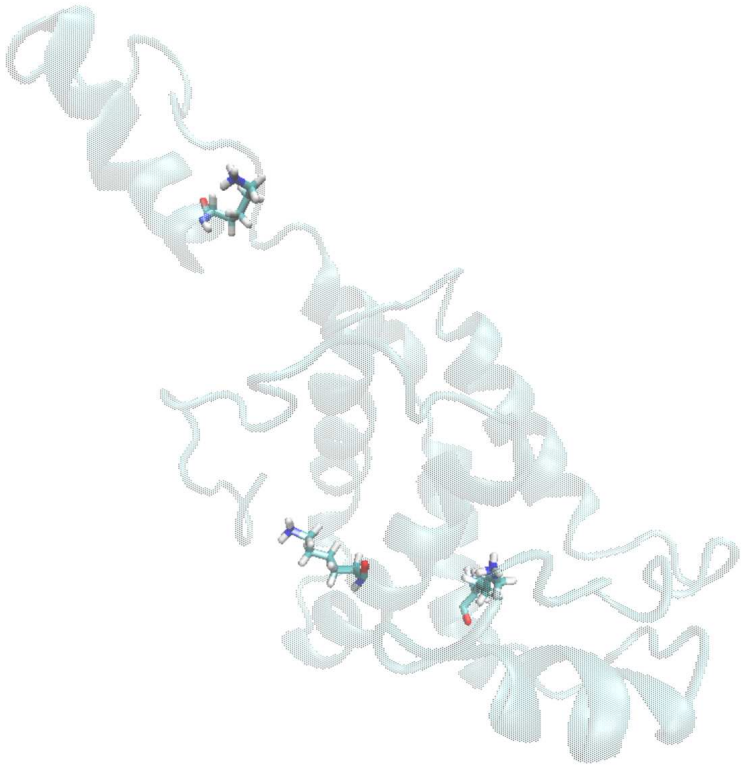


Figure 3. *Molecular modeling of Ara h2 showing Lys residues using VMD software.*[26]

BS^3 is a homobifunctional NHS ester crosslinking reagent that adds an eight-atom bridge (11.4 Å) between conjugated molecules.[61] This reagent is forming irreversible bond with the protein since the hydrocarbon chain is non-cleavable. The sulfonate groups give a negative charge to the molecule making it water soluble. NHS esters are reactive towards primary amino groups ($-NH_2$) in pH 7 to 9 buffers to form stable amide bonds. The recommended buffers to use are amine free such as Tris, glycine, or imidazole buffers so that the amines from the buffer do not cross-react with the NHS ester. The NHS ester forms amide linkages with α-amines and ε-amines of proteins like primary amines on the side chain of lysine residues and on the N-terminus of each polypeptide making them a good target by NHS ester reagents.[60] A reaction schematic can be seen in Figure 4 involving BS^3 cross-linker.

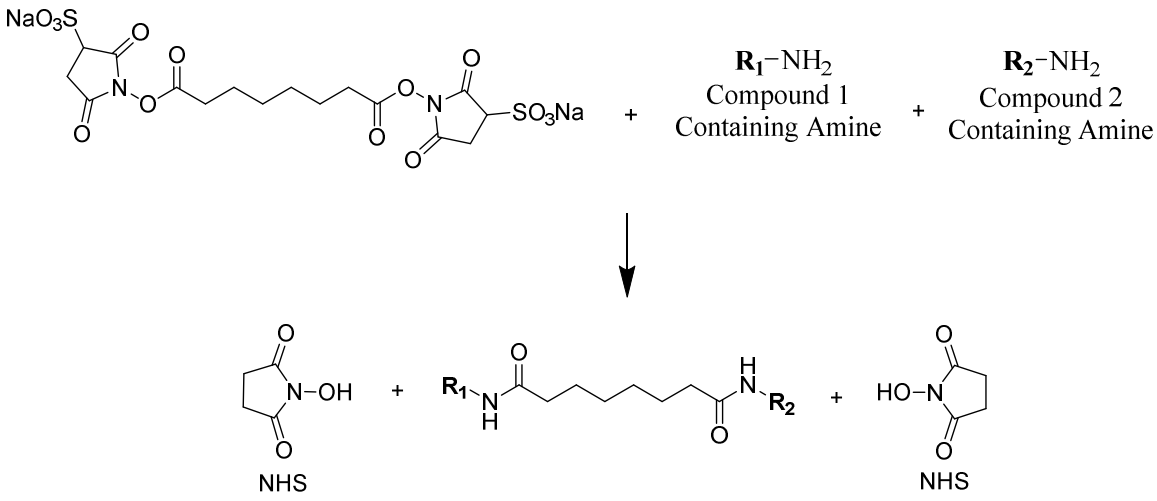


Figure 4. Reaction schematic of cross-linker BS^3. An amide bond is formed leading to conjugation of two molecules. The cross-linker is non-cleavable.

DST is a homobifunctional NHS ester crosslinking reagent that contains a central diol that is cleavable by sodium periodate thus making the conjugation reversible.[62] The reagent adds 6.4 Å of space between conjugated molecules. DST is not so soluble in aqueous buffers thus it needs to be pre-dissolved in organic solvents such as THF, DMF, or DMSO prior to addition with molecules that need to be conjugated. The recommended buffer to use are amine free such as Tris, glycine, or imidazole buffers with optimal pH of 7 to 9. As mentioned before, conjugation with this cross-linker can be cleaved using 0.015 M sodium periodate.[60] A reaction schematic can be seen in Figure 5 involving DST cross-linker.

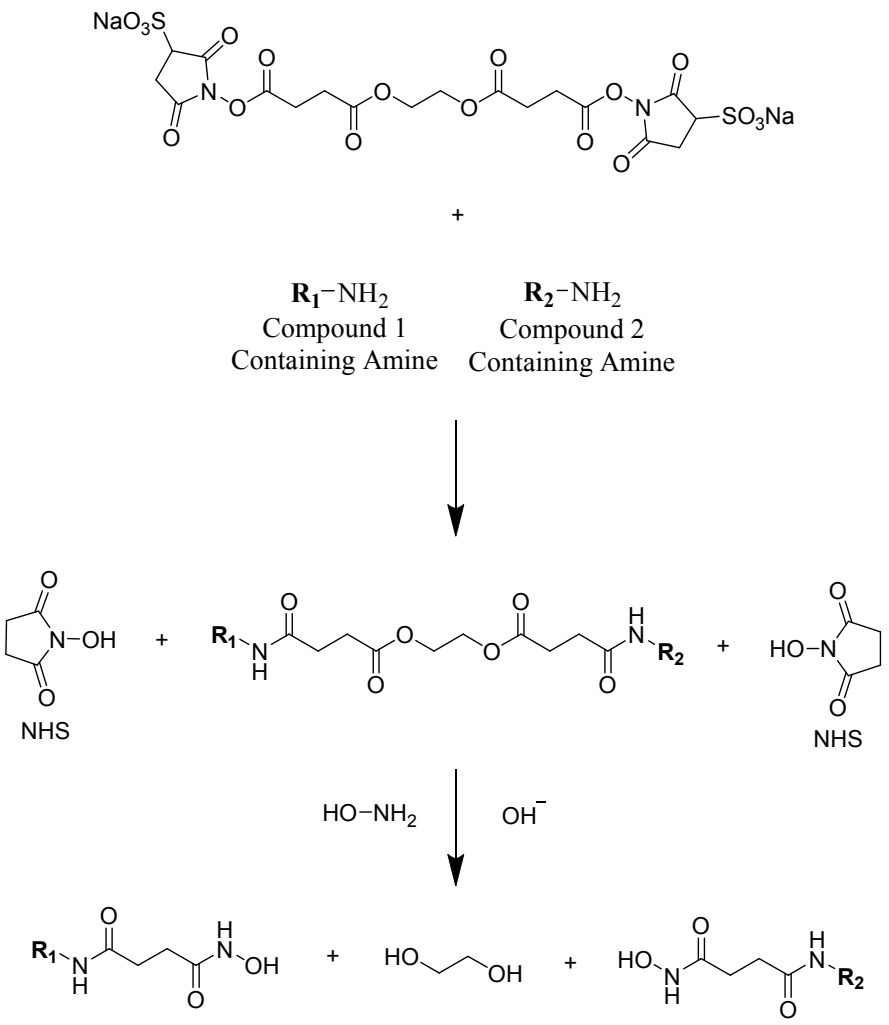


Figure 5. Reaction schematic of cross-linker Sulfo-EGS. An amide bond is formed leading to conjugation of two molecules. The ester groups are cleavable in basic conditions using hydroxylamine.

Sulfo-EGS is a homobifunctional crosslinking agent that contains NHS ester groups on the ends as well. Middle of the reagent is constructed from an ethylene glycol group esterified on either side with succinic acid. Sulfo-EGS is water soluble due to the negatively charged sulfonate groups on its NHS rings. This reagent adds a space of 16.1 Å between the conjugated molecules.[59] The NHS esters are reactive towards amines thus an amine free buffer is recommended with optimum pH range of about 7 to 9 of the buffer. The

central bridge of EGS provides two cleavable ester sites at pH 8.5 by incubation with 1-M hydroxylamine for 3 to 6 hours at 37° C.[60,63] A reaction schematic can be seen in Figure 6 involving sulfo-EGS cross-linker.

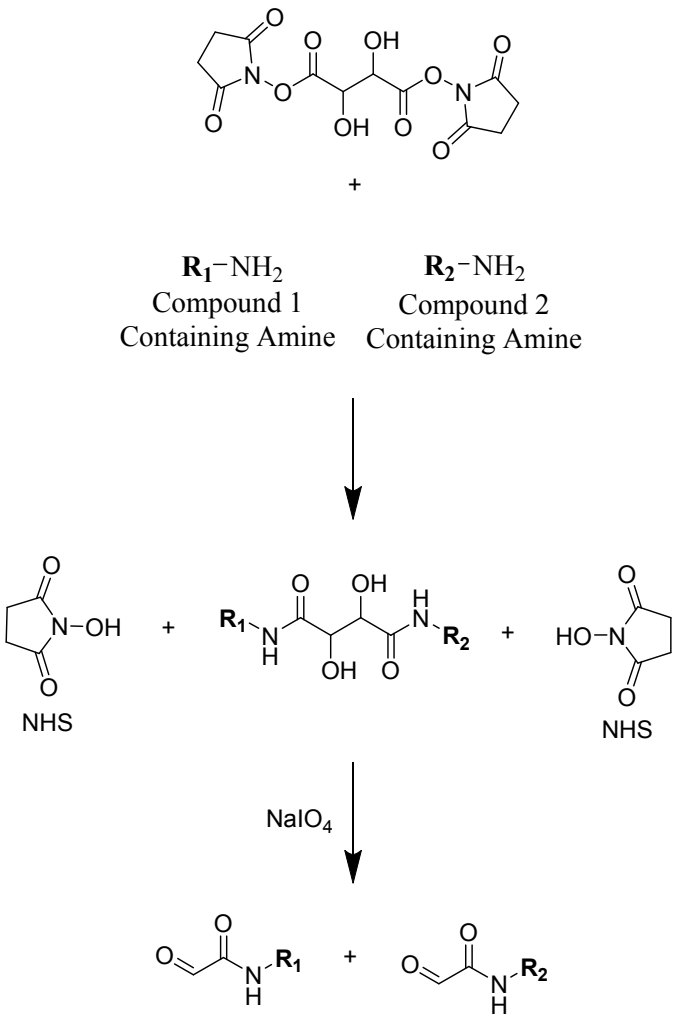


Figure 6. Reaction schematic of cross-linker DST. An amide bond is formed leading to conjugation of two molecules. The central diol is cleavable using sodium periodate.

$BM(PEG)_3$ contains bifunctional thiol reactive maleimides on each end that produces stable thioether linkages with sulfhydryls.[64] The cross-linker is hydrophilic with spacer arm of 17.8 Å. $BM(PEG)_3$ is recommended in pH range of 6.5 to 7.5 for reaction towards sulfhydryl groups and pH of >8 for primary amines, however, reaction rate is 1000 times slower with amines. Sodium phosphate (0.01-0.1 M) buffer works well for this reaction making sure to avoid amine-containing buffers along with sulfhydryl-containing disulfide reductants such as DTT or 2-mercaptoeathanol. A 3D molecular modeling of Ara h2 showing disulfide bonds can be seen in Figure 7 that was created using VMD software.[26] Maleimide moiety can hydrolysis resulting in opening of the maleimide ring rendering it nonreactive.[60] A reaction schematic can be seen in Figure 8 involving $BM(PEG)_3$ cross-linker.

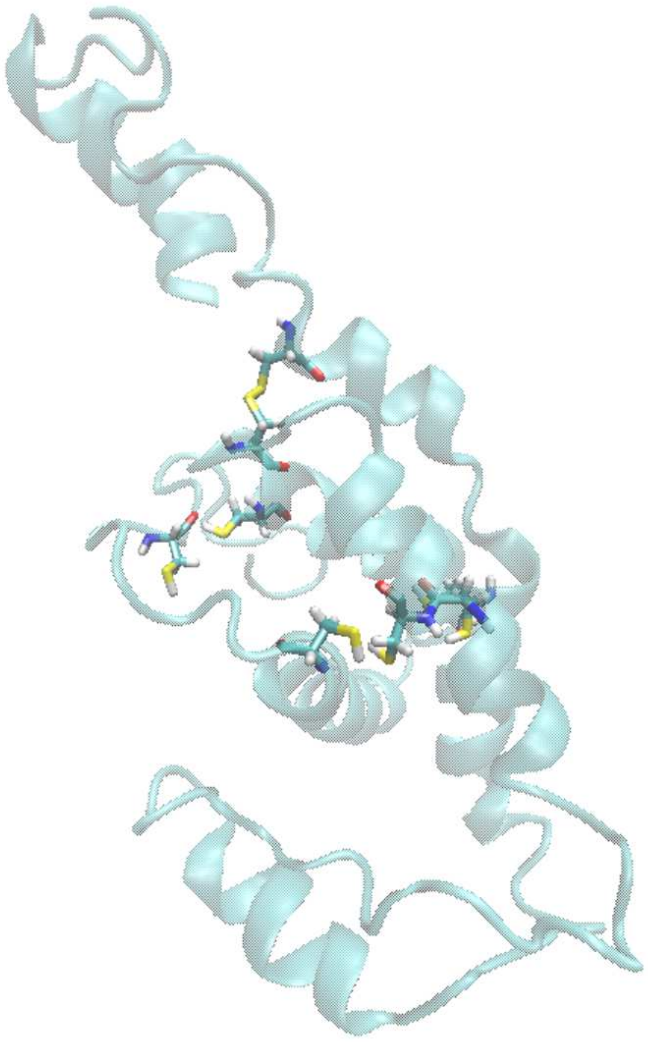


Figure 7. Molecular modeling of Ara h2 showing Cys residues using VMD software.[26]

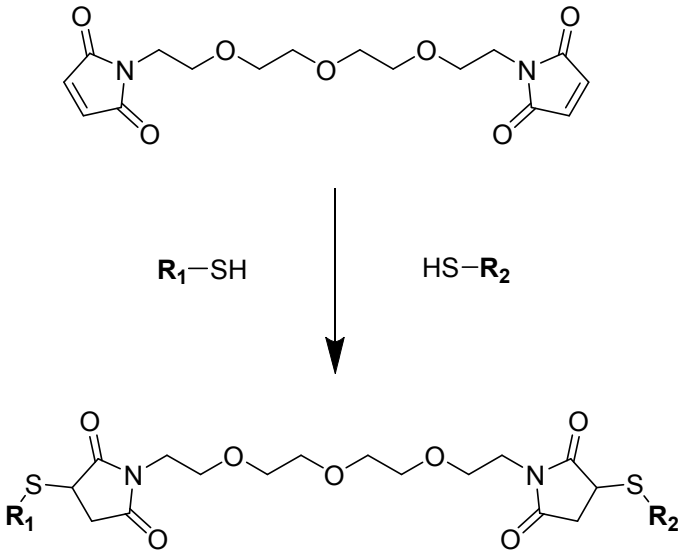


Figure 8. Reaction schematic of cross-linker BM(PEG)$_3$. Formation of thioether linkages on molecules or proteins that contain thiol groups.

EDC is a carbodiimide with a zero-length crosslinking arm that forms an amide or phosphoramidate linkage between a carboxylate group and an amine or a phosphate and an amine, respectively. A 3D molecular modeling of Ara h2 showing Asp and Glu residues can be seen in Figure 9 that was created using VMD software.[26] EDC reacts with carboxylic acid to form an amine-reactive *O*-acylisourea intermediate that will react with a nucleophile such as a primary amine to form an amide bond and release an isourea by-product as seen in Figure 10. Consideration should be given to the intermediate since it is unstable in aqueous solutions. Oxygen atoms can also act as a nucleophile as in water molecules thus resulting in activation of EDC and hydrolysis of the activated ester intermediate, regenerating the carboxylate group and release of an *N*-substituted urea.[65] Buffers such as MES [2-(*N*-morpholino)ehane sulfonic acid] at 0.1-M works well at acid pH conjugation, or a phosphate buffer at 0.1-M works for neutral pH conditions.[60] Overall,

any buffer that does not contain amines or carboxylic groups or other components that may react with the carbodiimide can be used.

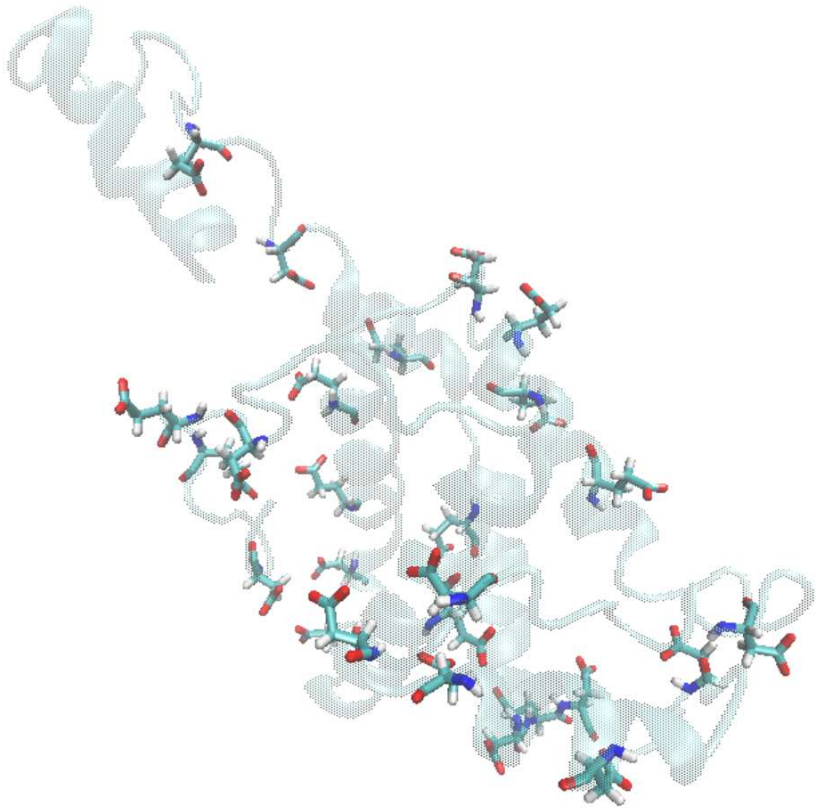


Figure 9. Molecular modeling of Ara h2 showing Asp and Glu residues using VMD software.[26]

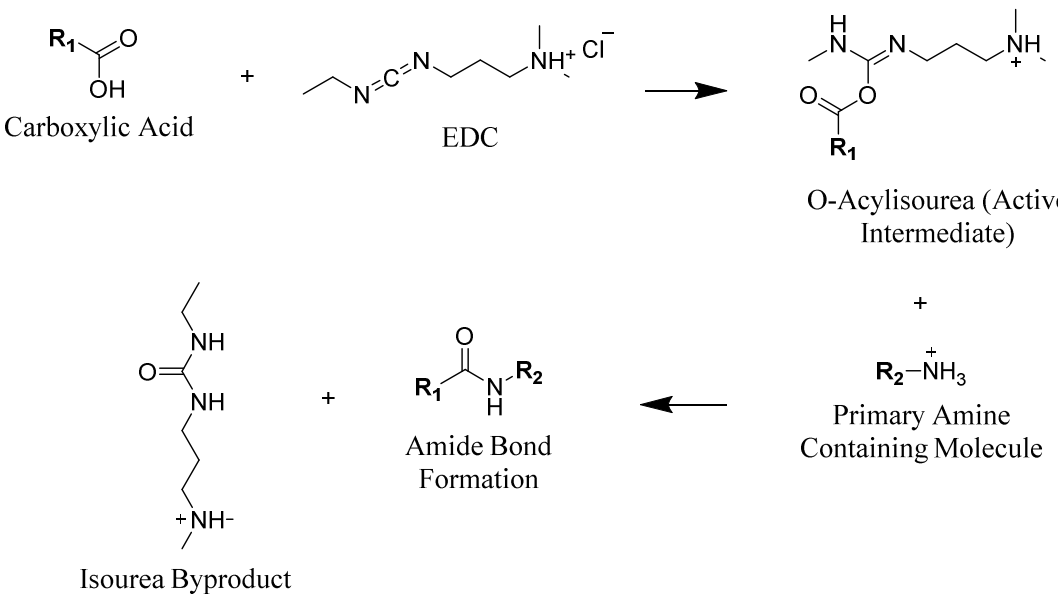


Figure 10. Reaction schematic of cross-linker EDC. Amide bond formation using EDC between carboxylic acid and primary amine.

The carboxylate activation is most efficient at pH 3.5 to 4.5, while amide bond formation occurs with highest yield at pH 4 to 6 thus a two-step coupling can be used (Figure 11). Since there are many by-products that can be formed, sulfo-NHS can be used to increase the solubility and stability of the active intermediate.[66] The active ester, *O*-acylisourea, is slow to react with amines and can hydrolyze in aqueous solution so introduction of sulfo-NHS to the active intermediate allows sulfo-NHS ester to form. This sulfo-NHS ester intermediate is more effective at reacting with amine and thus a higher yield of amide bond formation can be seen.[60,67,68]

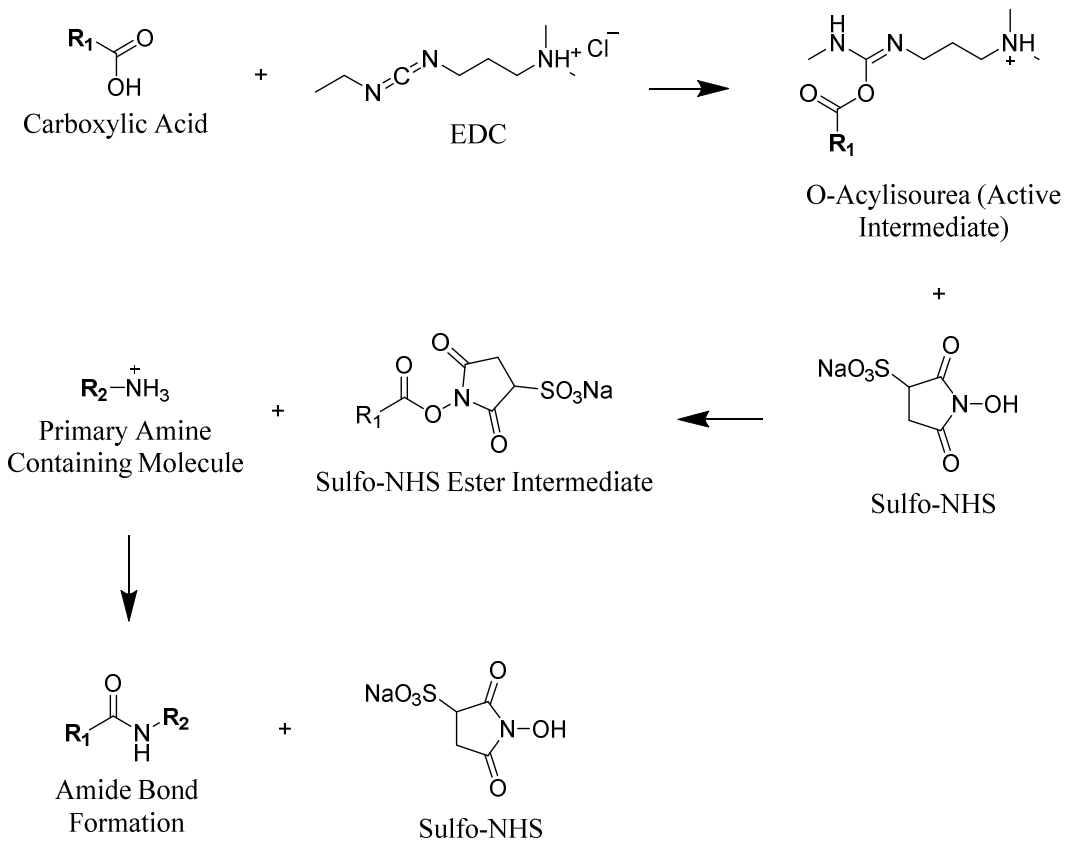


Figure 11. Two-step coupling reaction schematic of EDC and sulfo-NHS. Efficiency of reaction with EDC can be increased by introduction of sulfo-NHS and forming a sulfo-NHS ester intermediate which is more stable and reactive towards primary amines.

Research Design and Methods

Protein purification method 1. Ara h2 protein was purified according to Sen et al.[20] with few personal modifications. Roasted, unsalted peanut seeds (*Arachis hypogaea, hypogaea hypogaea*) were obtained from Good Earth Peanut Co., Skippers, VA, USA. Peanut seeds were crushed after freezing them with liquid nitrogen using a mortar and pestle. Crushed peanuts were placed in a cellulose extraction thimble which was placed in a soxhlet extraction for defatting with three changes of diethyl ether. Defatted peanut seeds

were dried by spreading them out on a paper towel over night under the hood. The dried defatted peanut seeds were crushed into a fine powder using mortar and pestle.

Peanut flour was dissolved in TBS buffer (65 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, pH 8.3) with 200 mM NaCl at a ratio of 1 gram to 50 mL buffer. The mixture was sonicated using Sonics Vibra-Cell at 39% power for 10 mins over ice followed by 1 hour of stirring at room temperature. The dissolved peanut suspension was then followed by vacuum filtration thru six layers of cheesecloth. The filtered solution was centrifuged (Sorvall RC 5B PLUS, SS-34 rotor) at 30,000 X g for 30 mins at 4° C. The supernatant was fractionated using ammonium sulfate precipitate $(NH_4)_2SO_4$ (MW 132.14) going from 0 to 40% carrying the supernatant and then carrying the pellet in 40% to 70% precipitate. The ammonium sulfate was added slowly over 30 min period and stirred for total of 1 hour over ice followed by centrifugation at 30,000 X g for 30 mins (SS-34 rotor).

The pellet was dissolved in 10 mL of TBS buffer without NaCl followed by sonication for 1 min at 39% power (over ice) and centrifugation (3,000 X g for 15 min) to remove undissolved particles. The supernatant was dialyzed (SnakeSkin Dialysis Tubing, 7K MWCO) overnight against TBS buffer to remove salts from the mixture. The supernatant was loaded onto a flex-column 1.5 x 30 cm (Kimble Chase) filled with approximately 50 mL of Q Sepharose Fast Flow (GE Healthcare) that had been equilibrated with TBS buffer. After loading the supernatant, 90 mL of TBS buffer with 40 mM NaCl was used to elute proteins of noninterest. Then a linear gradient of 400 mL using 200 mL TBS buffer with 40 mM NaCl to 200 mL TBS buffer with 500 mM NaCl was used to elute protein of interest at a flow rate of 2 mL/min. Fractions were collected for 1 min 15 secs per tube to give approximately 2.5 mL per tube.

The fractions were analyzed using coomassie G-250 dye bonding assay for protein in a clear 96-well plate. Ratio of 50 µL protein sample to 150 µL coomassie dye was used followed by reading of the plate at 595 nm (Thermo Scientific Multiskan Spectrum). Positive fractions were analyzed using SDS-PAGE (see below "SDS-PAGE protocol") for Ara h2 followed by dialyses (7K MWCO) overnight at 4° C against 25 mM Tris-HCl (pH 7.4) and 3 M NaCl. The dialyzed sample was loaded onto a flex-column 1.5 x 30 cm (Kimble Chase) filled with approximately 50 mL of Phenyl Sepharose 6 Fast Flow (high sub) (GE Healthcare) that had been equilibrated with 25 mM Tris-HCl (pH 7.4) with 3 M NaCl. After loading, a linear gradient of 200 mL using 100 mL of 25 mM Tris-HCl buffer with 3 M NaCl to 100 mL of 25 mM Tris-HCl buffer with 0 M NaCl was ran at 1.5 mL/min. Fractions were collected for 1 min 20 secs per tube to give approximately 2.0 mL per tube. Fractions were analyzed again for Ara h2 using coomassie G-250 dye bonding assay and SDS-PAGE. Ara h2 containing fractions were pooled and dialyzed (7K MWCO) overnight at room temperature against 15 mM ammonium bicarbonate (pH 7.0). The dialyzed sample was lyophilized (FreeZone 2.5 L Benchtop Freezer Dry System, Labconco) and stored at -20° C.

Protein purification method 2. A second, novel method for purifying Ara h2 was developed to increase yield and efficiency of the purification process. This method of purification was inspired by Jumnongpon et al.[69] as they purified cocoa protein from cocoa beans. Cocoa beans consist of 52% albumin and 43% globulins and peanut protein Ara h2 is from the albumin family thus it warranted us to utilize this purification method.

Peanuts were defatted and crushed into fine powder as described above in method 1. The peanut proteins were, however extracted using 0.5% SDS-0.05 M phosphate buffer,

pH 6.9 instead of TBS buffer. Peanut powder (0.1 g) was weighed into 1.5 mL Eppendorf tubes and combined with 1 mL of extraction buffer (multiple batches done at once). The Eppendorf tubes were placed in a water sonication bath (Model 50T, VWR) for 1 hour changing the water to keep the water temperature constant at room temperature. After 1 hour, the samples were centrifuged (Allegra 21 Centrifuge, Beckman) at 3,000 X g for 10 mins taking the supernatant out. The precipitates were further extracted by adding 1 mL of extraction reagent buffer to the Eppendorf tubes followed by 1 hour water sonication bath and centrifugation at 3,000 X g for 10 mins. The supernatant was combined and pellets discarded.

The supernatant was filtered using a Whatman syringe filter 1.0 µm GF/B w/GMF and placed in dialysis tubing (7K MWCO) to be dialyzed against 15 mM ammonium bicarbonate (5 L bucket) with minimum of 6 changes at 4° C. The extract was then lyophilized and protein powder (0.050 g) dissolved in 10 mL of TBS buffer (65 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, pH 8.3). The solution was then loaded (10 mL) onto a Q Sepharose Fast Flow (GE Healthcare) column that was packed with approximately 100 mL of resin in Flex-Column 2.5 x 30 cm (Kimble Chase). The column was washed with 100 mL TBS buffer containing 40 mM NaCl at flow rate of 2 mL/min to elute proteins of noninterest. Then a linear gradient of 400 mL using 200 mL TBS buffer with 40 mM NaCl to 200 mL TBS buffer with 500 mM NaCl was used to elute protein of interest at a flow rate of 2 mL/min. Fractions were collected for 1 min 15 secs per tube to give approximately 2.5 mL per tube.

The fractions were analyzed for protein as described above in Method 1 using coomassie G-250 dye bonding assay followed by SDS-PAGE gel to identify fractions

containing Ara h2. Fractions of interest were collected and dialyzed (7K MWCO) overnight at room temperature against 15 mM ammonium bicarbonate (pH 7.0) followed by lyophilization. The sample was resuspended in dH_2O (Milli-Q ultrapure) and subjected to centrifugal filters of 30K MWCO to remove proteins greater than or equal to 30 kDa (Amico Ultra-15, Ultracel-30K, Merck Millipore). The centrifuged protein was verified for purity by SDS-PAGE, lyophilized and stored at -20° C.

BS^3 reaction. Protein powder that had been lyophilized was dissolved in 20 mM HEPES, pH 8 buffer to give a concentration of 12 mg/mL as confirmed by BSA standard curve. This dissolved protein was used as a stock to separated and diluted by two-fold to give two different concentrations, 6 mg/mL and 12 mg/mL. BS^3 (Lot #PD198244, prod. # 21580, Thermo Scientific) was dissolved in 20 mM HEPES, pH 8 buffer to obtain concentrations of 100, 50, 25, and 12.5 mM. These concentrations were created in duplicates to accompany the two separate protein concentrations. The cross-linker was prepared immediately before adding to the protein to prevent from hydrolysis of NHS ester.

The dissolved protein and BS^3 solution was added at a 1:1 volume ratio (35 µL:35 µL) followed by incubation for 30 mins at room temperature followed by SDS-PAGE analysis (Figure 12a). After 30 mins of incubation at room temperature, samples were placed at 37° C for continued reaction with cross-linker followed by SDS-PAGE analysis after 2 days (Figure 12b) and 6 days (Figure 12c) (Gel from Thermo Scientific, 2x sample buffer & marker from Novagen). Due to 1:1 ratio, the final protein concentration and BS^3 cross-linker concentration was cut in half. This gave final protein concentration of 6 mg/mL from 12 mg/mL and 3 mg/mL from 6 mg/mL. The final concentration of cross-linker was 50, 25, 12.5, and 6.25 mM from initial 100, 50, 25, and 12.5 mM, respectively.

Another condition of increasing the pH from 8 to 10.7 was tested to see if cross-linking can be enhanced with BS^3. Same conditions as above were used, however, only one protein concentration of 6 mg/mL was tested instead of both. Again, fresh batch of BS^3 was prepared at concentrations of 100 mM to 12.5 mM as above. The reaction was completed in duplicate so that one set can be placed at room temperature and the other in the incubation at 37° C. The reaction mixture was allowed to incubate followed by SDS-PAGE analysis after 1 day (Figure 13a) and 4 days (Figure 13b) (Gel from Thermo Scientific, 2x sample buffer & marker from Novagen). Same ratio of protein to cross-linker was used as above at 1:1 volume ratio (35 µL: 35 µL) diluting the protein and cross-linker concentration by half. This gave final protein concentration of 3 mg/mL from 6 mg/mL and final concentration of cross-linker of 50, 25, 12.5, and 6.25 mM from initial 100, 50, 25, and 12.5 mM, respectively.

Since the NHS ester is reactive towards sulfhydryl and primary amine groups, a reducing agent 2-mercapaptoethanol was added to reduce disulfide bonds and open the structure so that more possible reactive sites can be accessed by BS^3. Lyophilized protein powder was dissolved in 20 mM HEPES, pH 8.0 buffer and separated in duplicates. To one protein solution, 1.4 µL of 2-mercaptoethanol was added after 15 mins of incubating the protein at 37° C. Fresh BS^3 was prepared immediately before adding to the protein at concentrations from 35 mM to 65 mM in 5 increments. Protein and cross-linker were added at 1:1 volume ratio (50 µL:50 µL) followed by incubation at 37° C.

Due to 1:1 ratio, the final protein concentration and BS^3 cross-linker concentration was cut in half. This gave final protein concentration of 2 mg/mL from 4 mg/mL of protein. The final concentration of cross-linker was 32.5, 30.0, 27.5, 25.0, 22.5, 20.0, and 17.5 mM

from initial 65, 60, 55, 50, 45, 40, and 35 mM, respectively. Since no reaction was observed within 30 mins or 2 days of incubation, an SDS-PAGE analysis was performed after 5 days of incubation (Figure 14) (Gel from Thermo Scientific, 2x sample buffer & marker from Novagen). Before loading the protein onto the gel, 0.5 µL of 1 M glycine was added to 10 µL of protein and cross-linker solution to quench any unreacted cross-linkers in the solution. Then 10 µL of 2x Laemmli sample buffer was added to the quenched solution which turned green/yellow due to pH change. Thus, to bring the solution back to blue, 1 µL of 1 M NaOH was added to the solution.

Sulfo-EGS reaction. Protein powder that had been lyophilized was dissolved in 20 mM HEPES, pH 8 buffer and 20 mM HEPES, pH 10.7 buffer to give concentrations of 8.6 mg/mL and 11.6 mg/mL, respectively, as confirmed by BSA standard curve. Sulfo-EGS (Lot #CK2606, prod. #C1129, ProteoChem) was dissolved in 20 mM HEPES, pH 8 buffer and 20 mM HEPES, pH 10.7 buffer to obtain concentrations of 10, 5, 2.5, and 1.25 mM. These concentrations were created in four sets to accompany the two-different pH and two different temperature conditions. The cross-linker was prepared immediately before adding to the protein to prevent hydrolyzes of NHS ester.

The dissolved protein and Sulfo-EGS solution was added at a 1:1 volume ratio (37.5 µL:37.5 µL). Due to 1:1 ratio, the final protein concentration and Sulfo-EGS cross-linker concentration was cut in half. This gave final protein concentration of 4.3 mg/mL from 8.6 mg/mL and 5.8 mg/mL from 11.6 mg/mL. The final concentration of cross-linker was 5, 2.5, 1.25, and 0.625 mM from initial 10, 5, 2.5 and 1.25 mM, respectively. One sample with buffer at pH 8 was incubated at room temperature (Figure 15a) and another at 37° C (Figure 15b). Same was done for reaction mixture with buffer at pH 10.7, one at room

temperature (Figure 15c) and another at 36° C (Figure 15d). An SDS-PAGE analysis was done 54 days after due to insufficiency of SDS-PAGE gels at the time (Gel from Thermo Scientific, 2x sample buffer & marker from Novagen).

DST reaction. Protein powder that had been lyophilized was dissolved in 20 mM HEPES, pH 10.7 buffer to give concentration of 4 mg/mL, as confirmed by BSA standard curve. Since the NHS ester is reactive towards sulfhydryl and primary amine groups, a reducing agent 2-mercapaptoethanol was added. The protein was split with one having 2-mercaptoethanol (added after incubating protein at 37° C for 15 mins) and one without. DST (Lot #DD2687, prod. #C1133, ProteoChem) was dissolved in 20 mM HEPES, pH 10.7 buffer. DST is not soluble in HEPES buffer so DMSO (7% final concentration) was used to dissolve the cross-linker. The same amount of DMSO was also added to the control protein.

A serial dilution was carried out from 25 mM to 3.125 mM with 4 total concentrations. The dissolved protein and DST solution was added at a 1:1 volume ratio (50 µL:50 µL). Due to 1:1 ratio, the final protein concentration and Sulfo-EGS cross-linker concentration was cut in half. This gave final protein concentration of 2 mg/mL from 4 mg/mL. The final concentration of cross-linker was 12.5, 6.5, 3.1, and 1.6 mM from 25, 12.5, 6.3, and 3.1 mM, respectively. Protein was incubated at 37° C followed by SDS-PAGE analysis 2 days later (Figure 16) (Gel from Thermo Scientific, 2x sample buffer & marker from Novagen). Before running the gel, 0.5 µL of 1 M glycine was added to quench

any unreacted cross-linkers in the solution followed by 2x Laemmli sample buffer and 1 µL of 1 M NaOH to turn the solution back to blue from the pH change.

BM(PEG)$_3$ reaction. Protein powder was dissolved in 20 mM HEPES, pH 8 to give a concentration of 4 mg/mL as confirmed by BSA standard curve. Since BM(PEG)$_3$ is a cross-linker that conjugates between sulfhydryl groups, the protein had to be reduced and this was carried out by using immobilized TCEP disulfide reducing gel (Thermo Scientific). To test the reducing activity of TCEP, Ellman's reagent was used which did confirm the gel was active after color change to yellow. The dissolved protein was added to TCEP resin that was equilibrated with 20 mM HEPES, 20 mM EDTA, pH 8 by vortexing for 1 min followed by centrifuge at 1,000 X g and removing supernatant. The protein (250 µL) and resin (500 µL) mixture was mixed in an Eppendorf tube and stirred for 2 hours at room temperature followed by centrifuge at 1,000 X g for 1 min, carrying the supernatant. Another 100 µL of buffer was added to the Eppendorf tube to extract any remaining protein from the resin mixture.

BM(PEG)$_3$ (Lot # RA230660, Prod # 22337, Thermo Scientific) was dissolved in DMSO (13.3%) followed by serial dilution to obtain following concentrations: 153.26, 76.63, 38.32, 19.16, 9.58, 4.79, 2.39, 1.20, 0.60, and 0.30 mM. Cross-linker was prepared and covered in foil to protect from light immediately before adding to already reduced protein. Added 130 µL of reduced protein and 20 µL of cross-linker at varying concentrations. Samples were incubated at room temperature followed with SDS-PAGE analysis after day 1 (Figure 17a) and day 7 (Figure 17b). (Gel from Bio-Rad, 5x sample buffer & marker from Bio-Rad). Final protein concentration was 3.5 mg/mL and final

cross-linker concentrations were: 20.43, 10.22, 5.11, 2.55, 1.28, 0.639, 0.319, 0.160, 0.0798 and 0.0399 mM.

EDC reaction. In order to modify/mask IgE binding epitopes of Ara h2, a peptide called TV1 (sequence: MDAEFRHDS-NH_2, MW: 1107.2) was reacted in the presence of EDC. This was carried out in order to crosslink with one of the amino acids on the epitopes or at least mask the epitope sites with the peptide. Protein was reacted with cross-linker EDC and sulfo-NHS via 2-step reaction since it is more efficient as described above. Since Ara h2 has high percentage of glutamic acid and aspartic residues, it was used in the activation reaction with EDC. The activation buffer is recommended to have pH between 4.5-7.2 (without amines or carboxylate in the buffer) so 4-Morpholineethanesulfonic acid (MES) was used. Activation buffer consisted of 0.1 M MES, 0.5 M NaCl, pH 5.0 that was used to dissolve the protein for a protein concentration of 8.0 mg/mL as confirmed by a BSA standard curve.

Since coupling buffer is recommended to be at pH 7-8, a phosphate-buffered saline (PBS) was used (100 mM sodium phosphate, 150 mM NaCl, pH 7.2). The TV1 peptide was dissolved in PBS buffer to give a concentration of 1.5 mM. EDC was equilibrated to room temperature before opening. To the activated protein in MES buffer, 2 mM of EDC and 5 mM of sulfo-NHS was added and allowed to react for 15 mins at room temperature. The reaction was quenched of unused EDC by adding 2-mercaptoethanol at a final concentration of 20 mM. Peptide containing solution was added to the quenched activated protein solution and allowed to react for 2 hours at room temperature. Final concentration of protein was 4 mg/mL since 1:1 volume ratio of Protein and TV1 peptide was used. The whole reaction was quenched by adding hydroxylamine at final concentration of 10 mM

which hydrolyzes nonreacted NHS present on the protein and results in hydroxamate. An SDS-PAGE analysis (Figure 18) was done after quenching the reaction using gel from Thermo Scientific, 2x sample buffer & marker from Novagen.

SDS-PAGE protocol. Two different brands of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were used, however, both contained the same polyacrylamide gradient of 4% to 20%. A 4-20% Mini-PROTEAN TGX (Tris-Glycine eXtended) Precast Protein Gels, 12-well, 20 µL from Bio-Rad and another a 4-20% Precise Tris-Glycine Precast Gels, 12-well, 20 µL from Thermo Scientific. A 2x Laemmli sample buffer or 5x Laemmli sample buffer was used that was made according to Cold Spring Harbor Protocols (120 mM Tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% w/v bromophenol blue, pH 6.8). Two different protein markers were used for reference on the SDS-PAGE gels. Commercially available Protein Marker, (Novagen) with molecular weights of 15, 25, 35, 50, 75, 100 and 150 kDa was used when gel from Thermo Scientific was run or Precision Plus Protein Standards Dual Color (Bio-Rad) with molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa was used when gel from Bio-Rad was used. Gel was running at 110 V until the dye front reached the bottom of the gel. Gel was stained with staining solution created with Coomassie brilliant blue R-250 followed by de-staining until bands were visible. Gel was imaged using Azure Biosystems c300.

Results and Discussion

NHS ester reactions. BS^3 reaction without any addition of 2-mercaptoethanol was tested to see if protein can be modified without reduction. The protein used for this batch

was not pure with just Ara h2 as it has other proteins as seen in Figure 12(a, b, c), lanes 2 & 8 (control lanes) for concentrations of 12 mg/mL and 6 mg/L, respectively. The purpose of this reaction was a prove of concept to see if any cross-linked dimer or oligomers are observed with BS^3 cross-linker. As seen by the SDS-PAGE gel in Figure 12(a, b, c) that there was disappearance of Ara h1 as the concentration of cross-linker was increased from left to right. This is possibly proving that Ara h1 was cross-linked and due to high MW, it could have precipitated out of solution without being loaded onto the gel.

Protein Ara h2 after 30 mins of incubation at room temperature Figure 12(a) with BS^3 appears to have little effect as noted by the same density of the protein bands as concentration of cross-linker is increased from left to right on the SDS-PAGE. There is slight decrease in intensity of Isoform Ara h2.02 in Figure 12(a), lane 12 which could possibly have cross-linked and precipitated out of solution. However, after placing this same protein/cross-linker mixture at 37° C followed by SDS-PAGE analysis 2 days (Figure 12(b)) and 6 days (Figure 12(c)) after showed Ara h2.01 and Ara h2.02 band intensity decrease, almost nothing to very faint. This was only observed at the highest cross-linker concentration of 50 mM BS^3 (Figure 12(b, c) lanes 6 & 12). There are two possible reasons that Ara h2 band intensity decreases with no possible dimerization or multimerization. One reason is that the protein could have cross-linked with decreased solubility causing the protein to precipitate out of the solution. The other reason is that the high concentration of BS^3 could have some effect on the SDS-PAGE gel.

BS^3 was tested with different pH as well to see if that would promote cross-linking among peanut proteins thus pH was changed from 8 to 10.7. Protein and cross-linker were incubated for 1 day (Figure 13(a)) and 4 days (Figure 13(b)) followed by SDS-PAGE

analysis. Batch was split in half with one at room temperature and other at 37° C. It can be seen that as concentration of BS^3 is increased from left to right, there is a decrease in protein band intensity of Ara h2, Ara h1 and Ara h3. As for the highest concentration of BS^3 there is a disappearance of all the proteins from that lane as compared to the control lane. This could be due to the protein being cross-linked and precipitating out of the solution or it could be that particular concentration of BS^3 could be have a reaction with the SDS-PAGE gel, however, this is undetermined and an area for future investigation.

Since BS^3 reaction without 2-mercaptoethanol showed little evidence of dimerization or oligomerization, another set of reactions were set up that included the reducing agent 2-mercaptoethanol. Since this batch of protein was different, both non-reducing and reducing conditions were used. It can be seen from Figure 14(a) and 14(b) that there is no dimer or oligomers observed on the SDS-PAGE gel. What can be noticed is that the decrease in intensity of protein is observed compared to the control. This is observed in both the reducing and non-reducing conditions of the protein. This means that it must be BS^3 interfering with the SDS-PAGE gel and causing the band intensity to decrease and not the reducing agent. There is a decrease in Ara h1 as noticed with protein band disappearance. Thus, it is highly likely that BS^3 has reactivity towards Ara h1 and possibly some reactivity towards Ara h2 at 50 mM BS^3 concentration. Since Ara h1 is also a major peanut allergen, this can be of a further study by isolating and reacting Ara h1 with cross-linker. Further testing is required to confirm if Ara h2 concentration has decreased

due to modification or cross-linker by testing patient's IgE reactivity to the modified protein in an ELISA or Western Blot assay.

Protein reaction with Sulfo-EGS cross-linker showed no new formation of dimer or oligomer (Figure 15). The SDS-PAGE gels from Figure 15(a, b, & c) show no reactivity as the band intensity of Ara h2 protein (or any other protein on the gel) have not decreased along with no new bands as compared to the control. Even trying two different pH (8 & 10.7) and two different temperature (room temperature & 37° `C) showed no sign of cross-linking. Gel from Figure 15(d) (lane 6) shows possible crosslinking with new bands appearing between 60-70 kDa as compared to the control. Lane 7 which is the next higher concentration of cross-linker shows decrease in Ara h2.02 band however no new band formation to indicate cross-linking. Upon repeating the SDS-PAGE (data not shown) with same condition, there was no evidence of those bands, which indicate that those bands between 60-70 kDa were from some other impurities. It could be possible that upon repeating the SDS-PAGE gel, the cross-linked protein might have precipitated out and was not being loaded into the well.

DST cross-linker was applied which was dissolved in 7% DMSO. This same percentage of DMSO was also added to the control Ara h2 protein as well. DST was reacted with Ara h2 for 2 days (Figure 16) followed by SDS-PAGE gel to observe for cross-linking between protein. Protein was split into duplicate with one set having 2-mercaptoeathanol and the other without it. As seen on the gel, there is no new formation of dimer or oligomer. What can be seen is in lanes 5 & 10 a decrease in band intensity of Ara h2 which is at the highest concentration of DST used (12.5 mM). This can be due to two reasons; if Ara h2 did cross-link, it could have precipitated out or DST itself at that concentration could be

interacting with the SDS-PAGE gel. Again, this has to be further tested by using IgE from patient allergic to peanut in an ELISA and Western Blot assays to see if the reactivity is the same.

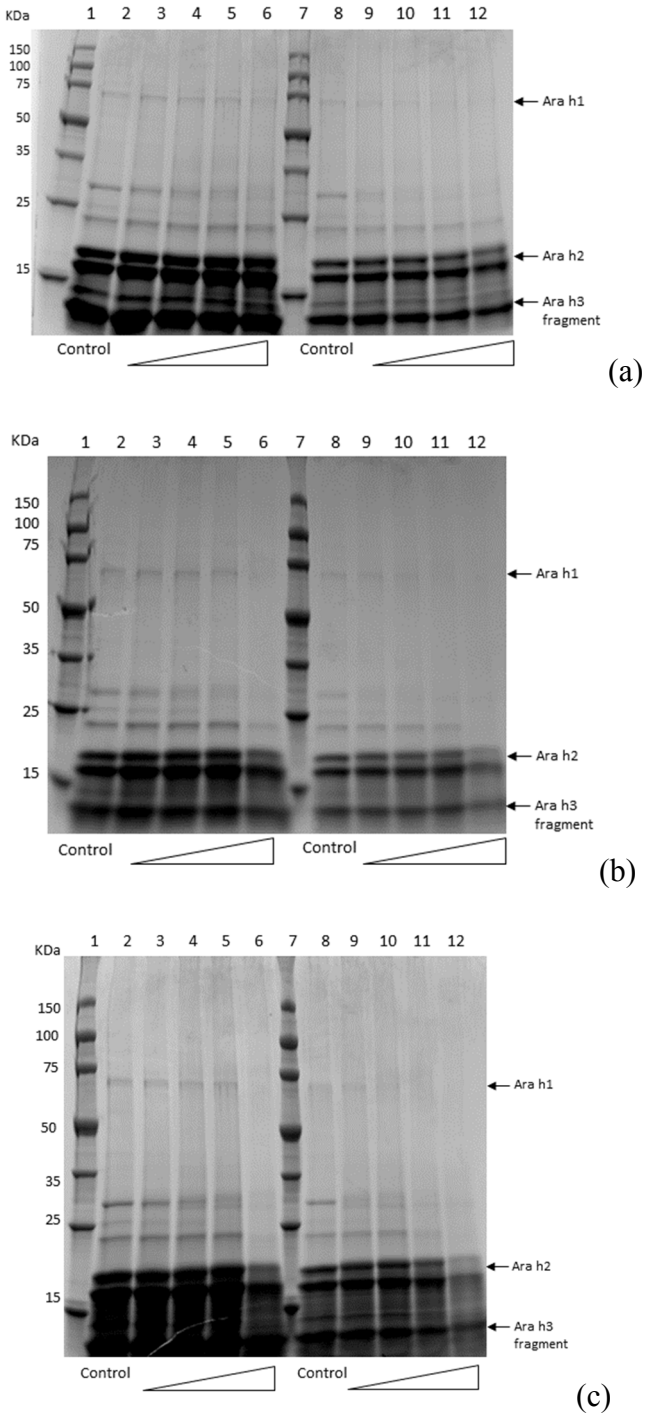


Figure 12. SDS-PAGE of BS^3 cross-linker in pH 8. SDS-PAGE after **30 mins (a)** of reaction with **BS^3** in 20 mM HEPES, **pH 8** at **room temp**. SDS-PAGE after **2 days (b)** of reaction with **BS^3** in 20 mM HEPES, **pH 8** at **37°C**. SDS-PAGE after **6 days (c)** of reaction with **BS^3** in 20 mM HEPES, **pH 8** at **37°C**. Lanes: (1 & 7) molecular marker (2 & 8) control (3 to 6 & 9 to 12) 6.25 mM to 50 mM of BS^3 left to right. Concentration of protein in lanes (2 to 6) is 6 mg/mL and (8 to 12) is 3 mg/mL.

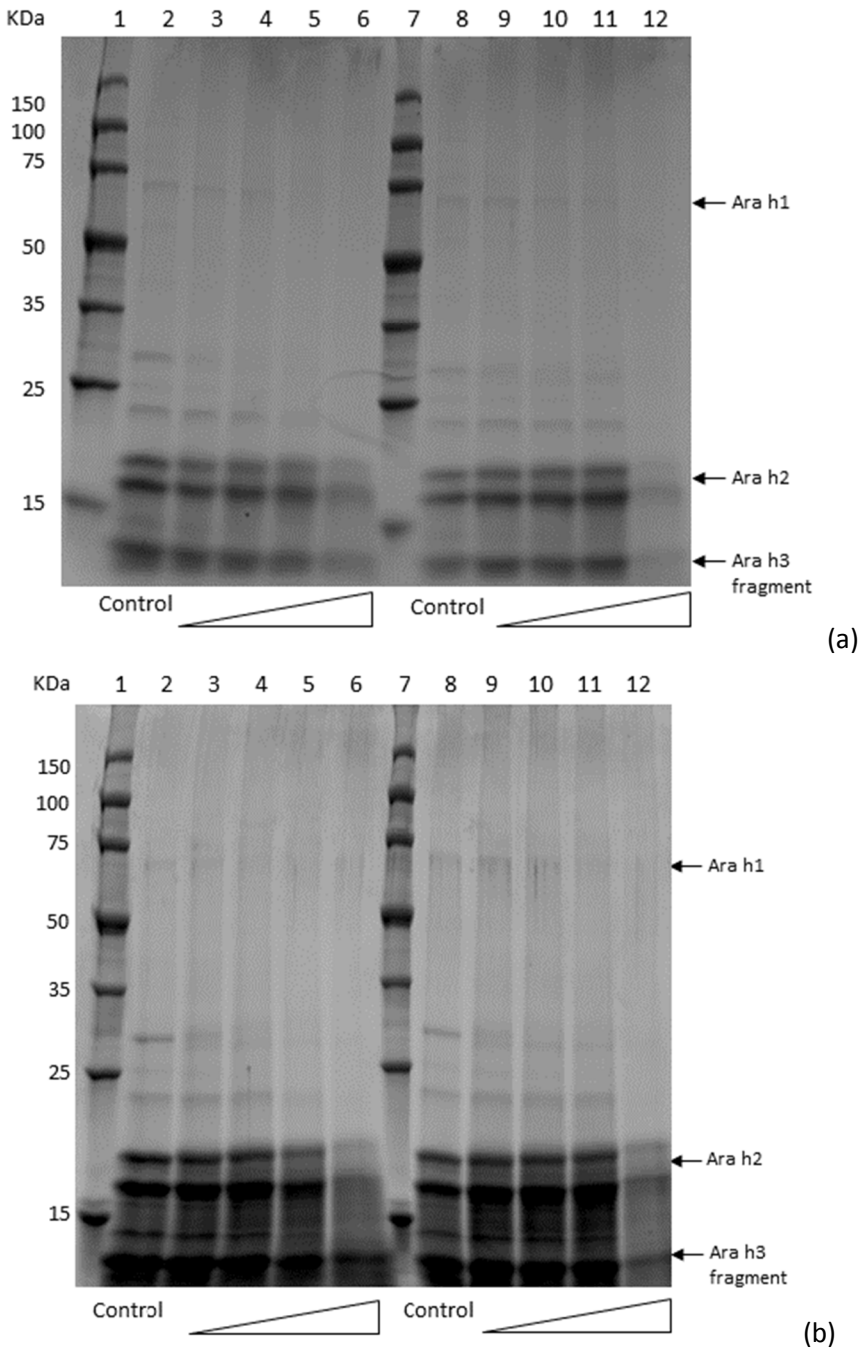


Figure 13. SDS-PAGE of BS^3 cross-linker in pH 10.7. SDS-PAGE after **1 day (a)** and **4 days (b)** of reaction with **BS^3** in 20 mM HEPES, **pH 10.7** at **room temp** and **37°C**. Lanes: (1 & 7) molecular marker (2 & 8) control (3 to 6 & 9 to 12) 6.25 mM to 50 mM of BS^3 left to right. Lanes (2 to 6) are samples incubated at 37°C and (8 to 12) are samples incubated at room temp. Final concentration of protein at 3 mg/mL.

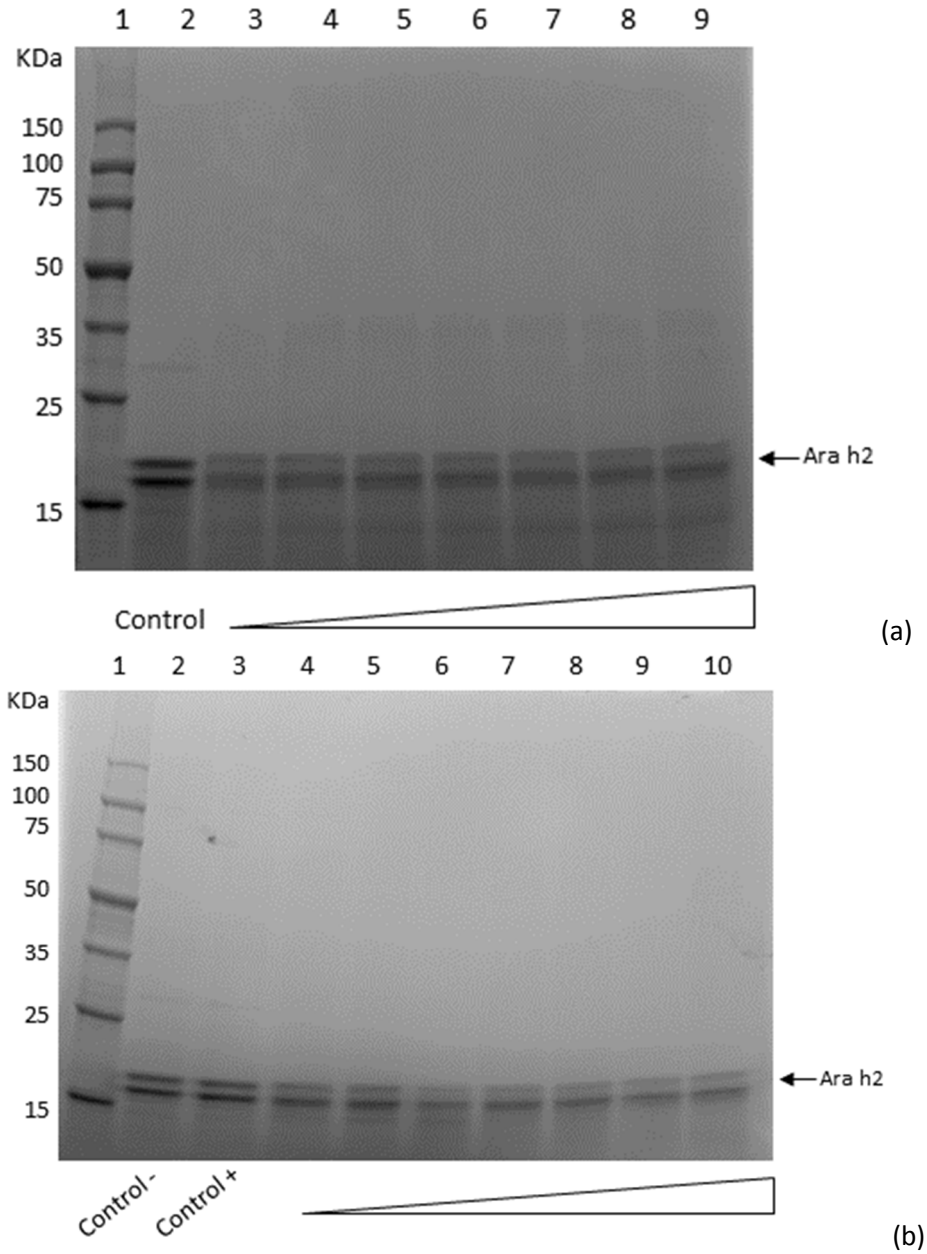


Figure 14. SDS-PAGE of BS^3 cross-linker in pH 8 with and without 2-mercaptoethanol. SDS-PAGE after **5 days** of reaction with **BS^3** in 20 mM HEPES, **pH 8** at **37°C**. **(a)** Protein plus BS^3 without 2-mercaptopethanol **(b)** Protein plus BS^3 plus 2-mercaptoethanol. Lanes: **(a)**(1) molecular marker (2) control without 2-mercaptoethanol and (3 to 9) 17.5 mM to 32.5 mM in 5 increments. **(b)**(1) molecular marker (2) control without 2-mercaptoethanol (3) control with 2-mercaptoethanol (4 to 10) 17.5 mM to 32.5 mM left to right. Final concentration of protein at 2 mg/mL.

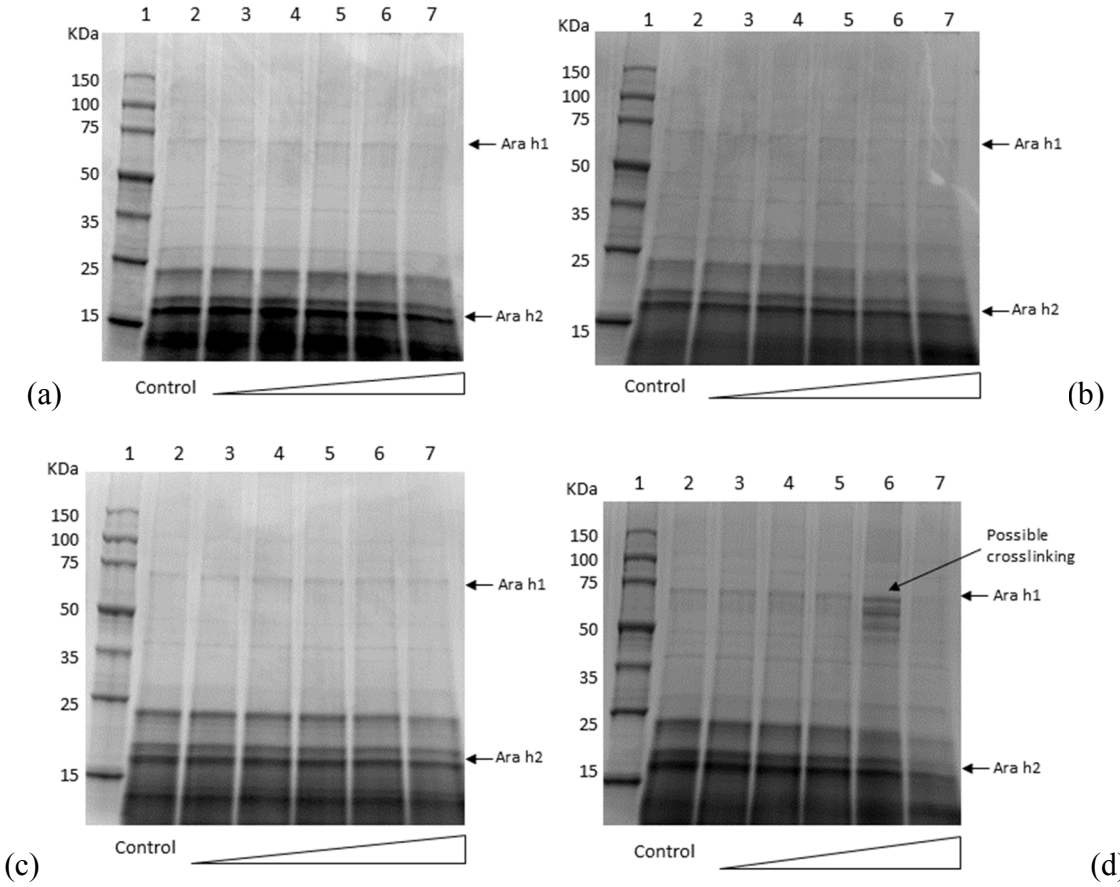


Figure 15. SDS-PAGE of Sulfo-EGS cross-linker. SDS-PAGE after 54 days of reaction with **Sulfo-EGS** in 20 mM HEPES, **pH 8** and **pH 10.7** at **room temp** and **37°C**. **(a)** pH 8 at room temp. **(b)** pH 8 at 37°C **(c)** pH 10.7 at room temp. **(d)** pH 10.7 at 37°C. Lanes: (1a,b,c,d) molecular marker (2a,b,c,d) control (3 to 7) 0.625 mM to 5 mM of Sulfo-EGS left to right. Final concentration of protein (a,b) is 4.3 mg/mL and (c,d) is 5.8 mg/mL.

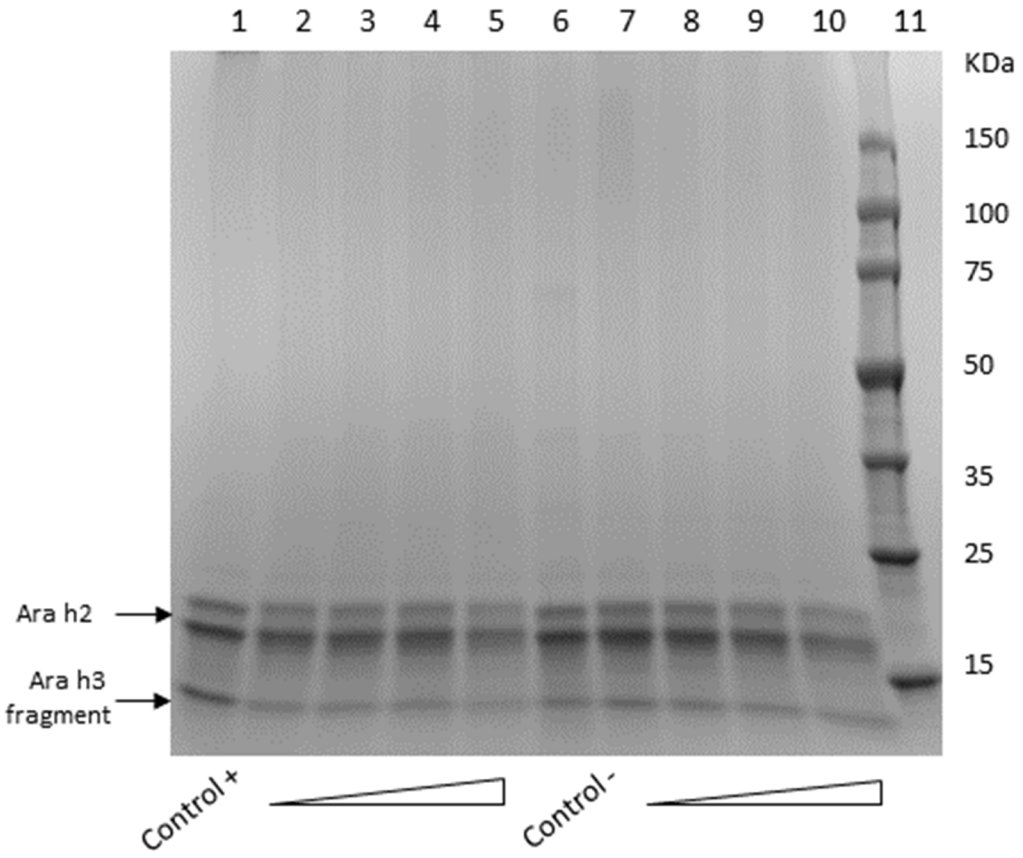


Figure 16. SDS-PAGE of DST cross-linker. SDS-PAGE after **2 days** of reaction with **DST** in 20 mM HEPES, **pH 10.7** at **37°C**. Lanes: (1) control with 2-mercaptoethanol (6) control without 2-mercaptopethanol (11) molecular marker ((2 to 5) with 2-mercaptoethanol & (7 to 10) without 2-mercaptoethanol) 1.6 mM to 12.5 mM of DST left to right. Final concentration of protein at 2 mg/mL.

Sulfhydryl reaction using maleimide. $BM(PEG)_3$ was reacted with Ara h2 protein for 1 day (Figure 17(a)) and 7 days (Figure 17(b) followed by SDS-PAGE analysis. As seen by the SDS-PAGE gel after 1 day and 7 days of incubation, there is no formation of dimer or oligomer. As a reminder, no reducing agent was added to this batch since TCEP resin was used to reduce protein before addition of cross-linker. There is a possibility that the cross-linker might have attached to one or more sulfhydryl groups of cysteine residues but not cross-linked with another protein which could be due to steric hindrance. The cross-

linker could have been inserted between the sulfhydryl groups but since the cross-linkers are of small molecular weight, they are not seen by SDS-PAGE analysis.

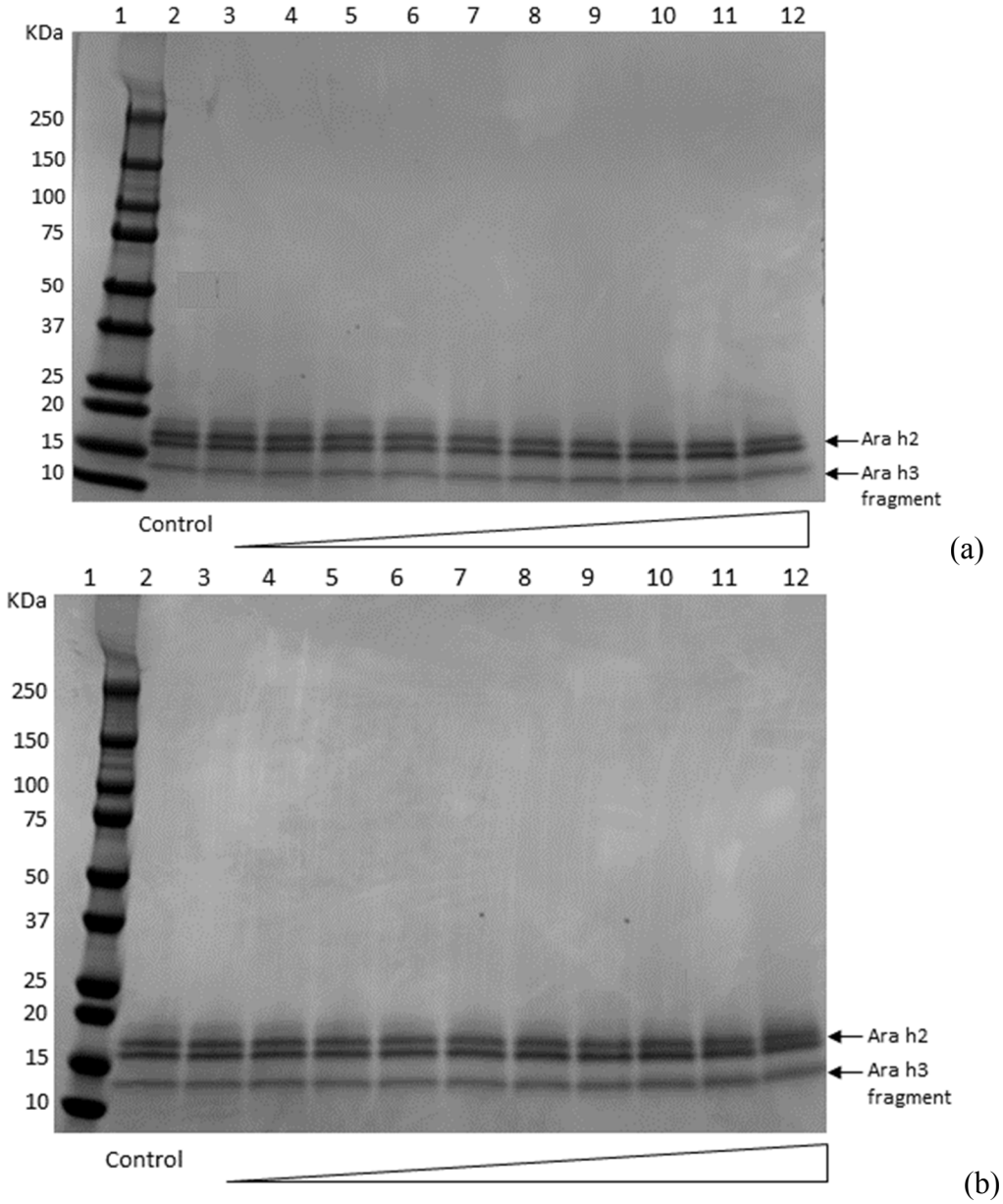


Figure 17. SDS-PAGE of $BM(PEG)_3$ cross-linker. SDS-PAGE after **1 day (a)** and **7 days (b)** of reaction with **$BM(PEG)_3$** in 20 mM HEPES, **pH 8** at **room temp**. Lanes: (1) molecular marker (2) control (3 to 12) 0.0399 mM to 20.43 mM of $BM(PEG)_3$ from left to right. Final concentration of protein at 3.5 mg/mL.

Amide bond formation between carboxylic acid and primary amine. EDC was used to try and conjugate Ara h2 with TV1 peptide using 2 step conjugation reaction with sulfo-NHS. As seen by SDS-PAGE in Figure 18 that there is no dimer or oligomerization observed. There are multiple possibilities why this is observed. Since the peptide is small with low molecular weight, it possibly could have attached itself to the protein but just not displayed on the SDS-PAGE gel.

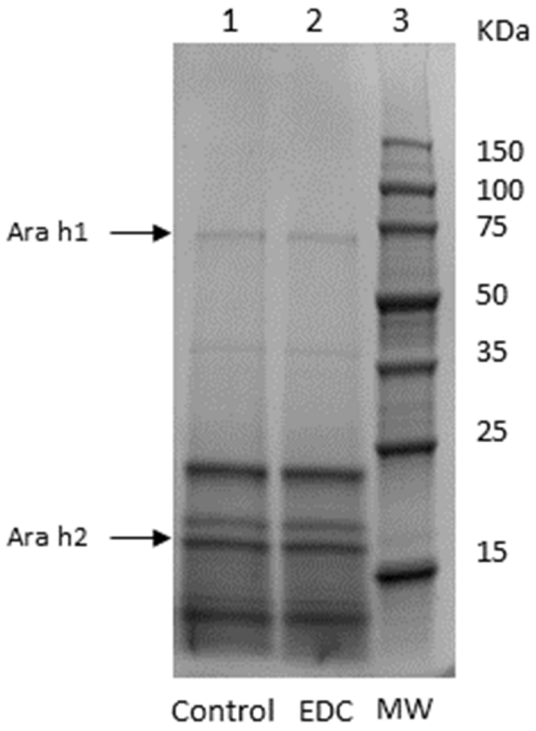


Figure 18. SDS-PAGE of EDC cross-linker. SDS-PAGE of reaction of Ara h2 with TV1 peptide using cross-linker EDC with sulfo-NHS in a 2-step reaction. Lanes: (1) control (2) EDC + TV1 peptide (3) molecular marker. Final concentration of protein at 4 mg/mL.

One test that can be employed on all the cross-linker is to do mass spectroscopy to see if the weight of the protein changed before and after addition of cross-linker. Another test can be tried to validate for the secondary structure of protein by using Circular Dichroism spectroscopy. Again, all these cross-linker could have attached to the protein and disrupted molecular binding between the proteins without actually conjugating with another protein but due to low molecular weight of cross-linker, they are not detectable by SDS-PAGE analysis. Thus, to really confirm a change in protein structure or addition of one or two cross-linkers without conjugation to another protein, an ELISA or Western Blot analysis can be carried out with IgE from patient serum that are allergic to peanut allergy.

Method 1 vs method 2. Most important part of cross-linking Ara h2 was to purify Ara h2 from peanuts at a continuous and efficient way so other parts of the research are not hindered. Basically, purification of protein from peanut seeds was the rate determining step in this study. The procedure that was used to in the beginning of this study was method **1**. This procedure involved 2 different chromatography columns with multiple steps utilizing many resources and time. Thus, method **2** was derived which involved only one chromatography column with fewer steps involving fewer resources and saving time.

Both method **1** and method **2** begins by defatting crushed peanut seeds using Soxhlet extraction in ether solvent. The next step was dissolving this defatted peanut powder in buffer and this is where the two methods make their first split. Method **1** used TBS buffer and Method **2** used phosphate buffer with 0.5% SDS. Method **2** originally used two different buffers to extract proteins: 0.5% SDS + 0.02 M phosphate buffer, pH 6.9 ("0.5% SDS" buffer hereafter) and 8 M Urea + 1% 2-mercaptoethanol ("8 M Urea" hereafter). Both buffers showed different proteins that were extracted as seen by SDS-

PAGE (Figure 19). Lane 2 & 3 contain protein that had been extracted, dialyzed (overnight against dH_2O), and lyophilized using 8 M Urea and 0.5% SDS, respectively. Protein powder extracted using 8 M Urea (0.0038 g) and 0.5% SDS (0.0037 g) was dissolved in 1 mL of 20 mM HEPES, pH 8. Lanes 4 & 5, 8 M Urea and 0.5% SDS, respectively, were proteins loaded with the extraction buffer. It can be seen that extraction with 0.5% SDS buffer showed Ara h2 being extracted with very few other protein as compared to extraction with 8 M Urea buffer. Thus, 0.5% SDS buffer was used for method **2** instead of 8 M Urea.

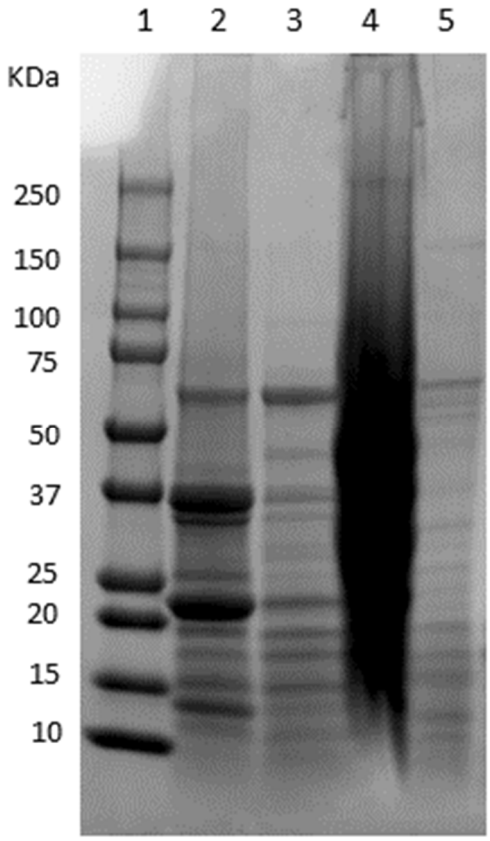


Figure 19. SDS-PAGE analysis of different buffers used to extract protein. Lanes: (1) molecular marker (2) 8 M Urea (dialyzed, lyophilized, and re-dissolved protein powder) (3) 0.5% SDS (dialyzed, lyophilized, and re-dissolved protein powder) (4) protein in 8 M Urea buffer (5) protein in 0.5% SDS buffer.

In comparison, Method **1** protein extract was filtered using cheese cloth followed by ammonium sulfate precipitate twice, first going from 0 to 40% carrying the supernatant and then carrying the pellet in 40% to 70%. This part of the procedure was omitted in Method **2.** Instead, the protein with extraction buffer was just subjected to centrifuge carrying the supernatant and passing it through syringe filter instead of cheese cloth. Next step in Method **1** requires dialyzing the pellet against TBS buffer so that it can be loaded onto Q Sepharose column. Method **2** also took the protein extract and was subjected to dialysis against 15 mM ammonium bicarbonate buffer. Protein from Method **1** was loaded onto the column after dialysis whereas protein that was on the lyophilizer was dissolved in TBS buffer and then loaded onto the Q Sepharose column.

After loading protein onto the column, both were subjected to running gradient of TBS buffer containing 40 mM to 500 mM NaCl after 100 mL of TBS buffer with 40 mM NaCl was ran through to elute protein. Method **1** requires fractions that contain Ara h2 to be dialyzed against Tris buffer containing 3 M NaCl and then loaded onto a phenyl sepharose column for another column chromatography. Method **2** takes proteins that contain Ara h2 after doing coomassie G-250 dye bonding assay to find those fractions as seen in Figure 20 that shows which fractions were collected followed by SDS-PAGE analysis (Figure 21). It can be seen that Ara h2 (Figure 12, lane 4) along with other lower molecular weight proteins elute out of Q Sepharose column earlier followed by heavier proteins like Ara h1 (Figure 21, lane 11). The fractions containing Ara h2 are then subjected to centrifugal filter of 30K MWCO to eliminated proteins above 30K and thus purifying Ara h2. It can be noted that Method **2** can also be used to purify Ara h1 as well if needed.

A summary of the two-different methods is presented in a flowchart analysis in Figure 22 along with the amount of time needed to complete each step.

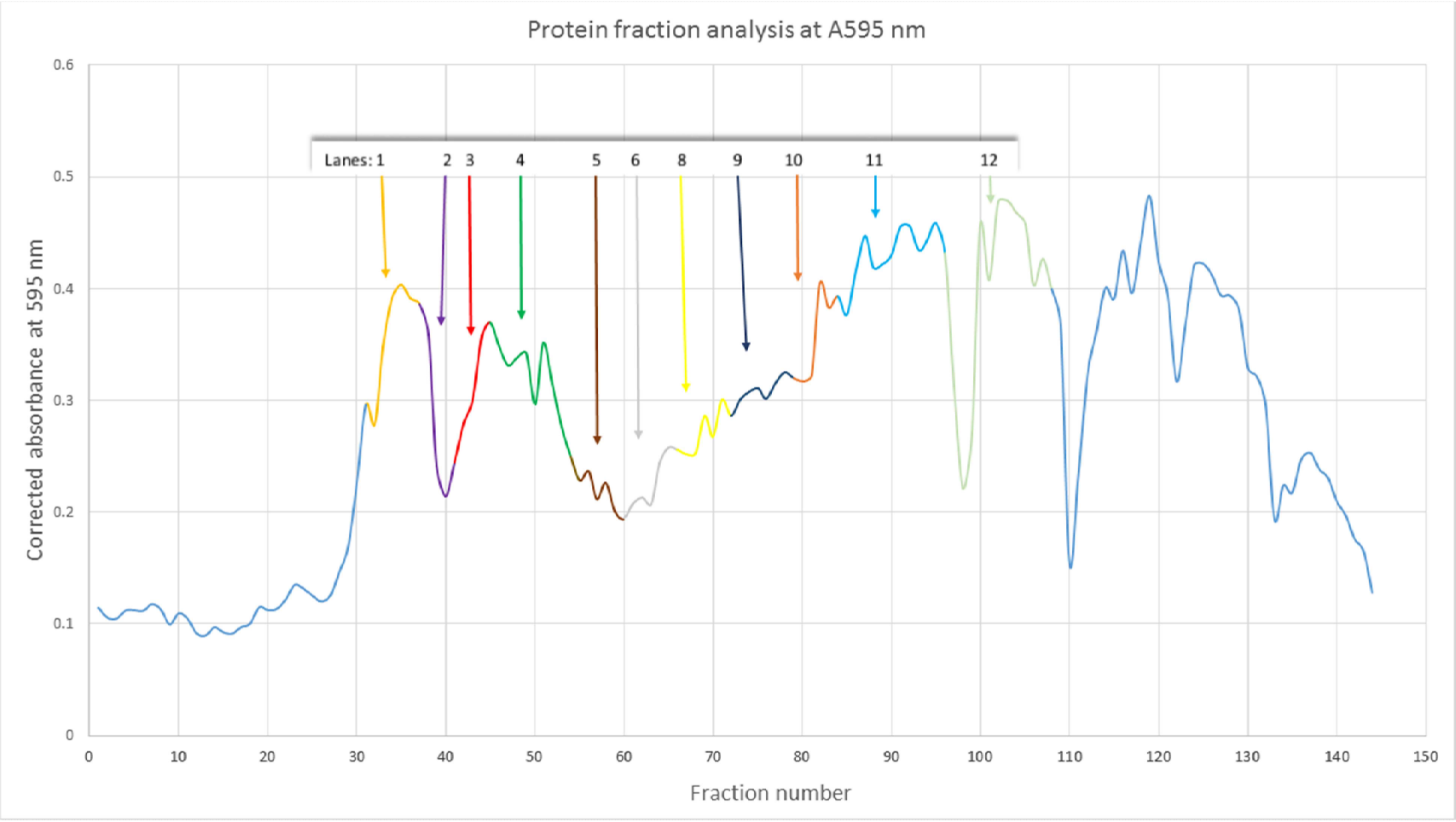


Figure 20. Analysis of fractions at absorbance 595 after Q-Sepharose column chromatography. Lanes of SDS-PAGE are labeled corresponding to the fractions that were collected and loaded onto the following lane well. SDS-PAGE (Figure 21).

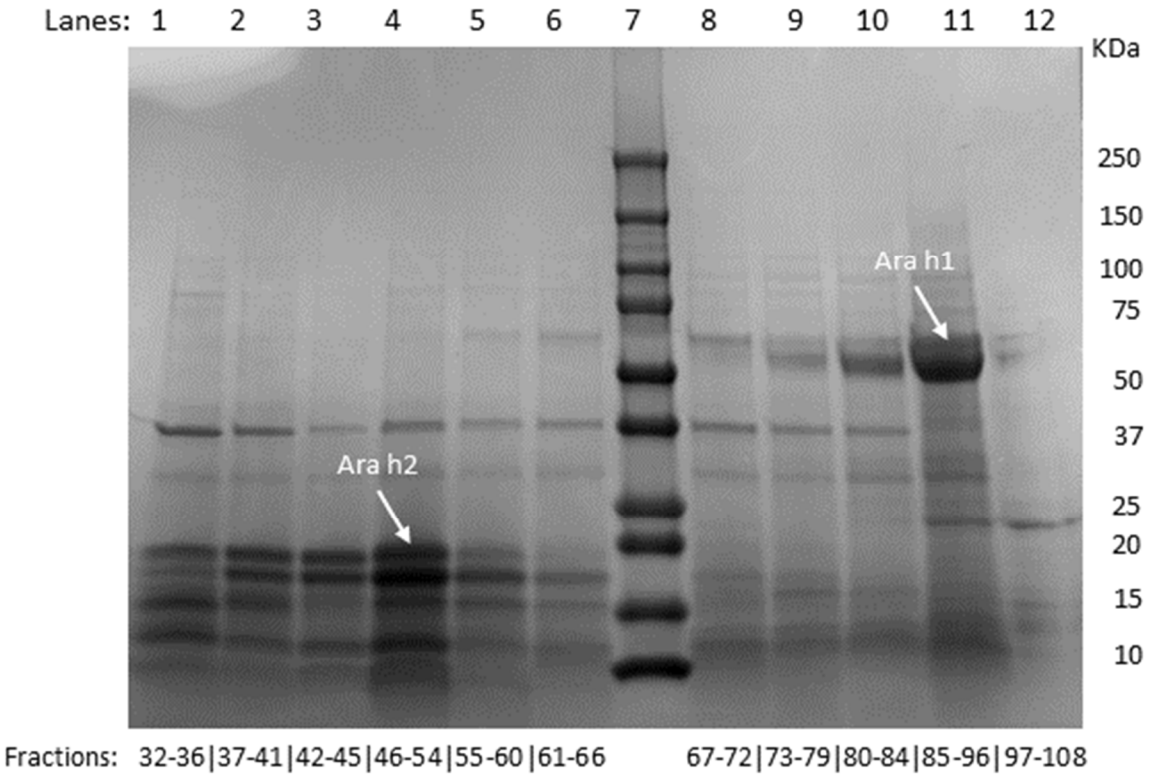


Figure 21. SDS-PAGE of fractions collected after Q-Sepharose column chromatography using Method **2**. Fractions are labeled under the lanes. Lane (7) molecular marker.

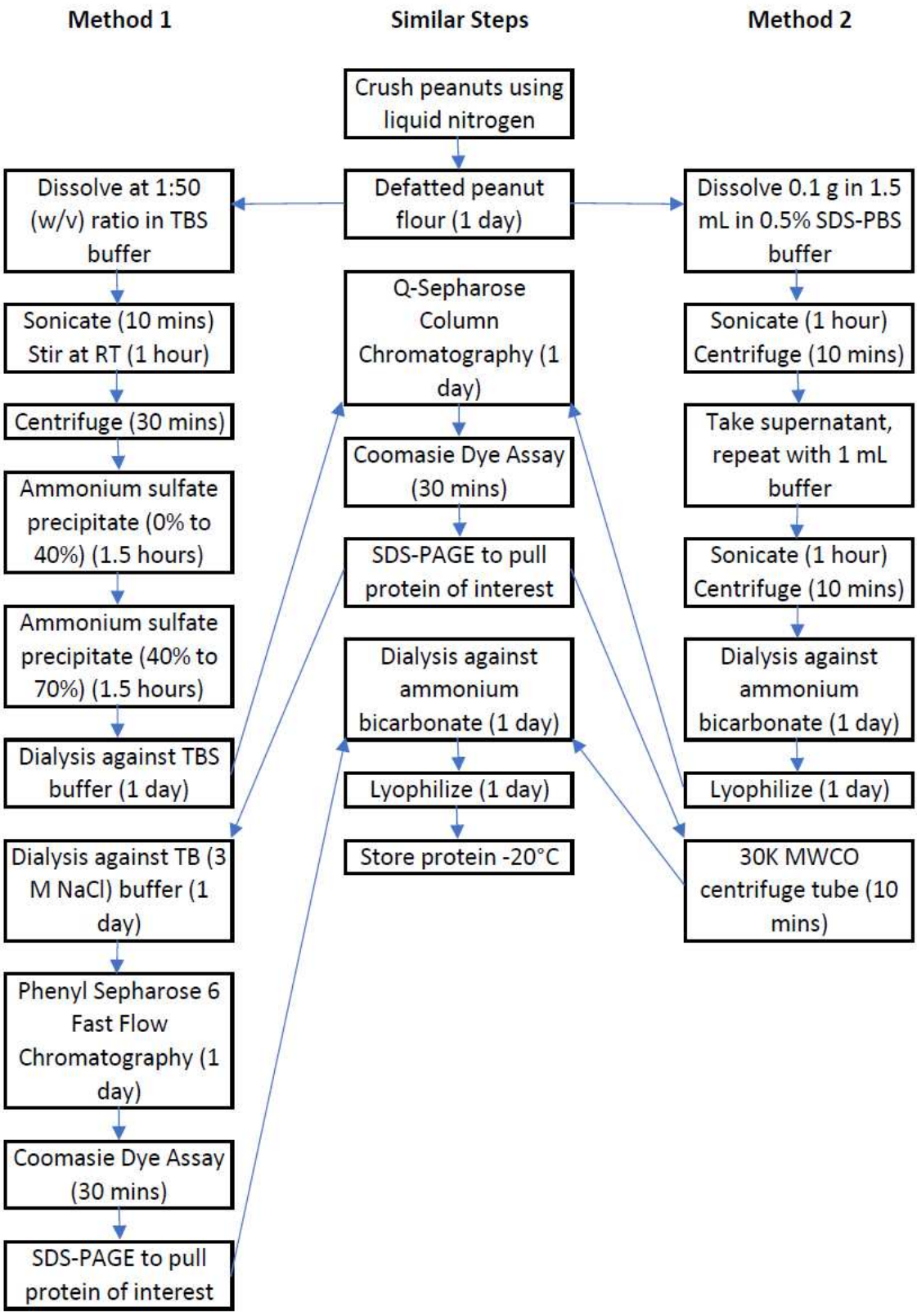


Figure 22. Flowchart comparison of Method **1** and Method **2**.

Conclusion

Overall, the reaction of cross-linkers with Ara h2 was negative to visualize a dimer or oligomer formation on SDS-PAGE; however, a new purification method was developed that requires less steps and time to purify Ara h2 and Ara h1. Cross-linkers containing NHS esters could have reacted with Ara h2 protein; however, further characterization of cross-linked product is needed to confirm. What was observed was that at higher concentration of cross-linkers like BS^3 there was precipitation of protein out of the solution. This was seen for both Ara h2 and Ara h1 along with other proteins. The precipitation can be confirmed since the protein band intensity decreased as concentration of cross-linker was increased. Reaction with bifunctional thiol reactive maleimides was inconclusive according to SDS-PAGE gel. Reaction with EDC cross-linker was not visualized as well on the SDS-PAGE requiring further characterization. EDC reacts with a carboxylic acid group, which Ara h2 has plenty of as seen in Figure 9. The peptide that was used, TV1, could possibly be attached on Ara h2. As mentioned before, the proteins could have one of the cross-linkers attached without being visualized on the SDS-PAGE and that further tests are required to characterize the structures of protein comparing before and after addition of cross-linker.

One of the possibility of not seeing cross-linking among Ara h2 protein could be the use of roasted peanuts. As stated in the introduction, roasted peanuts are subjective to the Maillard reaction which modifies lysine and arginine residues via glycosylation bond with sugars. Since Ara h2 protein was extracted from roasted peanut (most amount of protein extractable compared to raw protein), it is a possibility that Ara h2's lysine sites could be glycosylated making them unreactive towards NHS esters. Since there are only

3 lysine in Ara h2 (Figure 3), the reaction could be very slow requiring very precise energy and collision with cross-linker in the solution. Use of raw peanuts is a possibility in modifying Ara h2 since those proteins will not be glycosylated.

If the cross-linkers were to have reacted with Ara h2, there would have been a dimer or trimer formation seen on the SDS-PAGE. This dimer or trimer would have been isolated and characterized followed by purification of the allergoid. The allergoid would then have been sent out for in vivo testing to see if it has an effect on allergenicity in animals allergic to peanuts. If the in vivo study would have been followed, the allergoid would have proceeded to further studies and possibly as a modified allergen for use in EPIT studies on human.

Chapter 3

Dipeptidyl Peptidase-IV Inhibition for Treatment of Type 2 Diabetes

Introduction

Diabetes mellitus. Undoubtedly diabetes is one of the most severe disease in the human history. Diabetes has been found dating back around 1500 B.C. by the ancient Egyptians, who found effected people to urinate frequently and lose weight. The term diabetes mellitus was first coined by Greek physician Aretaeus who lived from about 80 to 138 C.E. Since the first measurements of glucose in urine in 1776 till now, there have been major improvements in understanding and controlling diabetes.[70] Diabetes mellitus is a disease where the blood glucose or blood sugar levels are too high. The homeostasis of glucose is disrupted because the body is not producing sufficient amounts of insulin or cannot properly use insulin. Glucose from the food is important to be transported from the blood to the inside of the cells to produce ATP. This is assisted by the hormone insulin which is produced by the organ pancreas. There are two types of diabetes mellitus; type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), both based on the amount of insulin produced by a person. With T1DM, body does not make insulin and with T2DM, which is the more common type of diabetes, the body does not make or use insulin well enough.

T2DM is one of the most important diseases of our time, with reported incidence increasing yearly among global population dramatically. It is projected that by 2025 there will be a 13.2 million increase in the number of people suffering from the disease specifically in the U.S.; with T2DM being the most prevalent and commonly seen in 90-

95% of the cases. This disease represents to be a global epidemic problem increasing morbidity, mortality and cost of care as diabetes has also been strongly associated with cardiovascular and cerebrovascular diseases. Initially at an earlier stage slow advancing insulin resistance is seen, which later advances to glucose intolerance and finally T2DM occurs over several years.[71]

According to the American Diabetes Association, 29.1 million Americans (9.3% of population) had diabetes in 2012 with 21.0 million diagnosed and 18.8 million undiagnosed. This is an increase of 1.0% from 25.8 million in 2010 to 29.1 million in 2012. The incidence of new diabetes cases was about the same in 2012 from 1.9 million in 2010 to 1.7 million in 2012. However, the number of Americans age 20 and older with prediabetes went up from 79 million in 2010 to 86 million in 2012. Diabetes is also high in certain ethnicity as 7.6% are non-Hispanic whites, 9.0% Asian Americans, 12.8% Hispanics, 13.2% non-Hispanic blacks and 15.9% American Indians/Alaskan Natives. The breakdown among Asian American is as follows; 4.4% Chinese, 11.3% Filipinos, 13.0% for Asian Indians and 8.8% for other Asian Americans. The breakdown among Hispanic adults is 8.5% Central and South Americans, 9.3% Cubans, 13.9% Mexican Americans and 14.8% Puerto Ricans.[72]

Diabetes is ranked number 7th as the leading cause of death in the U.S. in 2010, with 69,071 death certificates listing it as the underlying cause of death, and a total of 234,051 death certificates listing diabetes as an underlying or contributing cause of death. It is mentioned that deaths by diabetes is underreported due to the fact that only 35% to 40% of people with diabetes had it listed on their death certificate as the cause of death and that about 10% to 15% had it listed as the underlying cause of death.[72]

There are also two other categories called prediabetes and gestational diabetes. Prediabetes just means that you are at a borderline for T2DM and this can be prevented with proper exercise and diet. Gestational diabetes is when the blood sugar levels are high during pregnancy. About 7 out of 100 pregnant women in the U.S. get gestational diabetes and this is when a woman is pregnant for the first time. This increases the chances of developing T2DM later on and also increases in the child for obesity and T2DM. However, the focus of this study was on the control of T2DM since it is the most occurring type of diabetes. A simple blood test can show if you have diabetes which will be explained later on. Without enough insulin, the glucose from the food stays in the blood and over time can cause serious problems described hereafter.

Important hormones of pancreas in maintaining homeostasis of blood glucose. Before dwelling in the symptoms of diabetes, it's important to know the functions of pancreas and its secretion of important hormones for the homeostasis of glucose in the body. The pancreas secretes two important hormones, insulin and glucagon, along with digestive functions. There are other hormones secreted by the pancreas like amylin, somatostatin, and pancreatic polypeptide, however, their functions are not well known. The pancreas is composed of two major types of tissues; the acini and islets of Langerhans. The acini secrete digestive juices into the duodenum and the islets of Langerhans secrete insulin and glucagon directly into the blood. Insulin is released from the beta cells which make up the majority of cells (60% to 70%) in islets of Langerhans and glucagon is released from alpha cells which make up the second most abundant (25%) of islets of Langerhans.[73]

Insulin is stimulated by many stimuli with glucose being one of them. Glucose enters the beta cells via the glucose transporter type 2 (GLUT-2) and it is phosphorylated

by the enzyme glucokinase which serves as the glucose sensor for the beta cells and triggers the release of insulin as the glucose exceeds about 5 mM concentration. Another important factor according to Tan[74] is that orally administered glucose causes a greater increase in insulin than when it is administered via intravenous. The reason for this difference is due to increased production of incretin hormones such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide also called gastric inhibitory polypeptide (GIP) (discussed in greater detail below). These hormones are secreted by the gastrointestinal tract when food or glucose is orally consumed and causes direct activation of G-protein coupled receptors expressed on islet beta cells and thus indirectly increases insulin production. This is one of the ways to control insulin production and in turn controlling blood glucose levels.[74,75]

Insulin is a polypeptide made of two peptide chains, A and B, linked together by disulfide bridges. It is synthesized as preproinsulin, which is then cleaved in the endoplasmic reticulum to form proinsulin, which is then self-cleaved in the Golgi apparatus to form insulin and C-peptide. Insulin causes the storage of excess nutrients while inhibiting mobilization of endogenous substrates like glycogen. Insulin mediates glucose entry into most cells, in particular the muscle (skeletal and cardiac) cells. The brain and red blood cells are exceptions in which glucose is imported via diffusion down a steep concentration gradient. Insulin also stimulates glycogen production from glucose in muscle and liver while inhibiting glycogen breakdown in the liver. In adipose tissues, insulin inhibits triglycerides from breaking down by suppressing hormone-sensitive lipase activity. This decrease in free fatty acid release from adipose tissues causes a decrease in beta oxidation by the liver and thus a decrease in the production of ketone bodies. It is also

commonly seen in patients having T1DM who produce no insulin where the ketone bodies are increased because free fatty acid release is increased causing diabetic ketoacidosis.[74]

The other hormone released by the pancreas is Glucagon, which is a 29-amino acid long peptide produced from proglucagon. Glucagon has almost opposite affect than insulin in a sense that when blood glucose levels fall, glucagon helps to increase the blood glucose concentration by converting stored glycogen via glycogenolysis in the liver to glucose. Glucagon binds to the hepatic membrane receptor and causes a cascade of intracellular events via cyclic adenosine monophosphate (cAMP) as a second messenger, to regulate number of enzyme kinases or phosphatases. It is also important to note that patients with liver disease are hard to treat for hypoglycemia with glucagon. Another function of glucagon is the activation of myocardial adenylate cyclase which causes an increase in the cardiac output and should be monitored in T1DM.[74]

The cascade of insulin resolved glucose clearing from the blood to the tissues is known as the insulin signal transduction pathway. The activation of insulin receptors by insulin causes phosphorylation events of many, if not all, downstream components. The insulin-stimulated phosphorylation of phosphatidyl inositol-3-kinase (PI3K) stimulates the phosphorylation of two downstream proteins—protein kinase C (PKC)-ζ and protein kinase B (Akt). Phosphorylated PKC-ζ and Akt initiate two separate pathway branches, both of which ultimately lead to the translocation of glucose transporter 4 (GLUT4) from the cytoplasm to the plasma membrane. Once at the plasma membrane, GLUT4 facilitates the uptake of glucose into skeletal muscle and adipose cells causing a decrease in the blood glucose levels.[76]

Importance of controlling symptoms related to diabetes mellitus. Diabetes is known to cause number of complications like hypoglycemia, hypertension, dyslipidemia, stroke, blindness/eye problems, kidney disease and amputations. The common symptoms of diabetes are polyuria, polydipsia, feeling very hungry even after having a meal, extreme fatigue, blurry vision, cuts and bruises that are slow to heal, weight loss even though you are eating more (T1DM) and tingling, pain, or numbness in the hands or feet (T2DM). These symptoms are easy to detect in T1DM but are so faint in T2DM that they are sometimes unnoticed. Thus, it is important to detect early and treat diabetes which can decrease the risk of developing the complications of diabetes. There are several ways to diagnose diabetes which should be done in a health care setting such as doctor's office or at a certified lab that measures blood glucose levels.

In 2011, about 282,000 patients who visited the emergency room and were adults aged 18 and older had hypoglycemia as the first-listed diagnosis and diabetes as another diagnosis.[72] Hypoglycemia is a condition that is caused by a decrease in blood glucose levels of less than 70 mg/dL (normal <140 mg/dL). Some of the signs and symptoms of hypoglycemia include huger, nervousness, shakiness, perspiration, dizziness or light-headedness, sleepiness and confusion. Hypoglycemia can be grouped into two broad categories; autonomic and neuroglycopenic. Autonomic consists of sweating, heart palpitations, shaking, dizziness, hunger, etc. and neuroglycopenic includes confusion, drowsiness, speech difficulty, odd behavior, incoordination, etc.[77]

People with chronic kidney disease have a higher frequency of hypoglycemia than people with diabetes who do not have chronic kidney disease. Chronic kidney diseases affect how the drug is cleared and thus the drug's half-life. It also decreases degradation of

insulin in peripheral tissues resulting in a lowered insulin requirements and thus having longer lasting effects of insulin in the body. As the body gets older, the kidney functions decrease and it can be concluded that elderly patients are at increased risk of hypoglycemia. Their kidneys are insufficient at drug clearance thus increasing drug interactions due to long lasting effect of drug in the body and decreased cognitive functioning. This leads to very important concern in elderly patient since hypoglycemia unawareness and deteriorated cognitive function are critical factors to be carefully considered in treating older patients.[77]

In 2011, diabetes was listed as the primary cause of kidney failure in 44% of all new cases registered. There were 49,677 people who started treated for some sort of kidney failure due to diabetes alone. Another shocking number is in 2011, 228,924 people of all ages with kidney failure due to diabetes were living on chronic dialysis or with a kidney transplant. According to the Renal Data System between 1990 and 2006, there was an increase of about 9% per year in end stage renal disease (ESRD). The population sample was of Caucasian's in the USA aged 20 to 49 years old with T1DM predominant diabetes type and almost the exclusive type. The patients developed ESRD as a result of having at least 15 years or sufficient duration of diabetes. Incident cases of ESRD attributed to diabetes numbered 3,359 in 1990, 3,972 in 1995, 4,287 in 200 and 4,600 in 2006.[78]

Another complication associated with diabetes is hypertension. Hypertension is high blood pressure in the blood vessels that has a force greater than normal. Continues high pressure can strain the heart, damage the walls of the blood vessels, and increase risk of heart attack, stroke, kidney problems and death. From 2009 to 2012, adults of age 18 years and older, 71% had blood pressure greater than or equal to 140/90 mmHg (normal

<120/80 mmHg) or used prescription medications to control high blood pressure.[72] In fact, vascular complications are found to have an effect not only on the peripheral organs but also on cerebral circulation. The elasticity of smooth muscle cells is decreased with chronic hyperglycemia thus reducing the ability of blood vessels to maintain sufficient blood and nutrient supply to the brain tissue. Patients with T2DM and hypertension as a conjugate diagnosis showed a decreased in cortical gray matter thickness and cerebrovascular reactivity in a spatially overlapping region of the occipital lobe compared to healthy age-matched controls with just hypertension. Impact of these regions have shown a decrease in cognitive thinking thus making prevention and control of diabetes an even more important factor.[79]

Along with hypertension, dyslipidemia was diagnosed in 65% of patients with blood low density lipid (LDL) cholesterol greater than or equal to 100 mg/dL or used cholesterol-lowering medications who were 18 years and older from 2009 to 2012.[72] Normal level of LDL should be <100 mg/dL, (high density lipid) HDL should be <40 mg/dL and triglycerides should be <150 mg/dL. Diabetic dyslipidemia is where the fasting and postprandial triglycerides are elevated, low HDL-cholesterol, elevated LDL-cholesterol and the predominance of small dense LDL particles. This causes a 2 to 4 time increase risk of stroke and death from heart disease (increase in cardiovascular disease (CVD)) in patient with diabetes compared to non-diabetic patients. When insulin is produced in insufficient quantity, very low-density lipoprotein (VLDL) is increased as a main transporter of fasting triglycerides. Insulin suppresses lipolysis in adipose tissues by inhibiting the activity of hormone sensitive lipase, which catalyzes the mobilization of free

fatty acids from stored triglycerides, which act as substrates and regulatory factors for VLDL assembly and secretion.[80]

Overall, insulin is involved at all stages of VLDL production and secretion. Also, in the liver, insulin inhibits the transcription of microsomal triglyceride transfer protein, which in turn transfers triglycerides to nascent apolipoprotein B (apoB), which is the predominant surface protein of VLDL. When insulin is insufficient, liver increases the production of free fatty acid for energy which leads to decreased degradation of apoB, thus causing an overproduction of VLDL. As these VLDL molecules increase, they cause a cascade of events that leads to reduced HDL and increased small dense LDL levels.[80]

CVD due to dyslipidemia and thus the death rates related to CVD were increased by 1.7 times from 2003 to 2006 among adults aged 18 years or older with diagnosed diabetes than among adults without diagnosed diabetes. Under CVD falls heart attacks and stroke. In 2010, hospitalization rates for heart attack were 1.8 times and stroke hospitalization rates were 1.5 times higher among adults aged 20 years or older with diagnosed than among adults without diagnosed diabetes. People with diabetes also had blindness and eye problems. In 2005 to 2008, of adults with diabetes aged 40 years or older, 4.2 million (28.5%) people had diabetes retinopathy, which is damage to the small blood vessels in the retina that may result in loss of vision.[72]

Some of the pharmacotherapies for diabetes treatment include the following categories: sulfonylureas, meglitinides, biguanides thiazolidinediones, sodium-glucose cotransporter 2 (SGLT2) inhibitors, alpha-glucosidase inhibitor, glucagon-like peptide-1 analogs and dipeptidyl peptidase-4 (DPP-IV) inhibitors. The main focus of this research

will be on the inhibition of DPP-IV enzyme to treat T2DM but before that it's imperative to know the other ways of controlling diabetes.

Sulfonylureas and meglitinides class of drugs. These class of drugs interact with the sulphonylurea receptor (SUR) 1 (an integral component of pancreatic K_{ATP} channels) and close the adenosine triphosphate-sensitive potassium (K_{ATP}) channels in the pancreas. The blocking of K_{ATP} channels causes voltage-dependent Ca^{2+} channels (VDCCs), eliciting Ca^{2+} influx and a rise in intracellular Ca^{2+} that stimulates exocytosis of the insulin-containing secretory granules.[81] The only problem with blocking the K_{ATP} channels and increasing insulin is that these channels are also present in the coronary vascular smooth muscle cells and cardiomyocytes, but here the SUR components are of the SUR 2A and SUR 2B subtypes, respectively. The normal function of these channels in the heart are for adaptation to stress and have a central role in the signaling cascades underlying myocardial ischemic preconditioning and the cardiac responses to systolic overload. Thus, stimulation of such reactions by blocking the K_{ATP} channels in the heart can cause a response of a failing heart and can result in increased cardiovascular death.[82]

It has been shown that when sulfonylurea-induced and meglitinide-induced insulin secretion in pancreatic beta cells exposed chronically, there is reduced insulin content and a reduced number of functional K_{ATP} channels on the plasma membrane, as well as accelerated apoptotic beta cell death. These two groups of drugs thus might not be as effective when used over time as it can cause impaired acute inusinotropic action of these agents.[81] Currently there are 3rd generation sulfonylureas and several meglitinide class of drugs available that are approved by the FDA.

Biguanides class of drugs. This class refers to the group of drugs that control blood glucose level by decreasing amount of blood sugar produced by the liver, decreasing amount of sugar absorbed by the intestines and restoring body's sensitivity to insulin. There is only one drug approved in this class which is metformin. Metformin is used primarily to treat T2DM but can be used to treat T1DM in combination with other therapeutics like insulin. Metformin decreases blood glucose levels by routes other than pancreatic mechanisms. Metformin increases body's sensitivity to insulin and decreases rate of gluconeogenesis and small effect on glycogenolysis which in turn reduces the amount of blood glucose in the body helping control diabetes. Metformin is known to activate enzyme adenosine monophosphate kinase (AMPK) which inhibits key enzymes involved in gluconeogenesis and glycogen synthesis in the liver while also stimulating insulin signaling and glucose uptake by transporters in muscles. Metformin usually does not cause hypoglycemia which makes this class of drug a good choice. Metformin can cause serious side effect of lactic acidosis which is known to cause dizziness, severe drowsiness, muscle pain, tiredness, and other systemic symptoms, however, data has shown that these adverse effects are minute compared to the positive effects of metformin thus it is rarely discontinued from a patient's medication regimen.[83]

SGLT2 inhibitor class of drugs. Another class of prescription for controlling diabetes is SGLT2 inhibitors. SGLT is known to reabsorb glucose in the kidney and thus inhibiting it leads to excretion of glucose in the urine, hence decreasing plasma glucose level helping control diabetes. There are six active variants from SGLT1 to SGLT6, however, two types, SGLT1 and SGLT2, with SGLT1 (10% glucose reabsorption in kidneys) predominately expressed in intestine, kidney, heart, and skeletal muscle, and

SGLT2 (90% glucose reabsorption in kidneys) is exclusively expressed in kidney.[84,85] Under normal physiological conditions, 180 g of glucose is filtered through the kidneys daily and all of it is reabsorbed into the circulation by SGLT's. SGLT transport sodium and glucose into the cells using Na^+/K^+ ATPase pumps followed by glucose being further passively transported into the interstitium by GLUT-2.[84] Inhibition of SGLT2 improves hyperglycemia along with caloric and weight loss, small decreases in blood pressure and key property of low incidence of hypoglycemia due to insulin-independent mechanism of action. There are side effects like increased urinary and genital tract infections, diabetic ketoacidosis, skeletal effects (disruption of homeostasis of calcium and phosphorus), and some excess of bladder cancers seen exclusively with treatment using dapagliflozin.[85] Currently three compounds are approved for use which are dapagliflozin, canagliflozin, and empagliflozin.

Thiazolidinediones class of drugs. The thiazolidinediones are peroxisome proliferators-activated receptor γ (PPAR-γ) agonists that alter the transcription of genes influencing carbohydrate and lipid metabolism. Patients who are at the risk of prediabetes can take thiazolidinediones to slow down the development of T2DM and patients who are diagnosed with T2DM can take thiazolidinediones to improve glycemic control. However, the use of thiazolidinediones is advised as a cautionary since the side effects are great which include body-weight gain, congestive heart failure, bone fractures and possibly bladder cancer. This led to warnings and eventually restrictions on the use of rosiglitazone in the U.S. by the FDA.[86] Some of the members of thiazolidinediones include rosiglitazone, pioglitazone, lobeglitazone and troglitazone.

Alpha-glucosidase inhibitor class of drugs. Alpha-glucosidase inhibitors are effective against T2DM by controlling the post-prandial hyperglycemia. The enzyme responsible for absorbing the glucose in the intestine is alpha-glucosidase. This enzyme is a typical exo-type glycosidase enzyme that catalyzes the releases of alpha-glucosides from the non-reducing end of carbohydrates. It's one of the key enzyme involved in absorbing glucose in the intestine after a meal. So, by inhibiting alpha-glucosidase, glucose can be reduced and thus can be effective in the treatment and management of hyperglycemia and hyperlipidemia. However, alpha-glucosidase inhibitors are known to cause various side effects, such as flatulence, diarrhea, and abdominal discomfort.[87] Some of the drugs under this class are miglitol, acarbose and voglibose.

GLP-1 analogs class of drugs. GLP-1 analogs are another possibility of treatment of T2DM since *in vitro* and animal studies have reported that GLP-1 is associated with multiple positive effects on pancreatic beta cells. It has also been reported that GLP-1 regulates the expression of beta cell specific genes, regulates beta cell mass by inhibiting beta cell apoptosis and preventing beta cell glucolipotoxicity thus improved beta cell function.[88] The incretins are also responsible for improving the heart and endothelium. Incretins decrease CV risk markers, infarct size, atherosclerosis, endothelial dysfunction/inflammation and endoplasmic reticulum (ER) stress.[89] They also signal the brain to decrease food intake and increase cognitive function. Incretins are also helpful in increasing bone calcium levels and increasing glucose uptake in muscle cells indirectly. They are also shown to decrease gastric emptying which can help keep blood glucose levels from spiking rapidly causing hyperglycemia.[90]

Since GLP-1 plays an important physiological role in the body to control hyperglycemia along with other actives, it was a good pharmaceutical candidate to create analogs that mimicked GLP-1 peptide. Usually, GLP-1 peptide along with GIP is inactivated in T2DM by DPP-IV enzyme (discussed in greater detail below) making them useless in maintaining blood glucose homeostasis. These are naturally occurring hormones that are secreted in response to glucose ingested after a meal. These hormones are responsible for the incretin effect with more insulin secretion when ingested rather than taken intravenously. Some of the approved analogs for GLP-1 agonists are exenatide, liraglutide, lixisenatide, albiglutide and taspoglutide.

Recent research depicts the details of the pathophysiology of the disease showing that there is an intricate interaction of hormonal (hormones of the pancreas) and neural stimuli which are involved in the regulation of plasma glucose and maintaining homeostasis.[75] With the development of the enteroendocrine hormone equivalents and other compounds, which raise the concentration of these incretin hormones by delaying their degradation or by binding to their receptors, seem to aid in achieving a balance for the glycemic control.[91]

These incretin hormones are deactivated by DPP-IV in the body. The rapid degeneration of GLP-1 by DPP-IV enzyme dramatically reduced its action making it less effective. With advance in research there has been two ways found to work with this problem. One is the development of a mimic of the incretin GLP-1 (discussed above) resistant from degradation by DPP-IV enzyme and second is the development of the DPP-IV inhibitor (discussed next).[75,92]

DPP-IV enzyme inhibitor class of drugs. DPP-IV, EC 3.4.14.5, is an enzyme first described in 1967 as multifunctional membrane-anchored serine ectopeptidase belonging to the α,β-hydrolases (family S9B) and sequentially related to prolyl oligopeptidase (POP). The human 110 KDa DPP-IV is a 766-amino acid transmembrane glycoprotein consisting of mainly three parts: a cytoplasmic tail (residues 1-6), a transmembrane region (residues 7-28) and an extracellular part (29-766). It is the extracellular region that is responsible for cleaving incretin hormones. Specifically, it is residues 508 to 766 that have a catalytic triad Ser630-Asp708-His740 and α/β-hydrolase fold and other eight-bladed β-propeller chain (residues 56-497) which also contributes to the inhibitor binding site. DPP-IV is secreted as a monomer but forms a dimer which is responsible for the proteolytic activity as seen in Figure 23 using computational modeling. DPP-IV cleaves peptide/substrate after the 2nd amino acid from the N-terminal end, specifically if the 2nd amino acid is alanine (like in GLP-1) or proline.[90] The catalytic site lies in a large cavity between the two extracellular domains and can be accessed through two active site openings as seen in Figure 23.[93]

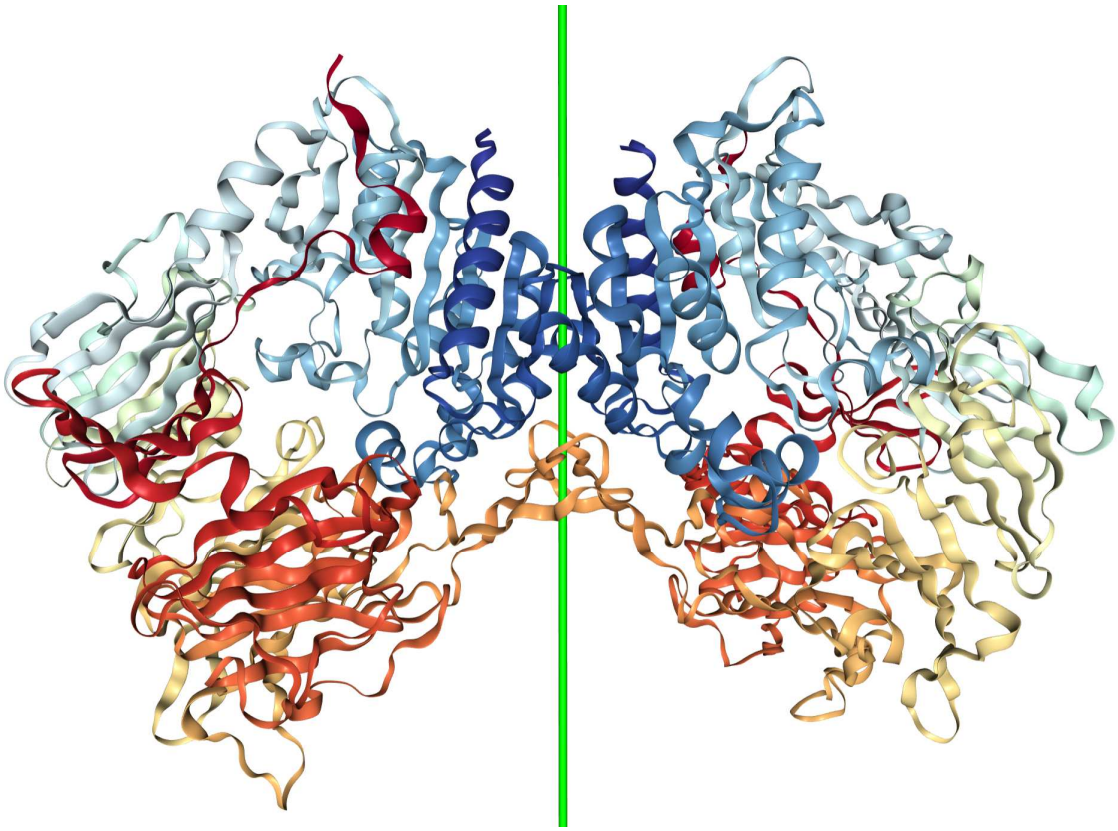


Figure 23. Molecular modeling of DPP-IV enzyme that is observed as a homodimer. Image using NGL viewer with UniProt P27487 from www.rcsb.org.[94,95]

DPP-IV inhibition prevents the inactivation of GLP-1, which increases levels of active GLP-1. As mentioned above, this effect increases insulin secretion and reduces glucagon secretion, thereby lowering blood glucose levels[92] It has been estimated that incretin hormones increase insulin response by 66% after an oral glucose administration[96] GLP-1 is a 36-amino-acid peptide produced by the L cells, which are localized predominantly in the lower part of the small intestine. In GLP-1 secretion, which is released minutes after meal ingestion; fats, carbohydrates and proteins all alike have the same stimulator effect. GIP is a 42—amino-acid peptide produced mainly by the K cells, which

are located mainly in the duodenum. GIP is stimulated by fat and carbohydrates, whereas protein does not stimulate GIP as much.[90,97]

Both GLP-1 and GIP are inactivated minutes after secretion (half-life in the order of 1 to 2 minutes) by DPP-IV. The use of DPP-IV inhibitors like sitagliptin and vildagliptin have clinically proven to significantly reduce HbA1c and offer many potential advantages over existing therapies which includes a lower risk of hyperglycemia, no weight gain along with no problems of gastrointestinal intolerance.[75] In some of the animal studies, administration of DPP-IV inhibitors depicted a long life of up to 5 hours and an inhibition of more than 90% of plasma DPP-IV. When tested on T2DM patients, DPP-IV inhibitors showed a potent and long-lasting inhibition soon after its intake. Some other studies experimented with sitagliptin or vildagliptin also showed results depicting a significant increase of GLP-1 plasma levels.[91] It has been shown that inhibition of DPP-IV increases the prandial levels of active GLP-1 approximately three-fold, from about 5-6 pmol/L to 15-20 pmol/L.[71]

When preclinical studies were administered using DPP-IV inhibitors, selective inhibition of DPP-IV was acceptable at the safety and tolerability criteria as compared to two other human dipeptidyl-peptidases DPP8 and DPP9 which belong to the same family as DPP-IV (post-proline serine peptidases). A study by Lankas et al.[98] showed that both DPP8 and DPP9 are very weak inhibitors with data showing very low affinity as compared to DPP-IV, which displayed highest selectivity. Acute toxicity in rodents was reported with inhibition of enzymes DPP8 and DPP9 so in order for a compound to be an effective DPP-IV inhibitor, the compound must show low affinity towards DPP8 and DPP9 and high

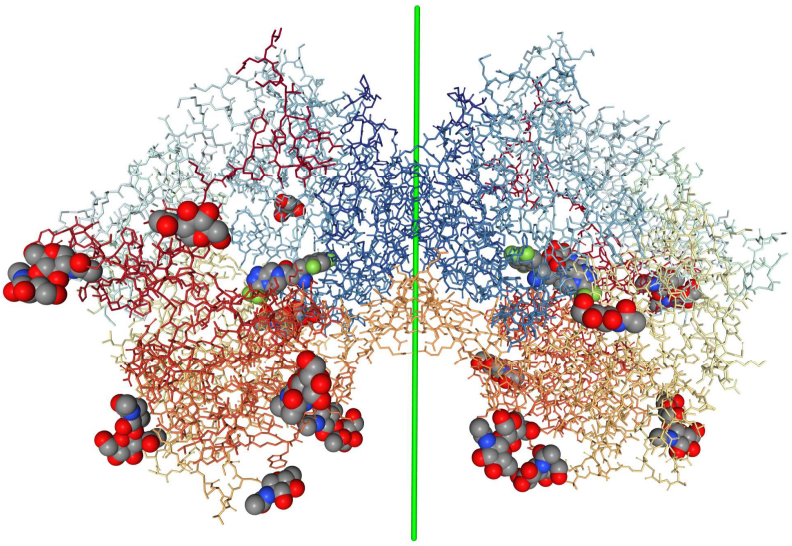
affinity towards DPP-IV. Thus, a lack of DPP8 and DPP9 inhibitory activity should be absent for the development of DPP-IV as an effective and safe antidiabetic therapy.[75,99]

DPP-IV is also known as the T-cell antigen CD26, and is a cell-surface protease localized in numerous sites, including the kidney and intestinal brush-border membranes as well as on hepatocytes. DPP-IV is also found throughout the entire vascular bed, located on endothelial cells as well as in a soluble form in plasma.[100] It is also widely distributed in kidney, liver, intestine, spleen, adrenal glands, lymphocytes, endothelial cells and placenta.[93] Not only is DPP-IV used to cleave integrins, but it is also used to metabolize neuropeptide Y, peptide YY, gastrin-releasing polypeptide, pituitary adenylate-cyclase-activating polypeptide, insulin-like growth factor-1, substance P and various chemokines. Metabolism and activation of peptide YY and neuropeptide Y which are involved in GI functions and regulation of food intake and obesity can be associated to the contributing factors of T2DM.[75] The following activities of DPP-IV show that it is involved in other homeostatic mechanisms like neurogenic inflammation, blood pressure and the immune system.[90]

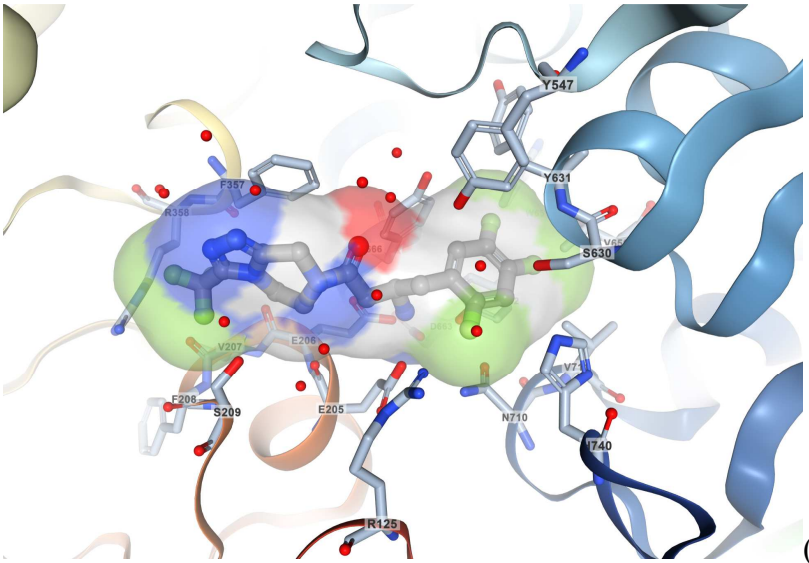
The available FDA approved drugs for inhibiting DPP-IV enzyme are as follows; sitagliptin, vildagliptin, saxagliptin, alogliptin and linagliptin. Sitagliptin was used as a control in our research, which is sold as the brand name Januvia. Sitagliptin phosphate monohydrate is described chemically as 7-[(3R)-3-amino-1-oxo-4-(2,4,5-trifluorophenyl)butyl]-5,6,7,8-tetrahydro-3-(trifluoromethyl)-1,2,4-triazolo[4,3-a]pyrazine phosphate (1:1) monohydrate [$C_{16}H_{15}F_6N_5O \cdot H_3PO_4 \cdot H_2O$] with the R enantiomer being the active form of Januvia. Pharmacophore of sitagliptin is the 3-(trifluoromethyl)-5,6,7,8-tetrahydro[1,2,4]triazolo[4.3-a]pyrazine as shown in Figure 24

labeled sitagliptin binding region (SBR) with DPP-IV enzyme. There are two other pharmacophore sites on sitagliptin that are labeled common binding region (CBR) with DPP-IV enzyme. The amino acids associated with SBR are serine (S), phenylalanine (F), and arginine (R) at positions, 209, 357, and 358, respectively. The serine and arginine are both donating hydrogens to trifluromethyl and triazole, respectively. The phenylalanine is involved in pi-pi interactions with the aromatic ring. The other amino acids involved are glutamic acids (E) at position 205 and 206 and tyrosine (Y), valine (V), tryptophan (W), tyrosine (Y), aspartic acid (D) and tyrosine (Y) at positions 631, 656, 659, 662, 663 and 666, respectively, on DPP-IV enzyme (Figure 24).[101]

Based on current literature and structural requirements for a compound to be a DPP-IV inhibitor, we were able to synthesize a lead compound that showed inhibitory activity. This lead compound was further used to build a library of DPP-IV inhibitors via structural activity relationship via *in vitro* DPP-IV enzymatic inhibition assays.



(a)



(b)

Figure 24. Molecular modeling of DPP-IV enzyme in complex with inibitors. (a) β-amino acid inhibitor and (b) amino acid residue interaction of DPP-IV enzyme with drug Sitagliptin.[94,95]

Chapter 4

Characteristic of DPP-IV Inhibitors and their Validation via *In Vitro* DPP-IV Enzymatic Assay

Novel Compounds Containing Cyanopyrrolidine and β-amino Alcohols Scaffolds

Inhibitors of DPP-IV required specific structural groups to prevent the function of this enzyme. Cyanopyrrolidinyl β-amino alcohols are novel scaffolds with potential for a wide array of pharmacological properties. These scaffolds were selectively accessed from simple and inexpensive commercially available chemicals in few synthetic steps with minimal purification. A 36-compound library of these scaffolds were provided to be examined as DPP-IV inhibitors.[102]

Cyanopyrrolidines are molecular scaffolds that are highly recurrent among natural products and commercial drugs. The scaffold has shown affinity to dipeptidyl peptidases as a proline mimetic group and it has been hypothesized that the activity arises from a transient covalent interaction of the nitrile with S630 that triggers a slow dissociation from the enzyme. Thus, most of the most potent DPP-IV inhibitors contain this scaffold (vinagliptin, saxagliptin, vildagliptin). Cyanopyrrolidines can be easily obtained from naturally available proline and many synthetic approaches are available. However, despite the diversity of methods many lack scalability and reproducibility.[102]

β-Amino alcohols are an important class of compounds in medicinal chemistry and drug discovery due to their high occurrence in commercially available drugs. They often behave as β-adrenergic blockers, widely used in the management of cardiovascular disorders, including hypertension, anginapectoris, cardiac arrhythmias, and other disorders

related to the sympathetic nervous system. Amino alcohols have also been used as protein kinase C inhibitors, glycosidase inhibitor and antimalarial agents. Among commercial drugs, this scaffold is present in Oxycontin, Coreg, and Toprol-XL. Other pharmaceutical drugs such as Zyvox and Skelaxin feature oxazolidones that can be formed through amino alcohol precursors. Moreover, these can be used as chemical synthons for the synthesis of more complex heterocyclic scaffolds. Thus, a novel family of inhibitors that shares the cyanopyrrolidine and β-amino alcohol scaffolds towards more potent and selective diabetes therapeutics were created and tested.[102]

Research Design and Methods

General Method A: Synthesis of mono-alkylated amino alcohols. In a 20 mL vial at room temp., epoxide (1.0 mmol, 1.0 equiv.) and amine (1.5 mmol, 1.5 equiv.) were added to 6.7 mL of DMF. The reaction was stirred at 60 °C for 12 h at which point the rxn mixture received deionized water (50 equiv.). The reaction was allowed to stir at 60 °C for an additional 12 h. The resulting crude was stripped of solvent and the residue loaded directly onto a silica gel column for purification.[102]

General Method B. Synthesis of double-alkylated amino alcohols. In a 20 mL vial at room temp., epoxide (2.0 mmol, 2.0 equiv.) and amine (1.0 mmol, 1.0 equiv.) were added to 5 mL of deionized water. The reaction was stirred at room temp. for 12 h. The resulting crude was stripped of water and the residue loaded directly onto a silica gel column for purification.[102]

Compounds. (2*S*)-1-((2-hydroxy-2-phenylethyl)glycyl)pyrrolidine-2-carbonitrile (**1aa**): 2-((2-hydroxy-2-phenylethyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**1ab**): (2S)-1-

(bis(2-hydroxy-2-phenylethyl)glycyl)pyrrolidine-2-carbonitrile (**1ba**): 2-(bis(2-hydroxy-2-phenylethyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**1bb**): (2S)-1-((2-(4-fluorophenyl)-2-hydroxyethyl)glycyl)pyrrolidine-2-carbonitrile (**2aa**): (2S)-1-((2-(4-chlorophenyl)-2-hydroxyethyl)glycyl)pyrrolidine-2-carbonitrile (**3aa**): (2S)-1-((2-(4-bromophenyl)-2-hydroxyethyl)glycyl)pyrrolidine-2-carbonitrile (**4aa**): 2-((2-(4-bromophenyl)-2-hydroxyethyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**4ab**): (2S)-1-(bis(2-(4-bromophenyl)-2-hydroxyethyl)glycyl)pyrrolidine-2-carbonitrile (**4ba**) (Figure 4.9): 2-(bis(2-(4-bromophenyl)-2-hydroxyethyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**4bb**): (2S)-1-((2-hydroxy-3-phenoxypropyl)glycyl)pyrrolidine-2-carbonitrile (**5aa**): 2-((2-hydroxy-3-phenoxypropyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**5ab**): (2S)-1-(bis(2-hydroxy-3-phenoxypropyl)glycyl)pyrrolidine-2-carbonitrile (**5ba**): 2-(bis(2-hydroxy-3-phenoxypropyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**5bb**): (S)-1-(((S)-2-hydroxy-3-phenoxypropyl)glycyl)pyrrolidine-2-carbonitrile (**6aa**): (S)-2-((2-hydroxy-3-phenoxypropyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**6ab**): (S)-1-(bis((S)-2-hydroxy-3-phenoxypropyl)glycyl)pyrrolidine-2-carbonitrile (**6ba**): 2-(bis((S)-2-hydroxy-3-phenoxypropyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**6bb**): (2S)-1-((2-hydroxy-3-(o-tolyloxy)propyl)glycyl)pyrrolidine-2-carbonitrile (**7aa**): 2-((2-hydroxy-3-(o-tolyloxy)propyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**7ab**): (2S)-1-(bis(2-hydroxy-3-(o-tolyloxy)propyl)glycyl)pyrrolidine-2-carbonitrile (**7ba**): 2-(bis(2-hydroxy-3-(o-tolyloxy)propyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**7bb**): (2S)-1-((3-(benzyloxy)-2-hydroxypropyl)glycyl)pyrrolidine-2-carbonitrile (**8aa**): 2-((3-(benzyloxy)-2-hydroxypropyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**8ab**): (2S)-1-(bis(3-(benzyloxy)-2-hydroxypropyl)glycyl)pyrrolidine-2-carbonitrile (**8ba**): 2-(bis(3-(benzyloxy)-2-

hydroxypropyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**8bb**): (2S)-1-((2-hydroxy-3-(4-methoxyphenoxy)propyl)glycyl)pyrrolidine-2-carbonitrile (**9aa**): 2-((2-hydroxy-3-(4-methoxyphenoxy)propyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**9ab**): (2S)-1-(bis(2-hydroxy-3-(4-methoxyphenoxy)propyl)glycyl)pyrrolidine-2-carbonitrile (**9ba**): 2-(bis(2-hydroxy-3-(4-methoxyphenoxy)propyl)amino)-1-(pyrrolidin-1-yl)ethan-1-one (**9bb**): (2S)-1-((3-(4-fluorophenoxy)-2-hydroxypropyl)glycyl)pyrrolidine-2-carbonitrile (**10aa**): (2S)-1-((3-(3,4-difluorophenoxy)-2-hydroxypropyl)glycyl)pyrrolidine-2-carbonitrile (**11aa**): (2S)-1-((2-hydroxy-3-(3,4,5-trifluorophenoxy)propyl)glycyl)pyrrolidine-2-carbonitrile (**12aa**): (2S)-1-((2-hydroxy-3-(naphthalen-1-yloxy)propyl)glycyl)pyrrolidine-2-carbonitrile (**13aa**): (2S)-1-((2-hydroxy-3-phenylpropyl)glycyl)pyrrolidine-2-carbonitrile (**14aa**): and (2S)-1-((3-(furan-2-ylmethoxy)-2-hydroxypropyl)glycyl)pyrrolidine-2-carbonitrile (**15aa**).

***In vitro* assay.** The enzymatic inhibition of DPP-IV was measured *in vitro* by the endpoint fluorometric assay using substrate Gly-Pro-AMC (MW: 329.4). The enzyme cleaves the bond between Pro-AMC thus releasing the fluorescent aminomethylcoumarin (AMC) resulting in an increase in fluorescence intensity. The inhibitors, DPP-IV enzyme and substrate were dissolved in the 100 mM HEPES buffer (pH 7.5, 0.1 mg/ml BSA). Inhibitor stock concentration was dissolved in 100% DMSO followed by working serial dilution starting at 1% DMSO. The final concentrations of enzyme and substrate used were 0.003 ng/µL and 50 µM, respectively.

The assay was performed in an 384-Well plate (from BD Falcon, catalog # 353232, well 120 µL assay plate white, standard surface, nonsterile) with final volume of 100 µL in all wells. All samples were assayed in triplicates. A background well was created with

10 µL of Gly-Pro-AMC (from MP Biomedicals, LLC, catalog # AMC039) substrate plus 90 µL buffer; a 100% activity well was created with 10 µL of DPP-IV enzyme (from Sigma-Aldrich, Inc., catalog # D7052) and 10 µL of Gly-Pro-AMC substrate plus 80 µL buffer; and inhibitor wells were created with 10 µL of DPP-IV enzyme, 10 µL of inhibitor and 10 µL Gly-Pro-AMC substrate plus 70 µL buffer. The order of the addition to the well was buffer, enzyme, inhibitor and finally the substrate to initiate the reaction. The plate was covered and centrifuged for 30 seconds at 800xg followed by incubation for 30 mins at 37° C. The fluorescence was the measured on the SpectraMax M5 with excitation wavelength of 360 nm and emission wavelength of 460 nm with an auto cut-off wavelength setting on the instrument at 37° C. IC_{50} values were analyzed using Graphpad Prism 6 by a fit of reaction rates to a four-parameter Hill equation by nonlinear regression. Equation used to calculate IC_{50}:

Y=Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*HillSlope))

Where IC50 is the concentration (same unit as X) of inhibitor that gives a response half way between Bottom and Top (plateaus in the units of the Y). HillSlope describes the steepness of the family of curves. A HillSlope of -1.0 was used.

Results and Discussion

Two different series were created, cyano (mono-alkylated (*aa*) and double-alkylated (*ba*)) and descyano (mono-alkylated (*ab*) and double-alkylated (*bb*)). Table 2 shows series 1 to 9 and Table 3 shows series 10 to 15 of DPP-IV inhibitor. It can be seen from series 10 to 15 that *ab*, *ba*, and *bb* were not synthesized as the Structural Activity

Relationship (SAR) from series 1 to 10 showed no activity for compounds containing these characteristics.

It was anticipated that series *aa* would establish the baseline activity for these inhibitors and that is what was observed as aromatic cyanopyrrolidinyl amino alcohols demonstrated to have significant potency (IC_{50} under 0.2 uM). All mono-alkylated compounds with cyano group *aa* showed greater activity than all double-alkylated *bb* series. It was observed that analogue **1aa** (42 nM) (Figure 25) had significant more inhibition against DPP-IV than the other substituted aromatic analogues (**2aa**, **3aa** and **4aa**) (Figure 26) where the epoxide used was styrene oxide. Moreover, from those the *p*-fluorophenyl analogue **2aa** (6511 nM) surprisingly displayed much lower inhibition followed by *p*-chlorophenyl **3aa** (123.5 nM), then *p*-bromophenyl **4aa** (121.6 nM) and highest activity when there was no substitution **1aa** (42.19 nM). What was surprising was **4ba** (129.6 nM) showed similar IC_{50} to **4aa** analog along with some activity in descyano mono-alkylated **4ab** (1299 nM) and no activity for descyano double-alkylated **4bb** (Figure 27). Compounds in entry 4 containing bromine on the aromatic were the only series that showed activity in *ba* and *ab* series as all other compounds containing *ba* and *ab* series showed >10K nM IC_{50}.

Phenylgylcidyl ethers were used to react with amino alcohol series and it was found that the respective cyanopyrrolidine amino alcohols were obtained in great yields and as single isomers (Table 1, Entry 5). The resulting amino alcohols were obtained as mixtures of diastereomers at the alcohol and cyano centers, but due to their remote relationship, they are seen as one isomer by NMR spectroscopy. Despite the remoteness between the chiral centers on the proposed target, we anticipated that we might need to construct this scaffold

as a single diastereomer to validate the effect of the remote chiral center. Thus, (S)-phenylglycidyl ether was reacted with the amine series to get products (Table 1, Entry 6). The scaffold remote relationship between its chiral centers suggested that as a mixture of diastereomers, the scaffold inhibition would be potentially comparable. We corroborated this hypothesis as we found that the inhibitory activity of **5aa** (91.02 nM) (mixture of diastereomers) (Figure 28) was very similar to the enantiopure amino alcohol **6aa** (81.72 nM) (Figure 29). The introduction of phenoxy ethers is known to enhance activity among several β-adrenergic blockers [103]. Consequently, we found the SAR of phenoxy in series **5aa** and **6aa**, *o*-methylphenoxy in **7aa** (98.93 nM) (Figure 30), benzyloxy in **8aa** (80.24 nM) (Figure 31) and *p*-methoxy in **9aa** (74.32 nM) (Figure 32) to have similar inhibitory properties as to the previous analogues but failed to improve **1aa** activity.

Based on the current SAR data found, we envisioned that introducing fluorine atoms on the phenoxy group might further increase the inhibitory properties. We found that *p*-fluorophenoxy **10aa** (112.6 nM) had comparable activity. Moreover, 4,5-difluorophenoxy **11aa** (77.96 nM) proved to be more active (analogues to SAR from reported studies), but 4,5,6-trifluorophenoxy **12aa** (141.19 nM) proved to decrease the inhibitory activity. Figure 33 shows the dose-response curve comparing the number and placement of fluorine on the phenoxy group. Further assessment of the SAR demonstrated that naphthyl cyanopyrrolidinyl amino alcohol **13aa** (100.6 nM) is comparably active. Interestingly, removing the oxygen from the benzyloxy analogue **14aa** (2680 nM) fully drops DPP-IV activity. Low activity from heterocyclic analogue **15aa** (>10K nM) further proved the need for a more lipophilic side chain.[102] Figure 34 displays the dose-response curve comparing analogous compounds **13aa**, **14aa** and **15aa**.

It was found that the remaining analogues had significantly lower inhibitory activity. Moreover, Series *ab/bb* also failed at providing significant activity against DPP-IV. The SAR data from this preliminary biochemical study shows that analogue **1aa** is the most active (42 nm), and analogues that introduce complexity around the phenyl ring have comparable inhibitory properties. It has been demonstrated that the monoalkylated scaffold is by far the most active scaffold. Despite reported evidence of a potential decomposition pathway through an acid-catalyzed intramolecular cyclization, stability studies displayed complete structural stability for the inhibitor against analogous conditions. This study also has shown that fluorine incorporation can improve activity, but does not play a significant role as seen in other DPP-IV inhibitors.[102]

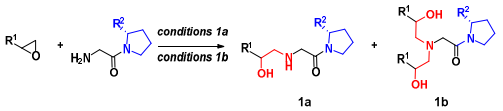
Conclusion

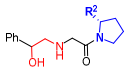
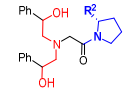
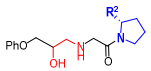
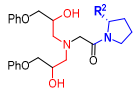
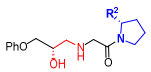
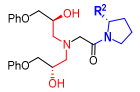
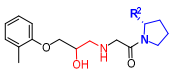
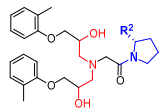
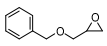
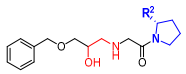
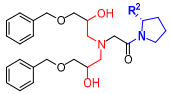
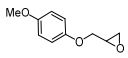
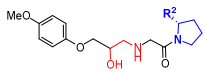
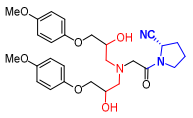
Novel cyanopyrrolidinyl β-amino alcohols were derived and successfully tested *in vitro* against DPP-IV enzyme. All of the mono-alkylated amino alcohols showed greater activity than all of the double-alkylated amino alcohols against DPP-IV enzyme. It was also shown that all compounds that contained the cyano group on the pyrrolidinyl amine showed greater activity than compounds without the cyano group. Compounds containing phenyl glycidyl ethers showed greater activity than styrene oxides. When styrene oxide was substituted with halogens, bromine showed activity greater than chlorine followed by fluorine; however, no substitution showed the greatest activity. When fluorine was substituted on the phenyl glycidyl ether, it was found that para substitution was favored followed by meta which was better than no substitution. It can also be concluded from the SAR that a lipophilic side chain is more favored over a hydrophilic side chain on the epoxide.

Since these novel molecules contain the β-amino alcohols, the next step is to test them against β-adrenergic receptors as potential β-blockers which would make these compounds a dual acting drug that would be beneficial in maintenance of diabetes and hypertension. Also, since literature has shown toxicity to the animal when enzymes DPP8 and 9 were inhibited along with DPP-IV, it is important for these compounds to be tested against DPP8 and 9 enzymes as it will help in synthesizing a more potent second-generation library of DPP-IV inhibitors. Not only is activity against DPP8 and 9 important for second-generation library, but it is important so that these molecules can be further studied *in vivo*.

Table 2

SAR activity of compounds 1 to 9.[102]

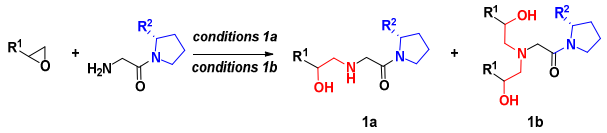


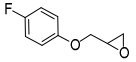
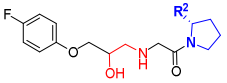
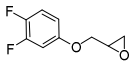
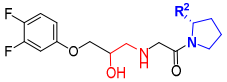
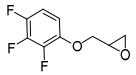
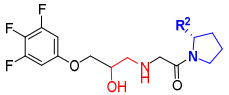
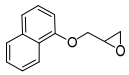
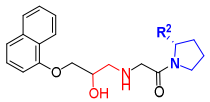
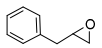
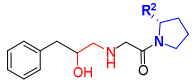
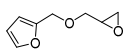
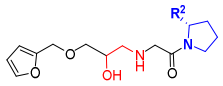
Entry	Epoxide	Amine	Product 1a	Product 1b	IC_{50} (nM) 1a	IC_{50} (nM) 1b
1 = X = H 2 = X = F 3 = X = Cl 4 = X = Br		R^2 = aa/ba = CN R^2 = ab/bb = H			1aa = 42 1ab = >10K 2aa = 6511 3aa = 123.5 4aa = 121.6 4ab = 1299	1ba = >10K[a] 1bb = >10K 4ba = 129.6 4bb = >10K
5		R^2 = aa/ba = CN R^2 = ab/bb = H			5aa = 91.02 5ab = >10K	5ba = >10K 5bb = >10K
6		R^2 = aa/ba = CN R^2 = ab/bb = H			6aa = 81.72 6ab = >10K	6ba = >10K 6bb = >10K
7		R^2 = aa/ba = CN R^2 = ab/bb = H			7aa = 98.93 7ab = >10K	7ba = >10K 7bb = >10K
8		R^2 = aa = CN R^2 = ab = H			8aa = 80.24 8ab = >10K	8ba = >10K 8bb = >10K
9		R^2 = aa/ba = CN R^2 = ab/bb = H			9aa = 74.32 9ab = >10K	9ba = >10K 9bb = >10K

Conditions 1a: 1.5 equiv. of amine, 50 equiv. of H_2O in DMF at 60 °C . Conditions 1b: 0.5 equiv. amine, H_2O, rt. a. 10K = 10,000

Table 3

SAR activity of compounds 10 to 15.[102]



Entry	Epoxide	Amine	Product 1a	Product 1b	IC_{50} (nM) 1a	IC_{50} (nM) 1b
10		R^2 = aa = CN		Not synthesized entries 10 to 15	10aa = 112.6	
11		R^2 = aa = CN			11aa = 77.96	
12		R^2 = aa = CN			12aa = 141.9	
13		R^2 = aa = CN			13aa = 100.6	
14		R^2 = aa = CN			14aa = 2680	
15		R^2 = aa = CN			15aa = >10K	

Conditions 1a: 1.5 equiv. of amine, 50 equiv. of H_2O in DMF at 60 ^oC . Conditions 1b: 0.5 equiv. amine, H_2O, rt. a. 10K = 10,000

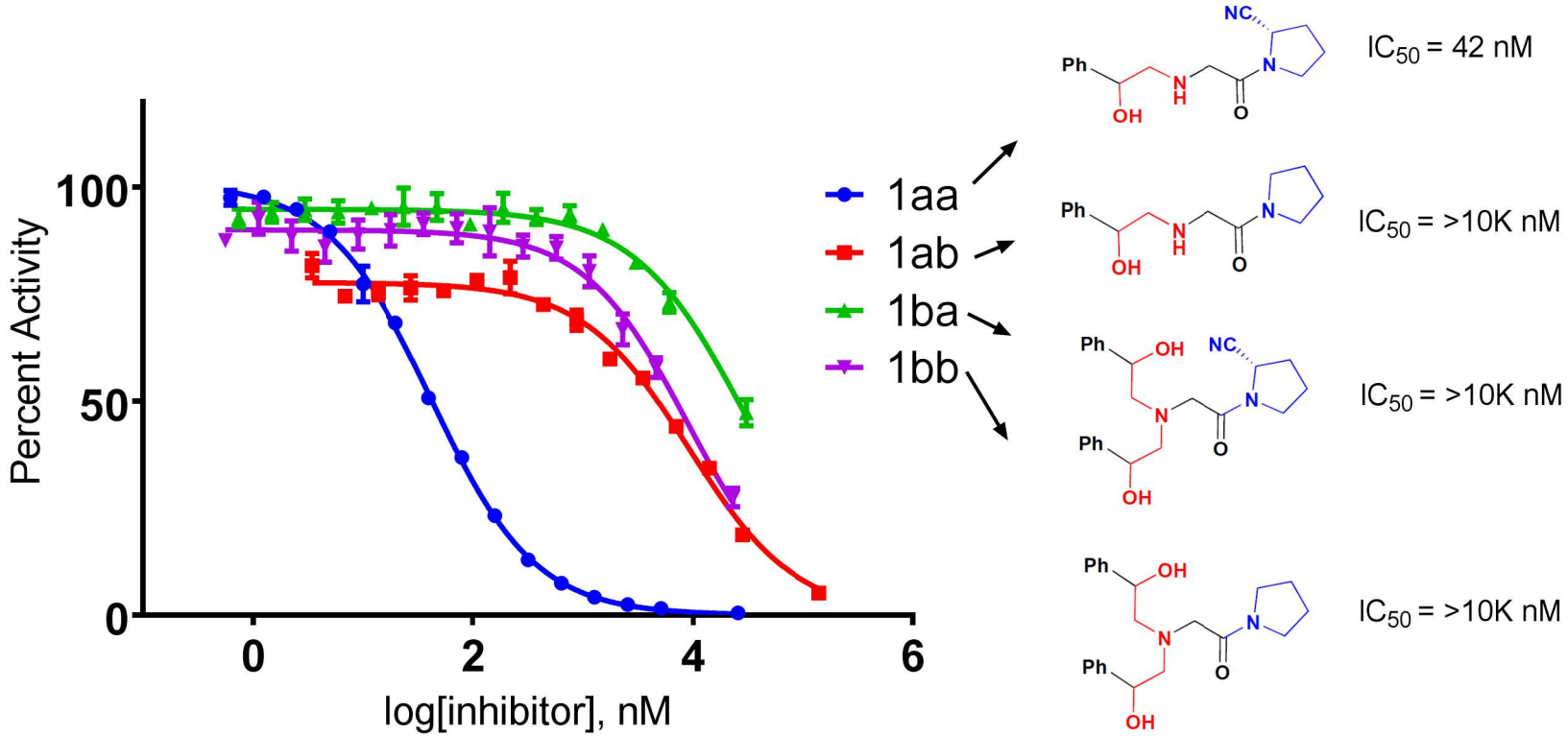


Figure 25. Dose-response curve showing IC_{50} of compounds 1aa, 1ab, 1ba, and 1bb. The values represent the mean ± S.E.M (n=3)

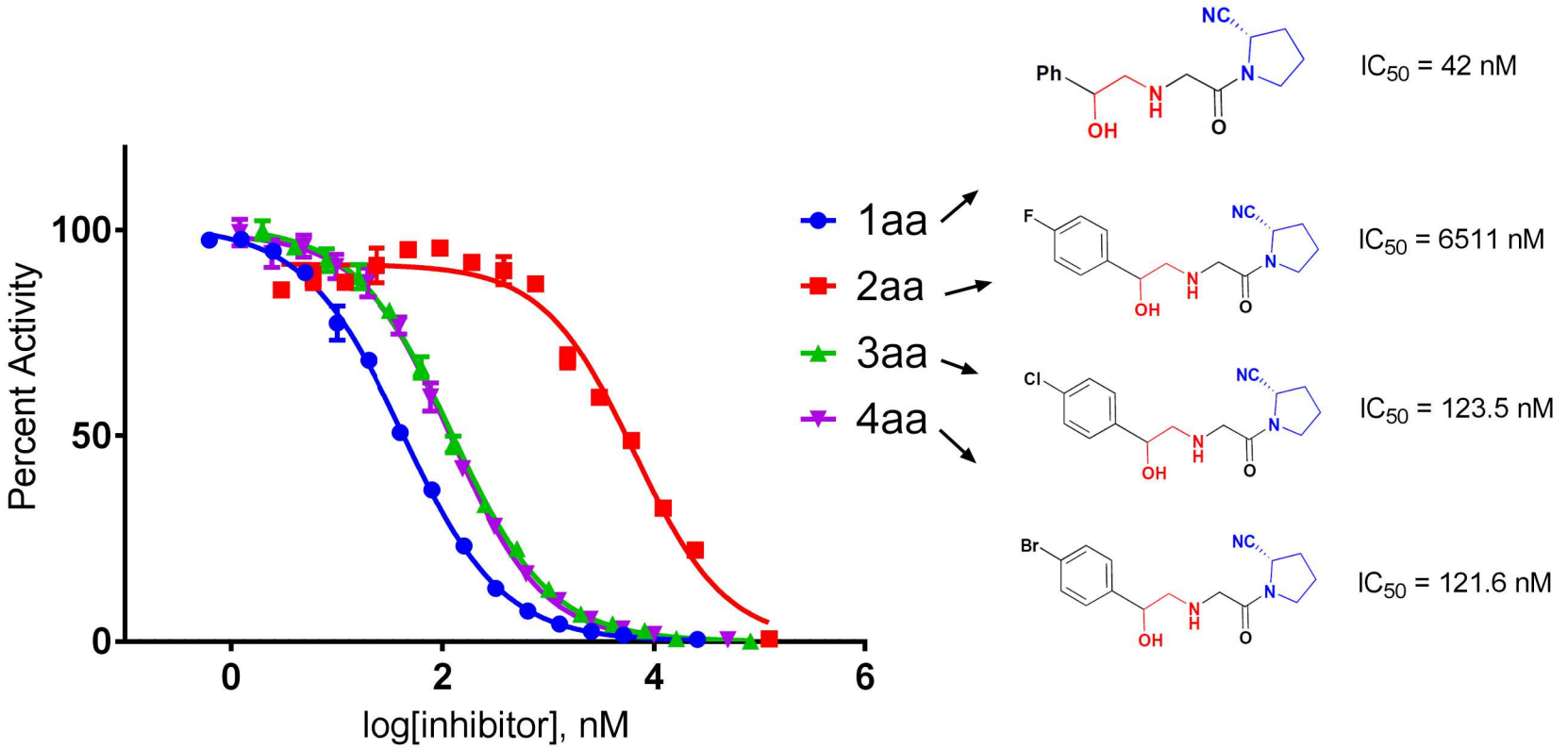


Figure 26. Dose-response curve showing IC_{50} of compounds 1aa, 2aa, 3aa, and 4aa. The values represent the mean ± S.E.M (n=3)

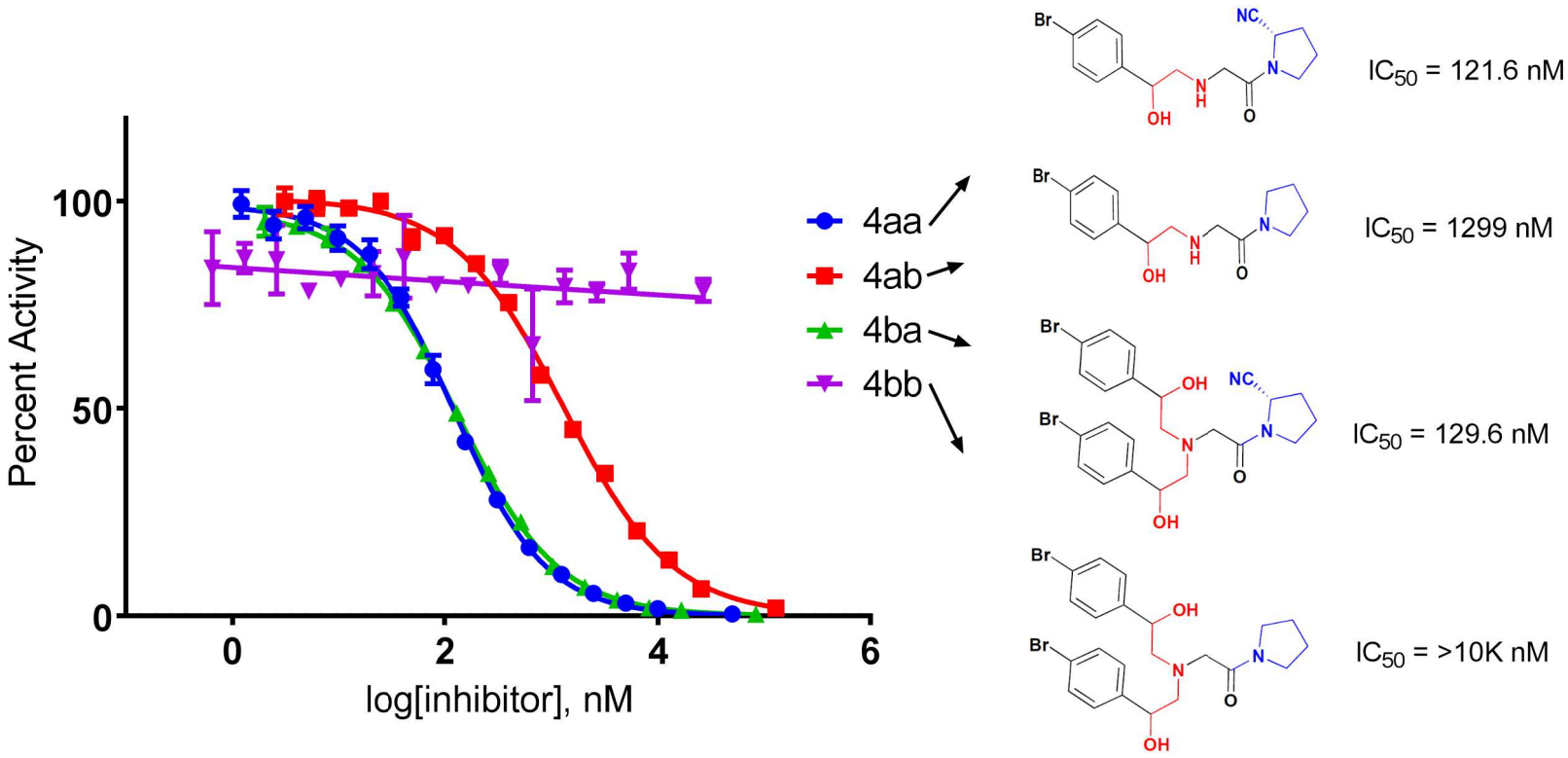


Figure 27. Dose-response curve showing IC_{50} of compounds 4aa, 4ab, 4ba, and 4bb. The values represent the mean ± S.E.M (n=3)

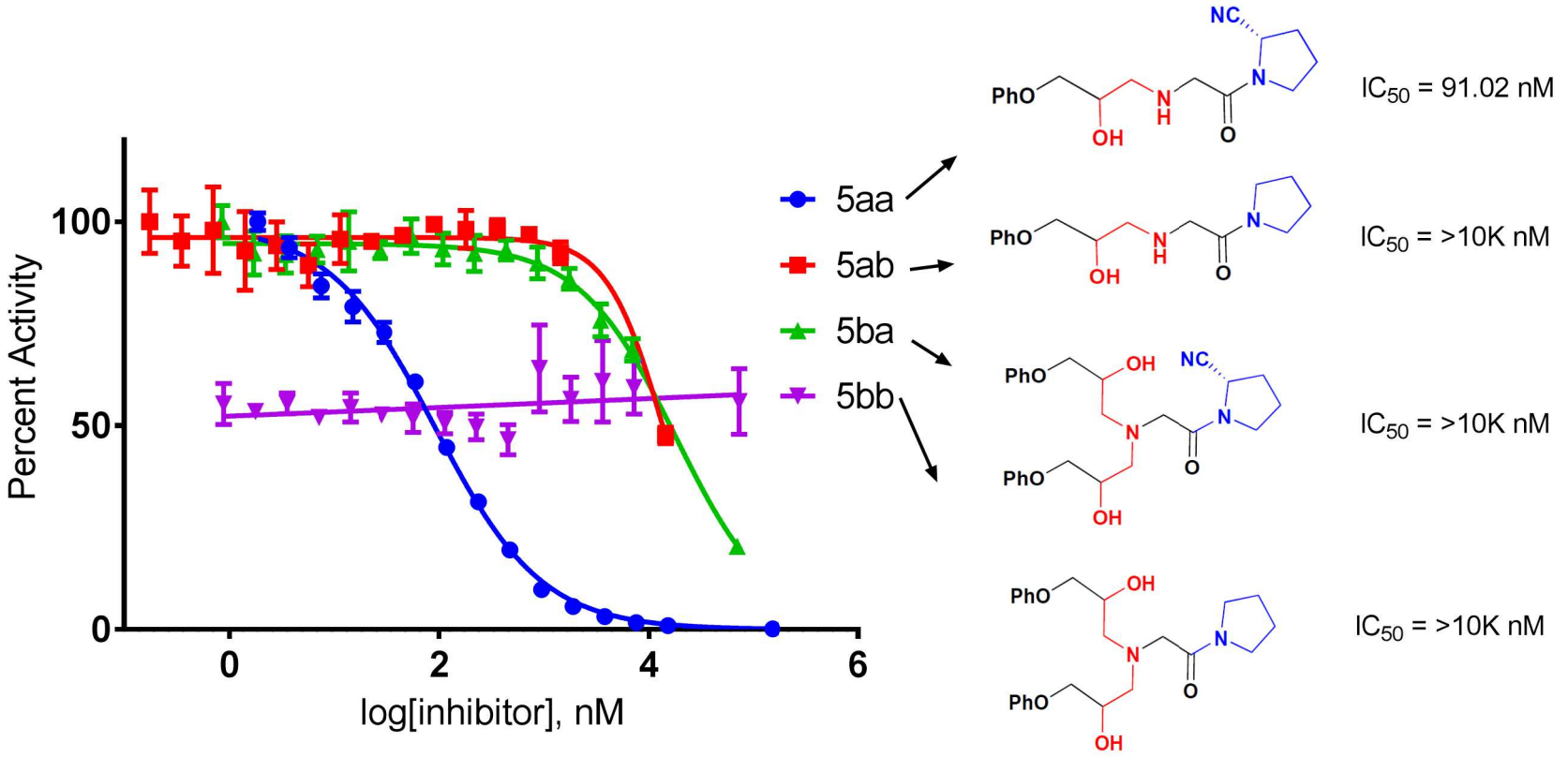


Figure 28. Dose-response curve showing IC_{50} of compounds 5aa, 5ab, 5ba, and 5bb. The values represent the mean ± S.E.M (n=3)

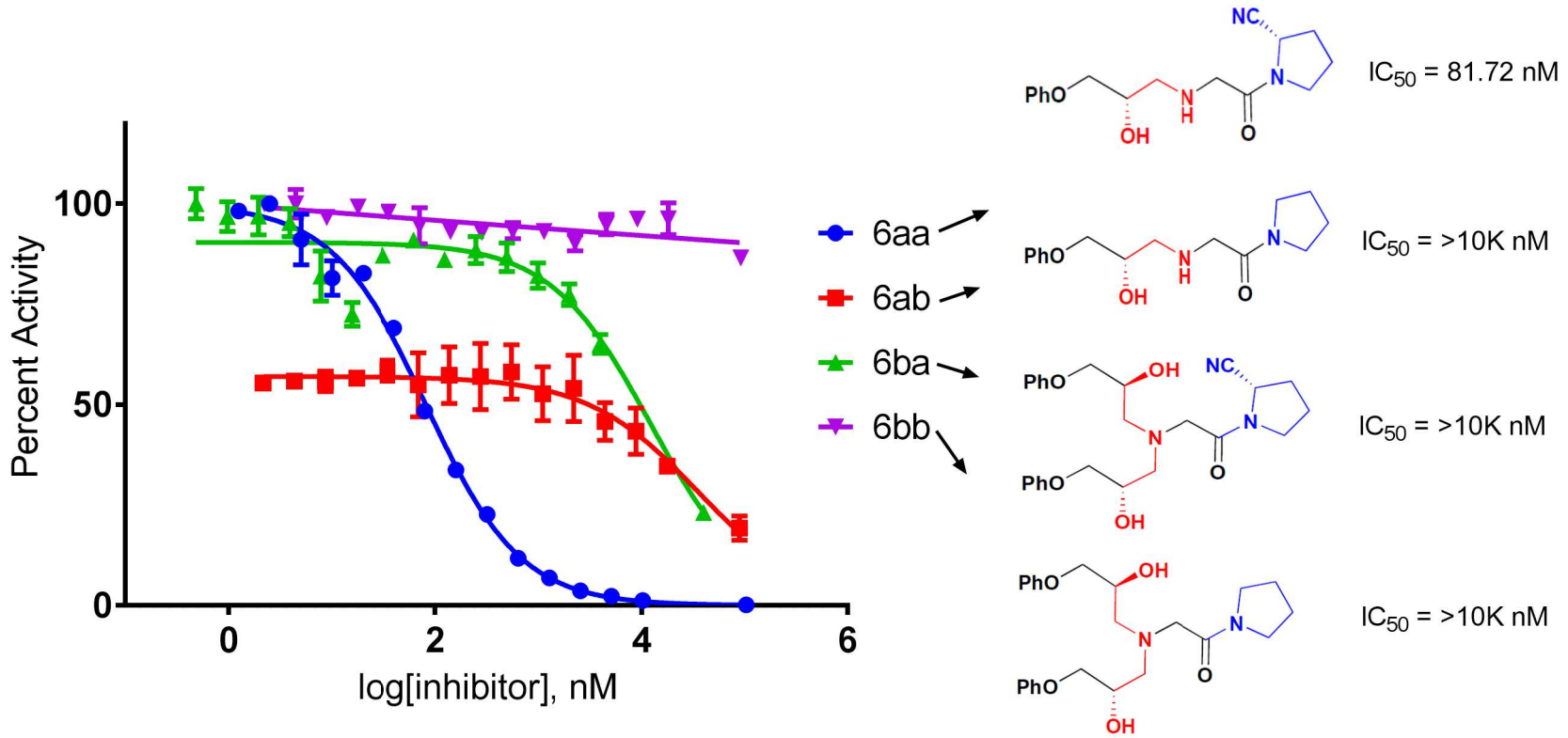


Figure 29. Dose-response curve showing IC_{50} of compounds 6aa, 6ab, 6ba, and 6bb. The values represent the mean ± S.E.M (n=3)

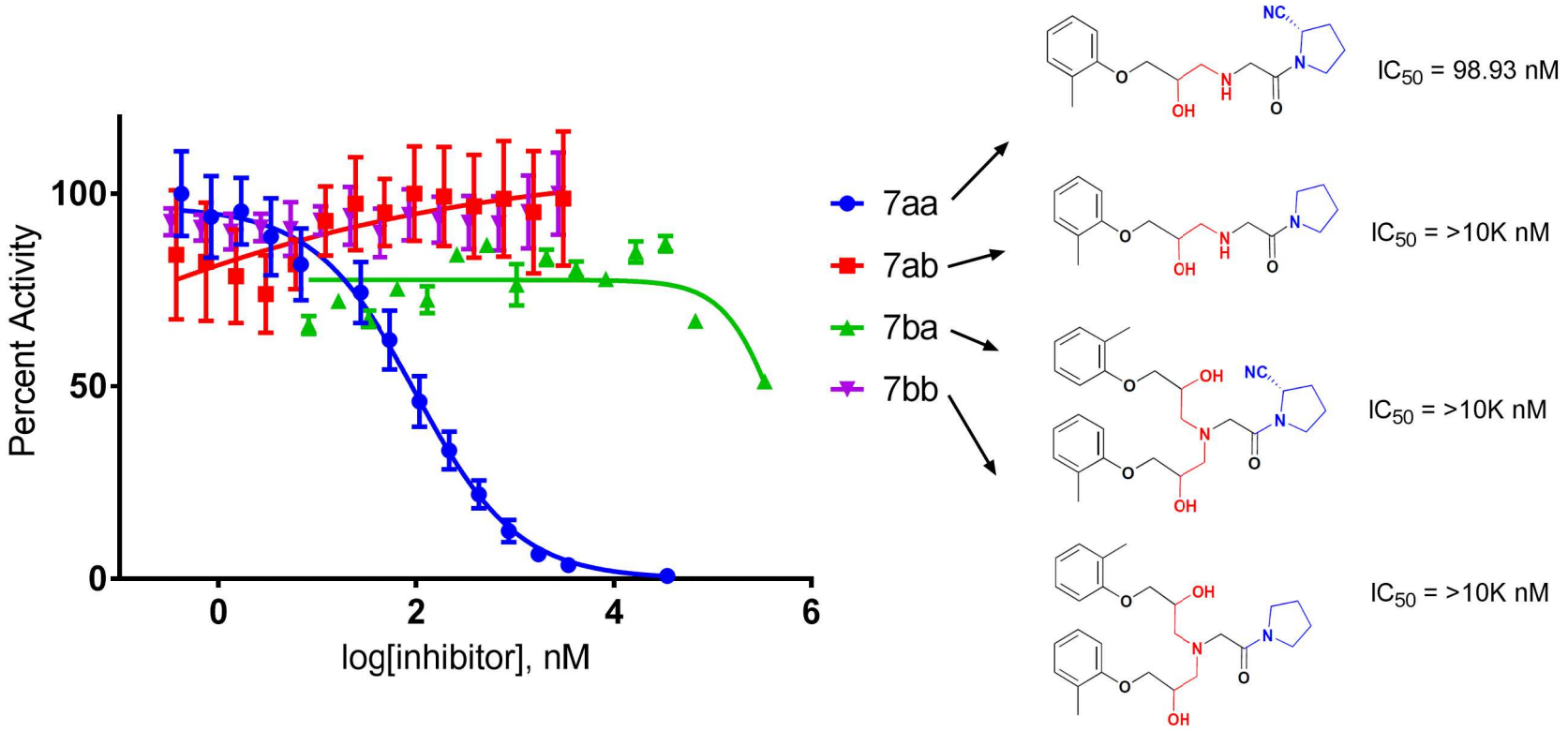


Figure 30. Dose-response curve showing IC_{50} of compounds 7aa, 7ab, 7ba, and 7bb. The values represent the mean ± S.E.M (n=3)

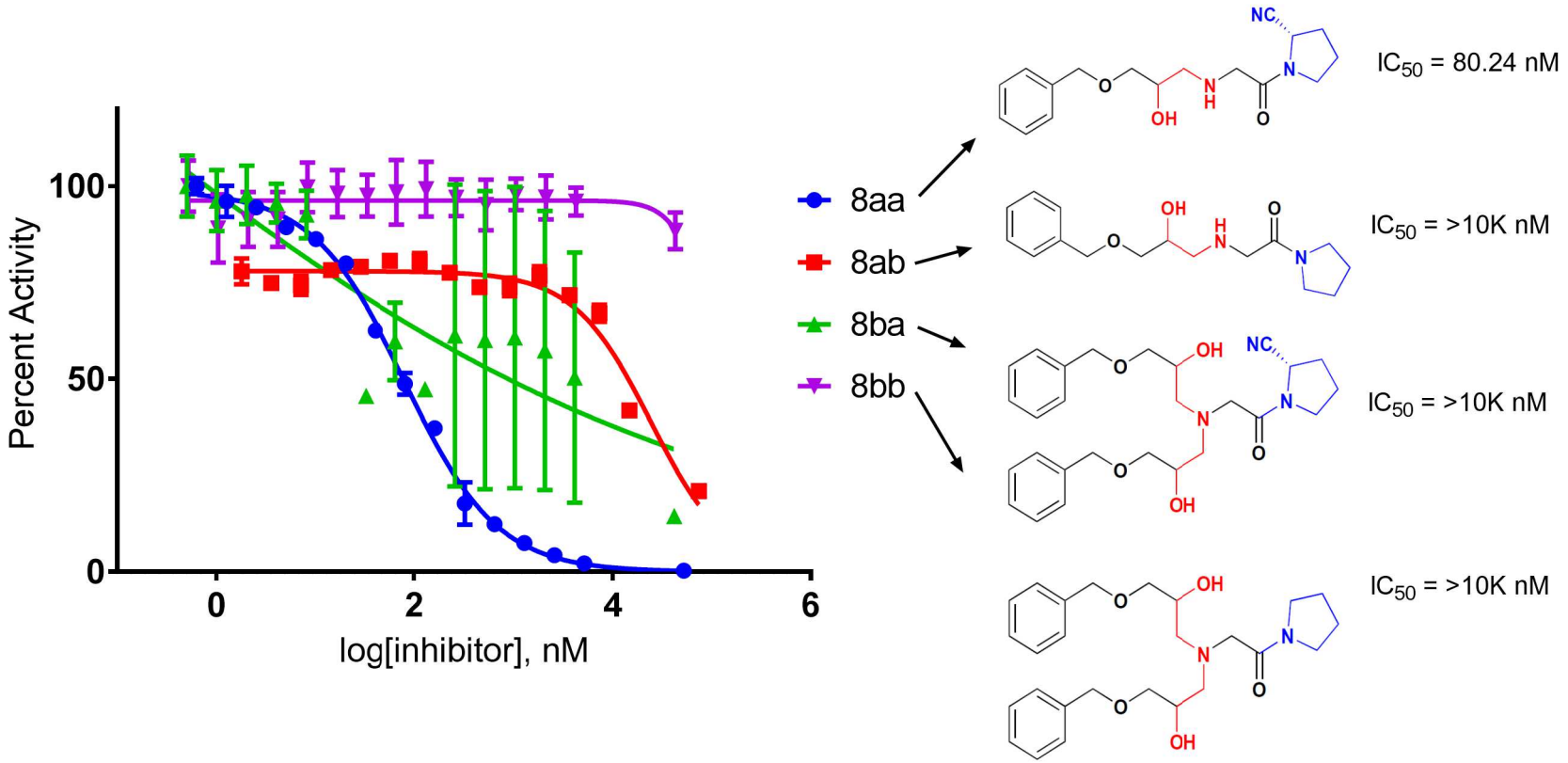


Figure 31. Dose-response curve showing IC_{50} of compounds 8aa, 8ab, 8ba, and 8bb. The values represent the mean ± S.E.M (n=3)

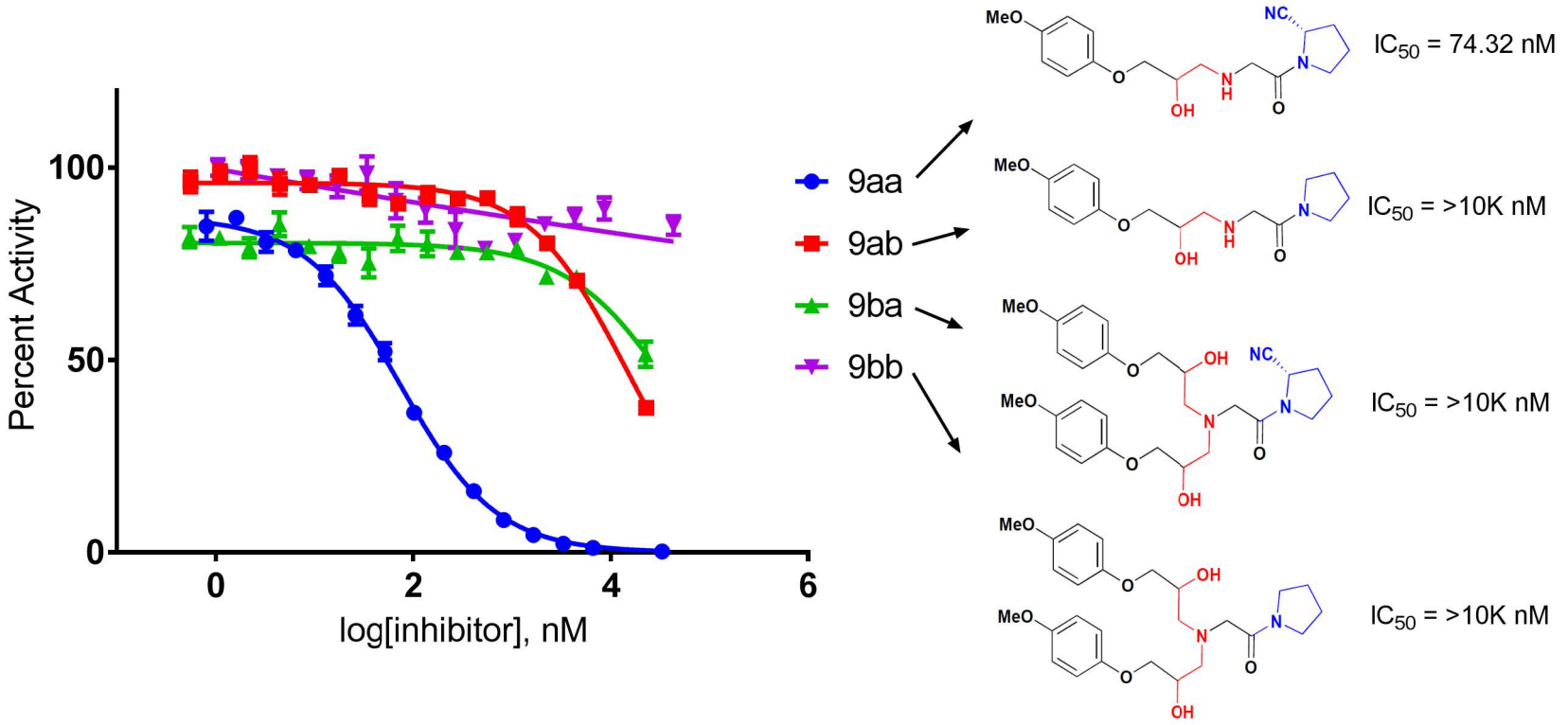


Figure 32. Dose-response curve showing IC_{50} of compounds 9aa, 9ab, 9ba, and 9bb. The values represent the mean ± S.E.M (n=3)

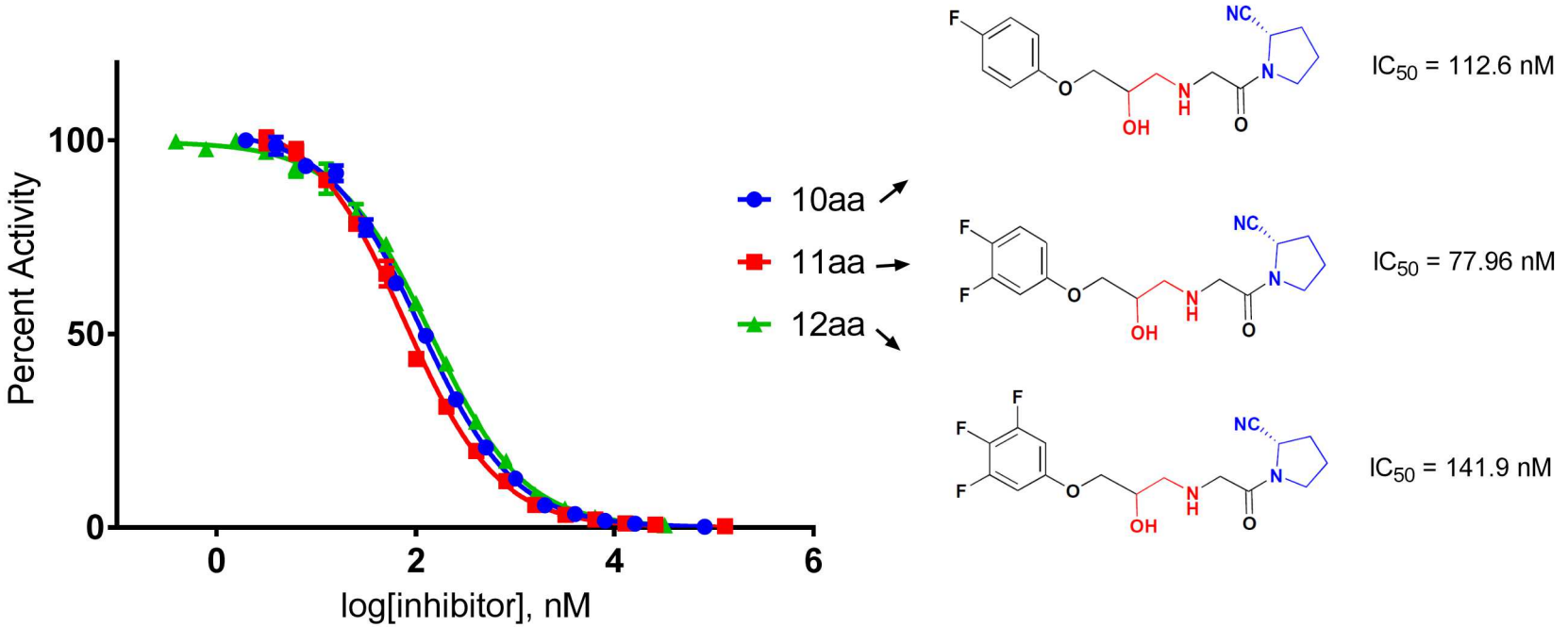


Figure 33. Dose-response curve showing IC_{50} of compounds 10aa, 11aa, and 12aa. The values represent the mean ± S.E.M (n=3)

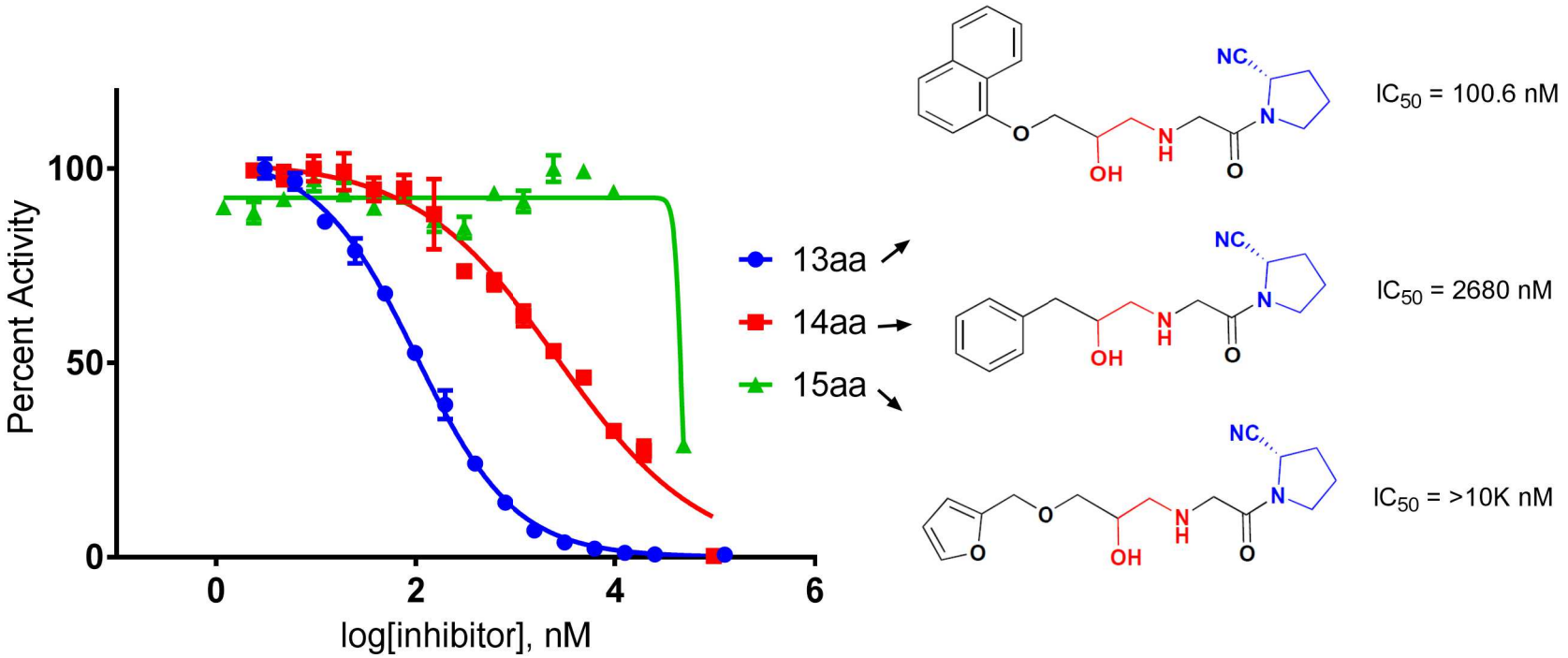


Figure 34. Dose-response curve showing IC_{50} of compounds 13aa, 14aa, and 15aa. The values represent the mean ± S.E.M (n=3)

Chapter 5

Summary Remarks

Peanut Allergies

Problem of peanut allergenicity was investigated since the rate of prevalence and burden on the western world has increased in recent decade. We looked to find a solution that would alleviate majority of people allergic to peanuts in the most efficient way. Since majority of allergic reactions are due to macromolecule protein, we looked to manipulate proteins of peanut, specifically Ara h2, since it has been shown in literature that IgE to Ara h2 has been found in majority of people allergic to peanut. Before we could manipulate Ara h2, we needed great amounts of protein to work with in an efficient manner. Thus, we were able to come up with a novel purification method that allowed us to purify Ara h2 efficiently.

After having sufficient amount of Ara h2 to work with, we had the task of modifying the protein without completely destroying it. We looked to mask various epitopes of Ara h2 by cross-linking the proteins together using various cross-linkers that included BS^3, DST, EDC, Sulfo-EGS and $BM[PEG]_3$. These cross-linkers were utilized according to literature and cross-linked with Ara h2 at various conditions that manipulated temperature, pH and native/reduced form of protein. After cross-linking, protein was visualized on SDS-PAGE gels for visual confirmation of polymerization of the protein, which was negative. On various cross-linkers, as concentration of cross-linker was increased, there was disappearance of protein as seen on the lanes of SDS-PAGE gel. This

could have been due to the insolubility of the newly formed cross-linked product that was not able to dissolve in sample buffer and thus unable to be visualized on the SDS-PAGE.

Further characterizations are required to confirm if Ara h2 had not indeed been cross-linked with various cross-linkers. One method is to determine the protein concentration of Ara h2 in control sample compared to various concentrations of cross-linker and protein samples combined. This can be done using ELISA that includes IgG antibodies specific to Ara h2 to see how the concentration has decreased as concentration of cross-linker has increased. Another approach is to determine changes in the secondary structure of Ara h2 using CD, however, with caution since Ara h2 exists as two different isomers. If there is indeed a cross-linking between molecules of Ara h2, we should be able to confirm this change in mass using mass spectrometry. Finally, to confirm a decrease in allergenicity of Ara h2, ELISA or Western blot can be utilized with IgE antibodies since these antibodies are responsible for causing allergic reaction in the body.

Overall, various cross-linkers were tested, however, more empirical proof is required to confirm a change in structure of Ara h2 along with confirmation of decreased binding to IgE specific to Ara h2.

Diabetes Mellitus

Diabetes is an increasing problem everywhere in the world. The number of newly diagnosed cases of diabetes has been increasing, however, there is also an increase in new drugs that are either in clinical trials or have been recently approved for treatment of diabetes. We decided to look into treating diabetes, specifically type 2 diabetes by the mechanism of inhibiting enzyme DPP-IV in the body. This enzyme is known to cleave

incretin hormones in the body that are responsible for aiding in decreasing blood glucose levels. It has been shown in literature that by inhibiting this enzyme in the body, it is possible to control T2DM. However, we were interested in not just controlling diabetes but to help people who are also diagnosed with hypertension associated with diabetes. We wanted to synthesize novel compounds that would target DPP-IV enzyme and β-adrenergic receptors as one compound containing two different pharmacophores targeting two different protein structures in the body.

We were able to incorporate cyanopyrrolidine and β-Amino alcohol scaffolds to make a novel 36-compound library. These compounds were successful tested for SAR activity against DPP-IV enzyme *in vitro* assays. A series of characteristics were determined that will now allow for synthesis of second generation library. We are in the implementation of next step which is taking the most potent DPP-IV inhibitor compounds and testing them against DPP8 and DPP9. We are also in the process of testing the most potent compounds against CHO cell lines that express β-adrenergic activity *in vivo* assay. This data will allow us to move into *in vivo* studies that would test for blood glucose levels in murine studies along with β-adrenergic receptor blockers in the murine.

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