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Effect of Enzymatic Hydrolysis on the Allergenic Capacity of Shrimp Tropomyosin

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EFFECT OF ENZYMATIC HYDROLYSIS ON ALLERGENIC CAPACITY OF SHRIMP TROPOMYOSIN

by

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Abstract

Food allergy is caused by allergenic proteins within food reacting negatively with IgE antibodies in the human body. Shrimp, part of the shellfish is one of the big eight allergenic foods that can cause anaphylaxis. This study is an attempt to investigate a method of reducing shrimp allergenicity using plant-based enzymes. Shrimp was marinated for 3, 6 or 16 hours in three enzyme solutions of 3% and 5% for Papain, Bromelain and 4% and 8% for Ficin. The effect of marination times with these concentrations on the allergenic proteins was tested using BCA Assay, Lowry Assay, Indirect ELISA and SDS-PAGE, (Specifically, examined for total protein, total degree of hydrolysis, IgE binding strength). The results show that though the tropomyosin was hydrolyzed by the enzymes the IgE binding was not reduced. This leads to the conclusion that enzymatic hydrolysis and marination at the parameters of this experiment is not an effective way to reduce shrimp allergenicity, and that avoidance of the food product should continue to be the main strategy to avoid a reaction.

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Introduction

Shrimp allergies and tropomyosin

What is a food allergy? A food allergy is an adverse reaction of the immune system to certain food proteins. When the immune system makes antibodies to protect the body from pathogens, sometimes those antibodies mistake food proteins as foreign particles that need to be removed. There are eight common food allergens, known as the "big eight," which are proteins in: cow's milk, eggs, peanuts, wheat, soy, fish, shellfish, and tree nuts. In people with these allergies, even a tiny amount of the food can trigger an adverse immune response (Chang et al., 2010). During an immune response, Immunoglobulin E antibodies stimulate mast cells to release histamine and other chemical factors into the bloodstream, thus causing an adverse physical reaction. Symptoms of an allergic reaction can range from mild (runny nose, itchy skin or rash) to severe, (swelling of the face, lips or tongue, wheezing, stomach pain, among others.) (Chang et al., 2010). The major preventative tactic is avoidance of trigger foods and Epi-pen treatment for emergency exposures. However, avoidance can be difficult due to the prevalence of certain ingredients in food such as corn, or milk (Capobianco et al., 2008).

Shellfish allergies are one of the eight most common allergies known to produce anaphylaxis, a serious allergic reaction that is rapid in onset and may cause death if not treated right away. Sixty percent of shrimp allergies are found in adults (Food Allergy Research and Education) of which 2% of the United States population is effected. However, the allergy is most common in Asia with 30% population effected. The major allergen in shrimp and shellfish is the 34 kilodaltons (kDa) myofibrillar protein, tropomyosin. Tropomyosin has specific allergenic regions called epitopes that are bound

by Immunoglobulin E (IgE) antibodies, which result in allergenic reaction. Tropomyosin is both heat and acid stable, so it does not denature easily (Kamath et al., 2013). This makes it difficult to reduce its allergenic capacity through cooking. The allergenic capacity of a food is its ability to cause an allergic reaction. Some foods that cause common allergies can be altered to have a reduced allergenic affect. For example, peanuts when roasted increase the allergenic capacity of that food, however when they are boiled the allergenic capacity decreases (Jimenez-Saiz et al., 2014).

Food allergies are often caused by proteins within the food. There are 2 types of protein structures in relation to epitopes: conformational and linear. Conformational epitopes are the allergenic areas of the protein that exist when the protein is in its fully structured state and not denatured. Antibodies recognize this type of epitope due to its three-dimensional shape and its protein structure. Linear epitopes are allergenic areas of protein based on their amino acid sequence. It is recognized by antibodies by its primary structure, so denaturing its tertiary or secondary structures is ineffective against linear epitopes. The amino acid sequence of the epitope would have to be cut to reduce its IgE binding (Albrecht et al., 2009).

Albrecht et al. (2009) identified linear epitopes in the tropomyosin of shrimp based on the retention of its IgE binding when its secondary and tertiary structures are denatured. Invertebrate tropomyosin is highly conserved across invertebrate species and displays homologies responsible for cross-reactive allergies (Capobianco et al., 2008). A cross reactive allergy means the proteins in one substance are like the proteins in another, so the immune system sees them as the same. As a result, if you're allergic to one, you are most likely allergic to others.

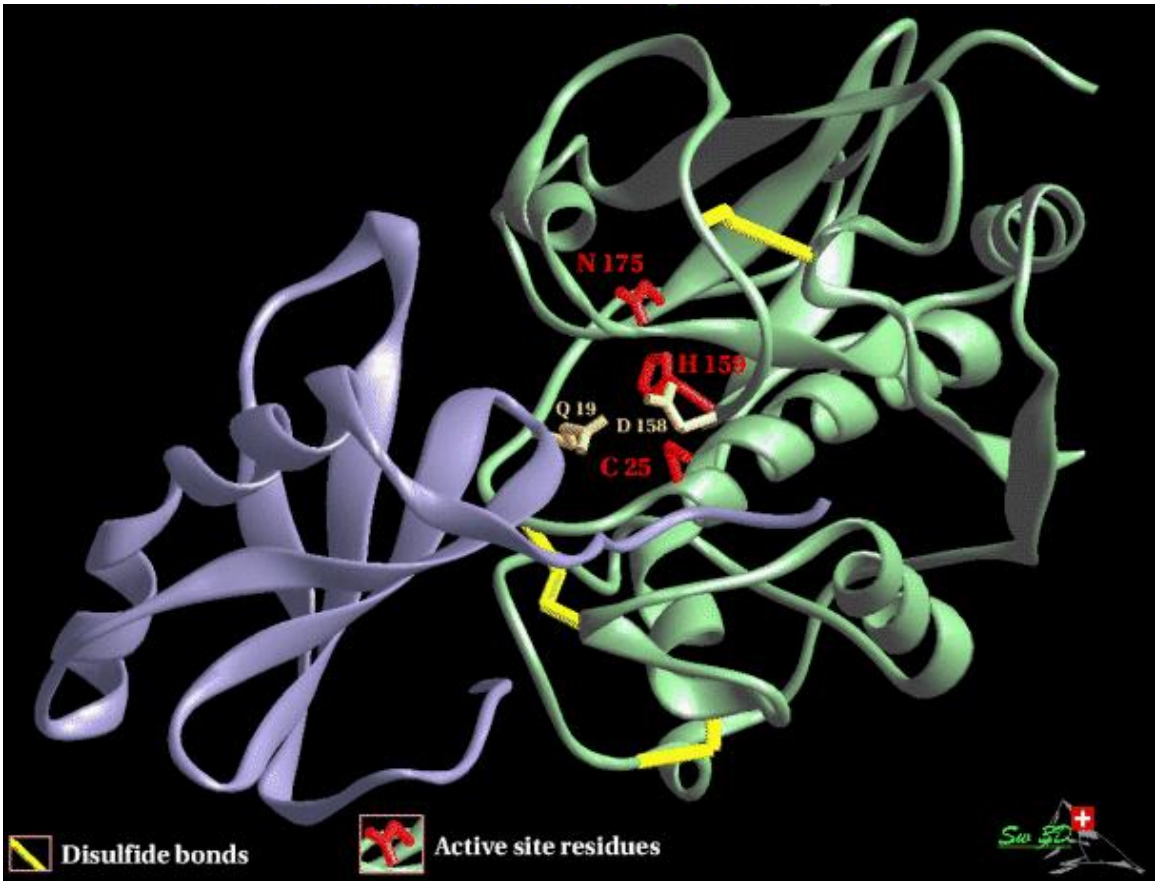
We are looking for ways to reduce the allergenic capacity of tropomyosin, to create a product that can be eaten by those with the allergy without detrimental effect. The food product will hopefully be used in clinical trials for oral immunotherapy under a clinician. My hypothesis is that protein degradation will increase and IgE binding strength will decrease with the increase in duration of marination and enzyme concentration.

Enzymes

Different enzymes preferentially hydrolyze different types of protein. Based on the literature, three enzymes (papain, bromelain, and ficin) that cleave myofibrillar proteins were used in this experiment. Papain, bromelain and ficin are cysteine proteases, and are mostly studied for meat tenderization. These enzymes have a low specificity and are able to hydrolyze a wide range of bonds, such as peptide, amide, ester, thiol ester and thiono ester bonds (Bekhit et al., 2014). These cysteine proteases have a common structure of a monomeric polypeptide with 2 domains with the active site in the cleft between them. Studies have shown that some digestive enzymes do not reduce allergenic reaction as well as enzymes such as Papain (Panda et al., 2015). Based on this study as well as other previous studies investigating other enzymes hydrolyzing wheat flower (Li et al., 2016), three cysteine fruit enzymes Papain, Bromelain and Ficin were selected for this study.

The mechanism of the enzyme activity has been studied extensively in papain. The cysteine and the histidine in the active site form an ion pair which allows the enzyme to cleave bonds. Papain is a relatively simple globular enzyme, consisting of a single 212

amino acid residue chain (Figure 1). Papain folds into two distinct, evenly sized domains, each with its own hydrophobic core. The substrate binding pocket is situated between these two domains. Cyst25 and Hys159 are the residues actually involved in protease activity (Sullivan et al., 2010). Papain digests most proteins, and has a very broad specificity. It is known to cleave peptide bonds of basic amino acids as well as leucine and glycine residues. Papain has a high binding affinity for amino acids with large hydrophobic side chains. Because of this broad binding affinity Papain has many varied uses in commercial products (Proteopedia).



(Figure 1: Papain active site)

The second enzyme used in the experiment was bromelain extracted from the stem of a pineapple. Bromelain from the stem is a glycosylated single-chain protein with

a lower peptide specificity than fruit bromelain. Cysteine is the most effective compound to activate bromelain (Manzoor et al., 2016).

The third enzyme used in the experiment was Ficin. Ficin's active site is centered around a cysteine with a histidine residue in close proximity. It is similar to papain in this respect because both enzymes are part of the plant protease family that has their enzymatic activity depend on the cysteine residue. (Papain is the most extensively studied enzyme in this family). Ficin gives the most balanced degradation of both myofibrillar and collagen proteins of the three enzymes used. The maximum activity of ficin is in the pH range of 5-8, and it requires a cysteine or other reducing agent to activate it (Bekhit et al., 2014). According to the literature, all three enzymes work as meat tenderizers and should be able to cleave tropomyosin (Buyukyavuz et al., 2014).

According to the literature, these enzymes should hydrolyze the epitopes of tropomyosin due to their broad specificity and efficiency at cutting myofibrillar proteins. The epitopes of tropomyosin have components with large hydrophobic sidechains, which are specific areas of attack for the enzymes. The hypothesis to the enzymes will hydrolyze tropomyosin at the epitope regions theoretically reducing allergenic activity. Since we do not possess the technology to locate where exactly the enzymes will be hydrolyzing and how large the fragments will be we will not know how small the fragments would have to be in order to no longer have an allergenic effect.

One previous study investigating enzymatic hydrolysis of buckwheat observed how effective 7 commercial proteases were at reducing buckwheat allergenicity. The enzymes were grouped on their characteristics as serine-type peptidase, cysteine-type

peptidase, aminopepdidase, and aspartic-type peptidase. Both the serine-type peptidase and cysteine-type peptidases cause partial decrease in the allergenic capacity. The aminopepdidase and aspartic-type peptidase enzyme did not reduce the allergenic capacity, and in some cases it increased (Sung et al., 2014). Other studies combine some form of processing such as blanching in addition to enzymatic treatment which enhanced the allergenic reduction (Yu et al., in 2011).

Some previous methods for reducing shrimp tropomyosin allergenicity are boiling, combined ultrasound and boiling(CUB), as well as high pressure steaming(HPS) (Yu et al., 2011). According to this study, the most effective treatment was HPS to promote degradation of tropomyosin by digestive protease. Another study done by Toomer et al., (2015) showed tropomyosin to be pepsin and pancreatin stable for up to one hour after initiating digestion.

Materials and Methods

Materials

Frozen shrimp *(Penaeus monodon)* were purchased from a local store and kept at -20°C until use. Papain, bromelain, and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St Louis, MO, USA). Ficin was purchased from MB Biomedicals (Solon, OH, USA). Bicinchoninic (BCA) Assay, as well as Modified Lowry Assay kits were purchased from Thermo Fisher Scientific (Rockford, IL, USA). The pre-cast gels, nitrocellulose membranes (0.2 um) and sample and running buffers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from BioRad

(Hercules, CA, USA). Horseradish peroxidase (HRP)-labelled goat anti-human IgE was purchased from Life-Technologies (Grand Island, NY, USA). Sera from two individuals with confirmed IgE antibodies from shrimp tropomyosin were purchased from PlasmaLab (Everett, WA, USA). Trichloroacetic Acid, 2M NaOH, and 20mM phosphate buffer with 1M NaCl were also solutions that were used.

Marination

Initially the experimental design was to use fruit juice extracted from the fruits containing the enzymes papain, bromelain and ficin from papaya, pineapple and figs respectively. However, this was both energetically and financially expensive as well as being ineffective. From our preliminary tests we realized that the extracts from the fruit themselves do not work to hydrolyze the tropomyosin. Some reasons we hypothesized for this could be that the specific enzymes we are hoping to use are not in high enough concentrations to cut the proteins. Another hypothesis was that the selected enzymes, specifically papain, bromelain and ficin are interacting with other enzymes or substrates in the fruit extract itself, rendering it ineffective. Therefore, commercially available enzyme products were used for this study.

An extraction using 20 mM Phosphate Buffer containing 1M NaCl was performed to remove the soluble and insoluble protein from the shrimp. The extract was nonspecific and contained all proteins present in the sample not just tropomyosin. The black tiger shrimp (species), purchased from a local seafood store, were thawed, shelled, and split lengthwise down the middle. They were also weighed, and the weights were used to calculate the amount of enzyme needed.

Papain and bromelain were used in 3% and 5% solutions for this experiment. Ficin was used in solutions of 4% and 8%. Before marination, the pH of the solutions was adjusted to pH 9 for the bromelain and papain solutions and to pH 7.5 for the ficin solution using 2M NaOH. Buffer was not used to control for the enzyme activity, without the possible interference of buffer. These pH values represent the level for optimum enzyme activity for each enzyme. This was determined through literary references as well as trials done prior to the experiment.

The shrimp were dipped in these enzyme solutions and marinated for 3, 6 and 16 hours at 4°C under slight agitation with a rotary shaker. A total of 63 shrimp were marinated.

Extraction

After marination the shrimp were rinsed and mashed into paste. Five grams of each mashed sample was taken and added to 10 ml of 20mM phosphate buffer containing 1M NaCl and homogenized for approximately one minute. The homogenate was then combined with the remaining 40ml of buffer and spun for 1 hour. This phosphate buffer was used because, its high salt content would cause the soluble proteins to leach out into the solution. The homogenate was centrifuged at 7800rpm for 20 minutes using a centrifuge **(model #)** and the supernatant was collected and frozen for further use.

Determination of Soluble Protein Concentration

Bicinchoninic Acid (BCA) Assay reagent reacts with copper ions and peptide bonds to produce a purple end product (Thermo Fisher Scientific). The advantage of BCA is that the reagent is fairly stable under alkaline conditions, and can be included in the copper solution to allow a one-step procedure.

The Albumin standard (BSA) was prepared through a series of dilutions, using phosphate buffer as diluent, and placed in the first column of wells. The working range of standards was 2000-20ug/ml. One hundred microliters of sample in duplicates were taken and placed in the first row of wells, with 50 ul taken to make a series of dilutions, with the highest dilution being 8x more diluted than the initial sample. A 1:50 ratio of working reagent is made, using BCA Reagent A and BCA Reagent B, and 200ul is added to each well. Then the plate was shaken for 1 min and incubated at 37°C for 45 minutes. The absorbance was measured using a plate reader at 562nm.

Determination of Trichloroacetic acid (TCA) Soluble Peptide Concentration

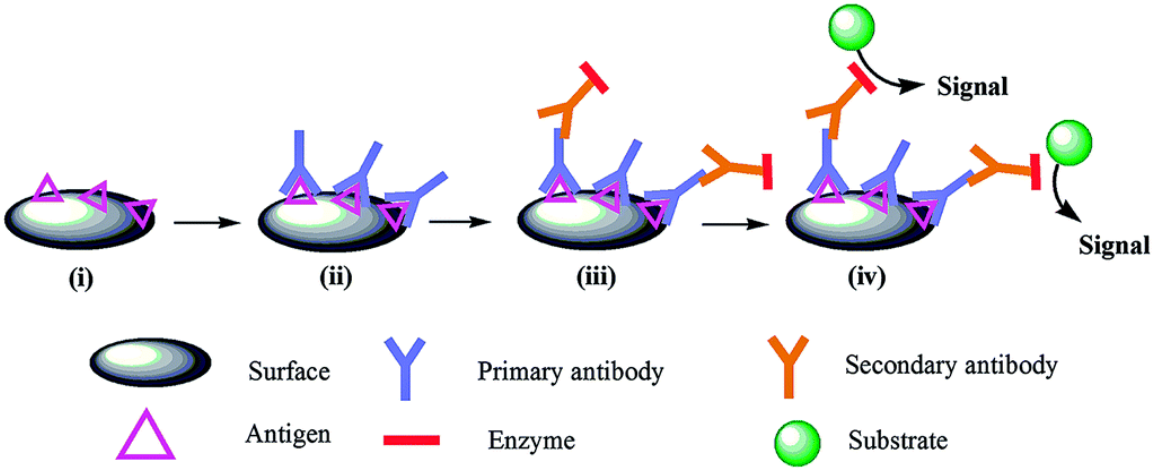
The Modified Lowry assay was used instead of the BCA assay for determining TCA soluble peptide concentration because the Lowry Assay is more alkaline than the BCA assay. The acid from the TCA solution would not detrimentally affect the colorimetric reaction by copper reduction. Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue. The necessary buffers were prepared. 20% TCA was made from 6.1 M stock solution, 20ml of TCA in 80ml of deionized water. 10% TCA was also made to be used as diluent for stock solutions.

Both the protein extracts and the Lowry reagent were brought to room temperature. Two hundred microliters of sample were combined with 200ul of 20% TCA. These samples

were then vortexed and left to sit at room temperature for 15 minutes. This procedure would precipitate out the whole proteins, leaving the peptides in solution. The mixture was then centrifuged at 20,000 rpm for 5 minutes and the supernatant was kept. The BSA standard using the same dilutions as the BSA assay was prepared, using deionized water for the peptide analysis instead of the phosphate buffer used in the total protein quantification assay. A 1:3 dilution was used for each of the samples and 40ul were taken to be analyzed in separate wells. Two hundred microliters of the modified Lowry reagent was added to the wells and mixed for 30 seconds. The plate was covered and allowed to sit at room temperature in the dark for exactly 10 minutes. Twenty microliters of 1XFolin reagent was then added to the wells and mixed for 30 seconds. The plate was incubated again at room temperature in the dark for 30 minutes and the absorbance was measured at 750nm.

Indirect Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA allows us to determine the strength of the IgE binding between a protein and an antibody. This is important because it shows whether or not the antibody can still attach to the epitope region of the protein whether it is hydrolyzed or not. The higher the absorbance level, the deeper the color which means there is more binding present. The protein in the sample is bonded to the high-binding plate. The primary antibody (the IgE) is then added that is human sera from individuals who have shrimp allergy, which binds to the protein. The secondary antibody, an enzyme-labeled, anti-species globulin conjugate, is added and binds to the primary antibody. The addition of an enzyme substrate chromogen reagent causes color to develop directly proportional to the amount of bound antibody in the sample. (Indirect ELISA)



(Figure 2: Indirect ELISA scheme)

The percentages used in the following methods were optimized to the human sera that were found using checkerboard ELISA's.

A number of buffers were prepared, and standard Indirect ELISA procedure was followed. Briefly, 100 ul of shrimp extract, as well as 2 blanks of just diluent, were placed in duplicate in a high binding polystyrene 96 well plate at a concentration of 5 ug/ml with coating buffer used as diluent. The plate was incubated at 37°C for 2 hours. The plate was washed (flooded with liquid then removed) with 1X Phosphate Buffered Saline Tween-20 (PBST) 5 times and blocked with 2% Bovine Serum Albumin (BSA) in PBST blocking buffer at 200 ul per well. It was then incubated at 37°C for 2 hours. After blocking, the plates were washed again 5 times with PBST. Pooled human plasma containing IgE antibodies for shrimp allergens was diluted with 1% BSA in PBS diluent solution at a ratio of 1:20 and 50 ul added to each well. The plate was then incubated at 37°C for 1 hour. After washing with PBST five times, 50 ul of secondary antibody goat anti human IgE conjugated to HRP (1:1500) was added to each well then the plate was incubated for 1 hour at 37°C. HRP is horseradish peroxidase which is the enzyme bound

to the secondary antibody to produce color. The wells were again washed 5 times with PBST and Tetramethylbenzidine (TMB) was added at 50 ul per well. TMB is the substrate solution that causes color development when bound to the enzyme on the secondary antibody.After a 15-30 minute incubation for color development an equal volume (50 ul) of 2 M H2SO4 to the wells as stop solution. The absorbance was measured at 450nm.

Sodium Dodecyl Sulfate Polyacrylamide (SDS-PAGE) gel electrophoresis

SDS-PAGE gel electrophoresis is used to separate proteins based on their molecular weight with the help of an electrical field. Since they will have different molecular weights they will have different migration rates through the porous gel sieve. The movement of any charged species through an electric field is determined by its net charge, its molecular radius and the magnitude of the applied field. Proteins with the same molecular weight would migrate at different speeds based on their net charge and 3 dimensional structure. SDS is used along with boiling water and Dithiothreitol (DTT) or Mercaptoethanol (BME) to denature the protein's tertiary structure; the SDS coats the protein with a uniform negative charge in such a way that makes the protein's charge proportional to its molecular weight. The differing pH of the Tris-HCl buffer allows it to conduct the electrical current and keep the protein moving from the cathode to the anode. The stacking gel ensures that all of the proteins arrive at the running gel at the same time so proteins of the same molecular weight will migrate as tight bands. Underneath the stacking gel is the running gel, which since it has an increased acrylamide concentration, slows the movement of the proteins according to their size, and causes the separation.

The higher molecular weight proteins move more slowly through the porous acrylamide gel than lower molecular weight proteins (Osawald et al 2016).

The triplicate protein extracts were pooled together and standardized to 1000 mg/ml. The final concentration that was used was 15ug/ml. Twenty microliters of each of the samples were taken and combined with 20ul of 2X Laemmli buffer with 5% BME. These samples were the boiled for 5 minutes then placed on ice. The running buffer was then prepared by adding 100ml of 10X Tris/glycine/SDS (TGS) Running buffer to 900ml of deionized water. The 12% gel was rinsed with running buffer and the assembly was filled with running buffer until just below the outer gel plate. Ten microliters of the molecular weight protein standard was loaded into the first gel lane. Fifteen microliters of samples were then loaded into subsequent gel lanes. The gel was run for approximately 40 minutes. Once the protein had reached the end of the gel they were removed from the apparatus and stained in small tanks using 20ml of GelCode Blue Stain reagent for approximately 1 hour. Then the gels were rinsed with deionized water 3 times for 5 minutes and left to sit overnight in deionized water.

Statistical Analysis

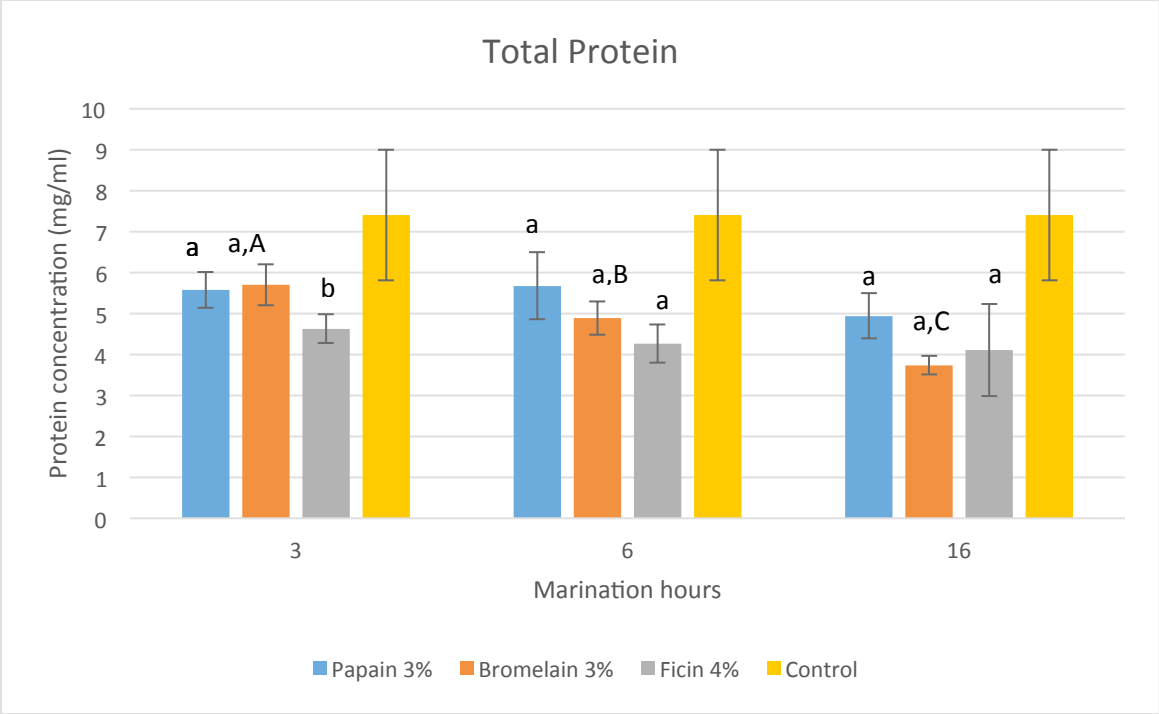
Each combination of the levels of independent variables tested in this experiment (enzyme concentration, and time) was replicated 3 times and all quantitative data was reported as a mean ±standard deviation. The statistical analysis was preformed using a one-way analysis of variance (ANOVA) test to determine if there were significant effects of the independent variables on the dependent variables. The level of significance used was $p \leq .05$. Data was analyzed using a free social sciences software online.

Results

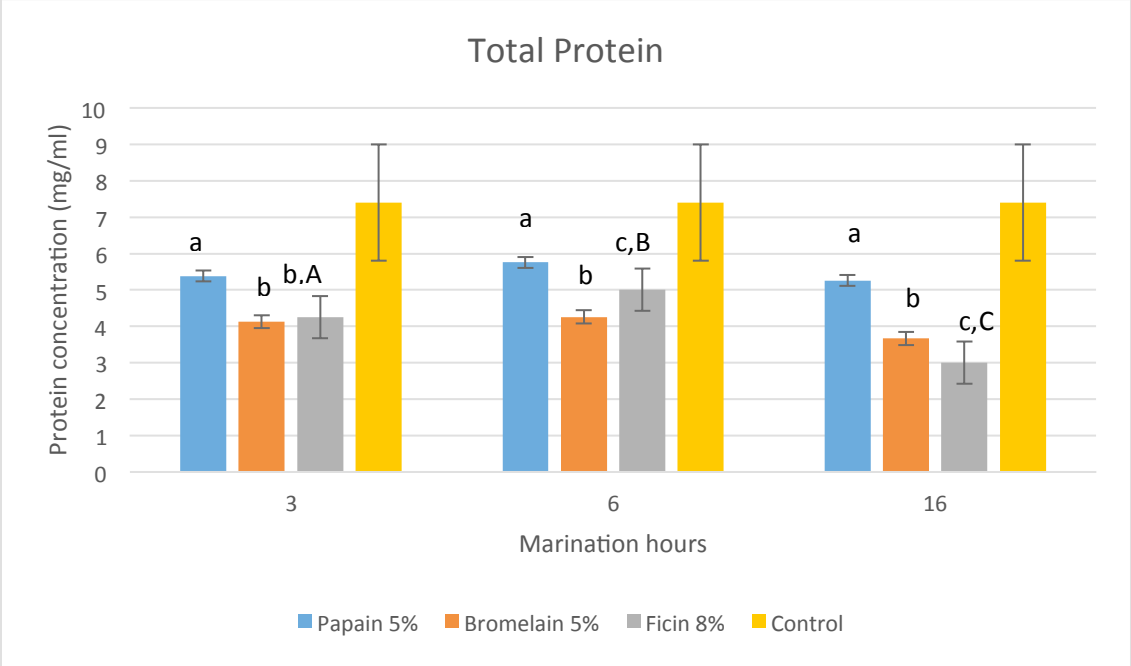
Total Protein Concentration (from BCA Assay)

The lowest protein concentration was observed in the 16 hour marination samples. The 3% papain and bromelain solutions at 16 hours shows values of 5mg/ml and 3.75mg/ml, which is consistent for the 5% at 16 hours as well. The protein concentrations for ficin at 3% and 5% decreased from 4 mg/ml to 3mg/ml. (Figures 2 and 3) The protein concentrations after 3 and 6 hours were comparable to each other ($p=0.04$). The untreated controls have an average total protein concentration in the 7mg/ml range, which is fairly high and as expected. The treatment samples all have fairly consistent protein concentrations within the range of 4-5 mg/ml of protein. This shows that there was some hydrolysis of the proteins by the enzymes, however, not as much as expected. Papain has the highest treatment total protein concentration, with bromelain and ficin showing similar results. Bromelain at 3% enzyme concentration has a steadily decreasing trend.

The lower total protein concentrations in the treatment levels than in the control can be seen in other studies as well. Lasekan et al 2017 found that after shrimp tropomyosin was exposed to acidic conditions the total soluble protein concentrations were lower in the treatment than the control. An explanation used in this study was that the proteins could have been denatured, which results in poor solubility in the extraction buffer. Another explanation could be that vinegar leached out into the extraction solution which would reduce it pH, buffering capacity and ability to solubilize myofibrillar proteins (Lasekan et al 2017).



(Figure 3: Total Protein recovery from shrimp after marination with enzymes at lower concentrations) (The small letters, a,b,c denote statistically significant differences between enzymes in a specific marination time. The large letters A,B,C denote statistically significant differences between marination times of a specific enzyme.)



(Figure 4: Total Protein recovery from shrimp after marination with enzymes at higher concentrations)

(The small letters, a,b,c denote statistically significant differences between enzymes in a specific marination time. The large letters A,B,C denote statistically significant differences between marination times of a specific enzyme.)

Since all of the protein concentrations are similar it can be deduced that the enzyme hydrolytic rate is less than 3 hours. If I were to do future experiments, I would use the three hour marination because marinating beyond that time beyond that doesn't affect the protein concentration in a statistically significant way. All of the treatment values compared to the control (untreated sample) were statistically significant ($p<0.05$), however when comparing the time variables to each other and the enzymes to each other a variety of values were obtained. The 5% values when compared by enzyme in time to each other were all statistically significant ($p<0.03$). Bromelain 3% and ficin 8% across the time variables were also statistically significant. The marination time had no effect on the control, so the average control value is used.

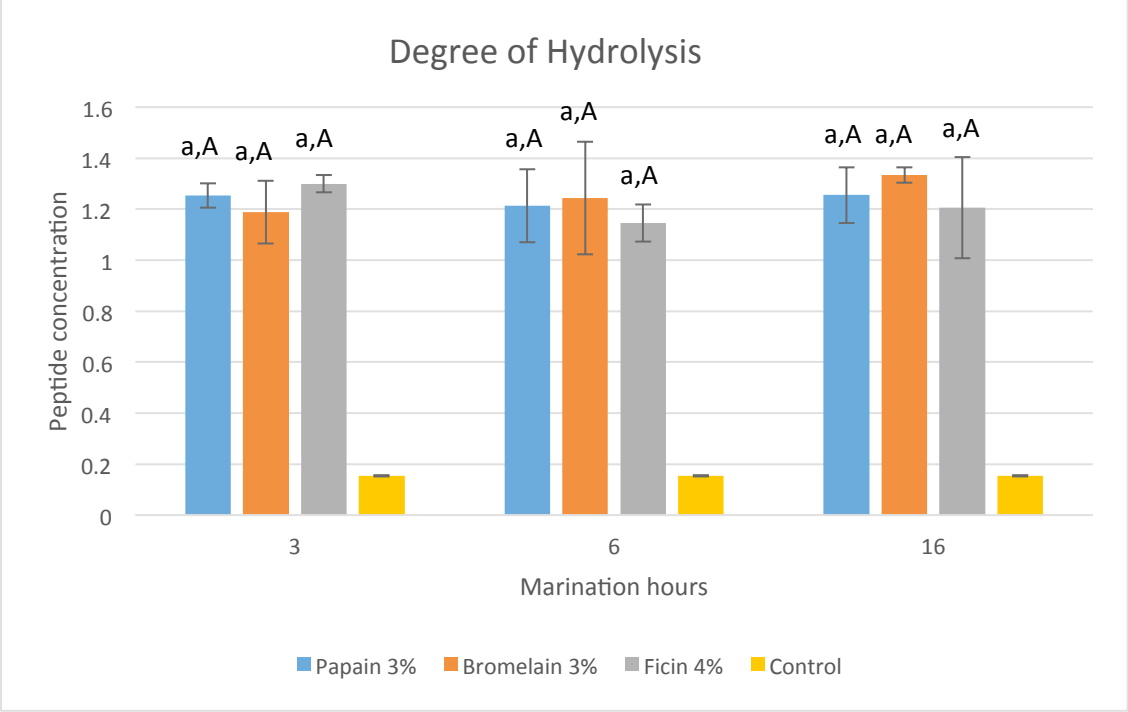
The BCA assay as well as the Lowry assay to check for degree of hydrolysis show that enzymatic hydrolysis is occurring, however there is no way of knowing whether tropomyosin is hydrolyzed or not. However, these tests are still needed in order to perform the ELISA by giving the protein concentrations so the proper dilutions can be made in order for the ELISA to work.

Degree of Hydrolysis

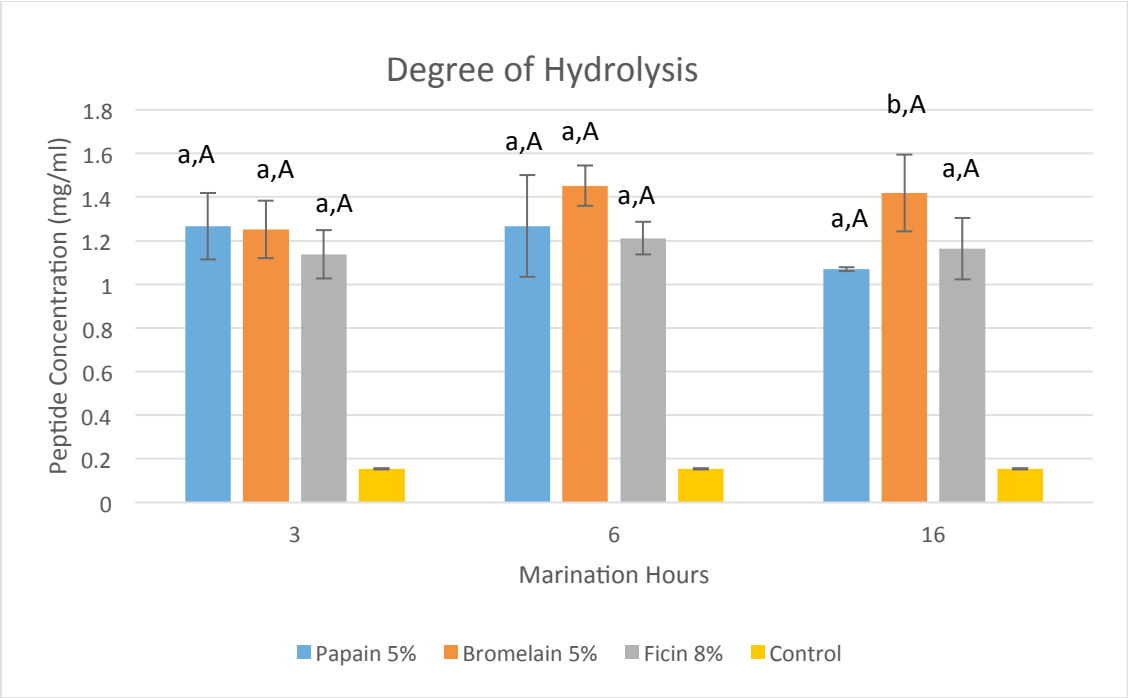
The degree of hydrolysis was examined using the Lowry assay. The hypothesis was that there should be an increased concentration of hydrolyzed protein with increased marination time. Additionally, it was hypothesized that there would be an increase of soluble peptides due to the increased enzyme concentration. These hypothesized results

should lead to decreased IgE binding as tested for by Indirect ELISA. Overall, there does not appear to be any consistent trends in the data, though bromelain did show an increased concentration for the 3% over time. The concentrations have similar results.

This data fits what is expected after looking at the total protein concentrations. Since the treatment total protein concentrations were lower than the control by around 2-3 mg/ml of protein, it makes sense that the degree of hydrolysis is similar with an overall concentration of around 1.5 mg/ml. The soluble peptide concentrations are similar both across enzyme concentrations as well as across the different enzymes (Figure 4 and 5). The data in figure 5 has slightly more variability across the table than the data in figure 4. This suggests that the enzymes have similar capabilities when it comes to hydrolyzing the protein. It also suggests that their hydrolysis rates are similar both in the time as well as temperature component There were no statistically significant differences between the data for figure 4 ($p>.05$).



(Figure 5: Total Degree of hydrolysis recovery from shrimp after marination with enzymes at lower concentrations) (The small letters, a,b,c denote statistically significant differences between enzymes in a specific marination time. The large letters A,B,C denote statistically significant differences between marination times of a specific enzyme.)



(Figure 6: Total Degree of hydrolysis recovery from shrimp after marination with enzymes at higher concentrations) (The small letters, a,b,c denote statistically significant differences between enzymes in a specific marination time. The large letters A,B,C denote statistically significant differences between marination times of a specific enzyme.)

While ficin had lower total protein concentrations, the soluble peptide concentrations are comparable with the other enzymes. There is certainly some hydrolysis happening, there is not as much as expected. Only the 5% concentration at 16 hours was statistically significant ($p=.04$), all other values were not ($p>.05$). Compared to the control all of the values were statistically significant ($p<.05$).

ELISA

Table 1: Absorbance values from Indirect ELISA

	Enzyme Concentration	Time marinated (hours)		Absorbance values
Enzyme	0	3	6	16
Papain	3%	0.94 ± 0.21 (a)	0.85 ± 0.02 (a)	0.76 ± 0.05 (a, A)
	5%	0.82 ± 0.06 (a)	0.87 ± 0.09 (a, A)	0.74 ± 0.002 (a, A)
Bromelain	3%	0.75 ± 0.04 (a)	0.84 ± 0.14 (a)	0.93 ± 0.06 (a, B)
	5%	0.86 ± 0.07 (a)	0.65 ± 0.02 (b, B)	0.75 ± 0.05 (c, A)
Ficin	4%	0.79 ± 0.16 (a)	0.91 ± 0.02 (a)	0.76 ± 0.09 (a, A)
	8%	0.8 ± 0.06 (a)	0.87 ± 0.13 (b, A)	0.64 ± 0.01 (c, B)
Control	0.88 ± 0.16			

(Chart 1)(The small letters, a,b,c show statistical significance across time during a specific marination enzyme concentration. The large letters show significance across a marination concentration during a specific time)

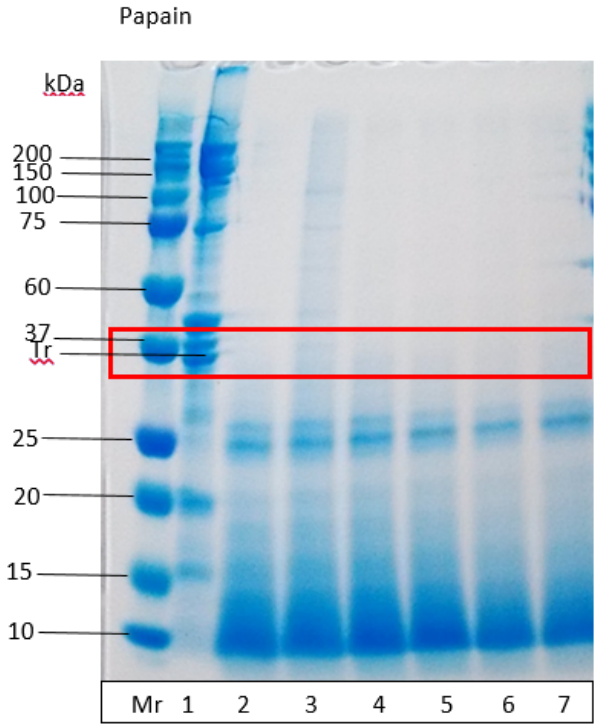
The Elisa data shows that there is little to no reduction of IgE binding to the tropomyosin taking place. There are no trends throughout the data. The high absorbance values show there is some slight increased binding of the IgE to the tropomyosin, however, the overall trend is that there is no decrease in the binding, and as such no decrease in the IgE binding.

The p-values for all of the enzymes compared to the control values were not statistically significant, except for the 5% bromelain treatment($p=.049$). The p values when the enzymes were compared to each other at 3% at 16 hours as well as the 5%

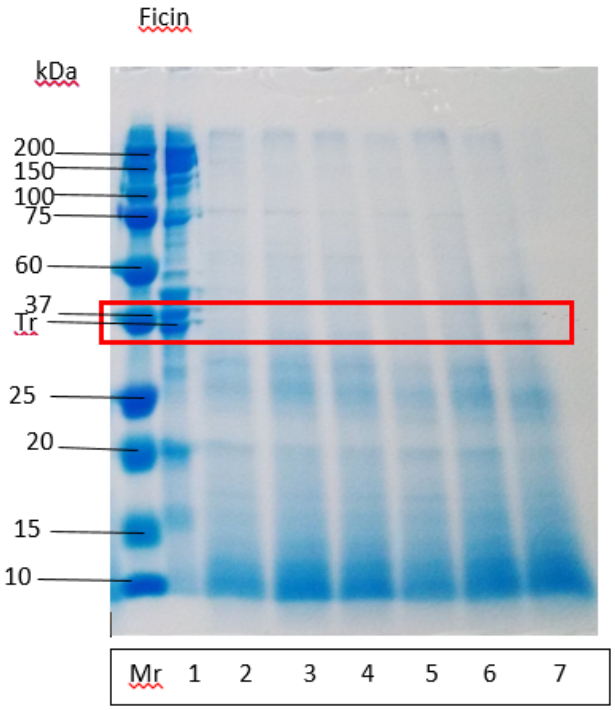
treatments at 6 and 16 hours were also statistically significant ($p<.05$). During the ELISA test, some of the plates were varied in the absorbance values, though not in relation to the control.

SDS Page

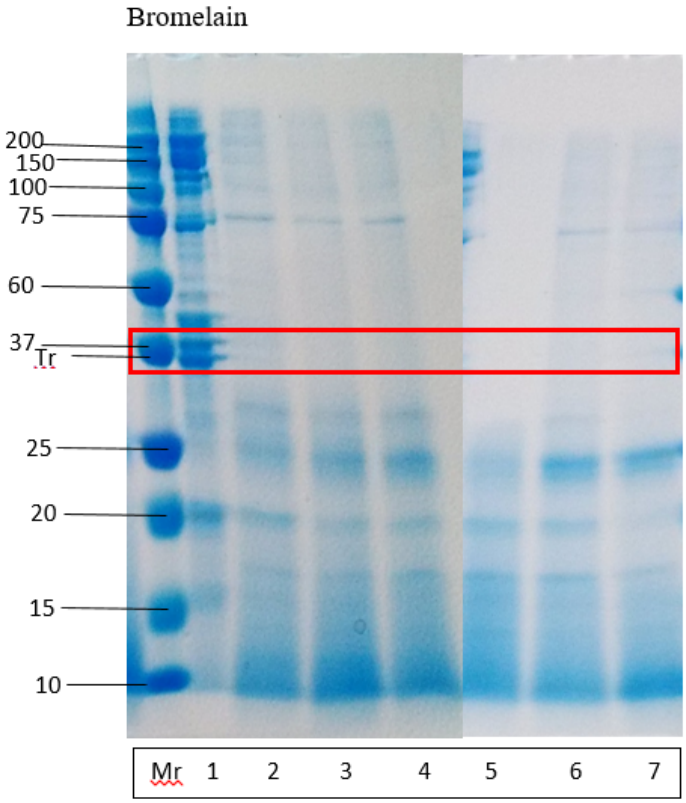
Figure 7 shows that the enzymes did a good job of hydrolyzing the higher density proteins, including tropomyosin, which is inside the area boxed in red. As shown all of the enzymes do a comparable job hydrolyzing the protein. The papain lanes (figure 6) have much darker bands at the bottom than either ficin or bromelain (figure 7, 8), indicating that there is a much higher concentration of smaller peptides. This does not appear to be the case with bromelain or ficin, with bromelain having the lightest bands. This would suggest that bromelain does a better job of hydrolyzing protein than the other two enzymes. The enzyme concentrations of 3% or 5% do not change, both are equally effective.



(Figure 7 numbers are designated as follows. **Mr.** Molecular weight marker, **1.** Control, raw sample, **2.** Papain 3% at 3 hour marination, **3.** Papain 3% at 6 hour marination, **4.** Papain 3% at 16 hour marination, **5.** Papain 5% at 3 hour marination, **6.** Papain 5% at 6 hour marination, 7. Papain 5% at 16 hour marination. The boxed region shows where tropomyosin falls on the gel.)



(Figure 8 numbers are designated as follows. **Mr.** Molecular weight marker, **1.** Control, raw sample, **2.** Ficin 4% at 3 hour marination, **3.** Ficin 4% at 6 hour marination, **4.** Ficin 4% at 16 hour marination, **5.** Ficin 8% at 3 hour marination, **6.** Ficin 8% at 6 hour marination, **7.** Ficin 8% at 16 hour marination. The boxed region shows where tropomyosin falls on the gel.)



(Figure 9 numbers are designated as follows. **Mr.** Molecular weight marker, **1.** Control, raw sample, **2.** Bromelain 3% at 3 hour marination, **3.** Bromelain 3% at 6 hour marination, **4.** Bromelain 3% at 16 hour marination. **5.** Bromelain 5% at 3 hour marination, **6.** Bromelain 5% at 6 hour marination, **7.** Bromelain 5% at 16 hour margination. The boxed region shows where tropomyosin falls on the gel.)

Discussion

There are no consistent trends in the total protein and degree of hydrolysis. The degree of hydrolysis is comparable for all of the enzymes, times and concentration variables. The total protein content shows no consistent trends, although the 16 hour enzyme marination does show lower total protein levels. There is also a slight increase in degree of hydrolysis for Bromelain and Ficin at 5% concentration. Some reasons for this could be the rate or reaction for the enzymes, which could have been lesser or greater than our marination times. Other reasons could be that the shrimp muscle proteins were

not accessible enough to the enzymes so only a bit of the protein was hydrolyzed. Shaking the solution during marination increased the protein concentrations compared to just letting them sit. Therefore, if the shrimp were crushed more it is likely that the protein concentration would increase as well. These results do not seem to match previous studies. In a study with peanuts the degree of hydrolysis as well as total soluble protein increased with increased time and enzyme concentration (Yu et al 2015, Li et al 2013).

The marination samples were placed in the refrigerator at a temperature of 4°C. The temperature was discovered to have gone lower than expected. This was not planned and could have introduced a margin of error. Low temperatures cause the formation of intramolecular hydrogen bridges which convert enzymes to a catalytically inactive state, in which the active centers either lose their specific configuration or are no longer exposed to the substrate. The conversion of enzymes to the catalytically inactive form happens at temperatures of –10–0°C (Kavanau et al 1950). This suggests that though there was hydrolysis of the protein it may not have been as effective as it could have at a slightly higher temperature. A possible explanation for the similar values could be that peptides leached into solution when hydrolyzed, which we did not test, so could show varying concentrations of peptides. The lack of expected hydrolysis could be due to the specificity of the enzymes for a certain portion of protein, or the slightly lower than anticipated temperature of the marination. Lower temperatures decrease enzymatic activity, thus reducing hydrolysis. Proteases are a class of enzymes used to cut other proteins. Some proteases have different hydrolysis mechanisms for cutting or have

specificity for certain residues. Other factors that could influence the concentrations are the protein-protein interactions in solution.

As exemplified in the chart 1 in the results section, the results did not support my hypothesis that protein IgE binding would be reduced with increased marination time and enzyme concentration. The ELISA shows that there was little to no reduction in IgE binding of the protein. This suggests one of two things. Either the enzymes are not cutting at the epitopes of the tropomyosin, causing the protein to still have full IgE binding, or the enzymes are cutting at the epitopes however the hydrolyzed pieces are still large enough to be bound by IgE, thereby it still has its allergenic properties. However, when looking at the gel we notice that the enzymes did hydrolyze the protein, because the tropomyosin bands at 34 kDa no longer show in the treatment lanes. There is a high concentration of peptides that can be seen at the bottom of the gel. The enzymes may have cut the epitopes of the protein however, since the ELISA still shows reactivity, there could still be enough of an epitope to have IgE binding capability. We do not have the tools to tell exactly where the enzymes are cutting. Based on literary references the enzymes should be cutting at the epitopes. Though they are hydrolyzed we suspect that they still react with the IgE binding (Albrecht et al., 2009, Bekhit et al., 2014). This is supported in other areas of research because the enzymes can hydrolyze the protein but not reduce allergenicity. In some cases the IgE binding capability is even increased because of enzymatic hydrolysis exposing more epitope regions (Panda et al., 2015).

Yu et al., 2011 found that even though the SDS-PAGE band may have disappeared that does not necessarily mean that the IgE binding has been reduced as shown through Western Blott and other analysis. Enzymatic treatment of the raw kernels

of peanut reduced allergen city in some instances and increased it in others. The peptide fragments may or may not still retain their allergenic binding capabilities. This is also seen in another study (Sung et al., 2014).

As shown in figures 6, 7 and 8 of the SDS-PAGE it exhibits that the bands of tropomyosin are no longer there. However there are dark smears at the bottom of the gel, implying that the peptides are still there. Papain has the darkest smears with Ficin and bromelain becoming lighter. This implies that bromelain did the best job of hydrolyzing the protein because the peptides lighter than 10 kDa and single amino acids are not seen on the gel.

Another reason for the relatively low numbers for results could be due to the storage temperature and freeze-thaw cycle effects on the protein. These factors could have impacted the activity of the protein and therefore the results. The coagulation of protein could have impacted the concentrations used in the later tests that were performed. The impacts of the freeze-thaw cycle and the protein-protein interactions were minimized by only unfreezing small portions of extract at a time. Additionally, diluting the total protein concentrations so that the protein no longer formed gels within the extracts.

Conclusion and Next Steps

I can conclude from the results of this experiment that I should reject my hypothesis, which is that these enzymes, papain bromelain and ficin, reduce the allergenic capacity of shrimp tropomyosin through enzymatic hydrolysis. Our alternate hypothesis is that these enzymes do not reduce the allergenic capacity of shrimp

tropomyosin through hydrolysis of the peptide bonds. Some enzyme hydrolysis occurred as can be seen in the gel images as well as the Lowry Assay results. However the epitopes were still allergenic, despite possibly being hydrolyzed. Therefore, in conclusion, enzymatic hydrolysis through the marination with the parameters that were chosen for my experiment is not an appropriate method to reduce IgE binding.

If I were to go back and do this experiment again, I would choose a different marination temperature. Due to an unforeseen complication, the marination was at a lower temperature than anticipated, so I would correct that in future experiments. Lower temperatures impact protein structure as well as lower enzyme activity. Future steps would be to do mass spectrometry with the protein to see what kind of peptides there are at the bottom of the gel to see if there are still epitopes intact in the peptide fragments there. This would enable us to observe exactly where the enzymes are hydrolyzing and if they are effective at denaturing the epitope region.

List of references

1. Chang HJ. Food Allergies. Jama. 2010;303(18):1876.

2. Shellfish - Food Allergy Research & Education. Shellfish - Food Allergy Research & Education. [accessed 2017 Mar 16]. https://www.foodallergy.org/allergens/shellfish-allergy

3. Kamath SD, Rahman AMA, Komoda T, Lopata AL. Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies. Food Chemistry. 2013;141(4):4031–4039.

4. Jiménez-Saiz R, Benedé S, Molina E, López-Expósito I. Effect of Processing Technologies on the Allergenicity of Food Products. Critical Reviews in Food Science and Nutrition. 2014;55(13):1902–1917.

5. Albrecht M, Kühne Y, Ballmer-Weber BK, Becker W-M, Holzhauser T, Lauer I, Reuter A, Randow S, Falk S, Wangorsch A, et al. Relevance of IgE binding to short peptides for the allergenic activity of food allergens. Journal of Allergy and Clinical Immunology. 2009;124(2).

6. Capobianco F, Butteroni C, Barletta B, Corinti S, Afferni C, Tinghino R, Boirivant M, Felice GD. Oral sensitization with shrimp tropomyosin induces in mice allergen-specific IgE, T cell response and systemic anaphylactic reactions. International Immunology. 2008;20(8):1077–1086.

7. Sullivan G, Calkins C. Application of exogenous enzymes to beef muscle of high and low-connective tissue. Meat Science. 2010;85(4):730–734.

8. Bekhit AA, Hopkins DL, Geesink G, Bekhit AA, Franks P. Exogenous Proteases for Meat Tenderization. Critical Reviews in Food Science and Nutrition. 2014;54(8):1012–1031.

9. Papain. Papain - Proteopedia, life in 3D. [accessed 2017 Mar 16]. http://proteopedia.org/wiki/index.php/Papain

10. Manzoor Z, Nawaz A, Mukhtar H, Haq I. Bromelain: Methods of Extraction, Purification and Therapeutic Applications. Brazilian Archives of Biology and Technology. 2016;59.

11. Buyukyavuz A. Effect of Bromelain on Duck Breast Meat Tenderization [thesis]. TigerPrints; 2014.

12. Yu H-L, Cao M-J, Cai Q-F, Weng W-Y, Su W-J, Liu G-M. Effects of different processing methods on digestibility of Scylla paramamosain allergen (tropomyosin). Food and Chemical Toxicology. 2011;49(4):791–798.

13. Toomer OT, Do AB, Fu TJ, Williams KM. Digestibility and Immunoreactivity of Shrimp Extracts Using an In Vitro Digestibility Model with Pepsin and Pancreatin. Journal of Food Science. 2015;80(7).

14. Sung D-E, Lee J, Han Y, Shon D-H, Ahn K, Oh S, Do J-R. Effects of enzymatic hydrolysis of buckwheat protein on antigenicity and allergenicity. Nutrition Research and Practice. 2014;8(3):278.

15. Yu J, Ahmedna M, Goktepe I, Cheng H, Maleki S. Enzymatic treatment of peanut kernels to reduce allergen levels. Food Chemistry. 2011;127(3):1014–1022.

16. Panda R, Tetteh AO, Pramod SN, Goodman RE. Enzymatic Hydrolysis Does Not Reduce the Biological Reactivity of Soybean Proteins for All Allergic Subjects. Journal of Agricultural and Food Chemistry. 2015;63(43):9629–9639.

17. Li Y, Yu J, Goktepe I, Ahmedna M. The potential of papain and alcalase enzymes and process optimizations to reduce allergenic gliadins in wheat flour. Food Chemistry. 2016;196:1338–1345.

18. Pierce BCA Protein Assay Kit - Thermo Fisher Scientific. [accessed 2017 Apr 10]. https://www.bing.com/cr?IG=D9A6A2ADF6B149C88481A744388F183B&CID=0F39FBD5986262752FE4F1B599F26397&rd=1&h=eZD0cLOJZuuAWG0ag-vFIV77BcHhBrHl40EfOqnYvJc&v=1&r=https%3a%2f%2ftools.thermofisher.com%2fcontent%2fsfs%2fmanuals%2fMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf&p=DevEx,5049.1

19. Indirect ELISA, conventional but efficient. Indirect ELISA. [accessed 2017 Apr 10]. http://www.elisa-antibody.com/ELISA-Introduction/ELISA-types/indirect-elisa

20. Oswald DN, Oswald DN, S, S, Salunke A, S, C, J, Akhil S, Welch DA, et al. How SDS-PAGE works. Bitesize Bio. 2016 Jul 7 [accessed 2017 Mar 16]. http://bitesizebio.com/580/how-sds-page-works/

21. Lasekan A, Cao H, Maleki S, Nayak BB. Shrimp tropomyosin retains antibody reactivity after exposure to acidic condition. Journal of the Science of Food and Agriculture. 2017.

22. Yu J, Hernandez M, Li H, Goktepe I, Robinette C, Auerbach A, Peden D, Ahmedna M. Allergenicity of roasted peanuts treated with a non-human digestive protease. Food Research International. 2015;69:341–347.

23. Li H, Yu J, Ahmedna M, Goktepe I. Reduction of major peanut allergens Ara h 1 and Ara h 2, in roasted peanuts by ultrasound assisted enzymatic treatment. Food Chemistry. 2013;141(2):762–768.

24. Kavanau JL. Enzyme Kinetics And The Rate Of Biological Processes. The Journal of General Physiology. 1950;34(2):193–209.

Authors Biography

Angela Silke was born in Portland Maine on June 1, 1995. She was raised in Dixmont, Maine and graduated from Nokomis Regional High School in 2013. Majoring in Biology with a Pre-Medical concentration, Angela has a minor in Anthropology. She is a member of Alpha Lambda Delta. She received the Carolyn Reed Fellowship.

Upon graduation Angela plans to live at home while looking for job prospects and fiddling with her fish tank and gaining more experience in life and the medical field before re-applying to medical school.