The Effect of Alcalase Concentration on the Proteins from the Shells of *Litopenaeus setiferus* (White Shrimp)

Liam Quan

Heathwood Hall Episcopal School, Columbia, SC

Chitin is a naturally abundant polymer that also happens to be biodegradable. Chitin can be used in a variety of different products such as biodegradable plastics, papers, medical products, foods, and medical treatments. To extract chitin, shells must be demineralized and deproteinized. The goal of this experiment was to examine the effect of the protease Alcalase in the deproteinization of *Litopenaeus setiferus* shells. The hypothesis was that if the concentration of Alcalase increased, then the absorbance of proteins in the spectrophotometer reading would increase. The null hypothesis was that if the concentration increased there would be no change in absorption. Shrimp shells were first cooked in boiling water for 20 minutes then grinded up with liquid nitrogen. Then, some were demineralized using hydrochloric acid while others were untouched by this step. Demineralized shrimp shells and untouched were put into several different solutions of 0%, 0.5%, 1.5%, and 2.5% concentration. Afterwards, they were put in a water bath to kill the Alcalase. All samples were read through a UV Visible Spectrophotometer to find that the data was too red-shifted and inaccurate above 2 Au to give any factual conclusion. These problems were conjectured to be attributed by polarity, the machine itself, and the HCl bath. The data could be interpreted to disprove the null hypothesis, as 0.5% looked to be the optimal concentration, if the red-shift and absorption above 2 Au was caused by the reasons above.

Introduction

As plastic continues to cover the oceans and remain stagnant, a new material that is biodegradable is needed to replace the water-insoluble material. Chitin is part of the answer. Chitin is a homopolysaccharide that is found all over several different animal groups, especially arthropods, which are animals that are characterized by their sturdy exoskeleton[1]. After cellulose, chitin is one of the most abundant organic materials being found in even common foods like crab, shrimp, and lobster[2]. In fact, according to the National Oceanic and Atmospheric Administration(NOAA), the supply of shrimp exceeds the demand, the average person eating 4 pounds of shrimp per year[3]. Chitin can be found combined with calcium and other minerals to make the exoskeletons of arthropods and crustaceans. Recent studies have shown that a polymer can be created out of chitin as a substitute for plastic, as chitin is biodegradable and water insoluble, allowing liquids to be placed in chitin and the polymer to be disposed of naturally. CruzFoam is a company that is using chitin to produce surfboards, showing how incredibly versatile chitin is. The very problem with chitin is the process of refining it, which requires, "harsh chemical reactions used today to also sever the long polymer chains that make the material sturdy"[2].

The standard process being used to extract chitin consists of first grinding up the shells, then taking away the minerals with hydrochloric acid and the proteins with hot sodium hydroxide. Chitin can also be deacetylated with another bath of highly concentrated and hot sodium hydroxide to create chitosan, which is more soluble than the previous polymer[4]. Chitosan can be more commonly used for medical purposes, for instance pill capsules. Chitosan can also be used in medical dressings to help clot blood. While a simple process, this does leave a large portion of hazardous waste which is why this study focuses on finding a more environmentally friendly alternative, in this case proteases. This study focuses solely on trying to find another way to deproteinize shrimp shells, research on an HCl replacement must be done in the future. Proteases, or an enzyme that can break down proteins, can be disposed easily down the sink once they are killed, averse to harsher chemicals that have been used in the past.

The independent variable was the concentration of the enzyme, Alcalase, while the dependent variable was the absorbance of the liquid at different wavelengths. *Litopenaeus setiferus* shells were grinded up and split into 2 groups, one that would be demineralized and another that would not. Both groups underwent deproteinization at 4 different concentrations and the methods used were stated in a study led by *Islem Younes*[5].

The purpose of this experiment is to find the best concentration of Alcalase to denature and breakdown proteins found in shrimp shells. The hypothesis is as follows: if the concentration of Alcalase increases then the sample will absorb at a higher rate at a wavelength of 280 nm, as proteins absorb at 280 nm[6]. The null hypothesis is no matter the concentration of Alcalase the results will be the same, if any proteins are broken down at all. All the samples were run through a UV visible spectrophotometer to find the different absorptions of the groups and subsequent wavelength to pair up with the materials in the shell homogenate.

Methods

First the pound of raw shrimp shells were put in a pot of boiling water for 20 minutes[5]. Salt was added to the water to help prevent an unnecessary amount of water drain from the shells and or the structure of the shell to break down further. The purpose of this step was to try and clean the shrimp of outer impurities and remove some of the proteins on the outer layer. The shells were then air dried for one day then soaked in liquid nitrogen. At first the shells were beaten with a mallet until it was realized that this method would not grind up the shells to the desired size. Still frozen with liquid nitrogen, the shells were put in the Vitamix Blender for ~2 minutes to further process the shrimp shells[5].

Once at the desired size, 100 grams of the shrimp homogenate was measured out and placed in a 1 liter bath of 1.5 M HCl for 10 hours. To ensure that the mixture was constantly stirred, the beaker was placed on a hot plate with a magnetic stir bar and stirred at a medium speed. The homogenate was then rinsed and decanted 4 times with distilled water until the water was clear before covering in more distilled water and left mixing overnight. This entire process was to demineralize the shrimp homogenate, the major minerals in *Litopenaeus setiferus* being calcium and phosphorus[7]. In the morning the pH of the water was tested and found to be 1. The shells were then placed in cheesecloth and put over a funnel. 1 gallon of distilled water was used to rinse the shells mixture until it reached a pH of 6. At this point the cheesecloth was pressed to drain the water and the homogenate was measured again. 65.78 grams of product was recovered, meaning roughly 33%of the shell was not recovered or was composed of minerals and other solids that could be dissolved by hydrochloric acid.

The 28 vials were then labeled in preparation for the enzyme solution. One group consisted of the shrimp shells that were demineralized with HCl and the enzyme/buffer solution, another group acted as a control with just buffer and enzyme in it, and the last group was filled with shrimp shells that were not demineralized and the enzyme/buffer solution. Every group had 4 different concentration points, 0%, 0.5%, 1.5%, and 2.5%. Groups

had 3 vials at each concentration, except the group of just buffer and enzyme, which was only repeated 1 time. To then prepare each data point, a solution was created by filling a 200mL beaker with distilled water and adding a pH buffer of 10 until the water reached a pH of 10. 1.25 mL, 0.75mL, and 0.25mL of Alcalase was pipetted into the 3 respective groups, 2.5%, 1.5%, and 0.5% concentration respectively. Then, water was poured into each vial until 50mL of volume was attained. All of the groups were placed into a water bath of 50ºC for 1 hour, 50º being the optimum heat for the enzyme to work. After 1 hour each cap was unscrewed then screwed back on to allow the gas to escape. Once each vial had a chance to let the gas escape the water bath was increased to a temperature of 90ºC for 20 minutes to kill the enzyme[5]. All the groups were then placed in the refrigerator overnight until they could be brought to the lab for testing.

For use of the spectrophotometer, 1 standard cuvette was filled with 2 mL of the solution at the top of the sample to avoid solids, ran through the machine, then rinsed with distilled water before being used again with the next sample. This machine shined a light through the sample and tested wavelengths from about 200 nanometers to 1100 nanometers, allowing a wide spectrum of ultraviolet to infrared light to be tested. The spectrophotometer was blanked with the vial that consisted of just the buffer from the second group mentioned earlier.

Precautions were taken to ensure that the remaining solutions were disposed of in the correct fashion. Because hydrochloric acid was being used, the counters were washed with baking soda thoroughly. Lastly, all the materials, like vials and beakers, were labeled to ensure that they were not tampered with.

Results

Each line in the graphs consists of the average of all the vials in the concentration group and preparation. It should also be noted that the spectrophotometer had a hard time reading samples of a wavelength shorter than about 280 nm and reading accurate data that absorbed more than 2 Au.

Discrediting everything before about 280 nm, it appears that adding more enzymes resulted in a shift in wavelength, not necessarily absorbance because every line reached 2 Au. The groups that had enzyme in them all absorbed only around 2 Au, while the group with 0% enzyme absorbed less than 1 Au. This seems to be supporting the null hypothesis, as the enzyme groups all absorbed within a range to be counted as 2 Au, yet the red-shifting proposes that something different was being absorbed in every group.

Figure 2 shows every group generally absorbing more than Figure 1. Even the group with 0% of the enzyme in it absorbed more, meaning that this set of data had more of something in the solution. This could be minerals that rose off the shells or even proteins. The groups with enzyme in it all generally absorbed above 2 Au, which makes it impossible to conclude that they absorbed more total than Figure 1, but the drastic difference can lead to the assumption that they did, in fact, absorb more. The same trend of red-shifting also occurred in this data. Because the peaks were not at 280 nm the null hypothesis is supported.

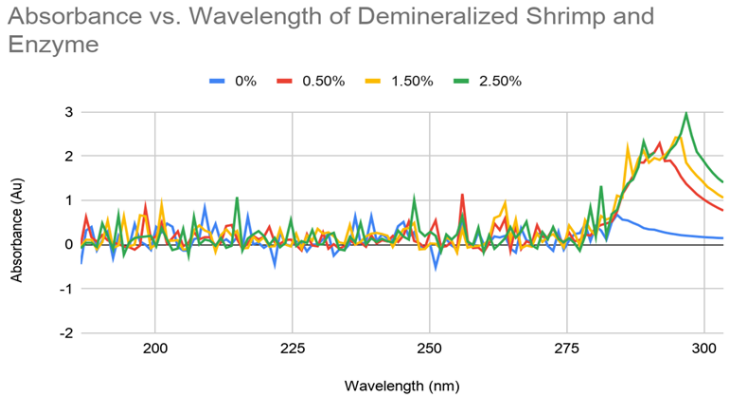

Figure 1: Absorption of Demineralized Shrimp and Enzyme

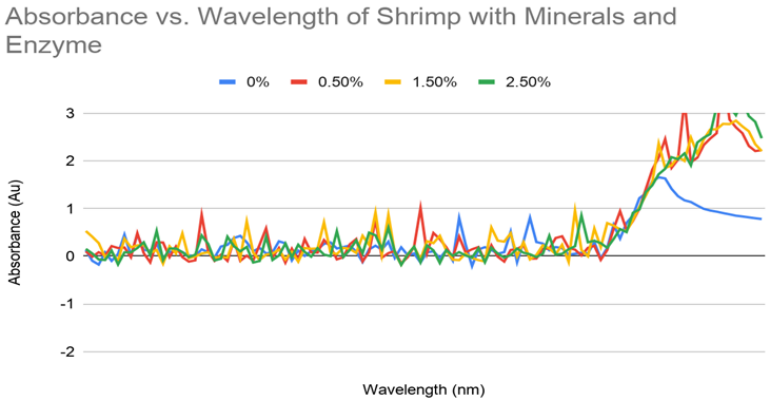

Figure 2: Absorption of Shrimp With Minerals and Enzyme

The group of data with 0% of the enzyme was not included in this figure because all the data was blanked with that sample. The red-shifting also occurred within this set of data. As the concentration of enzymes increased, however, more absorption was read. This data closely mimics the other two trials, especially the wavelengths where the most absorption occurs. At this point, it seemed like the spectrophotometer was reading the enzyme and not protein in all the figures, supporting the null hypothesis and showing that no protein may have been absorbed at all.

For the next two figures, the data from Figure 3 was subtracted from the former two to produce graphs that show something that can not be enzyme but hopefully proteins and amino acids. In Figure 4, when the control was taken away, there is an inverse from the figures above; the 0.5% of enzyme absorbed slightly more than the other two total. Because this figure is based on data that is originally in the margin of error of the machine and there is not a large variance between the lines, it cannot be concluded that the null hypothesis was disproved or supported by this figure. While even after 280 nm there is negative absorption, that can be attributed to the fact that the control absorbed more at the wavelength.

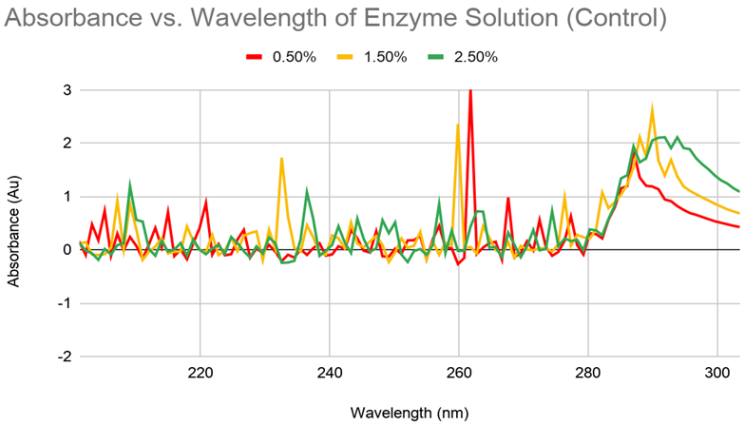

Figure 3: Absorption of Enzyme Solution (Control Group)

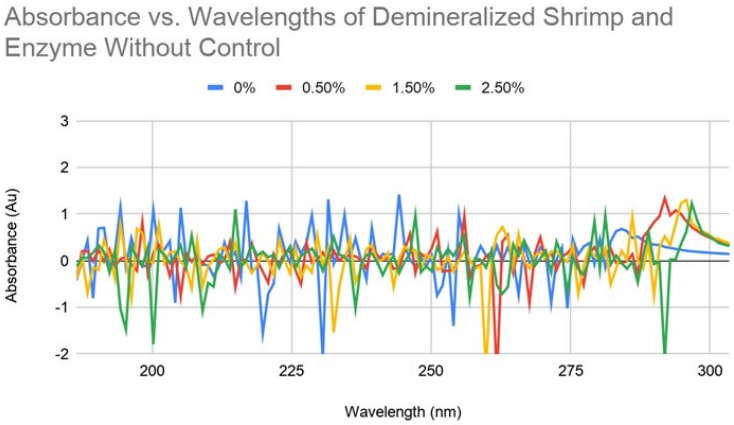

Figure 4: Absorption of Demineralized Shrimp and Enzyme Without Control

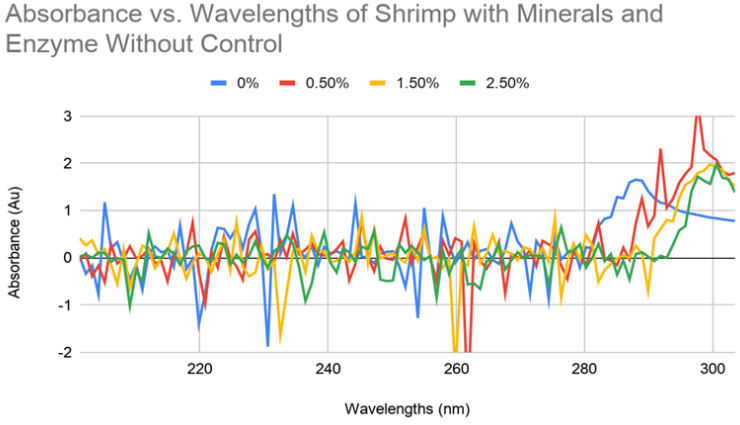

Figure 5: Absorption of Shrimp with Minerals and Enzyme Without Control

While it must be taken into account that this figure is showing data also originally above 2 Au, the differences in this graph compared to Figure 4 can be assumed to be, indeed, different. The drastic differences in Figure 5 show that something was absorbed and not enzyme. Based on this data, 0.5% concentration seems to be the most efficient concentration in breaking down something unknown, disproving the null hypothesis. At first it can be assumed that broken down or denatured proteins were being picked up in this reading but the red-shifting as more enzyme was added seems to disprove that theory.

Conclusions

The purpose of this study was to find the optimal concentration of Alcalase to remove the proteins attached to chitin. Nothing can be completely concluded with this data because the amount of data collected does not outweigh the margin of error the spectrophotometer produces when reading above 2 Au and before 280 nm. After further research was conducted it was discovered that red-shifting of around 10 nm can occur as tryptophan and tyrosine, the amino acids that make up proteins, change the wavelength they absorb on slightly when exposed to a different polarity, specifically a red-shift with a nonpolar solution[6]. Because the red-shifting can be explained, the null hypothesis is not completely supported by the data. Glycerol, the majority ingredient in Alcalase, is non-polar. When the nonpolar Alcalase was added to the polar water and buffer solution the polarity could have decreased enough to red-shift the reading, which is very possible because of how little the data was shifted. Combined with the fact that denatured proteins and broken down proteins also can slightly alter the wavelength of absorption it is very possible and likely that the data was red-shifted away from 280 nm[6]. If the data indeed red-shifted due to the difference in polarity and amino acids, then Figure 5 disproves the null hypothesis and shows that 0.5% of the enzyme could be optimal. Figure 4, however, would still show that the change in enzyme concentration slightly changed the amount of proteins altered, if any at all. This could actually be a result of the hydrochloric acid as HCl itself denatures proteins[8]. Figure 4 involved the shrimp that were demineralized by HCl, but during that process proteins could have broken down as well and been washed away when that mixture was drained. That could explain why the groups of demineralized shrimp and enzyme were proven to be mostly enzyme by Figure 3 and Figure 4. Figure 5 showing most protein, and assuming the red-shift was due to reasons stated above, this could be attributed to the fact that the enzyme had more proteins to break down because the shrimp in this test were not demineralized and therefore didn't have the opportunity to be denatured by HCl. The last point to address is the fact that the spectrophotometer had trouble reading absorptions above 2 Au. Figures 4 and 5 are greatly affected by this as their parent figures reach an absorption above 2 Au. Rather than depend on the exact absorbance all the figures read at above 2 Au, looking at the trends that exist in that margin of uncertainty cannot be denied. Figures 2 and 5, which consisted of shrimp that did not experience an HCl bath, have drastic absorbances above 2 Au while Figures 1 and 4, which were affected by HCl, showed lower absorbances. In Figures 4 and 5, a 0.5% concentration of Alcalase produced the most absorbance, which, when combined with the explanations above, could show that this was the optimal concentration after all.

In this study the main limitation was the spectrophotometer itself and the sample size. To actually conclude anything stated above, further research must be conducted. Addressing the red-shift, not only should the same test be run again with more samples, but the polarity of the solution should be tested and altered several times to see if polarity affects the wavelengths. Denatured proteins should be isolated and tested several times to see how much their wavelength differs. The same should be done with broken down protein strands. Addressing the absorbance limitations, a different spectrophotometer could be used. This study used a fast acting spectrophotometer, but there are machines that take longer but can read higher absorbances. Samples should be tested after being soaked in HCl and compared to the ones after the whole process. Younes also ran an ash content analysis which resulted in more information of shrimp shell composition using machines that are not easily accessible. A group of samples should also be tested with only being affected by Alcalase and not demineralized. Lastly, another test should be run using the standard NaOH to compare. All together, it would be helpful to see the same study conducted in this paper but in a greater multitude to find more conclusive data

Notes and References

1. Barnes, Robert D. (2019). Encyclopædia Britannica. Illinois: Encyclopædia Britannica, Inc. [Cited 25 January 2020]. Available from: www.britannica.com/animal/arthropod.
2. Dahl, Carmen. (2019). "How Seafood Shells Could Help Solve the Plastic Waste Problem." *Science News* Vol. 195: No.11
3. NOAA. (2017). National Oceanic and Atmospheric Administration. US: NOAA. [Cited 25 January 2020] Available from: www.fisheries.noaa.gov/2011-top-10-favorite-seafoods-united-states.
4. Roy, Jagadish & Salaün, Fabien & Giraud, Stéphane & Ferri, Ada & Guan, Jinping & Chen, Guoqiang. (2017). Solubility of Chitin: Solvents, Solution Behaviors and Their Related Mechanisms. In Zhenbo Xu *Solubility of Polysaccharides.* IntechBooks, London, UK, pp 109-123.
5. Younes, Islem, and Marguerite Rinaudo. (2015). "Chitin and Chitosan Preparation from Marine Sources. Structure, Properties and Applications." *Marine Drugs*, vol. 13, no. 3, 2015, pp. 1133–1174.
6. Schmid, F.-X. (2001). Biological Macromolecules: UV-visible Spectrophotometry. In e LS , (Ed.). doi:10.1038/npg.els.0003142
7. Dechapinan, Saranya, et al. "Calcium from Pacific White Shrimp (Litopenaeus Vannamei) Shells: Properties AndFunction as Fortificant in Soy Milk." *Food and Applied Bioscience Journal*, 2017, pp. 176–195.
8. Grant, Janine. (2019). LiveStrong. New York City (NY): Leaf Group [Cited 2 February 2020]. Available from: www.livestrong.com/article/501637-hydrochloric-acid-protein-digestion/.