Purified Duck Lysozyme Presents a Higher Rate of Reaction-Cleaving Bacteria than Hen-Stock Lysozyme

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Abstract

The interest in the influential enzyme lysozyme (E.C. 3.2.1.17) has been on the rise because of its antibacterial properties by hydrolyzing glycosidic bonds. This makes it a perfect food preservative and pharmacological component. For this reason, finding the best source of this enzyme is in high demand. Hen egg white is a very common source of lysozyme, which makes up 3.5% of its components. A very similar lysozyme to the hen's is that of the duck egg white, with 2.1% of total proteins. Nonetheless, the differences in enzymatic activity between these two are very under-researched. The purpose of this experiment is to successfully extract lysozyme from duck egg white and compare it to a stock solution of hen lysozyme for their catalytic activity on the bacteria *Micrococcus lysodeikticus*. For this, the purity and presence of lysozyme were analyzed by gel electrophoresis, protein concentration assay, and spectrophotometry to first identify if there was extracted lysozyme and if the isolation of this was successful. This was followed by dialyzing the samples to prepare them for enzymatic activity analysis at their accurate units of activity conditions. The enzymatic activity for the hen and duck lysozyme was compared using the values of Vmax, Km, and Kcat obtained from the Michaelis-Menten and Lineweaver-Burk plots obtained from another spectrophotometry analysis. The results showed that the analysis of extracted lysozyme from duck was not successful because too many samples were lost and not pure enough. However, from the data set from another group with duck egg white, the comparison with our measures of hen lysozyme yielded that duck has a much higher rate of reaction than hen, but the latter one is more specific to the bacteria used, higher Kcat. Further studies should replicate this experiment to see if these results are due to human error or if this is true for this lysozyme.

Introduction

At our molecular level, as living organisms, our functioning depends highly on one of our basic components, proteins. Many of these proteins' purpose is to act as enzymes, which help aid and catalyze numerous biochemical reactions. The big diversity of enzymes is what dictates their role such as antimicrobial activity (Oh and Park, 2018). This is a critical role in the defense–immune – response that organisms have to fight against pathogens. Some of the widely known enzymes with antimicrobial properties are lactoperoxidase, glucose oxidase, and a very important one that can hydrolyze polysaccharides, and lysozyme (Khorshidian, N., et al., 2022). This experiment will focus on the latter one.

Lysozyme is of great importance. It is a bacteriolytic enzyme, which means it is involved in the destruction of bacterial cells, specifically by targeting their cell walls (*PROSITE*, n.d.). Its antibacterial mechanism involves the substrate peptidoglycan. Its activity is known to be determined by the rate of hydrolysis that happens in the cell wall of the β-(1,4)-linkages between N-acetyl-muramic acid and N-acetyl-D-glucosamine residues (Phillips, 1967a). This also serves as evidence of the specificity of lysozyme to more gram-positive bacteria, since these are characterized by having a thicker and bigger peptidoglycan cell wall, consisting of 20 to 80 nm thickness, than gram-negative bacteria, this has a thickness of 2 to 3 nm (Sizar, O., & Unakal, C. G., 2022). The mechanism of lysozyme catalytic activity is depicted in Figure 1. This mechanism is highly influenced by non-covalent bonding, such as hydrogen bonding, that provides stability and specificity, see Figure 2 for one example of this.

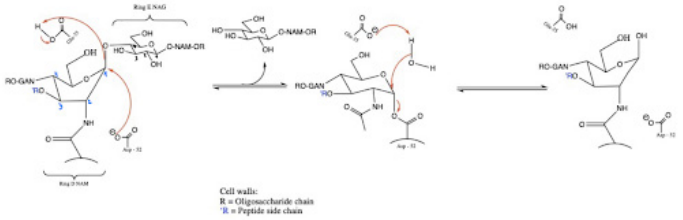

Figure 1. Mechanism of cleavage of Lysozyme hydrolyzing the NAM-β-O-NAG bond. The reaction depicted happens with two steps, first aspartate 52 with a nucleophilic attack to the carbon forming the acetal in the NAM ring and having the sugar from the NAG leave as the leaving group by protonating it with glutamate 35 acting as an acid. Then, when water is introduced, glutamate 35 acts as a base to make water a better nucleophile by deprotonation of it. This allows for the water to attack the initial carbon, generating the aspartate ion and the NAM ring, with a free enzyme. The numbers in the ring depict how this is a 1 → 4 cleavage. This is the proposed mechanism obtained from Jakubowski, H., B2. Lysozyme.

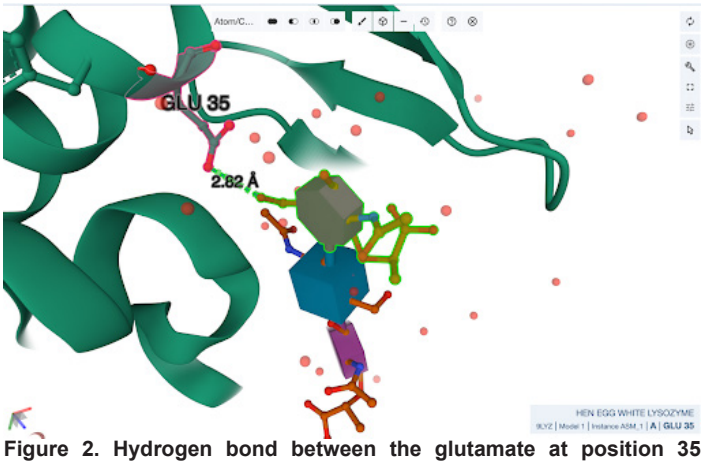

Figure 2. Hydrogen bond between the glutamate at position 35 from hen egg white lysozyme to the bacterial cell wall from the complex NAM-NAG-NAM. Screenshot from RCSB Protein Data Bank for binding of lysozyme to NAM-NAG-NAM complex, PDB ID: 9LYZ. In this case, the NAM ring is the one highlighted in green in purplish color and the NAG is the one in blue. The bond distance of the NAM to the glu 35 is 2.82 Å. This non-covalent interaction is of high importance for the hydrolysis of the NAM-NAG-NAM complex which allows for glutamate to get deprotonated and make the NAM a more electrophilic molecule.

The lysozyme function is also reflected in its nomenclature, E.C. 3.2.1.17 (Brenda Professionals, 2022). Each division refers to a lysozyme belonging to the group of hydrolases (3), its subclassification is glycosylases (2), followed by a glycosidase (1), and its index is, as commonly known, lysozyme (17). The lysozyme weighs about 14.6 kDa (Phillips, 1967b) and due to its antibacterial properties is commonly seen used as a food preservative (Proctor, V. A., & Cunningham, F. E., 1988a). This is because lysozyme can get added to the food preventing any bacteria from growing on food products, and the extent to which types are useful for its vast variety including all sorts of items such as milk, meats, and vegetables. Its applications have given tremendous results that lysozyme is even applied for pharmacological uses (Proctor, V. A., & Cunningham, F. E., 1988b). This is to say the enzyme can be used to even prevent bacteria from infecting a wound and to aid in anticancer drugs, and eye drops, among others. Since the discovery of this enzyme by Alexander Fleming in 1922, the interest in lysozyme has only grown exponentially (Tan, S. Y., & Tatsumura, Y., 2015). In recent years, scientists have even tested the antibacterial effects of lysozyme on HIV infections (Hartono, Y. D., et al., 2011).

With all of the varied applications of this enzyme and its promising effects, the search for the most optimum purification has gotten the attention of many scientists. As of now, lysozyme is primarily extracted from hen egg whites, even though it can be found in many other substanc-

es such as honey and saliva (Gajda, E., & Bugla-Płoskońska, G, 2014). The reason why using hen egg white is common is because 3.5% out of 11% of the total protein in this mixture is lysozyme, making it a good source of it. Considering the immense research done on hen egg white lysozyme, there is still a lot unknown between the differences and similarities between duck and hen as sources of lysozyme. These two are known to have similar structures, and when comparing their amino acid sequence, it is visible that they share some similarities in the active site (Figures 3 and 4). It is important to mention that for duck lysozyme three different isoforms are present independently from one another, and the one with higher similarity to hen lysozyme is duck egg-white lysozyme III (Jollès, J., et al., 1967). Furthermore, it has been studied that 1.2% of proteins in ducks are lysozyme (Hermann and Jollès, 1970).

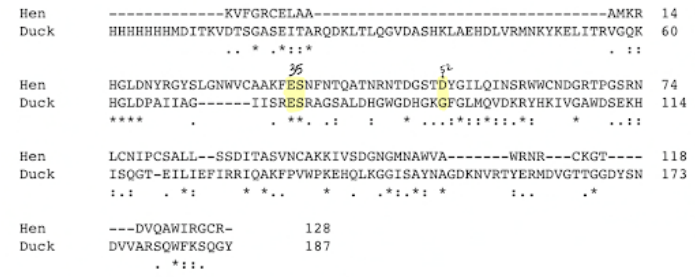

Figure 3. Comparison of sequences of hen and duck egg white lysozyme using Cluster Omega protein sequence alignment. The top sequence is the corresponding to the hen and the bottom one is corresponding to the duck. The different symbols represent the similarity between the two sequences. The asterisk (*) represents a 100% match between the two. A colon (:) represents highly similar amino acids. The period (.) is those amino acids that are slightly similar, and no symbol means no similarities. There are two regions highlighted with yellow that are the two amino acids used in the active site Glu 35 and Asp 52 in hen lysozyme. There is a conservation of the Glu 35 in duck, but no Asp 52, even though the Gly 52 present is slightly similar.

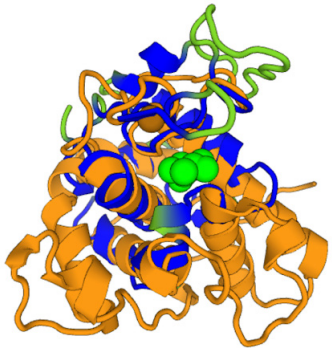

Figure 4. The structure of hen lysozyme depicted in green superimposed to the structure of duck lysozyme in orange. Using the Dali server for 3D global protein comparison, the structure of the lysozyme from the two sources can be aligned and allows for visualization of homology between these two. The areas colored blue are the regions where the structure is conserved among the two.

In this experiment, the aim is to extract lysozyme from duck egg whites and compare it to the lysozyme from a stock solution of hen lysozyme. To do this, first, the duck egg white will be isolated and purified using ion exchange chromatography, followed by purification and enzymatic activity check with gel electrophoresis and protein assays. After the best fractions of pure lysozyme are identified these will undergo a process of dialysis and concentration to prepare them for enzyme kinetics. In this step, the enzymes from both sources will be analyzed creating two plots: Michaelis-Menten and Lineweaver-Burk plots, to accurately depict their catalytic properties. Based on the percentage content of lysozyme for each source, it is hypothesized that the hen and duck will differ. In particular, the hen egg will have a higher rate of reaction when presented with the same bacteria.

Results

Successful purification of lysozyme from duck egg white

To use the lysozyme from the duck egg white, we first had to extract it and purify it. For this, ion exchange chromatography was used, followed by spectrophotometer analysis using bacteria. For detailed protocol refer to the methods section. The egg white was separated from other components of the egg, buffered, and run through the column with a negatively charged resin. The fractions obtained were then analyzed for an enzymatic activity to test if lysozyme was present. This was possible because of the hydrolytic activity that the lysozyme has with the bacteria, so by analyzing the absorbance at 450 nm of the latter one we can determine if they are being destroyed. This means if the bacteria are being cleaved, the absorbance decreases, as a result of more light going through and reaching the detector, resulting in a steeper slope. After obtaining the values of each slope for all samples, except for wash 4 and 5 – these were lost in transfer – the results were summarized in table 1.

	Flow through	Wash 1	Wash 2	Wash 3	Elution 1	Elution 2	Elution 3	Elution 4	Elution 5	Egg white
Slope (DA/min)	-0.000656	-0.000988	0.000004974	-0.000393	-0.004222	-0.002761	-0.000861	-0.000460	0.00001309	-0.00426

Table 1. Summary of slope (DA/min) for each fraction recorded. Calculated by spectrophotometer analysis at absorbance 450.4 nm, best for the bacteria *Micrococcus lysodeikticus* used.

From table 1, it is evident to say that lysozyme was successfully purified and extracted from the egg white of the duck. Elution 1 to 3 showed the highest enzymatic activity against the bacteria, with values of -0.004222, -0.002761, and -0.000861, respectively. This is expected because the pH used in the wash and flow-through steps was close to 8.0, considering lysozyme has a pI close to 10.0, it is only when the elution buffer (pH = 10.5) is introduced that the lysozyme loses it's positively charged and affinity to the resin, that it can get detached and eluted to the fraction vial.

Pure Lysozyme identified through gel electrophoresis

After identifying the samples with enzymatic activity, it is important to identify which of these have pure lysozyme with no contamination. We sought to recognize which samples had pure lysozyme by performing gel electrophoresis on all indicated fractions from table 1. We expected to observe lysozyme in elution 1 to 3, with no contamination primarily in elution 2. Differently, the flow through and the washes should show contamination of all the other proteins present in the egg white, decreasing as we move through the washes. To do this gel electrophoresis was done using the staining with Coomassie brilliant blue (refer to methods for a detailed protocol). The results are shown in figure 5. Elution 5 was not loaded due to limited space in the gel, and this was selected because of its very low enzymatic activity shown in table 1.

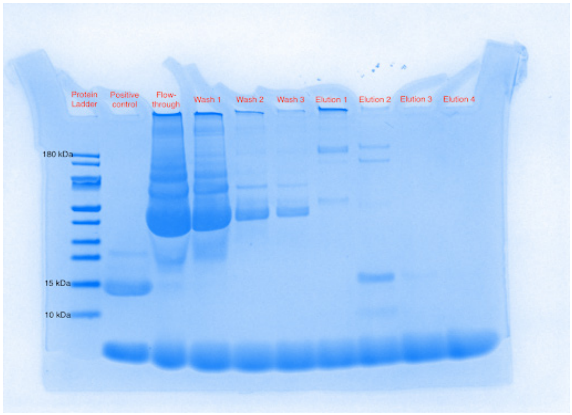

Figure 5. Pure Lysozyme was identified through gel electrophoresis stained with Coomassie brilliant blue. The first lane is the PageRuler Prestained Protein Ladder with relevant weights indicated. The second lane is a positive control, which is a lysozyme sample. Lane 3 is the flow through. Lanes 4, 5, and 6 are wash fractions 1 through 3. Lanes 7, 8, 9, and 10 are the elution fractions 1 through 4, the circled bands are lysozyme.

Firstly, the positive control, lane 2, showed the expected size for lysozyme at approximately 15 kDa. The flow through and the washes also showed multiple bands as expected, with the flow through having too many components from the egg white. The intensity and shape of the flow through bands is also an indicator that too much protein sample was loaded. For the flow through, it is expected to find enzymatic activity because it is a mixture of all the components (proteins, lipids, and more) of the egg white, except the lysozyme. For this reason, some of the other enzymes might be giving the activity seen. For wash 1, a possible explanation for finding enzymatic activity could be that the lysozyme from duck egg white might not fully adhere to the Sephadex, as is expected for hen lysozyme.

Elution 1 and 2 also showed bands, some at the expected range. And elution 3 showed a very faint band at approximately 15 kDa, which is the expected value for lysozyme for duck eggs. Surprisingly, we saw higher bands in elution 1 and 2, which could potentially be contamination.

Pure lysozyme concentration was analyzed with a protein concentration assay

Furthermore, to analyze the remaining aspect of the extracted and purified lysozyme a protein concentration assay was done on all fractions of figure 5. This helps provide a quantitative measure of the extracted lysozyme that compliments the qualitative measure of the gel in figure 5. It was expected to see a higher concentration of protein for elution 2, followed by 1 and 3. Table 2 shows the results of this after being detected at an absorbance of 280 nm in the plate reader, proteins' best.

	Flow through	Wash 1	Wash 2	Wash 3	Elute 1	Elute 2	Elute 3	Elute 4
260/280	0.117	0.62	0.658	0.843	0.77	0.67	0.795	0.974
mg/ml	0.091	1.938	0.212	0.085	0.081	0.187	0.028	-0.015

Table 2. A280 measure of protein concentration for flow through washes 1-3, and elution 1-4.

For the concentration, we see the highest value is for wash 1, and looking at the elute the highest is for elute 2, followed by 1 and then 3, as expected. The high values for flow through and washes are due to the big mixture of other proteins that are present. Surprisingly, the elution 4 concentration showed a value of -0.015, which equals 0, the negative value is a consequence of the intrinsic error of the machine. This quantitative information was important to determine which samples will follow for future experiments, starting with dialysis.

Fractions 2 and 3 were chosen to be put into a dialysis cassette and adjusted for the required pH to accurately measure their unit of enzymatic activity. This was done using the protein dialysis protocol that can be found in the methods. Furthermore, because elution 1 showed enzymatic activity (Table 1), showed higher bands possibly due to polymerization (Figure 5), and had protein concentration (Table 2), it was also taken further to analyze lysozyme activity.

Successful concentrating of purified Lysozyme with concentrator

After completing our three aims of seeing the enzymatic activity of extracted lysozyme (Table 1), analyzing which is pure with the gel electrophoresis (Figure 5), verifying the presence and obtaining protein concentration for each (Table 2), it is relevant to concentrate the protein to have a more accurate concentration. We sought to concentrate the lysozyme using PierceTM Protein Concentrators PES described in the methods section. To analyze the data, the protein concentration was measured before and after concentrating, with the protein concentration assay described previously. The results of this are shown in table 3.

Sample Read dilute	260/280	mg/mL
Elution 1 trial 1	-	-
Elution 1 trial 2	-	-
Elution 2+3 combined	1.053	0.0991946

Table 3. Results from protein concentration from plate reader before dialysis for samples of fraction elution 1 and elution 2 and 3. Elutions 2 and 3 were combined to achieve a higher volume for dialysis. Elution 1 had two trials because it was done alone and there was space in the plate reader. No values were obtained for the latter one because the sample was re-obtained because of a mistake after it was concentrated. Elution 2+3 resulted in 0.0991946 mg/mL.

Due to a mistake done in the lab, elution 1 was mixed with another group and then retrieved. After the protein concentration was measured these were concentrated with the concentrator, and the values for protein concentration can be seen in table 4.

Sample Read	260/280	mg/mL	Average for concentration (mg/mL)
Elution 1 trial 1	1.197	0.114	0.1575
Elution 1 trial 2	0.866	0.201	
Elution 2+3 combined	0.699	0.8272778	N/A

Table 4. Results from protein concentration from plate reader after dialysis for samples of fraction elution 1 and elution 2 and 3. These are the concentrated samples. The average protein concentration in mg/mL was calculated for the two trials of elution 1 to obtain a single value. This was not necessary for elution 2+3, only one trial was measured due to limited space in the plate reader when recording this.

The protein concentration assay after the concentrator yielded what was expected. Elution 2+3 had the highest concentration of protein, as compared to Elution 1, which is only one sample. And that the concentration of lysozyme after the concentrator increased. The %error for elution 2+3 was calculated using the expected concentration obtained from the formula M1V1=M2V2, using the initial volume when pipetted out of the dialysis bag of 5.8 mL, and the final after the concentrator of 0.645 mL. For Elution 1, no %error can be calculated because there was no initial concentration recorded. The results before and after concentrating are summarized in table 5.

	Protein Concentration (mg/mL)	Expected Concentration (mg/mL)	%Error
Dilute fraction 2+3	0.0991946	N/A	N/A
Concentrated fraction 2+3	0.8272778	0.89198	7%
Concentrated fraction 1	0.1575	N/A	-

Table 5. Summary of protein concentration for two fractions (1 and 2+3). The concentrated fraction of 2+3 ended up having a protein concentration of 0.8272778 mg/mL, with a 7% error. Fraction 1 has a protein concentration of 0.1575 mg/mL.

The process of concentrating the fractions' protein concentration was successful.

Determining enzyme kinetics for duck lysozyme

Once the extracted lysozyme from duck egg white was successfully extracted, purified, and concentrated, the protein is ready to be used for assessing and measuring enzyme kinetics. To do this, the spectrophotometer and LoggerPro were used again, plotting absorbance at 450 nm vs time (seconds). This measures the substrate, bacteria, and disappearance as the absorbance decreases with higher enzyme activity, allowing us to determine the velocity of the reaction. When processing the data and optimizing the enzyme and substrate concentrations, all values were recorded in an excel spreadsheet titled "BMB300_Lysozyme_kinetics". This worksheet can be found attached to the labnotebook in LabArchives in Lab 5E. After multiple trials the optimum concentration for the enzyme was determined to be 0.038 mg/mL and for bacteria to have an initial volume of 10 uL and a final of 40 uL, considering the stock bacteria concentration was 15 mg/mL, making the initial concentration 0.15 mg/mL and the final 0.6 mg/mL.

From the slopes of the absorbance vs time graph, the initial velocity can be calculated by multiplying the slope by -1. These values were then used to obtain the two graphs: Michaelis–Menten kinetics plot (Vo vs [S]) and the Inverted Lineweaver–Burk plot (1/[Vo] vs 1/[S]). The resultant graphs for fraction 1 can be seen in Figures 6 and 7.

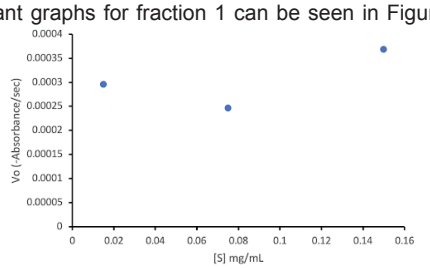

Figure 6. Michaelis–Menten plot for Lysozyme from duck egg white for fraction 1. The absorbance was measured at 450.4 nm. Each dot represents a different reading with the same enzyme concentration of 0.038 mg/mL and different substrate concentrations.

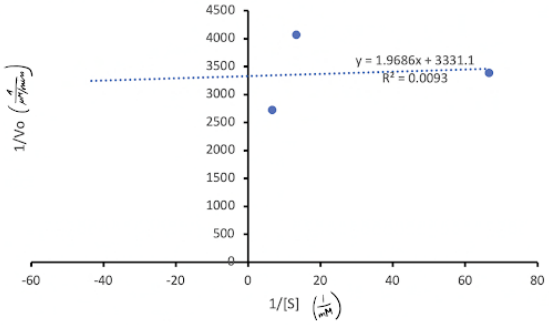

Figure 7. Lineweaver-Burk plot for Lysozyme from duck egg white for fraction 1. The reciprocal of the initial velocity is graphed out as a function of the reciprocal of substrate concentration. A trendline was added to the data set, with the slope depicted in the top right corner with an equation of y = 1.9686x + 3331.1, followed by the r-squared value of 0.0093.

Figures 6 and 7, show an evident pattern that there are not enough data points obtained to make an accurate decision of enzymatic kinetics for this fraction. From the graph, the slope is y =1.9689x + 3331.1, and the R-squared = 0.0093, which denotes a high variance in the data set. The same procedure was followed for fraction 2+3, and the results are shown in figures 8, 9, and 10.

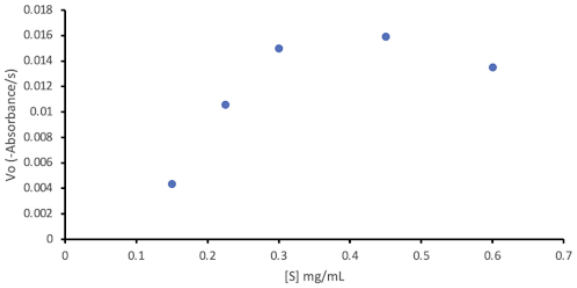

Figure 8. Michaelis–Menten plot for Lysozyme from duck egg white for fraction 2+3. The absorbance was measured at 450.4 nm. Similarly, each blue dot is one trial with constant [E] = 0.039 mg/mL. The curve shows an almost parabola.

Figure 8 shows the Michaelis-Menten plot for the duck Lysozyme, when [E] = 0.038 mg/mL, [S1] = 0.15mg/mL, and [S2] = 0.6 mg/mL. Because we see the rapid increase and then a sudden decrease, similar to a parabola, it is evidence to say the enzyme was too saturated. For this reason, we excluded the last two data points, depicted in figure 9. Values smaller for bacteria concentration should have been measured but due to the lack of the enzyme, this part couldn't be followed-up with more trials.

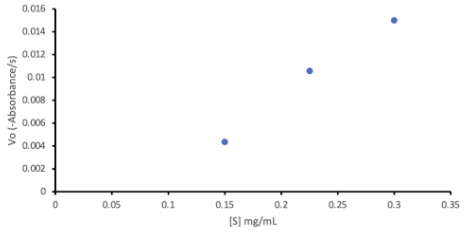

Figure 9. Updated Michaelis–Menten plot for Lysozyme from duck egg white for fraction 2+3. The absorbance was measured at 450.4 nm. The three blue dots represent three different trials with different substrate concentrations. The data values were for the initial substrate [S1] = 0.15mg/mL and final [S2] = 0.3 mg/mL.

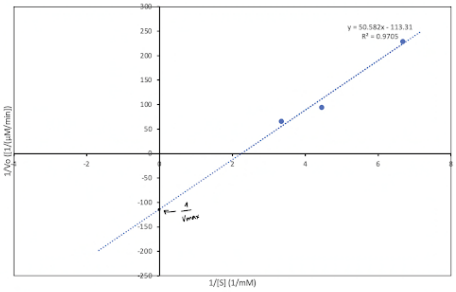

Figure 10. Lineweaver-Burk plot for Lysozyme from duck egg white for fraction 2+3. The trendline was added with the slope equation, y = 50.582x -113.31, followed by the r-squared value of 0.9705. The formula for Vmax is depicted in black notation.

From figure 10, which provides a better depiction of the enzymatic kinetics than figure 8 or 9, we see the y-intercept of the trendline is on the negative part of the axis. This is theoretically impossible. For this reason, since there was no more enzyme to keep trying and experimenting with different volumes, the duck lysozyme extracted cannot be used to compare it with the stock hen lysozyme. It is important to make an analysis of the units of activity of the fractions since this is the unit by which the enzyme's catalytic activity gets measured. The values for these two fractions are shown below in table 6 with the values for egg white before buffer. It is noteworthy to say it was diluted 2-fold (3 mL from the egg white with 3 mL from the buffer).

Fraction	[Protein] mg/mL	Units of Activity	Specific Activity Unity/mgs of proteins	Volume of fraction used in assay (mL)	Total protein mgs	Total activity units	%Recovery	Fold pure
Fraction 1	0.081	8.444	521.23	0.2	0.0162	8.444	-	-
Fraction 2	0.187	5.522	147.65	0.2	0.0374	5.522	-	-
Fraction 3	0.028	1.722	307.5	0.2	0.0056	1.722	-	-

Table 6. Summary of fractions data for units of activity. The first six columns were the only ones that could be completed.

When completing table 6, the last two columns could not be completed because when measuring protein concentration, it wasn't measured the egg white one. The others were attempted at completing.

Determining enzyme kinetics for hen stock lysozyme

Another goal of this experiment was to compare the enzymatic kinetics of duck lysozyme to hen egg white lysozyme. For this, to determine the hen lysozyme kinetics, a sample of stock lysozyme was obtained from Sigma and used the same way the duck lysozyme was analyzed to obtain the two plots. The enzyme concentration was kept the same, [E] = 0.038 mg/mL.

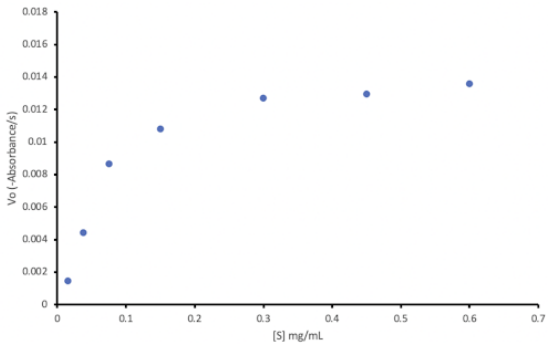

Figure 11. Michaelis–Menten plot for Lysozyme from hen stock. The absorbance was measured at 450.4 nm. Each dot represents different trials. At [E] = 0.038 mg/mL, with the initial substrate [S1] = 0.015 mg/mL and final [S2] = 0.6 mg/mL.

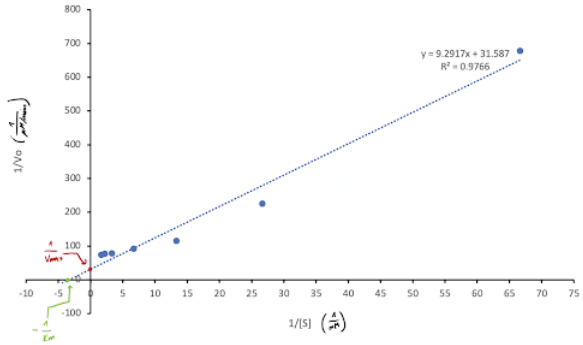

Figure 12. Lineweaver-Burk plot for Lysozyme from hen stock. The trendline is present with the slope equation, y = 9.2917x + 31.587, followed by the r-squared value of 0.9766. The Vmax formula is highlighted in red where the trendline intercepts with the y-axis. The Km formula is depicted in green, where the trendline intercepts with the x-axis.

The hen stock lysozyme yielded good results for both plots, which allows for further analysis of the Vmax, Km, and Kcat. The slope is used to calculate these. For Vmax, this is when x = 0. For Km is when y = 0, and Kcat is (Vmax)/[E]. The results are summarized in table 7.

Egg source	Lysozyme concentration (mg/mL)	Final [E] (mg/mL)	[S1] (mg/mL)	[S2] (mg/mL)	Km	Vmax	Kcat	Kcat/Km (Specificity constant)
Hen stock	10	0.038	0.015	0.6	0.29	0.032	0.84	2.90

Table 7. Results for enzymatic kinetic for hen lysozyme from Sigma. In the table, the values for the concentration of enzyme initial and final substrate are depicted, along with the experimentally calculated Km, Vmax, and Kcat.

The purpose of this experiment was to compare the enzyme kinetics for the lysozyme of hen and duck. For this reason, data for duck lysozyme from another classmate, Lorena Monroe, was shared with us. The data set for each plot was added to the existing plot for hen egg lysozyme (Figures 11 and 12) to compare the different graphs. This is also possible because the other group used a similar enzyme concentration of [E] = 0.04 mg/mL. This can be seen in figures 13 and 14.

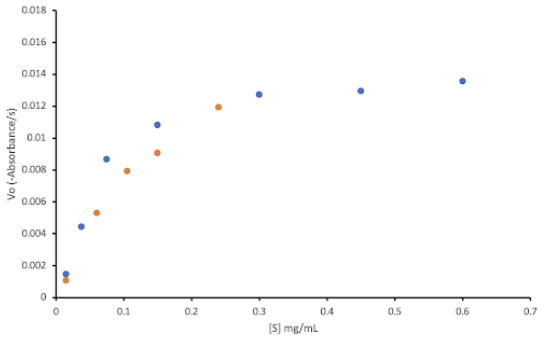

Figure 13. Michaelis-Menten plot for Lysozyme from a hen in blue and duck in orange. The initial velocity is graphed as a function of substrate concentration. For the hen lysozyme the [S1] = 0.015 mg/mL and [S2] = 0.6 mg/mL, and for the duck lysozyme [S1] = 0.015 mg/mL and [S2] = 0.024 mg/mL.

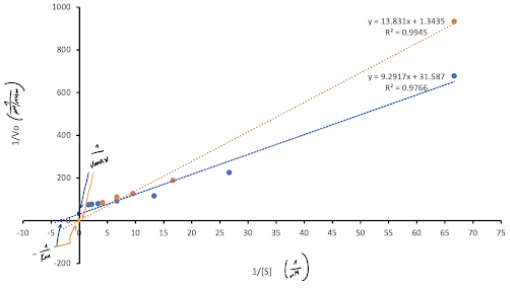

Figure 14. Lineweaver-Burk plot for Lysozyme from hen in blue and duck in orange. The trendlines are present for each sample with their corresponding slope and r-squared value on top of each line. The one on the top corresponds to duck lysozyme, and the bottom one to hen lysozyme, from this experiment. The Vmax formula is highlighted with corresponding colors for each with a dot to indicate where in the graph they are. The same is for the formula for Km.

The values for Km, Vmax, and Kcat were further calculated for the new data set for duck lysozyme. These are summarized in table 8.

Egg source	Lysozyme concentration (mg/mL)	Final [E] (mg/mL)	[S1] (mg/mL)	[S2] (mg/mL)	Km	Vmax	Kcat	Kcat/Km (Specificity constant
Duck stock	0.6225044	0.04	0.015	0.24	10.295	0.744	18.61	1.81

Table 8. Results for enzymatic kinetic for duck lysozyme from a shared data set of another classmate group. In the table, the values for the concentration of enzyme initial and final substrate are depicted, along with the experimentally calculated Km, Vmax, and Kcat.

Discussion

In this experiment, we showed that lysozyme can be extracted and isolated from duck egg white (Figure 5 and Table 2) and assess this purification by looking at three main properties. First, the presence of enzymatic activity using the spectrophotometer measuring absorbance over time, which we saw in Table 1. Then, the presence of pure lysozyme without contamination through gel electrophoresis (Figure 5), and the concentration of this protein through assay with a plate reader (Table 2). This was to set up a good depiction of the fractions that were extracted which led us to choose fractions 1, 2, and 3, to continue with dialysis and the concentrator, which resulted in a %error for the fraction 2+3 of 7%, which indicates a low variation between expected and experimental value. This helps support the result that the process worked. All of this was to obtain an accurate measure of protein concentration to characterize the lysozyme through monitoring its enzymatic activity against the bacteria Micrococcus lysodeikticus.

Firstly, these findings are important by themselves since it is possible to speculate about some properties of duck egg white lysozyme. As seen in figure 5, elution 1 did not have any bands at the 15 kDa mark but showed enzymatic activity in table 2. This leads us to hypothesize that lysozyme in duck eggs can polymerize, getting bigger, and thus, appearing higher up in the gel. Moreover, another reason is that the protein present so high up could be another type of lysozyme that we are not studying in this experiment or another protein in duck egg that has a pI similar to lysozyme (approximately 10.0) and would explain why it wasn't eluted with the column buffer, but it was with the elution buffer in the ion exchange chromatography. This is an interesting topic that could be explored in future research. Especially considering how under-researched the different isoforms of duck lysozyme are.

Another aim of this experiment was to compare the lysozyme kinetics extracted from duck to a sample from hen lysozyme. Even though our extracted duck did not yield good results for analysis as mentioned and seen in figures 6 and 10, and table 7, another group of data was used for comparing the duck and hen lysozyme. For this, looking at tables 7 and 8, it is possible to conclude that duck lysozyme has a much higher Vmax and Km, being 0.744, and 10.29, respectively. For hen lysozyme, these are 0.032 and 0.29. This result is surprising and contrary to what was hypothesized that hen lysozyme would have higher catalytic activity. Even looking at Kcat, the duck egg had a value of 18.61, while the hen had a value of 0.84. This is also evident from the Lineweaver-Burk plots (Figure 14), which shows that at higher concentration of substrate, the duck lysozyme seems to work faster hydrolyzing the bacteria as compared to the hen lysozyme.

However, another noteworthy element is that looking at the specificity constant, Kcat/Km, the hen egg white lysozyme has a higher one, 2.90, as compared to the duck lysozyme, 1.81. This is contradictory to what would be assumed considering the rate of the reactions for each. A possible explanation for this is that even though the duck lysozyme works faster than the hen, the hen lysozyme is more precise and has higher catalytic efficiency at cleaving the bacteria. The substrate used is more specific for hen than duck lysozyme. The hen enzyme does not need a high substrate

concentration to have a good reaction rate, and thus, why we got the results we did.

These values could have been a result of different sources of error throughout this experiment. For future research, these should be addressed. Starting by the fact that in all instances of this research, the buffers used were previously made, except for the one used in the dialysis, and the pH of these was not re-measured before starting. All conclusions are done assuming the pH stayed the same as when first made, which theoretically should happen. Nonetheless, it is important to measure the pH every time it's going to be used since ion exchange chromatography depends highly on it. Secondly, for protein kinetics, this was our first time performing it. Although we were familiar with the spectrophotometer, the optimization of our concentrations to stay in the range of 0.5-1.0 AU required too much of our enzyme. Next time, the values used here can be used directly or the practice should be done with a stock solution. Thirdly, there was a big delay in this experiment due to mistakenly having changed dialysis bags with another group. In the future, this should be avoided by being more careful in the handling of all reagents and samples. Another area to consider is the intrinsic error that comes from the apparatuses used. For example, when using the plate reader for elution 1, the two trials presented different protein concentrations. This could have been an error from loading differently, but also could be a source from the same machine. Similarly, this will also give negative values for protein concentration which is not possible.

After considering possible limitations to this experiment, many areas require expansion. One of the aims of this experiment was to compare duck lysozyme to hen, but this could be expanded to other bird species too, such as goose lysozyme. The comparison for duck should not only be limited to other species but further analysis of its different isoforms of lysozyme has to be sought out. As mentioned previously, these are not present together ever, which leads us to hypothesize that the different isoforms present different enzymatic activity. To determine which isoform is being used, sequencing could be done after extracting the lysozyme from different duck eggs and testing for these differences.

Lastly, previous research had hinted at differential gene expression and regulation of lysozyme from chicken and mice that contribute to macrophage-specific gene activation (Bonifer, C., et al., 1994; Short, M., et al., 1996). This relates to how the immune response gets activated and regulated by lysozyme in different species. It would be interesting to investigate further if this is the case for hens and ducks since both of these are bird species with more similarities.

Methods

Buffer preparation

For this experiment many buffers have to be used to ensure the pH remains at the desired one. For this, three different buffers were prepared and measured using the Henderson-Hasselbalch equation. These buffers are Tris-NaCl Column Buffer at pH 8., prepared using Trizma base, NaCl, and Trizma HCl; 0.2 M Carbonate Elution Buffer at pH 10.5, prepared with sodium carbonate and sodium bicarbonate; and lastly 0.1 M Potassium phosphate buffer at pH 7.0, using Potassium phosphate monobasic and dibasic. Each was prepared separately but followed the instructions according to the protocol in Lab 5A by WHC (Conrad et al., 2022). All of these started by calculating how much of each solid was necessary and mixing it with nanopure water up to 80% of the total volume. Then with a calibrated pH meter the pH was measured throughout the stirring and adjusted with either NaOH or HCl, and with KOH and H3PO4 for the potassium phosphate buffer to control for the same ions present in the solution. After adjusting pH, the volume was brought to its desired final one for each. All of these buffers were stored in a 4 °C refrigerator for future use in the experiment.

Enzyme Source Preparation

To create the source of lysozyme for this experiment egg white was extracted from a duck egg. To set up this sample, a duck was randomly selected and cracked, separating the egg white and the yolk according to WHC instructions in Lab 5B in LabArchives (Conrad et al., 2022a). The egg white was measured and transferred to a 1.2 mL Eppendorf microcentrifuge tube, to be centrifuged at top speed for 4 minutes. After this, the top supernatant was extracted and mixed with column buffer until a homogeneous mixture was reached. This sample was stored in ice. This is an overview of the protocol, for the full version refer to the student lab manual in the LabArchives notebook at https://mynotebook.labarchives.com/.

Ion Exchange Chromatography for Protein Purification

To separate and extract the lysozyme from the complex protein mixture in the duck's egg white, Ion Exchange Chromatography was used. This technique was done according to the WHC instructions in Lab 5B in LabArchives (Conrad et al., 2022b). The buffered egg white was prepared with 3 mL of column buffer and added to the column that was previously prepared with resin. This resin was done by mixing 0.5 g Dry CM-Sephadex 25 and 20 mL of Tris-NaCl column buffer at room temperature. The protein mixture, egg white, after adding to the column, a flow-through and 5 washes were extracted using the previously made, Tris-NaCl Column Buffer, pH 8.2. Then, it was followed by five elutions with 0.2 M Carbonate Elution Buffer, pH 10.5. All fractions were collected and stored in labeled vials. This technique helps separate only the lysozyme because it is the only protein with a pI high enough to remain with a pH of 8.2. The extracted lysozyme can be used for future manipulations.

Gel Electrophoresis and Coomassie Stained SDS gel

To test the presence of purified Lysozyme after extraction through Ion Exchange Chromatography, gel electrophoresis and Coomassie staining were used. The gel electrophoresis protocol was adapted from the Life Technology "XCell SureLock® Mini-Cell" (1) and Lab 5C from LabArchives (Conrad et al., 2022a). To set up this technique, an SDS gel was locked in the gel box with 500 mL of MES SDS Gel Running Buffer. To prepare the samples, 25 µL of each indicated fraction was mixed with 12.5 µL of 4x LDS sample buffer, "loading buffer", to ensure they would stay at the bottom of the tube and reduce the proteins' disulfide bonds. The samples were heated at 95 °C in an Eppendorf thermocycler. The samples and PageRuler Prestained Protein Ladder (10 to 180 kDa) were loaded, and the gel was run for 1.5 hours at 120 V. After the gel reached the bottom, the voltage was stopped, and the gel was cut out and stained with Coomassie brilliant blue for 15 minutes. It was washed three times with deionized water and visualized with the Molecular Imager Gel Doc XR+ with Image Lab Software.

Protein concentration assay

It is important to quantify the purity and concentration of the extracted lysozyme. For this, a protein concentration assay was done using the BioTek Synergy HTX Multimode Reader, which allows for analysis of the protein at A280. This machine provides an analysis at UV-Vis absorbance at 280 nm which is the protein's best absorbance. The protocol followed the guidelines in Lab 5B in LabArchives (Conrad et al., 2022c). 3 uL were loaded in each well for the samples, and an elution buffer was used as the blank sample.

Enzyme activity

To test if there was enzymatic activity in the fractions, an assay with spectrophotometer Vernier SpectroVisPlus connected to LoggerPro was used. This was following the instructions by WHC in Lab 5B (Conrad et al., 2022d). This method is possible to use because lysozyme is known to hydrolyze bacteria cells by disrupting the cell walls. As the enzyme cleaves these, the absorbance of the bacteria decreases, since there are fewer bacteria present in the sample; and this decrease can be concluded as enzymatic activity. Therefore, to study enzymatic activity, different cuvettes were prepared with 1.5 mg/mL Micrococcus lysodeikticus and phosphate buffer, and the spectrophotometer was set to record data f absorbance (nm) vs time (seconds) at 450.4 nm – best absorbance for this bacteria sample. The data collection started by putting the bacteria+buffer cuvette sample in the apparatus recording the baseline, then extracting it and adding 200 uL of the first protein fraction, and recording the absorbance again. The slope (DA/min) obtained with a linear fit that was seen right after introducing the enzyme, was multiplied by -1, which resulted in the value for the initial velocity. This process was repeated for all fractions, except for wash 4 and 5, which were lost when transferring the fractions.

Protein Dialysis

To prepare the pure and extracted lysozyme for future enzymatic assays, the lysozyme has to be brought back to a pH of 7.0 since the unit of activity for this protein

is considered to be at pH 7.0, with 25°C, when this is the same as when the absorbance decreases at 450 nm of 0.001 per minute. To do this, dialysis of the selected fractions – the ones that showed enzymatic activity and a positive band in the Coomassie-stained SDS gel – was used following instructions by WHC in Lab 5C (Conrad et al., 2022b). In order to change the pH, the phosphate buffer with a pH of 7.0 was used. A dialysis cassette was used, first submerged in the phosphate buffer to hydrate it for 30 seconds, and then the selected fraction of lysozyme – that was found in the elution buffer with a pH of 10.5 – was transferred into it and closed with clamps on both sides. This cassette was left for a week in the buffer. This cassette also allows further purification of the lysozyme by allowing small contaminants present to leave it and for the buffers to first reach an equilibrium between these. Then, the lysozyme would be found in a phosphate buffer with the corresponding pH of 7.0 to accurately assess enzyme activity.

Concentrating Lysozyme using a concentrator

The big aim of this lab is to test the protein kinetics. To do so, an accurate concentration of the lysozyme has to be obtained because the enzymatic activity of this is related and is a function of the protein concentration. Following Lab 5D by WHC, the protein was concentrated using the PierceTM Protein Concentrators PES, 3K MWCO, 5-20 mL (Conrad, et al. 2022). After dialyzing is completed, the sample is removed with a serological pipette, measuring the amount of sample, and transferred to the concentrator in the conical tube that has the ultrafiltration device. A small sample of this, 4 uL, was taken out and protein concentration was measured using the protein concentration assay described previously. The rest in the concentrator was centrifuged for 2 hours at a maximum speed of 4 °C. The retentate is removed from the tube, the final volume is measured using a pipette, and the protein concentration is measured again. All these values are used in the equation M1V1 = M2V2 to obtain the expected concentration and the calculated %error with expected and experimental values. Once the accurate and concentrated protein is ready and measured, it can be used for assessing its kinetic activity.

Protein Kinetics

To study the rate of the catalytic properties of the lysozyme, enzyme kinetics are analyzed by using the Michaelis-Mentel and Lineweaver-burk plots. This was measured by looking at the disappearance of the substrate using the same technique as in enzyme activity with the Vernier SpectroVis-Plus connected to LoggerPro. The protocol was followed according to Lab 5E (Conrad, et al., 2022). To prepare the cuvettes, the stock bacteria concentration was 15 mg/mL and the same bacteria Micrococcus lysodeikticus was used. Different concentrations of enzyme and substrate were tested to obtain the optimum concentrations for these. First, the enzyme concentration was calculated to be 0.038 mg/mL, then it was kept constant, and the bacteria (substrate) concentration was found. All of this is to keep a range of 0.5-1.0 AU, higher values would indicate a very saturated enzyme that doesn't allow for accurate measurements. After all values and calculations were completed the two graphs were plotted. From the Lineweaver-burk plot, the Vmax and Km were obtained. Then the Kcat was obtained by using the formula: Kcat = (Vmax)/[ET] and the specificity constant = Kcat/Km – which measures the efficiency of converting the substrate into products. In this experiment different conditions were tested depicted in the table below.

	Extracted Lysozyme Fraction 1	Extracted Lysozyme Fraction 2+3	Stock Lysozyme from Sigma
Source	Duck	Duck	Hen

Table. Summary of all conditions tested for enzyme kinetics. Of the extracted lysozyme from the duck egg used in this experiment fractions, 1 and 2+3 were analyzed separately. Lysozyme from a stock solution from Sigma company was also measured for enzyme kinetics to compare values between hen and duck.

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