LABORATORY REPORT

GROUP NUMBER T1

EXPERIMENT NUMBER 2

TITLE: Protein Separation – Column Chromatography

DATE SUBMITTED: 11/2/00

OVERALL GRADE 100/100
NICE REPORT
NICE GRAPHS- WHOEVER IS RESPONSIBLE FOR THE GRAPHS PLEASE SEE ME- I CAN USE SOME ADVICE FOR IMPROVING THE SUBMISSION OF GRAPHS VIA BB

ROLE ASSIGNMENTS

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SUMMARY OF CONCLUSIONS

The elution times of three proteins of known molecular weight (IgG, albumin, and lysozyme) were determined using Gel Exclusion Chromatography to derive the relationship between the ratio of eluted volume of a substance to void volume (V/V_o) and molecular weight of the substance. A linear regression fit model was performed which determined the equation of this relationship to be V/Vo = (-0.8768±1.07797) log MW + (6.2559±5.128944). Using this equation, hemoglobin molecular weight was determined using this GEC method and compared to the literature value of MW, as determined from gel electrophoresis. The average value of MW of hemoglobin, as determined from two trial, was is 52,485 g/mol with a standard deviation of +/- 639.47 g/mol (1.22%) and a 95% CI of 5.745 g/mol (10.95%) (assuming the linear regression equation was ideal, with no uncertainty). A t-test and the 95% CI of the literature value both indicated that the literature value of 64500 g/mol was significantly different from the experimental value as determined from the GEC method. A mass balance was performed on hemoglobin and results show that the calculated mass out (0.81±.0.18 mg 95% CI) was not significantly different from the calculated mass in (0.66 mg). Elution of combined protein sample (hemoglobin with lysozyme) showed that in this case when the absorbance peaks overlap, the elution peaks no longer correspond to that of the pure protein; instead, they become the sum of the two individual peaks. Thus the peak elution times of the sample is shifted and cannot be used to accurately determine the molecular weight of individual proteins.

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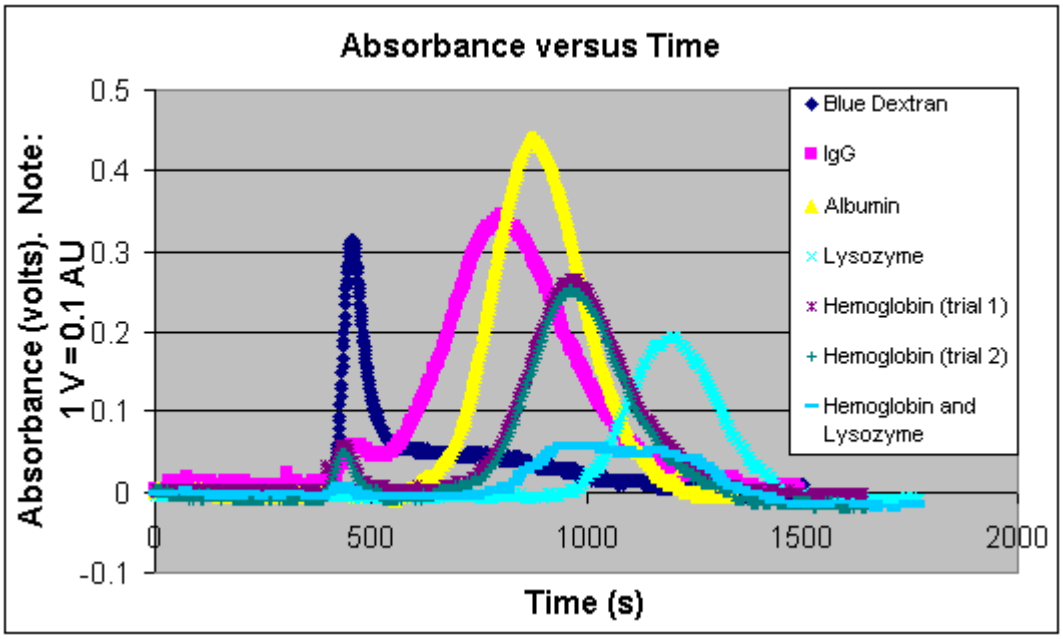
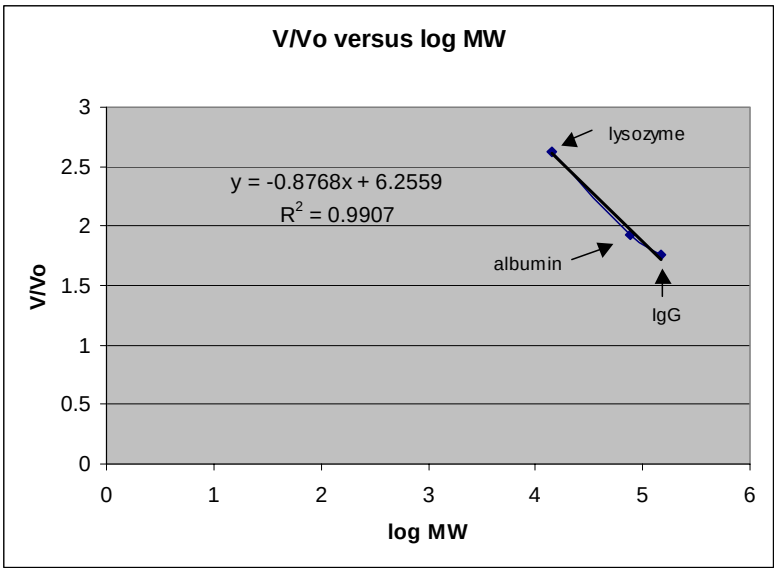
Results

Figure 1. Absorbance versus Time of All Proteins



The time at peak elution is denoted as "elution time" for the proteins. Volume at peak elution is calculated by multiplying the time by the flow rate of 1.91938 ml / min. Blue dextran volume is denoted as V_0. The volume of all other proteins is found in similar manner. V/Vo is plotted with respect to molecular weight below (Figure 2).

Figure 2. V/Vo versus log molecular weight



V/V_0 is plotted with V_0 (void volume) as the volume of blue dextran. A linear regression is performed on the data and the relationship between V/Vo and log MW is determined as follows (with 95% CI):

$$V/Vo = (-0.8768 \pm 1.07797) \log MW + (6.2559 \pm 5.12894) \qquad \text{Equation 1}$$

In this equation, molecular weight is in grams / mole.
Rearranged, this equation becomes:

MW = 10^(1/-0.8768±1.07797)(V/Vo – 6.2559±5.12894)
Equation 2

Figure 3. Comparison of Hemoglobin values

Hemoglobin	Estimated Elution Time (sec)	Elution Time (min)	Volume (mL)	V/Vo	MW (g/mole)	log MW
Trial 1-first lower peak (dimer)	441.004	7.350	14.108	0.963	1088259.617	6.037
Trial 2-first lower peak (dimer)	443.007	7.383	14.172	0.967	1075832.438	6.032
AVG-first lower peak (dimer)	**442.006**	**7.367**	**14.140**	**0.965**	**1082028.187**	**6.034**
Standard Deviation (+/-)	*1.416*	*0.024*	*0.045*	*0.003*	*8787.342*	*0.004*
% Standard Dev.	*0.320%*	*0.320%*	*0.320%*	*0.320%*	***0.812%***	*0.058%*
t	*12.706*	*12.706*	*12.706*	*12.706*	*12.706*	*12.706*
95% CI	12.725	0.212	0.407	0.028	78950.797	0.032
% CI	*2.88%*	*2.88%*	*2.88%*	*2.88%*	***7.30%***	*0.53%*
Trial 1-2nd higher peak (single)	971.257	16.188	31.070	2.121	52034.857	4.716
Trial 2-2nd higher peak (single)	968.252	16.138	30.974	2.114	52939.206	4.724
AVG 2nd higher peak (single)	**969.755**	**16.163**	**31.022**	**2.117**	**52485.084**	**4.720**
Standard Deviation (+/-)	*2.125*	*0.035*	*0.068*	*0.005*	*639.472*	*0.005*
% Standard Dev.	*0.219%*	*0.219%*	*0.219%*	*0.219%*	***1.218%***	*0.112%*
t	12.706	12.706	12.706	12.706	12.706	12.706
95% CI	*19.091*	*0.318*	*0.611*	*0.042*	*5745.401*	*0.048*
% CI	*1.969%*	*1.969%*	*1.969%*	*1.969%*	***10.947%***	*1.007%*

In the table above, peak elution times for hemoglobin were obtained from Figure 1, and V/Vo values were calculated using Vo value of 14.65124 ml / min. V/Vo values were substituted into Equation 1, and MW values were obtained. Average values of MW were determined for each peak. To evaluate the deviation in the data itself, standard deviation and 95% CI were determined based on the deviation of MW from average, assuming 100% confidence (no uncertainty) in Equation 1.

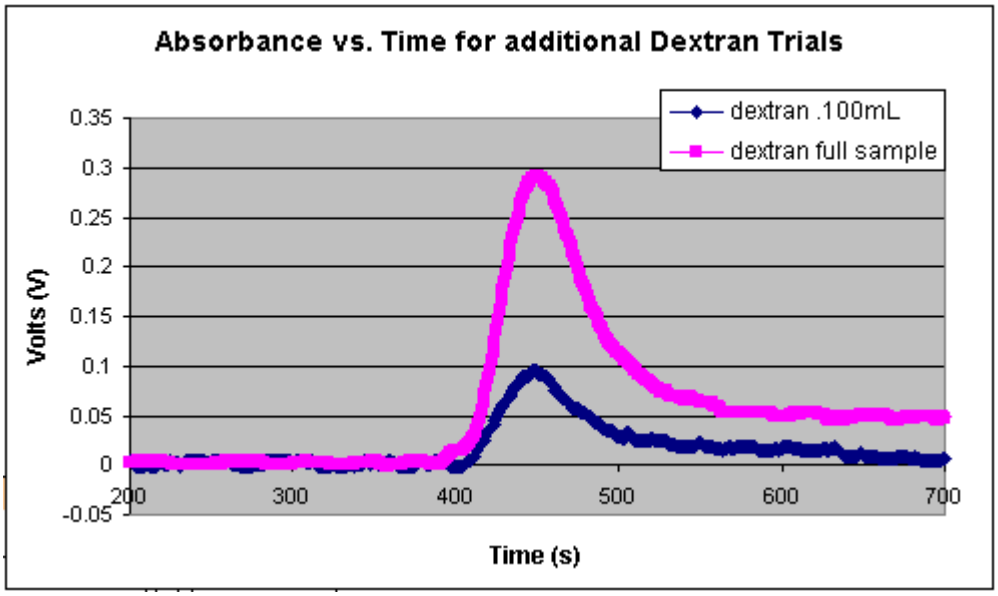
Figure 4. Comparison of experimental MW Hemoglobin to Literature

	MW Hemoglobin (g/mol)
Literature Value* (2nd peak)	64500
Experimental Value (2nd peak)	52485.084
t critical	26.57
t experimental	12.71
t(crit) > t (exp)	SIGNIFICANT DIFF.

The experimentally determined MW of the second peak for hemoglobin (presumably hemoglobin monomer, see explanation in analysis) is compared to literature value as determined by gel electrophoresis.

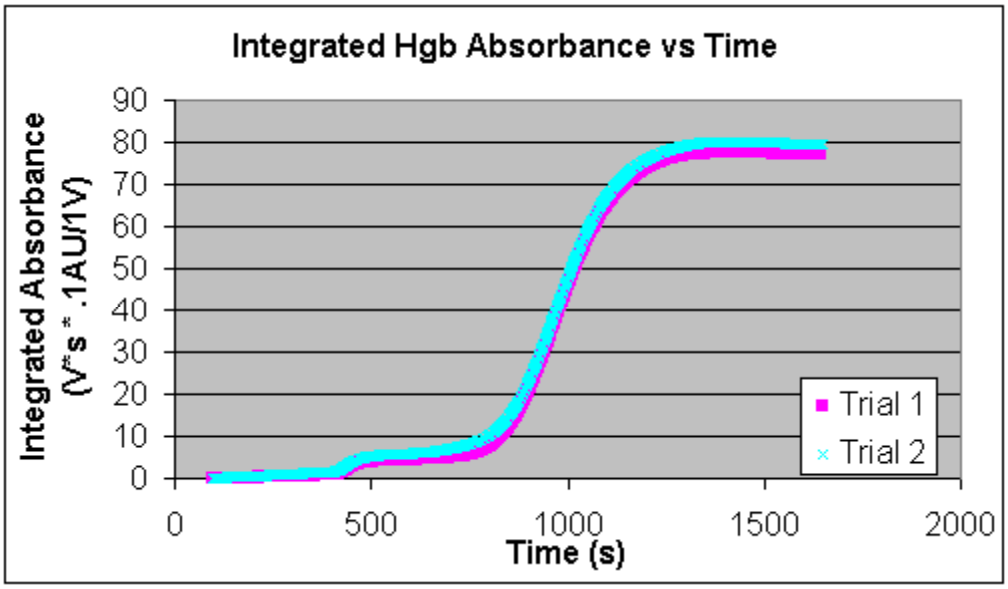
Experimentally determined hemoglobin monomer MW is significantly different from literature, assuming 100% confidence (no uncertainty) in Equation 1.

Figure 5. Data from Dextran trials



In order to determine the volume of the protein solution samples that pass through the column, we decided to use a known volume of dextran and compare that data to the data from a trial with a fully loaded loop. The lower of the trials was obtained by passing only 100µL through the column.

Figure 6. Integrated Hemoglobin vs Time



In order to do the mass balance, total output must be known. Above, the absorbance of hemoglobin as it passes through the column is integrated over time in order to give total absorbance.

HOW DID YOU DO IT? DID YOU INCLUDE THE LITTLE PEAK?

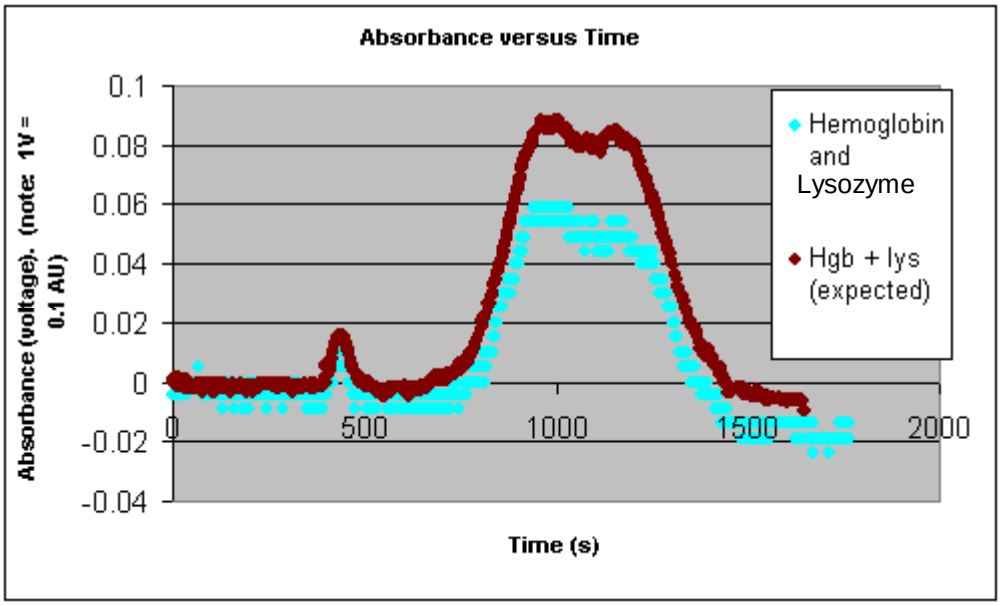
Figure 7. Tabulated Results of Mass Balance

Input		
Area (V*s) Dextran full	23.94	
Area (V*s) Dextran .1mL	7.28	
input volume (mL)	0.33	
input mass (mg)	0.66	
Output		
actual flow rate (ml/min)	1.91938	
Area (V*s) hgb	80.036	77.245
mass (mg)	0.828584	0.79969
%diff from input	22.97195	19.46255
avg output (mg)	0.814137	
avg output +/- (95% CI)	0.183567	
t-Test		
tcrit	12.70615	
texp	10.8177	
significantly different?	NO	

The input and output masses of hemoglobin are compared above. The input volume was found by integrating the two Dextran trials from 300-400s (this includes most of the primary peak). The ratio of the two areas were used to find the volume, since the input volume of one trial was known to be .1mL. The output mass was calculated by taking the area of the hemoglobin curve and multiplying by several conversion factors to get mass: $mass=(V*s)*(.1AU/1V)*(1min/60s)*(1.919mL/1min)*(.309AU*ml/mg)^{-1}$ Comparison of the input and output masses using a test of significance showed that there was no significant difference between the two.

THE LITTLE PEAK IS NOT HB SO IT HAS A DIFFERENT EXTINCTION COEFFICIENT SO YOUR CALCULATION IS SOMWHAT IN ERROR

Figure 8.



The above graph compares the experimental absorbance for the combined hemoglobin and lysozyme graph to an expected graph. The points for the expected data were calculated by combining the data from the individual hemoglobin and lysozyme trials and re-scaling to an arbitrary value so that it is easier to compare the peaks. The experimental data is somewhat more difficult to read due to excess signal noise.

LOVELY! GRADE 40/40

Analysis

As stated in the Results section, it is determined that the relationship between V/Vo and log MW is (with 95% CI)

$$V/Vo = (-0.8768 \pm 1.07797) \log MW + (6.2559 \pm 5.128944) \quad \text{(Equation 1)}$$

Using equation 1 and the constants Vo = 14.65124 ml and flow rate = 1.91938 ml / min (as determined from this experiment), time for elution can be correlated to the molecular weight of a protein. For example, for a protein with molecular weight of 90,000 Da, using equation 1, V/Vo is determined to be 1.91±16.72. Substituting for Vo to get V=28.01±244.97ml. Finally, since flow rate = 1.92 ml / min, time for elution is 14.60±127.62 (95% CI) minutes.

It is evident from the results that the mathematical relationship derived (Equation 1) has a very large 95% confidence interval, which is caused entirely by the uncertainty derived in calculating the regression slope and intercept. In the derivation of this equation, only three data points were used, and the R squared value was only 0.9907, resulting in little confidence in the regression. THIS IS A VERY GOOD VALUE- MOST RESEARCHERS WOULD SELL THEIR MOTHER FOR SUCH A VALUE!The uncertainty in the equation above is further exacerbated by the fact that the relationship between V/Vo and MW is logarithmic. In the example above, the uncertainty in the equation is magnified during the calculation of elution time, resulting in the tremendous 95% CI interval in the answer.

To improve the confidence of this equation, much more data needs to be obtained by eluting more proteins of known molecular weight through the column. In addition, the flow rate can be reduced, so that more time will be allowable for protein interactions with the porous matrix, resulting in more distinct retardation in elution time of different protein molecules.

The precision in determining the peak times for the two components of the hemoglobin can be evaluated by looking at the standard deviations and 95% confidence intervals associated with the elution time values. The average values with their respective standard deviations are 442.01 +/- 1.42 (.320%) seconds for the lower peak of the dimer and 969.755 +/- 2.125 (.219%) seconds for the higher peak of the monomer hemoglobin. Their respective 95% confidence intervals are 12.73 seconds (2.88%) and 19.09 seconds (1.97%). The standard deviation values for both hemoglobin types are small (under 1%),

as is the 95% CI values, which are both under 3%. This translates into low uncertainty within the trials and high precision in repeating and determining the peak time trials. As a result, the peak times for both hemoglobin types demonstrates high repeatability with great precision.

Despite the high precision and low uncertainty associated with determining the peak times (or elution time) values of the hemoglobin, the molecular weights which were calculated from these elution times by the derived equation of the relationship between V/V_o and the MW, shows greater standard deviation and 95% confidence interval values. If it is assumed that there is no uncertainty associated with Equation 1, it can be seen that from Figure 3, the average MW, determined from the two trials, of the first peak (dimer) is 1,082,028 g/mol, with a standard deviation of +/- 8787 g/mol (.81%) and a 95% confidence interval of 78950 g/mol (7.30%). The average value for the second peak (single hemoglobin) is 52,485 g/mol with a standard deviation of +/- 639.47 g/mol (1.22%) and a 95% CI of 5.745 g/mol (10.95%). IF YOUR CALIBRATION IS ANY GOOD THEN THE LITTLE PEAK CANNOT BE A HB DIMER SINCE ITS MW WOULD BE TWICE THAT OF HB NOT 20 TIMES- THIS IS PROBABLY AN IMPURITY These standard deviation and 95% confidence interval values for the MW determined are greater than those of the elution times, from which the MW were determined from. Again, this is due to the logarithmic relationship between the eluted volume and the MW. The elution time data collected is used to determine the eluted volume, using the eluent flow rate. As previously derived, the ratio of eluted volume to void volume (V/V_o) is proportional to log MW. As a result of this logarithmic relationship, any slight uncertainty in the elution time is carried into the eluted volume calculation and magnified greatly by the logarithmic equation. This explains the reason why the standard deviation and 95% CI values for MW are of considerable magnitude despite the small St. Dev. and 95% CI values associated with the elution times collected. Due to the logarithmic nature of this relationship, one can conclude that it is very hard to achieve high precision in determining the MW, using this means because any minute uncertainty in the elution time is amplified for the MW.

The MW value for the single hemoglobin, as determined by gel electrophoresis, is given as 64500 g/mol and compared with the experimental value of 52485 g/mol in Figure 4. As the t-test shows, if the uncertainty associated with Equation 1 is not taken into account, t-critical value is greater than the t-experimental value, thereby indicating that the "literature" and experimental MW values are significantly different. Despite the relatively large 95% CI of 10.95% associated with the experimental MW determined, the "literature" value still falls well outside of that interval. Based on the given literature value, the elution time of hemoglobin (2^{nd} peak) should be 15.563 minutes, which is 0.6 minutes shorter than the experimental elution time. It is possible that some property of hemoglobin causes extra affinity of the hemoglobin molecule to the column, retarding its elution time and therefore decreases the experimentally determined MW of hemoglobin. A more likely explanation, however, is that Equation 1 simply needs to be revised by the inclusion of more data points. IF YOUR CI IS MEANINGFUL, AND THE PEAKS ARE SIG DIFFERENT, LOWERING THE CI WOULD NOT MAKE THE MW ANY

BETTER. THERE HAS TO BE ANOTHER EXPLANATIONOtherwise, although the precision of this lab is relatively good, (more so in the case of the elution time values), gel exclusion chromatography is a poor method of trying to determine an accurate value of the MW of hemoglobin.

The results of the mass balance (Fig 7) show that there was no significant difference between the hemoglobin input and the output of the column: mass is conserved. Although the difference between the input and output was insignificant, the output was consistently greater than the input. One cause may have been due to beginning successive trials before the previous contents had been eluted. This means that it takes a significant amount of time for one trial to go to completion. YOU INCLUDED THE SMALL PEAK WHICH DOESN'T CONTRIBUTE MUCH TO THE INTEGRAL BUT MAY BE A GREATER FRACTION OF THE INPUT

Looking at the plot of the curves of hemoglobin (trials one and two), lysozyme, the actual hemoglobin and lysozyme mixture, and an expected hemoglobin and lysozyme mixture (Figure 1), it becomes evident that the mixed curves exhibit characteristics of both parent curves. The mixed-sample curves share similar peaks with the hemoglobin and lysozyme curves alike. The hemoglobin heavy dimer creates a primary peak of comparatively lower absorbance (0.0058AU at 443.007sec) than that of the single chain (0.0254AU at 967.251sec, and 0.0268AU). The lysozyme elution curve has, on the contrary, only one absorbance peak valued at 0.0196AU at 1204.302sec.

The difference in time of the single chain peak of the hemoglobin and the lysozyme peak, is maintained in the mixed-sample curve. Therefore mixed curve has three peaks. The first peak appears as a result of the hemoglobin dimer in the mixture and has an absorbance value of 0.001AU at 435.001sec. Because the lysozyme does not contribute to the peak, it occurs at the exact time as it would if the mixed-sample was just pure hemoglobin. This peak marks the elution of the hemoglobin dimer, which is heavier than the rest of the hemoglobin chain and heavier than the lysozyme protein. Because the lysozyme protein is so different in structure and weight than that of the hemoglobin dimer, elution time is greater.

The second peak appears as a result of the single hemoglobin chain and has an absorbance value of 0.0059AU from the time interval of 947sec to 1033sec. Even though this peak is representative of the hemoglobin single chain, it is more a plateau since the absorbance value remains the same for 86 seconds.

The third peak is a result of the lysozyme curve, having an absorbance value of 0.0054AU from 1147.3sec to 1174.3sec. This peak, much like the second one is also a plateaus because the absorbance stays the same for 30sec. The plateaus of the second and third peaks may be a result of the similar structure and weight of the hemoglobin single chain and the lysozyme protein.

The plateaus indicate the elution of both proteins at the same time, thus proving that the gel-exclusion chromatography is no longer a dependably method of separating proteins

when they are of similar size and/or molecular weight. Although on the mixed-sample curve the second and third peaks are visible, they are hardly distinct, providing no real data on where the first peak ends and the second begins. THIS MAY ONLY BE AT YOUR FLOW CONDITIONS AND USING THIS PARTICULAR GEL

Interestingly enough, the molecular weight of the hemoglobin single is calculated to be 52,112g/mol while that of lysozyme is 14,300g/mol. But the elution times of the two proteins only differ by a maximum of 227sec. Which suggests, that while the mass of the proteins differ greatly, they might be of similar size, since the relationship exists that the smaller the molecule size the greater the elution time, the heavier the molecule the lower the elution time.

VERY NICE ANALYSIS
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