Chem 237L Lab 5: Analysis of Protein mixture using SDS-PAGE to Observe Catalase and Myoglobin Separation

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Results

Summary:

- The objective of this lab is to analyse the separation technique of SDS-PAGE of a mixture of proteins containing myoglobin and catalase. Catalase activity was tested for using hydroxide and further tests were done using a spectrometer to uncover absorption values that will be later used to calculate Molecular weight and understand the separating nature of Size exclusion chromatography.

Sample Number	A [280]	A [410]
1	0.0074	0.0041
2	0.0100	0.0047
3	0.0086	0.0043
4	0.0077	0.0043
5	0.0078	0.0037
6	0.0086	0.0042
7	0.0171	0.0061
8	0.0594	0.0205
9	0.0273	0.0242
10	0.0325	0.0799
11	0.0488	0.1886
12	0.0358	0.1311
13	0.0196	0.0464
14	0.0146	0.0164
15	0.0117	0.0100
16	0.0047	0.0025

Table 1: Absorption analysis of the fraction samples using Cary60 spectrometer set at 280nm and 310nm

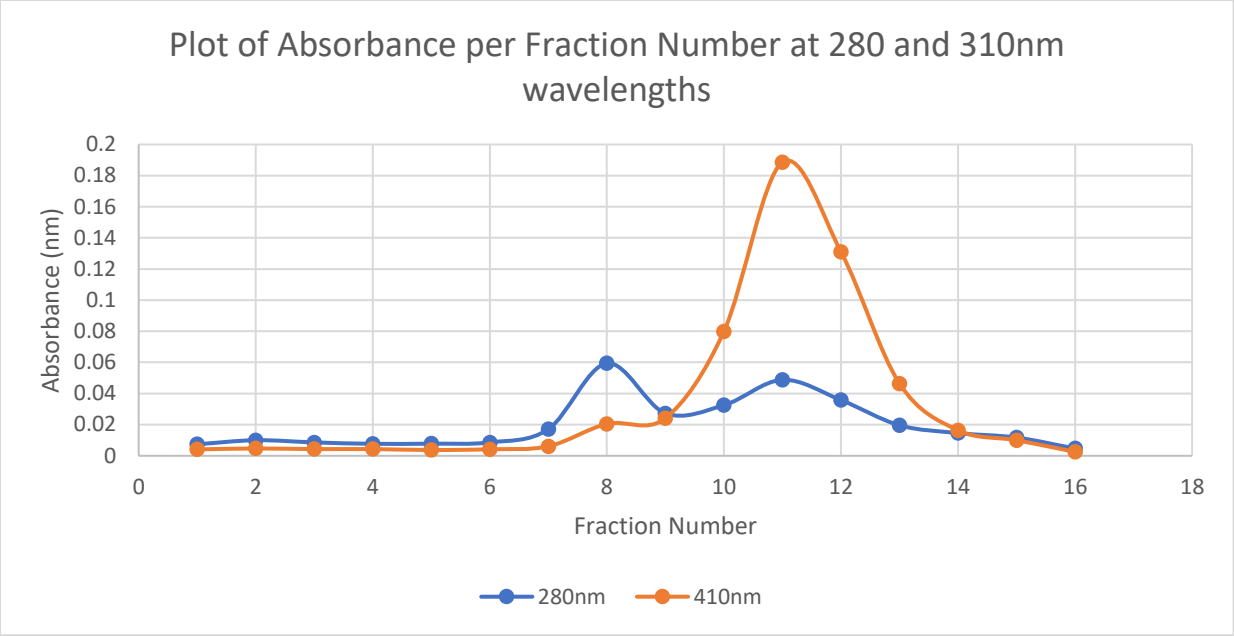


Figure 1: Absorbance value of Fraction number after protein separation to identify protein composition

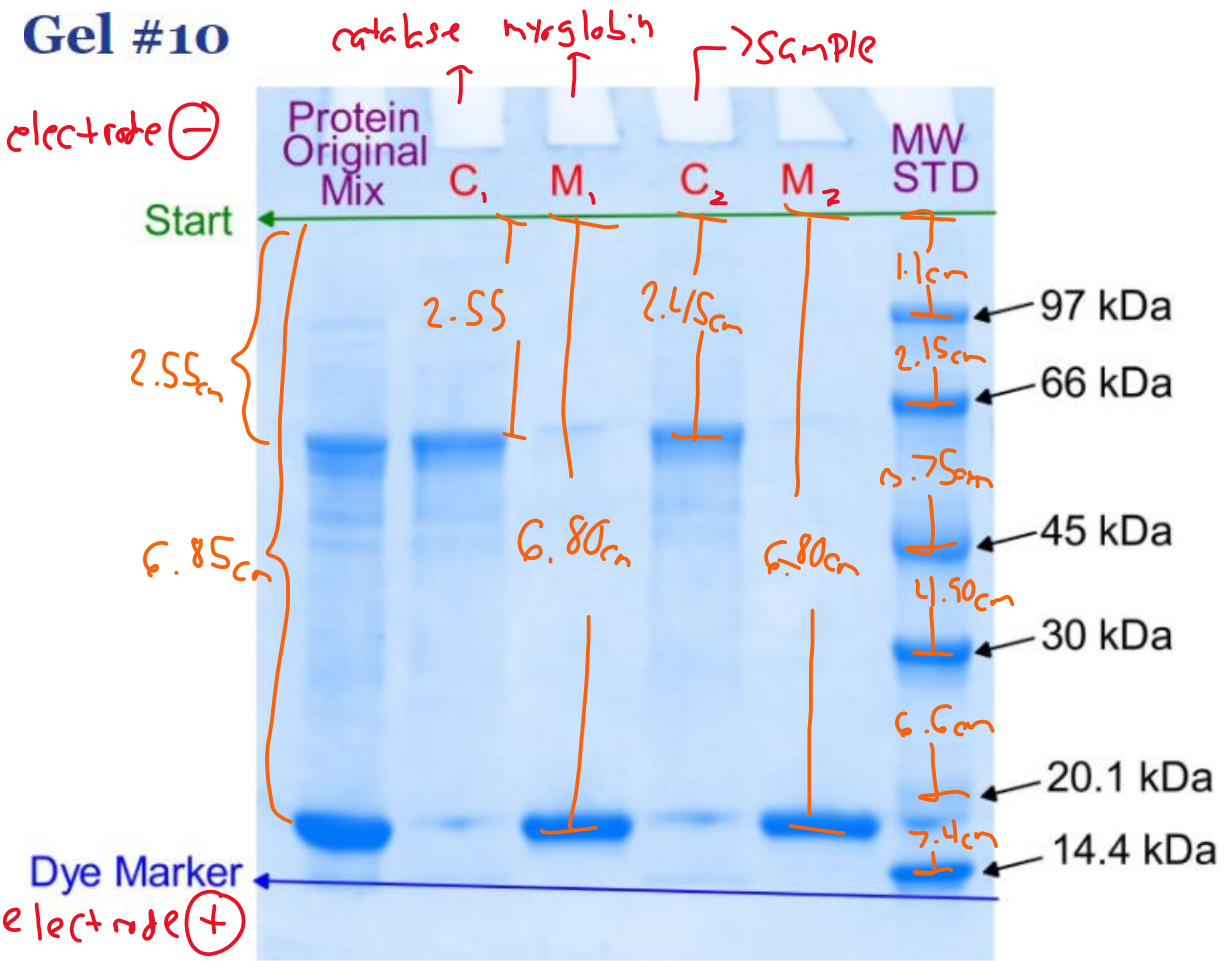


Figure 2: SDS-PAGE of protein mixture, Catalase-activity fraction labelled "C", myoglobin-presence fraction labelled "M", and the Molecular weight standards of proteins as a control.

migration distance of start to dye marker = 7.45, 7.51, 7.55, 7.59, 7.61, 7.70cm for the respective lanes

- Migration distance of Original Protein Mix (2 bands): 2.55cm and 6.85cm
- Migration distance of C1 and C2: 2.55cmand 2.45cm
- Migration distance of M1 and M2: 6.80cm and 6.80cm
- Migration distance of protein standard: 1.1cm,2.15cm,3.75cm,4.9cm,6.6cm,7.4cm respectively.

5.

- Rf of Original Protein Mix: Rf= $\frac{distance\ migrated\ by\ the\ protein\ bands\ of\ interest}{distance\ migrated\ by\ the\ dye\ marker\ front\ line}$
  - Rf = 2.55cm / 7.45cm = 0.34
- Rf of Original Protein Mix: Rf= $\frac{distance\ migrated\ by\ the\ protein\ bands\ of\ interest}{distance\ migrated\ by\ the\ dye\ marker\ front\ line}$
  - Rf = 6.85cm / 7.45cm = 0.92
- Rf of C1: Rf= $\frac{distance\ migrated\ by\ the\ protein\ bands\ of\ interest}{distance\ migrated\ by\ the\ dye\ marker\ front\ line}$
  - Rf = 2.55cm / 7.51cm = 0.34

- Rf of C2: Rf= $\frac{distance\ migrated\ by\ the\ protein\ bands\ of\ interest}{distance\ migrated\ by\ the\ dye\ marker\ front\ line}$
  - Rf = 2.45cm / 7.59cm = 0.32
- Rf of M1: Rf= $\frac{distance\ migrated\ by\ the\ protein\ bands\ of\ interest}{distance\ migrated\ by\ the\ dye\ marker\ front\ line}$
  - Rf = 6.80cm / 7.55cm = 0.90
- Rf of M2: Rf= $\frac{distance\ migrated\ by\ the\ protein\ bands\ of\ interest}{distance\ migrated\ by\ the\ dye\ marker\ front\ line}$
  - Rf = 6.80cm / 7.61cm = 0.89
- Rf of protein standards: Rf= $\frac{distance\ migrated\ by\ the\ protein\ bands\ of\ interest}{distance\ migrated\ by\ the\ dye\ marker\ front\ line}$
  - Rf = 1.1 cm / 7.7cm = 0.14
  - Rf = 2.15cm / 7.7cm = 0.28
  - Rf = 3.75 cm / 7.7cm = 0.48
  - Rf = 4.9 cm / 7.7cm = 0.64
  - Rf = 6.6 cm / 7.7cm = 0.86
  - Rf = 7.4 cm / 7.7cm = 0.96

6.

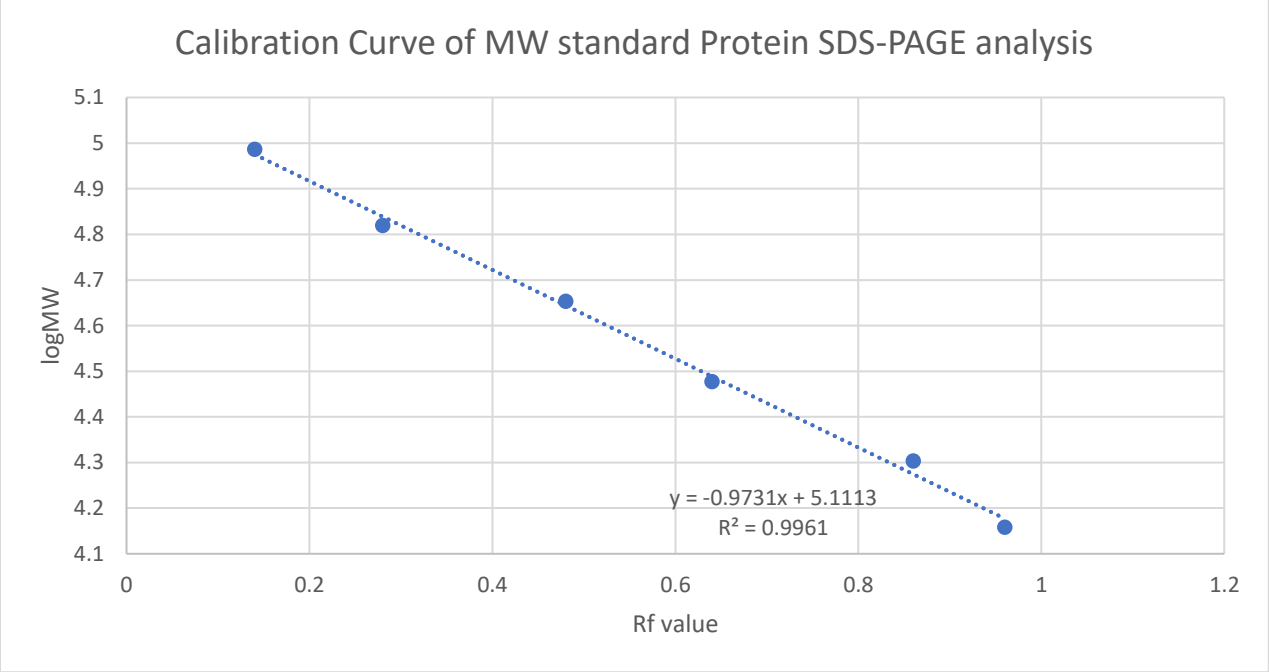


Figure 2: Calibration curve of protein SDS-PAGE analysis in order to identify composition of unknown protein mixture using log of MW and Rf value.

- MW of catalase: y=-0.9731x + 5.1113
  - = -0.9731(.32) + 5.1113
  - = 4.80 , because our y is log, $10^{4.8}$ = 62950 Da or 62.95 kDa
- MW of Myoglobin: : y=-0.9731x + 5.1113
  - = -0.9731(.90) + 5.1113
  - = 4.24 , because our y is log, $10^{4.24}$ = 17378 Da or 17.40 kDa

Questions

1.(a) Of the two proteins to be separated in the protein mixture which protein was expected to elute at a volume similar to void volume. Briefly explain the theory behind this expectation.

- The proteins found in the void volume are going to be larger than the upper fractionation limit of the resin and is going to elute first (Waterloo, 2021). With this, the protein in the M column migrated further down the gel with an Rf of .90 vs protein c having an Rf of .32, thus, protein M will be expected to elute at the void volume.

(b) From your chromatography results, what is the approximate void volume of the column used. Explain and show your calculations.

- As mentioned above void volume contains the protein that is above the fractionation range and thus will elute first, so according to Figure 1, the fraction at which the largest protein is starting to elute in is fraction 10. Each fraction is 0.5mL, thus void volume = 10 x 0.5mL = 5mL

(c) Use your approximated void volume to estimate the bed volume of the column used (show your calculations).

- Void volume is approximately 1/3 of bed volume, thus bed volume = 3 x 5mL = 15mL

(d) Relative to the bed volume at what volume did the second protein elute from the column. Does this agree with what you expected? Explain and show any related calculations.

- The second protein eluted from the column will be of a smaller molecular weight than the first as it will travel slower through the resin if it is below the fractionation volume. Thus, referring to figure 1, and based on Myoglobin having absorbance at 410 nm, the second protein will be the first hump at 280nm. This is located at fraction 8, so the second protein will elute at 8 x 0.5mL = 4mL.

2a. Molar extinction coefficient of Myoglobin

- 2 tyrosine and 2 tryptophan residues. (2 x 1490$M^{-1}cm^{-1}$) + (2 x 5500$M^{-1}cm^{-1}$) = 13980 $M^{-1}cm^{-1}$

2b. Molar extinction coefficient of Catalase

- 21 tyrosine and 6 tryptophan residues. (21 x 1490$M^{-1}cm^{-1}$) + (6 x 5500$M^{-1}cm^{-1}$) =64290 $M^{-1}cm^{-1}$

2c.

- Molar extinction coefficient of Catalase monomer = 64290 $M^{-1}cm^{-1}$. A tetramer would be of 4 subunits so 4 x 6492 $M^{-1}cm^{-1}$ = 257160 $M^{-1}cm^{-1}$

3. Steps

1. The absorbance reading of highest containing catalase fraction is 0.0594nm (fraction 8) at 280nm. Myoglobin's would be fraction 11 of 0.0488nm at 280nm.
2. Absolute absorbance value: average of fractions 1-7 before fraction 8:
    - (0.0074 + 0.01 + 0.0086 + 0.0077 + 0.0078 + 0.0086 + 0.0171) / 7 = 0.0096nm
    - The absolute absorbance = (average baseline) – (above value).
    - Catalase absolute absorbance = 0.0594nm - 0.0096nm = 0.0498nm
    - Myoglobin absolute absorbance = 0.0488nm – 0.0096nm = 0.0392nm
3. Beer-lambert equation = Abs = $l\boldsymbol{\varepsilon c}$
    - Catalase: 0.0498nm = (1cm) (257160 $M^{-1}cm^{-1}$) c
        - $c=1.93e^{-7}M$
    - myoglobin: 0.0392nm = (1cm) (13980 $M^{-1}cm^{-1}$) c
        - $c= 2.8e^{-6}M$
4. Catalase: C1V1=C2V2
    - $C1(100ul)=(1.93e^{-7}M)(1000ul)$
    - $C1=1.93e^{-6}M$

    Myoglobin: C1V1=C2V2
    - $C1(100ul) = (2.8e^{-6}M)(1000ul)$
    - $C1=2.8e^{-5}M$

4. Steps

1. MW of catalase = 240,000 g/mol and MW of myoglobin = 17,000 g/mol
2. Catalase: $1.93e^{-6}M$, myoglobin: $2.8e^{-5}M$
3. g/L conversion requires us to multiply MW by C
    - Catalase: 240,000g/mol x $1.93e^{-6}M$ mol/L = 0.4632g/L
    - Myoglobin: 17,000g/mol x $2.8e^{-5}$mol/L = 0.476g/L
4. mg/mL conversion requires x1
    - catalase: 0.4632mg/mL
    - myoglobin: 0.476mg/mL
5. catalase: 0.4632mg/mL x 0.5mL = 0.2316mg

myoglobin: 0.476 mg/mL x 0.5mL = 0.238 mg

5a.b.

- mg amount of total protein = 2.8mg/mL x 0.250mL = 0.7mg of protein. If catalase makes up 60% and myoglobin 40% then their concentrations = 0.7mg x 60% = 0.42mg of catalase and 0.7mg-0.42mg = 0.28mg of myoglobin.

6a.

- catalase: % recovery = $\frac{actual\ yield}{theoretical\ yield}\ x\ 100\%$ actual yield is catalase recovered at 0.2316mg and theoretical is starting amount at 0.42mg
    - = 0.2316mg/0.42mg x100 = 55.14%
- Myoglobin: % recovery = $\frac{actual\ yield}{theoretical\ yield}\ x\ 100\%$

- = 0.238mg/0.28mg x100 = 85%

b. the main contributor for such a low protein recovery is we recovered the protein using the data off of a single 0.5mL fraction, because we are not perfectly isolating these proteins in a single fraction, there will be some catalase in fractions 7 and 9 between the peak at 8, and same thing for myoglobin.

7.

- Size exclusion chromatography separated the proteins decently well as comparing the C and M wells to the original mixture, there are less "ghost lines" if you will where there is impure catalase and a more distinct line indicating a more pure separation as its showing all the protein of this weight is in a specific place. If there is bleeding that indicates proteins of varying densities. This type of bleeding would be present in an unsuccessful separation of the proteins.

8. calculations for molecular weight found in results 6. MW of catalase = 62950 g/mol and MW of myoglobin = 17378g/mol.

- Catalase % error = $\frac{Experimental-theoretical}{theoretical}\ x\ 100\%$
- = (62950 – 240000)/240000 x 100 = 73%
- Myoglobin = (17378 – 17000)/17000 x 100 = 2.22%
- % error for catalase is so high and the reason it is roughly ¼ of the expected value is because natively catalase exists as a tetramer meaning it contains 4 monomer subunits of catalase meaning our value is of a monomer of catalase.

9a.

- The protein eluted at 23mL will have a molecular weight at approximately 45000g/mol

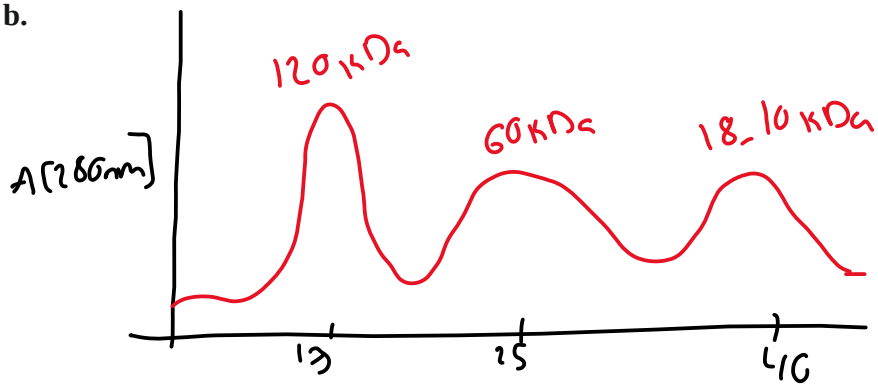
b.

- The molecular weight of the protein is approximately 45000g/mol

10a.

- Bed volume = (πr^2) x height = (π(.5cm)^2) x 50cm = 39.25mL
- Void volume is approximately 1/3 bed volume = 13.08mL

b.



Volume(mL)

c.

- The protein with 120kDa has the highest molecular weight and will elute into the void volume as it is above the fractionation range of 90 kDa. The 18 and 10 kDa proteins are going to elute into the bed volume and not separate as they are both under the fractionation range of 20 kDa. In terms of their steepness for the reasons above, the 120 kDa curve will be more pure of protein as it passes through first and is not affected by the pores and will fall quickly into elution. Finally the 60kDa will be trapped in the resin pores and will elute before the bed volume.

11.(a) What does it mean to have reducing conditions for an SDS-PAGE and what is its purpose?

- Reducing conditions are conditions that reduce the disulphide bridges within the protein structure to offer more accurate separation of size in SDS-PAGE analysis as disulfide bonds are important for protein structure and folding thus helping to unfold the protein. As SDS is a denaturing technique used to separate by Mw alone as the proteins will be denatured and converted to a net negative charge (Nowakowsi et al, 2014).

(b) What concerns are there for determining the molecular weight of a protein when a gel is run under non-reducing conditions.

- the protein not unfolding correctly allowing it to keep charges that affect the running of the gel in the electrophoresis tests. The concern would be of not having an accurate sample of protein to run experiments with.

Work Cited

Nowakowski, A. B., Wobig, W. J., & Petering, D. H. (2014). Native SDS-PAGE: high resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. Metallomics : integrated biometal science, 6(5), 1068–1078. https://doi.org/10.1039/c4mt00033a

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