ABSTRACT: This study was an attempt to isolate, purify, and characterize bovine myoglobin. Myoglobin was isolated from bovine muscle tissue using aqueous extraction. Next a G-25 desalting column was performed producing met- and oxy-myoglobin isolates. A UV-Vis scan was ran on met- and oxy-myoglobin isolates and the crude myoglobin supernatant to characterize the composition of the crude myoglobin supernatant. Then the crude myoglobin was purified using G-75 and CMC chromatography, in comparison. The quality of these methods of isolation was analyzed by SDS-PAGE. Since the presumed crude myoglobin isolate was red and the color of oxy-myoglobin is red, it was inferred the crude myoglobin supernatant was mainly oxy-myoglobin. This was further supported with a UV scan performed on the crude myoglobin supernatant, because the peaks observed at 424 nm, 541 nm, and 581 nm coincided with the predicted peaks and the oxy-myoglobin isolates peaks at 418 nm and 561 nm. The calculated total in the tissue sample was 3.46 mg and 0.489 and 1.84 mg in the CMC fraction and G-75 fraction respectively. The relative purity of the crude myoglobin supernatant was 0.00752. The relative purity of the CMC column was 0.084 and the relative purity of the G-75 column was 0.52. The fold purifications for the G-75 column and CMC column were 69 and 11 respectively. The calculated percent recovery was 28% for the CMC column and 260% for the G-75 column. From the SDS PAGE gel it was deduced all isolates had myoglobin since all isolates had bands that corresponded with the myoglobin standard. There were the fewest bands in the G-75 isolate. The estimated molecular weight of myoglobin was 13000 Da. From these results it was apparent the G-75 column was the best way to isolate myoglobin.

EXPERIMENTAL PROCEDURES
The procedure published by Pugh, et al. (1) was followed. Deviations from standard procedure are summarized as follows: The pH of the pi buffer used had a pH of 5.39.

Isolation
The settings used for the centrifuge was 4700 RPM for 10 minutes at 4°C.

UV-Vis scans
The crude myoglobin isolate was not diluted for analysis.

G-75 Column

The G-75 column did not utilize the published fraction size of 1.00 mL; instead, 2.00 mL fractions were collected from the column (1). The measurements of these fractions were approximate. The fractions were not diluted for analysis.

CMC Column
Approximately 5 mL of Pi + KCl buffer was used to elute proteins from the column. The fractions were not diluted for analysis.

SDS-PAGE
The sorting gel was 12% acrylamide and the loading stack gel was 4% acrylamide.
RESULTS AND DISCUSSION

The first step in the procedure was an aqueous extraction of myoglobin from masticated bovine tissue. The extraction was then isolated by centrifugation and was labeled the crude myoglobin supernatant. Then, a G-25 Sephadex desalting column was performed which produced two isolates. Aliquots of the crude isolate were reduced with sodium dithionite and oxidized with potassium ferricyanide, to produce oxy- and met-Mb, respectively. These were used to perform a qualitative analysis on the crude myoglobin supernatant.

Qualitative analysis of crude myoglobin supernatant. The presumed oxy-myoglobin isolate was red and the presumed met-myoglobin isolate was yellowish brown. According to Pugh et al. (1) these were the expected colors for each isolate. Thus, it was deduced the presumed oxy-myoglobin isolate contained mainly oxy-myoglobin, and the met-myoglobin isolate contained mainly met-myoglobin. The color of the crude myoglobin supernatant was red. From these qualitative data it was concluded that the crude myoglobin supernatant was composed of mostly oxy-myoglobin.

These qualitative data are a good starting point for analyzing the composition of the isolates and the effectiveness of the isolation methods used; however, more analyses were needed to confirm the results. One method used to characterize these isolates was UV-Vis scans.

![Figure 1: The dashed line (—) represents the absorbance of the crude myoglobin supernatant from 200 nm to 700 nm. The dotted line (●) represents the absorbance of the presumed oxy-myoglobin isolate from 200 nm to 700 nm. The solid line (——) represents the absorbance of the presumed met-myoglobin isolate from 200 nm to 700 nm. From the peaks present in the Soret region it was deduced that the presumed oxy-myoglobin isolate is composed of oxy-myoglobin, and the presumed met-myoglobin was composed of met-myoglobin. Moreover, from a comparison of the peaks of the three isolates it was inferred the crude myoglobin supernatant was composed mainly of oxy-myoglobin.](image)
**UV spectrum analysis in the Soret region.** As observed in Figure 1, the presumed met-myoglobin isolate had two peaks at 277 nm and 408 nm. According to Pugh *et al.* (1) a peak at 409 nm occurs when met-myoglobin was present. Thus, it was evident that there was mainly met-myoglobin in the presumed met-myoglobin isolate, because of the peak at 409 nm.

As observed in Figure 1, the presumed oxy-myoglobin isolate had a peak 424 nm. Also the UV scan did not give a clear reading before 400 nm. This indicates the presence of particulates. Thus, whether or not there are peaks before 400 nm was inconclusive. According to Pugh *et al.* (1) peaks occur at 348 nm and 417 nm when oxy-myoglobin was present. Hence, it was inferred that oxy-myoglobin could be present since there was a peak at 424 nm. Still, this inference was not definite since the peak at 424 nm was slightly more intense than the predicted peak at 417 nm and the presence of the 348 nm peak cannot be determined.

As observed in Figure 1, the crude myoglobin supernatant had two peaks at 418 nm and 337 nm. According to Pugh *et al.* (1) peaks occur at 348 nm and 417 nm when oxy-myoglobin was present. This indicates the crude myoglobin supernatant was composed of mainly oxy-myoglobin since there was peak at 418 nm. However there was no peak at 348 nm. The reason there was no peak at 348 nm could have been caused by experimental error.

Yet, Figure 1 was only useful for finding peaks in the soret region. According to Pugh *et al.* (1) there were peaks in the visible region that could be used to characterize the state of myoglobin. Thus, an enlarged image of 450nm to 700 nm was produced to show the visible region.

![Graph](image)

**Figure 2:** This figure was taken from the same UV spectrum seen in Figure 1. But 450 nm from 700 nm was enlarged to properly view the visible spectrum. The dashed line (—) represents the absorbance of the crude myoglobin supernatant from 450 nm to 700 nm. The dotted line (●) represents the absorbance of the presumed oxy-myoglobin isolate from 450 nm to 700 nm. The solid line (—) represents the absorbance of the presumed met-myoglobin isolate from 450 nm to 700 nm. From the peaks present in the visible region it was deduced that the presumed oxy-myoglobin isolate is composed of oxy-myoglobin, and the presumed met-myoglobin was composed of met-myoglobin. Moreover, from a comparison of the peaks of the three isolates it was inferred the crude myoglobin supernatant was composed mainly of oxy-myoglobin.
UV spectrum analysis in the visible region. Figure 2 was an enlarged version of Figure 1. In this Figure 2 it can be seen that there were peaks in the visible region. As observed in Figure 2, the presumed met-myoglobin isolate had a broad peak at 499 nm. The predicted peaks for presumed met-myoglobin were 504 nm and 635 nm (Pugh et al. 1). Thus, the peaks observed at 499 nm suggest that met-myoglobin was present. However, this peak was not well defined and the predicted peak at 635 nm was not present.

As observed in Figure 2, the presumed oxy-myoglobin isolate had a peak at 561 nm. This peak was located right between the two predicted peaks, 542 and 580 nm (Pugh et al. 1). This could indicate that the peak at 561 nm was these two overlapping peaks. This would also indicate experimental error was present in this scan and the presence of met-myoglobin.

As observed in Figure 2, the crude myoglobin isolate had peaks at 541 nm and 580 nm. This peak was pretty close to peaks predicted for oxy-myoglobin, 542 nm 580 nm (Pugh et al. 1). Therefor it was deduced that oxy-myoglobin was present in the crude myoglobin supernatant.

According to Pugh et al. (1) oxy-myoglobin is the reduced form of myoglobin and met-myoglobin is the oxidized form of myoglobin. Based on the trends observed in Figure 1 and Figure 2 it appears all myoglobin isolates were either completely reduced or oxidized because most of the expected absorbances were observed. Additionally, this also explained the observed peaks in both figures because when oxy-myoglobin is oxidized it forms met-myoglobin its absorption peaks change. The predicted shift of peaks was 348 nm and 417 nm to only 409 nm in the soret region, and 542nm and 635 nm to 542 nm and 580 in the visible region. These trends were observed except there was an overlap of peaks 542 nm and 580 nm of the presumed met-myoglobin isolate in Figure 2. Also in Figure 1 the presence of the 348 nm in presumed oxy-myoglobin isolates was not able to be determined. Therefore, oxidation had some of the expected effects of UV absorption. Deviations from the expected absorbances were probably due to experimental error.

In summary, the UV scans in Figure 1 and Figure 2 indicate there was oxy-myoglobin in the presumed oxy-myoglobin isolate and there was met-myoglobin in the presumed met-myoglobin isolate since they both had the peaks predicted by Pugh et al (1). Additionally, according to the UV scans the crude myoglobin supernatant did not appear to contain myoglobin in its reduced form, met-myoglobin. The crude myoglobin supernatant mainly had myoglobin in its reduced form, oxy-myoglobin.

However, the UV could only be used to characterize what was present in the crude myoglobin supernatant. The quantity and purity of the myoglobin still needed to be determined. The first step used to accomplish this was analysis by the Bradford assay.
Figure 3: Bradford standard curve used to analyze the crude supernatant. The graph shows the trend of concentration and the absorbance of those concentrations. The extinction coefficient was given by the slope of the line: 19.4 mL/mg·cm. The $R^2 = 0.9454$ indicates about a 95% fit of the line to the data.

Protein Determination. The first standard curve established an extinction coefficient for protein in the Bradford assay. This value was used to find the concentrations of nine samples of the original, diluted thymus extracts. Three of the nine samples were diluted by a factor of 25; three samples were diluted by a factor of 50; and three samples were diluted by a factor of 100. The first Bradford assay was employed to determine a general concentration of the protein in the crude isolation.

The values presented from the first standard curve were fairly different from previous studies. A Bradford standard curve from a previous study by Grimes et al. returned an extinction coefficient of 26.159 mL/mg·cm, a value significantly higher than the standard at hand (2). A similar trend was observed in the remainder of the Bradford standard curves; one reported an extinction coefficient of 43.983 mL/mg·cm (shown in Figure 4). The standard curve used in the CMC fraction analysis gave an extinction coefficient of 14.654 mL/mg·cm. The large deviations between the values from the standard curves, and the standard curves themselves may indicate error across the trials. This error could be experimental, but could also be due to the cycling of standards and reagents used in the assay. By keeping the standard curves specific to each of the experimental assays, the error was kept to a minimum.

Figure 4: Bradford Standard Curve for Analysis of G-75 Protein Content The curve represents a standard curve for use with the data from the second set of trials. The extinction coefficient was given by the slope of the line: 44.0 mL/mg·cm. The $R^2$ value indicates a significant departure of the trend-line from the trend, but this was common amongst other trials using the same standard and reagent.

The absorbances of the dilutions were used in conjunction with the extinction coefficient to give a value of concentration. For comparison to the mean across all trials, the averages for each set of trials are presented. The average concentration in the 1:100 dilution was 53.8 mg/ml. The average dilution in the 1:50 was 34.2 mg/mL. The average dilution in the 1:25 was 26.0 mg/mL. The clustering of the data points at each of the tested values indicates that there may have been an error in the creation of the
values. The values gave an average concentration of protein in the tested samples of 38.0 ± 11.7 mg/mL. Comparing across the separate sets of dilutions accounted for the large standard deviation.

![Figure 5: Bradford Standard Curve for Analysis of CMC Fraction Protein Content](image)

This standard curve shows the trend of concentration and the absorbance of the standard BSA stock solution. This graph pertains to the calculation of the protein concentration of the CMC column as the extinction coefficient was given by the slope of the line: 14.7 mL/mg*cm.

The literature presented a value for use in the calculation of the Myoglobin calculation. From the red color of the supernatant, it was assumed that the predominant species of myoglobin was the reduced form. Oxy-myoglobin has a peak absorbance at 417 nm, where the extinction coefficient is 7.57 mL/mg*cm (1).

Myoglobin Determination Following the preliminary determinations of both the protein and myoglobin content of the samples, a set of fixed wavelength ultraviolet readings were completed on the fractions collected from the column chromatography to construct the chromatograms. Both the CMC and the G-75 columns were characterized in this manner, using samples taken from the sets of 2 mL fractions.

![Figure 6: G-75 Chromatogram](image)

This chromatogram was created using 5 fractions from the G-75 size exclusion column. The A417 (♦) series follows the trend of absorbance at 417nm. The A280 (■) follows the absorbance at 280 nm. The former is scored against the left y-axis; the latter is scored against the right y-axis.

The G-75 chromatogram, Figure 6, depicts the trend of the absorbances at 417 nm and 280 nm. These values roughly reflect the concentrations of the protein, in the case of the 280 nm, and the myoglobin, at 417. The myoglobin absorbance at 417 is due to the heme group. In both chromatograms it was clear that the absorbance at both 417 nm and 280 nm stayed in proportion, reinforcing the assertion that the predominant species of myoglobin is the reduced form.

On the basis of the two chromatograms, it was clear that the CMC chromatogram was the more effective. Increasing support was lent by the trend of Figure 5 which gradually changes over the course of the fractions; the values only change slightly across the series and do so at a fairly equal absorbance. The equivalence of the rates, in conjunction with the gradual curve, leaves very little room for resolution and little to no identification of a clear
separation, which is the whole point of a column being utilized.

![CMC Chromatogram](image)

**Figure 7. CMC Chromatogram** This chromatogram was created using the 7 fractions from the CMC column. The A417 (♦) follows the trend of the absorbance at 417 nm. The A280 (■) follows the absorbance at 280 nm. The former is scored against the left y-axis; the latter is scored against the right y-axis.

The CMC Chromatogram, presented in Figure 7, indicated a more clear separation of the proteins. The separation of the fractions made it possible to decide which of the fractions contained the most myoglobin.

**Comparison of Purification Methods**

Table 1 shows the trends across the two purification methods. The total milligrams of protein of the first row showed that there was a clear reduction of total protein content in the sample following the purification steps. The total amount of protein in the crude supernatant measured 460 ±142 mg in a Bradford Assay of nine sample dilutions described above. After purification by the CMC column, the total protein was 5.83 ±1.07mg. After purification by the G-75 column, the total protein measured 3.58 ±0.272 mg. The reduction indicated that the column chromatography was separating out the different proteins.

The massive reduction of protein in the purification was acquired without a complete removal of the target myoglobin. The total myoglobin in the crude myoglobin supernatant was 3.46 mg. While there was a large reduction in total myoglobin in the CMC fraction, the change was not proportional to the even larger reduction of total protein; the total myoglobin from the CMC fraction was 0.489 mg. A similar result was acquired in the total myoglobin of the G-75 purification column. The G-75 fraction had a total myoglobin value of 1.84 mg. The total protein value was even more reduced in the G-75 purification than in the CMC purification.

By the merit of the first two sets of measurements, the G-75 was the better of the two purification methods, having left the total myoglobin value higher than the CMC, while also reducing the total protein even farther than the CMC.

When the purification methods were applied back to the original tissue from which the supernatant was extracted, the G-75, again, produced the more impressive result. From the crude supernatant, the ratio

<table>
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<th>Table 1: Values for Comparison of Purification Methods</th>
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<tr>
<td><strong>Crude Myoglobin Supernatant</strong></td>
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<tr>
<td>Total Protein (mg)</td>
</tr>
<tr>
<td>Total Myoglobin (mg)</td>
</tr>
<tr>
<td>mg Myoglobin per mg Tissue</td>
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<tr>
<td>Relative Purity</td>
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<tr>
<td>Fold Purification</td>
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<td>Percent Recovery of myoglobin</td>
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*These values are anomalous, expected to be due to error.
of milligrams of myoglobin to milligrams of tissue was $3.46 \times 10^{-4}$. The G-75 value for this ratio was comparable to this value, giving a little over half the value with $1.84 \times 10^{-4}$. The CMC fraction had a ratio value that was off by a factor of nearly ten from the crude isolate, $4.89 \times 10^{-5}$.

The most significant measure of the quality of the purification was more of a direct representation of how pure the fractions were following the chromatography, the relative purity. The relative purity of the crude myoglobin supernatant had a coefficient of 0.00752, representing less than one percent purity. Both of the purification methods surpassed this purity. The CMC Fraction purity was 0.084, over 11 times more pure than in the crude isolation, leaving the purification a success. The G75 Fraction was even more pure, the isolation representing over 50% purity with a relative purity coefficient of 0.52.

While the G-75 fraction consistently gave significantly high results of purification, its values were suspect to error due to those significantly high values. The final measure for comparison between the purification methods was the percent recovery of myoglobin. Despite how good a purification method may be, if the recovered amount is impractically small then the purification method is ultimately a failure. The percent recovery of myoglobin in the CMC chromatography column was 28% of the myoglobin used in the column. The percent recovery in the G-75 fraction was 260%.

It was at this point that the errors in the values for the G-75 purification were made evident. The values in which error was suspected were noted in Table 1. The trend of erroneous values appeared to arise following the value for total myoglobin. From this trend, it can be seen that the error had to do with the procedure surrounding the determination of the total myoglobin from the G-75 fraction.

Therefore, even though Table 1 presented values that supported the quality of the G-75 as a purifying procedure for myoglobin, the results are not solely definitive of the better purification mechanism. Another trial with a redetermination of the values for Table 1 or a separate, independent test would be necessary to reach a conclusion on

Following this quantitative set of values for the determination of the effectiveness of the purification methods, a qualitative determination was done to qualify the experimentally determined results. This qualitative test was done by an SDS-PAGE gel.
Figure 7: The PAGE gel was coded in the following way: Column A was the Blank; Column B was the MW standards, Column C was the myoglobin standards, column D was the crude myoglobin supernatant, column E was the G-75 column isolate, and column F was the CMC isolate. The bands for each column were numbered from top to bottom. The bands in column B and C were used to characterize the isolates.

Qualitative analysis of SDS-PAGE gel. As seen in Figure 7 there was a single band in lane C, which contained equine Mb. It is pure, as expected. Therefore, since bands D14, E8, and F9 lined up with C1 it was inferred they had some myoglobin present as well. However, it was noted that the bands C1, D14, E8, and F9 did not line up perfectly. This was probably due to a warped SDS-PAGE Gel

It also was noted that both columns E and F contained fewer bands than column D. This was expected because column D represents the crude myoglobin supernatant. While column E represents G75 isolate and column F represents the CMC isolate. The concentrations of the crude myoglobin supernatant, the G75 isolate and CMC isolate were 0.229 mg/ml, 0.12 mg/ml and .032 respectively. The CMC isolate had more bands than the G75 isolate. However the CMC isolate produced a darker band that corresponded with the myoglobin standard than the G75 isolated.

Moreover, the bands C1, D14, E8, and F9 approximately corresponded with band B6. Column B was the MW standards, and band B6 was lysozyme. According to Pugh el al. (1) lysozyme has a molecular weight of 14400 Da. Therefore bands C1, D14, E8, and F9 had a similar molecular weight.

The same was applied to the other bands. D1 and B1 correspond with each other. B1 was Phosphorylase b which has a molecular weight of 97400. Band D1 traveled less distance than band B1. Thus it was inferred band D1 has a molecular weight over 97400.

Bands D2 and F1 correspond to band B2. Band B2 was bovine serum albumin which has a molecular weight of 66200 Da. Thus it was deduced bands D2 and F1 had a molecular weight of approximately 66200 Da.

Band B3 was ovalbumin which has a molecular weight of 45000 Da. Additionally, bands D5, E1, and F2 corresponded with each other and were located between bands B2 and B3. Band D6 also was located between bands B2 and B3. Therefore molecular weight of bands D5, D6, E1, and F2 had a molecular weight between 66200 Da and 45000 Da. Bands D7, E3, F4, and F5 all corresponded with
Band B3. Thus it was concluded bands D7, E3, F4, and F5 had a molecular weight that was approximately 45000 Da.

Band B4 was carbonic anhydrase, which has a molecular weight of 31000 Da. Bands D8, D9, D10, E4, E5, and F6 are located between bands B3 and B4. Thus it was evident they all had a molecular weight between 31000 Da and 45000 Da. Additionally, band D8, E4 and F6 corresponded with each other thus it was apparent they had a similar molecular weight. Additionally, D8 and E5 correspond with each other therefore it was deduced they had a similar molecular weight. Moreover, bands D11, D12, E7, and F8 correspond with band B4 so it was inferred they all had a molecular weight of approximately 31000 Da.

Last, band B5 was Trypsin inhibitor which has a molecular weight of 21500 Da. Bands D14, E7, and F8 were located in between bands B5 and B6. Thus it was concluded they all had a molecular weight between 14400 Da and 21500 Da. Additionally, bands E8 and E9 corresponded with each other and hence had a similar molecular weights.

However, looking at the observed band patterns of the gel only gave a general idea of the purity of each isolate and the molecular weight of the isolates present. A graph comparing $R_m$ and the log of the molecular weight of the molecular weight standards was created. This was created to give a more accurate estimate of molecular weight of the bovine proteins.

![Figure 8](image-url)

Figure 8: The semi-log plot of $R_m$ and Molecular weight was produced from the SDS-PAGE Gel in Figure 7. The $R^2$ supports there is a negative linear trend between the log$_{10}$ of molecular weight and $R_m$ (0.9849). The equation displayed was used to estimate the molecular weight of the bands of the SDS-PAGE gel. The estimated molecular weight for myoglobin is 13000 Da.

**Applications of semi-log plot of molecular weight standard.** A semi-log plot was used because it produced the linear regression seen in Figure 8. This gave a linear trend line instead of an exponential decay. The linear equation produced from Figure 8 was used to estimate the Molecular weight for the myoglobin bands. The estimated molecular weights of D14, E8, and F9, were all 13000 Da. According to Joseph *et al.* (3) the molecular weight of bovine myoglobin was 16949 Da and according to Zaia *et al.* (4) the molecular weight for equine myoglobin is 16,951.49 Da. These differences in values could have been caused by warped SDS-PAGE gel.

**Quality of isolation methods.** From Figure 8 it can be observed that both chromatography methods removed contamination, because in both columns E and F there were fewer bands. However, the
bands in G-75 and the CMC did not have similar molecular weights. This indicates that the G-75 and the CMC column need to be improved for better isolation. Still the G-75 column had fewer bands than the CMC. This indicates that the G-75 is a better method of isolation. This agrees with the values obtain from the Bradford analysis and Chromatograph.

**Improving methods used.** To improve the quality of the G-75 column the column length should be increased and the width should be decreased. However, for the best purification, multiple different columns should be performed until the myoglobin is completely isolated.

**Conclusion.** According to UV-Vis scans the crude myoglobin supernatant contained myoglobin in its reduced form, oxy-myoglobin (Figure 1 and Figure 2). However, as observed in Table 1 this myoglobin was not pure. It also can be seen the G-75 column was a better method at purify myoglobin than CMC column because the G-75 had a higher relative purity, fold purification, and percent recovery. However, the error traced to the myoglobin determination step invalidated the experimental values of purity. This could have been due to experimental error or computational error, the source has yet to be determined. Moreover, the gel also indicated the G-75 is a better method of isolation than CMC because the G-75 had fewer bands. In conclusion, all analysis used support G-75 is better at isolating myoglobin than CMC.

**REFERENCES**


