Biology and Biochemistry Department
BIOC311
Biochemistry Lab

Experiment #7
Title: (A) The Isolation of Casein from Milk.
(B) The Isolation of Hemoglobin by Salt Precipitation.

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Objective:

(A) The objective of this experiment is to determine the amount of casein in store-bought milk and compare it to that of the theoretical yield.

(B) The objective of this experiment is to collect hemoglobin using a kosmotropic salt precipitate. (ammonium sulfate.)

Introduction:

(A) Casein is a phosphoprotein, which implies that phosphate groups on some of the amino acid side chains are linked to hydroxyl groups. Calcium caseinate, the calcium salt of casein, is present in milk. It's actually a mixture of at least three similar proteins, separated mostly by their molecular weight and phosphorus group composition. Alpha- and beta-casein have molecular weights in the 25,000s, with 9 and 4-5 phosphate groups per molecule, respectively. They're both insoluble in water. Casein is an 8,000-diameter protein with 1-2 phosphate groups per molecule. It encourages the formation of micelles, which assists in the water solubilization of the other two caseins. Calcium caseinate has an isoelectric point of 4.6 pH. As a result, it is insoluble in solutions with a pH lower than 4.6. Casein has a negative charge and is soluble as salt at this pH since milk has a pH of roughly 6.6. The negative charges on the casein micelles' outer surfaces are neutralized when acid is added to the milk, and the neutral protein precipitates, leaving the calcium ions in solution. (1)

(B) Protein solubility is influenced by a variety of factors, including the salt content in the solution. Salt stabilizes the different charged groups on a protein molecule at low concentrations, drawing protein into the solution and increasing protein solubility. This is referred to as "salting-in." A point of maximal protein solubility is frequently attained as the salt concentration is raised. With each rise in salt content, the amount of water available to solubilize protein decreases. Finally, when there aren't enough water molecules to interact with protein molecules, protein precipitates. Salting-out refers to the phenomena of protein precipitation in the presence of excess salt. To induce protein separation and purification by salting-out, a variety of salts have been used. Because of its great solubility and low cost, ammonium sulfate has been the most extensively employed chemical among these salts. Because enzymes are proteins, they may be purified using the same processes as proteins, with the exception that some consideration must be given to the possibility of irreversible activity loss due to denaturation under unfavorable circumstances. (2)

Materials:

(A)

- Milk
- Sodium acetate buffer

- Ethanol
- Ether
- Thermometer
- Centrifuge tubes and centrifuge
- pH meters

(B)

- Test tubes
- Graduated cylinders
- Pipettes
- Balance
- Centrifuge
- Protein solution (hemoglobin)
- Saturated ammonium sulfate solution.

Methods: (A)

1. 25 ml of milk was placed in a 125 ml beaker and warmed to 40°C. In another beaker, 25 ml of the acetate buffer was placed and warmed.
2. When the liquids have warmed, the acetate buffer was slowly added to the milk (dropwise) with stirring.
3. the resulting suspension was cooled to room temperature then left to stand for a further 5 min without disturbing it.

4. the suspension was transferred to a glass centrifuge tube and centrifuged for 5 minutes at 2500 pm.
5. the supernatant was discarded and the precipitate was washed with ~ 10 ml of water.
6. the pellet resuspended and recentrifuged as previously indicated. the supernatant was discarded and washed again with water. Do the water wash 3 times. (WASHI: 3 x water)
7. After the final water wash, the washed protein pellet was resuspended in ~ 20 ml of 95% ethanol. (WASH II: ethanol)
8. the precipitate was centrifuged and washed again this time with a mixture of 10 ml of ethanol and 10 ml of ether. the washed pellet was resuspended and recentrifuged. (WASH III: 50:50 ethanol: ether)
9. the precipitate was washed with 20 ml of ether and centrifuged. (WASH IV: ether)
10. a watch glass was labeled and weighed and the weight was recorded in our notebook.
11. the powder was carefully removed from the centrifuge tube and spread out on a the previously weighed watch glass and the remaining ether was allowed to evaporate overnight.
12. the recovered casein was weighed and the percentage yield of the protein was calculated.

(B)

1. 10 ml of hemoglobin was centrifuged at 3000 rpm for 5 minutes and a clear solution was obtained. the supernatant was used to Blank the spectrophotometer at 577 nm with water and the absorbance of the hemoglobin solution was recorded. This measurement was to be used in the calculation of the recovery of the protein.

2. 4 ml of the hemoglobin solution was pipetted into a centrifuge tube.
3. While continuous stirring, an equal volume of saturated ammonium sulfate solution was added drop-wise to the protein solution until precipitates started to form.
4. the mixture was centrifuged at 10,000 g for 15 minutes. the precipitate was collected by carefully discarding as much supernatant as possible.
5. the original hemoglobin solution was reconstituted by resuspending the precipitate in 4 ml of water.
6. the absorbance of the reconstituted hemoglobin solution was measured at 577 nm with a spectrophotometer, blanking with water.
7. the percent yield and percent error of hemoglobin were calculated.

Data and results:

Part A	
Weight of watch glass (empty)	29.6g
Weight of watch glass (with precipitate)	30.4g
Percentage yield	91.4%
Percentage error	9.4%

Table (1) weights, percentage yield, and percentage error of casein content in milk.

Part B	
The absorbance of pure hemoglobin	0.640nm
The absorbance of reconstituted hemoglobin	0.599nm
Percentage yield	93.6%
Percentage error	3.4%

Table (2) absorbances, percentage yield, and percentage error of hemoglobin content.

Discussion:

In part A, casein, a phosphoprotein was extracted from milk using multiple washes of water, ethanol, and ether. But, before that, the casein was suspended from the milk using a sodium acetate buffer. This makes the micelles on the casein become insoluble in water. The precipitate was then collected and washed with water multiple times, then ethanol and ether were added to aid drying. The results obtained were 0.8g casein/ 25ml of milk. These results were close to that of the theoretical ones written on the nutritional facts of the milk box. Given that the nutritional facts claim that there is 0.875g/25ml of milk. This gives a percentage yield of 91.6% and an error of 9.4%.

In part B, hemoglobin was purified using the salting-out technique. This is known because ammonium sulfate is considered a kosmotrope. This means it's a salt that aggregates protein structure and isolates it in a precipitate. In this part of the experiment, we tested the efficiency of this salt to purify and isolate hemoglobin. This was done by measuring the absorbance of pure hemoglobin and comparing it with the absorbance of reconstituted hemoglobin. The pure hemoglobin had an absorbance of 0.640nm and the reconstituted 0.590nm. This gave a 93.6% yield of hemoglobin after salting-out was done. A percent error of 3.4% was also recognized.

Conclusion:

In conclusion, there are many different methods to extract proteins, and if done precisely, they are efficient and accurate. Proven from our results, the errors combined were not more than 10%. This is excellent considering all the systematic errors that could've happened.

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References:

1. ***McMaster University, M. M. U. (2012). Proteins and Carbohydrates. Isolation of Casein and Lactose from Milk. CHEM2O06 - 1997/98 - experiment 11. Retrieved November 28, 2021, from https://www.chemistry.mcmaster.ca/~chem2o6/labmanual/expt11/2o6exp11.html.***
2. ***Ammoium sulfate precipitation. (n.d.). Retrieved November 28, 2021, from https://user.eng.umd.edu/~nsw/ench485/lab6a.htm.***

Appendix:

Questions:

****. How does your figure compare with the theoretical yield from 25 ml of milk? (Check the milk carton for the protein amount per 100 ml milk).**

- 25ml of Aljuniedi full fat 3% milk would usually yield 3.5g of protein per 100ml, and 0.875g per 25ml. Our results proved that this is 91.4% true being that we had 0.8g casein/25ml. But, this could be due to a systematic error and not an error in the brand's nutritional facts.

(A)

1. **Weight of isolated Casein = (Weight of watch glass + precipitate) - (Weight of empty watch glass)** = (30.4)-(29.6)=0.8g

2. **Theoretical weight of casein per 25 ml of milk = (theoretical weight of casein per 100 ml of milk * 25 ml) / 10**0 = (3.5g*25mL/100mL)= 0.875g

3. **Percentage Yield = Experimental weight of casein/ Theoretical weight of casein *100% =** ((0.8/0.875)*100%)=91.4%

4. **Percentage Error = ((Experiment weight of casein - Theoretical weight of casein|) / Theoretical weight of casein) *100 %** =9.6%

(B)

1. **Percentage Yield = Experimental Absorbance after salting out/ Theoretical absorbance of original Hb solution*100% =** (0.599/0.640)*100 = 93.6%

2. **Percentage Error = ((Experiment Absorbance after salting out - Theoretical absorbance of original Hb solution) / Theoretical absorbance of original Hb solution) *100 % =** ((0.599-0.640)(0.640)*100%= 6.4%