Chromatographic separation of hemoglobin from phenol red

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Chromatographic Separation of Hemoglobin from Phenol Red by Gel Filtration

Introduction

From catalysis to transport, proteins take part in countless cellular processes (Nelson & Cox, 2008). However, due to their small size, proteins can be challenging to work with in the lab. To overcome this hurdle, scientists have developed many fractionation techniques including centrifugation, precipitation, dialysis, and chromatography (Alberts et al., 2008).

Chromatography is one of the most versatile laboratory techniques as it is used in many fields of study (Partridge, 1952; Silva et al., 2014). There are several types of chromatography, and each separates molecules based on different properties. Size-exclusion gel filtration is a type of column chromatography which resolves molecules based on their size (Duong & Gabelli, 2013). In the column, there is a cross-linked polymer matrix with pores of a predetermined size (Scott, 2003). Small molecules are more likely to spend time in the pore, so they migrate slower and thus elute in later fractions (Nelson & Cox, 2008). Conversely, larger molecules are less likely to enter the pore, so they migrate faster and thus elute in earlier fractions (de Jong et al., 2015).

There are numerous types of column matrices, such as Sephadex G (composed of dextran and epichlorohydrin) and Sepharose (consisting of agarose) (GE, 2015). Each matrix has different sized pores and thus different

ranges of molecular sizes that it can separate, called fractionation ranges (de Jong et al., 2015).

In this lab, Sephadex G-25, which has a fractionation range of 1, 000-5, 000Da, was the column matrix (de Jong et al., 2015). Hemoglobin and phenol red were also used. Hemoglobin is a protein found primarily found in mammalian red blood cells, and is used to transport oxygen from an organism’s lungs to its tissues (Seeley, 2014). Phenol red is a chemical compound that changes color depending on the pH of the chemical environment, and so it is typically used as a pH indicator (Klein, 2012). Hemoglobin has a molecular weight of 64, 500Da while phenol red has a molecular weight of 354. 38Da (de Jong et al., 2015). It was expected that hemoglobin would be completely excluded from the pores of the Sephadex G-25 resin, allowing it to elute in early fractions. As well, it was expected that phenol red would be completely included in the gel, making it elute in later fractions.

Purpose

The goal of this lab was to separate a mixture containing hemoglobin and phenol red using size-exclusion gel filtration. As well, the aim was to compare the standard and literature wavelengths of maximum absorption of hemoglobin and phenol red.

Results

The results from this experiment are shown in Table 1 and Table 2.

Table 1. Fraction volumes from size-exclusion gel filtration using Sephadex G25.

Fraction	Volume (mL)
A	5. 5
B	2. 9
C	9. 0
D	14. 0

The size-exclusion gel filtration separated a mixture of hemoglobin and phenol red. In theory, fraction A contained void volume, fraction B contained hemoglobin, fraction C contained the buffer only, and fraction D contained phenol red.

Table 2. Peak absorbance and wavelength of standard hemoglobin and phenol red.

	Maximum absorbance	Corresponding wavelength (nm)
Standard hemoglo	2. 1	410

bin

Standard

phenol 1. 7 560

red

Using a Beckman spectrophotometer, the wavelength profile between 380nm and 600nm of two standard samples was determined. The standard hemoglobin sample contained 250µL of 0. 8% hemoglobin, while the standard phenol red sample consisted of 20µL of 0. 2% phenol red. Based on the graph, the maximum absorbance values and the corresponding wavelengths were determined.

Questions and Research

Question 1:

Search Engine: Web of Science

Search Terms: hemoglobin spectroscopy

Reference: DiFeo, T. J., Addison, A. W., & Stephanos, J. J. (1990). Kinetic and spectroscopic studies of haemoglobin and myoglobin from Urechis caupo. Distal residue effects. *Biochem. J* , *269* , 739-747.

Search Engine: Web of Science

Search Terms: hemoglobin Soret band absorptivity (sorted by relevance)

Reference: Akuwudike, A. R., Chikezie, P. C., & Chilaka, F. C. (2010). Absorption spectra of normal adult and sickle cell haemoglobins treated with hydrogen peroxide at two pH values. *Adv. Biomed. Res* , *1* , 55-60.

Search Engine: Google

Search Terms: phenol red maximum wavelength absorption

Reference: LobaChemie. (2015). Phenol Red. *Laboratory Reagents and Fine Chemicals* . Retrieved February 21, 2015, fromhttp://www. lobachemie. com/pH-Indicator-05180/PHENOL-RED-CASNO-143-74-8. aspx

Based on the graph from Blackboard, the wavelengths of maximum absorbance (λ_{max}) are shown in Table 2. For the standard hemoglobin sample λ_{max} = 410nm, and for the standard phenol red sample λ_{max} = 560nm.

Based on the literature, hemoglobin and related proteins are known to give three bands of maximum absorption: α-band, β-band, and Soret band (DiFeo et al., 1990). For normal adult hemoglobin (HgA), the wavelength of maximum absorbance in the Soret band is λ_{max} = 415nm (Akuwudike et al., 2010).

The wavelengths of maximum absorbance of phenol red changes based on pH. At a pH of 8. 2, phenol red has λ_{max} = 557-560nm while at a pH of 6, phenol red has λ_{max} = 430-435 (LobaChemie, 2015). This change in maximum absorbance with respect to pH is understandable because phenol

red is a common pH indicator, meaning it changes color based on pH (Nelson & Cox, 2008).

The literature and experimental wavelengths of maximum absorbance are summarized in Table 3. The experimental and literature values were fairly close, and their variance may be accounted for due to difference in the buffer and pH of the solvent.

Table 3. Literature and experimentalwavelengths of maximum absorbance.

	Experimental λ_{max} (nm)	Literature λ_{max} (nm)
Standard hemoglobin	410	415
Standard phenol red	560	557-560

The experimental wavelengths of maximum absorbance (λ_{max}) were obtained from Table 2. The literature λ_{max} of hemoglobin (HgA) was based on Akuwudike et al (2010). The literature λ_{max} of phenol red at pH = 8. 2 was based on LobaChemie (2015).

Question 2:

Based on the data collected in this experiment, it is impossible to determine whether the eluted hemoglobin is free of phenol red. To determine its purity, spectroscopy of the eluted hemoglobin (fraction B) would need to be conducted in order to draw a wavelength-vs-absorbance graph between 380nm and 600nm (similar to the one posted on Blackboard). If the eluted hemoglobin curve has only one peak (λ_{max} = 410-415nm), it contains only hemoglobin. However, it the curve has two peaks (λ_{max} = 410-415nm and λ_{max} = 557-5605nm), then the eluted fraction contains both hemoglobin and phenol red.

Question 3:

Search Engine: Web of Science

Search Terms: size-exclusion gel filtration (sorted by relevance)

Reference: Wang, Q., et al. (2005). Molecular characterisation of soybean polysaccharides: an approach by size exclusion chromatography, dynamic and static light scattering methods. *Carbohydrate research* , *340* (17), 2637-2644.

In this experiment, size-exclusion gel filtration was carried out, so hemoglobin and phenol red were separated based on size or more quantitatively based on molecular weight (Nelson & Cox, 2008; Wang et al., 2005). Smaller molecules, such as phenol red, spend more time in the pores and are eluted in later fractions (Nelson & Cox, 2008). Conversely, larger molecules molecules, such as hemoglobin, spend almost no time in the pores and are eluted in earlier fractions (Nelson & Cox, 2008).

Question 4:

Search Engine: Web of Science

Search Terms: chromatography theory (sorted by relevance)

Reference: Lee, W. C., Tsai, G. J., & Tsao, G. T. (1993). Analysis of chromatography by plate theory. *Separations Technology* , *3* (4), 178-197.

Search Engine: PubMed

Search Terms: chromatography theory column length (sorted by relevance)

Reference: Bedani, F., et al. (2006). A theoretical basis for parameter selection and instrument design in comprehensive size-exclusion chromatography× liquid chromatography. *Journal of Chromatography A* , *1133* (1), 126-134.

Based on the plate theory of chromatography, the resolution (R) is influenced by numerous factors including the plate number (N) (Lee et al., 1993). The plate number is affected by many elements of the column including column length and inner column radius (Bedani et al., 2006). Based on mathematical relationships, it has been determined that longer columns lead to increased resolution (Lee et al., 1993; Nelson & Cox, 2008). Thus longer chromatography columns will separate molecules better than shorter columns.

Question 5:

In order to separate β1, 3-galactosidase (75, 000Da) and β1, 2-xylosidase (MW 100, 000Da) the Sephadex G-100 resin would be ideal. This is because the molecular weight of both enzymes is within the fractionation range of this resin (4, 000-150, 000Da) (de Jong et al., 2015). The heavier protein (β1, 2-xylosidase) will elute before the lighter protein (β1, 3-galactosidase).

Question 6:

The fractionation range of Sephadex G-100 is 4, 000-150, 000Da (de Jong et al., 2015). Alcohol dehydrogenase (MW 140, 000Da) falls within this range, while aldehyde dehydrogenase (MW 200, 000Da) is above the range. Since both enzymes are not on the same extreme of the fractionation range, aldehyde dehydrogenase will elute very quickly and alcohol dehydrogenase will elute shortly after. Thus, Sephadex G-100 can be used to resolve alcohol dehydrogenase and aldehyde dehydrogenase. However, it would be ideal to use Sephadex G-200 because the molecular weight of both enzymes would be within the fractionation range of 5, 000-600, 000Da (de Jong et al., 2015).

Question 7:

Search Engine: PubMed

Search Terms: “ fast protein liquid chromatography” (sorted by relevance)

Reference: Sheehan, D., & O’Sullivan, S. (2004). Fast protein liquid chromatography. In *Protein purification protocols* (pp. 253-258). Humana Press.

Search Engine: PubMed

Search Terms: “ fast protein liquid chromatography” (sorted by relevance)

Reference: Goke, B., & Keim, V. (1992). HPLC and FPLC. Recent progress in the use of automated chromatography systems for resolution of pancreatic secretory proteins. *International Journal of Pancreatology* , *11* (2), 109-116.

Search Engine: PubMed

Search Terms: “ fast protein liquid chromatography” (sorted by relevance)

Reference: Lavanya, G., et al. (2011). Protein Chromatography. *J Biomed Sci and Res, 3* (3), 424-438.

Search Engine: Google

Search Terms: fast protein liquid chromatography

Reference: Bio-Rad. (2015). Fast Protein Liquid Chromatography. *Bio-Rad Life Science Research* . Retrieved February 22, 2015, fromhttp://www. bio-rad. com/en-ca/applications-technologies/fast-protein-liquid-chromatography

Search Engine: PubMed

Search Terms: fast protein liquid chromatography use

Reference: Verbeke, K., & Verbruggen, A. (1996). Usefulness of fast protein liquid chromatography as an alternative to high performance liquid chromatography of 99m Tc-labelled human serum albumin preparations. *Journal of pharmaceutical and biomedical analysis* , *14* (8), 1209-1213.

Fast protein liquid chromatography (FPLC) is an automated chromatography technique that uses columns with a small inner diameter (Goke & Keim, 1992; Sheehan & O'Sullivan, 2004). By means of pumps and valves, the FPLC system regulates how fast the liquid mobile phase moves down the agarose stationary phase a constant, and maintains a constant flow rate (Lavanya et al., 2011). As well, based on the readings of the UV detector and conductivity meter, the FPLC system is able to control how and when the eluted fractions should be separated (Bio-Rad, 2015). There are variations of FPLC that allow proteins to separated base on size, charge or hydrophobicity (Verbeke & Verbruggen, 1996).

FPLC, as its name suggests is quite useful in analyzing and purifying protein mixtures (Lavanya et al., 2011). For example, FPLC has been used to identify zinc-containing proteins in rat saliva; and it has also been used to separate and analyze pancreatic juice in order to examine disease-causing proteins (Lavanya et al., 2011).

Conclusion

In this lab, a mixture of hemoglobin and phenol red was resolved using size–exclusion gel filtration. Since Sephadex G-25 (fractionation range 1, 000-4, 000Da) was used as the column matrix, it was expected that hemoglobin would be completely excluded from the matrix pores, allowing it to elute in early fractions. As well, it was anticipated that phenol red would be completely included in the gel, making it elute in later fractions.

The hemoglobin/phenol red mixture was separated into four fractions and their respected volumes are shown in Table 1. In theory, fraction A contained

void volume, fraction B contained hemoglobin, fraction C contained the buffer only, and fraction D contained phenol red. Due to time and budget constraints, the absorbance of the eluted fractions was not analyzed. However based on an absorbance-vs-wavelength graph, the wavelengths of maximum absorbance (λ_{max}) for standard hemoglobin (λ_{max} = 410nm) and standard phenol red (λ_{max} = 560nm) were determined, as described in Table 2. These values were also compared to literature values, as shown in Table 3. Normal adult hemoglobin (HgA) is known to have maximum absorbance at λ_{max} = 415nm, while phenol red is known to have λ_{max} = 557-560nm. The experimental and literature values were fairly close to one another, and their slight variance may be accounted for due to difference in the buffer used and pH of the solvent.

For further research, spectroscopy of the eluted hemoglobin (fraction B) would need to be conducted in order to draw a wavelength-vs-absorbance graph between 380nm and 600nm. This would help to clarify whether the eluted hemoglobin is free of phenol red.

References

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Wang, Q., et al. (2005). Molecular characterisation of soybean polysaccharides: an approach by size exclusion chromatography, dynamic and static light scattering methods. *Carbohydrate research* , *340* (17), 2637-2644.