# Demonstration of Size-Based Separation of Molecules by Gel Chromatography: An Exercise for Biology Beginners

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**Abstract:** An introductory laboratory exercise has been designed for biology beginners to visualize and analyze the chromatographic separation of a mixture of blue dextran, α-chymotrypsinogen (protein) and potassium ferricyanide on a Sephadex G-75 column (60×1.0 cm). Separation of the two coloured components *i.e.* blue dextran (blue colour), α-chymotrypsinogen (colourless) and potassium ferricyanide (yellow colour) of a green-coloured mixture can be visually seen in the form of blue- and yellow-coloured bands distant by a colourless zone. The elution volumes of different components in the mixture were found similar to the elution volumes of these components, when loaded individually onto the same column. Such demonstration of separation of different components in a mixture on a gel chromatographic column is an interesting exercise for biology beginners (undergraduate students) to learn separation technique on the basis of size.
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## 1. Introduction

Protein separation techniques have been important since the 1940s in order to understand structure-function relationship of proteins. Now these techniques have become the integral part of biochemistry and biotechnology curricula as protein is the translational product in molecular biology experiments. Even biological researches have reached to an advanced level involving some of the biochemical techniques. Therefore, practical exercises on biochemical techniques have moved from specialized graduate courses to undergraduate biochemistry and a few biology programs for the entry level students. In order to move with the growing scientific pace, it is important to keep one abreast of these biochemical techniques. To include a laboratory separation technique in the undergraduate biology program, it is required that the laboratory exercise should be relatively inexpensive, require minimum instrumentation (equipment) and simple to handle.

Gel chromatography, also known as gel filtration, molecular sieve chromatography, gel permeation chromatography or size exclusion chromatography is one of several biochemical techniques used to isolate and purify a protein from a given mixture. The principle of separation is based on the difference in the size (molecular weight) of different biomolecules. The stationary phase used in gel chromatography is porous gel particles/beads which allow access to different sized molecules to different extents. Molecules, which are able to completely access these gel particles are retained in the stationary phase and elute later from the chromatographic column. On the other hand, molecules bigger than the pore size of the gel particles do not enter the gel beads and elute earlier. Therefore, separation of molecules differing in size takes place on the chromatographic column in such a way that those bigger molecules elute faster followed by smaller molecules. In other words, the elution volume of a molecule on a gel chromatographic column is inversely proportional to the size of the molecule.

Although a number of exercises on gel chromatography are available in the literature (Wallach, 1982; Versee, 1985; Dixon, 1985; Malhotra and Kumar, 1989; Rowe, 1993; Davis and Brunauer, 2008), they might be either difficult to follow or practice independently by biology beginners. The following laboratory exercise may be a good alternative for inclusion in the undergraduate biology curriculum in order to give a proper understanding of the gel chromatographic technique to these students. The approach is intended to be general but emphasis has been given to visualize the separation of coloured molecules in the given mixture. The students can prepare their own gel chromatographic column, learn column packing and equilibration, load the sample, collect and monitor the fractions, plot the elution profile and analyze their results (active learning about separation of molecules on the basis of size). They can work in pairs but each of them should be able to perform the experiment at

least once. This exercise can be completed in three days with three hours laboratory sessions. The whole exercise is comprised of three parts: (i) packing and equilibration of the gel chromatographic column, (ii) sample loading and elution of different components of the given mixture and (iii) elution of individual components from the same column and analysis of results. Although this laboratory exercise is not new and has been used since the 1960s, use of coloured mixture and visual separation of two coloured and one colourless component in the mixture will surely add to students' learning about size-based molecular separation through this technique. For students without any biochemistry/ biotechnology background, it would be necessary to use a visual mode for demonstrating the principle of a biochemical technique to make it more understandable.

## 2. Materials and Methods

### Chemicals

Sephadex G-75, α-chymotrypsinogen, blue dextran and potassium ferricyanide were procured from Sigma-Aldrich Inc., USA. All other chemicals used in this study were of analytical grade.

### Absorbance measurements

The concentrations of blue dextran, protein and potassium ferricyanide were determined by measuring the absorbance at 540, 280 and 420 nm, respectively, using Thermospectronic Genesys 10 UV spectrophotometer.

### Preparation of a gel chromatographic column

Sephadex G-75 powder form (5 g) was allowed to swell in 200 ml of water at 90°C for 3 h as recommended by the manufacturer. Fines were removed by repeated decantation before packing of the gel into the column. A glass burette was mounted onto a table in a vertical position with the help of an iron stand with two clamps. The radius (r) of the glass burette was determined at three different places along the height of the burette by collecting a known volume of water of 2 cm height (h) in the burette. The volume (V) of the collected water was taken as equal to the volume of a cylinder and the radius was obtained by substituting the values of V, π (3.14) and h (2 cm) into the formula, $V= \pi r^2 h$. The lower end of the burette received a disc of glass wool previously boiled in water and its surface was covered by a few glass beads. The burette was filled with buffer [0.02M sodium phosphate buffer, pH 7.0 containing 0.15M NaCl (PBS)] up to one fourth of its height and the gel slurry was poured slowly into the column with the help of a glass rod in a single operation. The gel was left for 1 h to settle under gravity and the outlet was opened slowly with a flow rate of 5 ml/h. The flow rate was increased gradually after the gel settled down. Three bed volumes of the buffer (PBS) were passed through the column at a flow rate of 40 ml/h to equilibrate and stabilize the gel bed. The column is supposed to be stable if there is no change in the gel length during operation.

### Sample application and elution

Before application of the sample, most of the buffer above the gel surface was removed and the outlet was closed. The sample containing mixture of blue dextran (3 mg), α-chymotrypsinogen (6 mg) and potassium ferricyanide (2 mg) or individual components in 1 ml of PBS were layered gently on top of the gel bed with the help of a micropipette and allowed to drain into the bed by slowly opening the outlet. Once the sample had passed into the gel, 1 ml of PBS was applied in the same way at least two times and finally connected to a reservoir containing PBS. The elution was performed with a constant flow rate (30 ml/h) and fractions of 2 ml size were collected in tubes. Absorbance was recorded at 540 nm for blue dextran (blue-coloured solution), 280 nm for protein (colourless solution) and 420 nm for potassium ferricyanide (yellow-coloured solution). Absorbance values were plotted against the elution volume to get the elution profile(s) of the given sample(s). The volume required to elute the component at its maximum elution (peak position) was taken as the elution volume of the component.

## 3. Results and Discussion

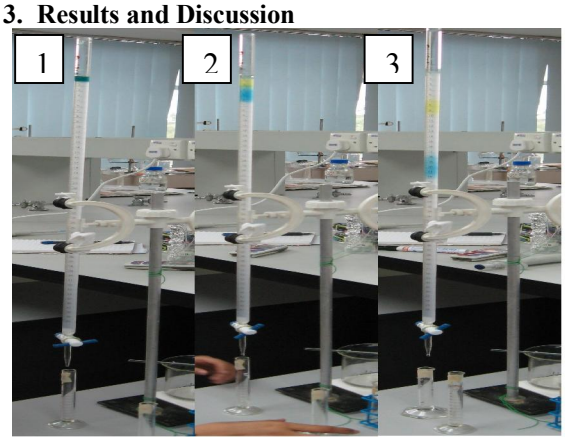


**Figure 1. Separation of various components of a given coloured mixture on a Sephadex G-75 column (60 ×1.0 cm).** The green-coloured mixture (3 mg blue dextran + 6 mg α-chymotrypsinogen + 2 mg potassium ferricyanide) in 1 ml of 0.02M sodium phosphate buffer, pH 7.0 containing 0.15M NaCl was applied onto the column and the elution was performed at a flow rate of 30 ml/h. Lane 1 shows photograph taken soon after loading the green-coloured sample. Lanes 2 and 3 show photographs taken 5 min and 20 min, respectively after application of the sample.

Figure 1 shows visual chromatographic separation of different components of the mixture (green in colour) containing blue dextran (blue in colour), α-chymotrypsinogen (colourless) and potassium ferricyanide (yellow in colour) on a Sephadex G-75 column. The first lane shows the sample (green-coloured mixture), when loaded onto the column. Lane 2 shows separation of different components of the mixture into distinct blue- and yellow-coloured bands. The blue-coloured band represented blue dextran while potassium ferricyanide band was of yellow colour. It is important to note that the colourless, α-chymotrypsinogen band was yet to be seen as separation was incomplete. Being bigger in size with very high molecular weight ($2 \times 10^6$), blue dextran was completely excluded by Sephadex G-75 gel particles and moved faster through interstitial spaces available in the column. On the other hand, potassium ferricyanide, being a low molecular weight (329) compound had both the inner spaces and interstitial spaces of the gel column available to it and therefore, took longer time to pass through the gel column. Lane 3 shows a very clear visual demonstration of the separation of two coloured components of the mixture, as they were seen to be well separated and far apart from each other on the column. The middle colourless zone between the blue- and the yellow-coloured bands was the protein, α-chymotrypsinogen with a molecular weight of 25000. Elution of these components from the column in different fractions also reflected their separation from each other as fractions collected very early were of blue colour representing elution of blue dextran, followed by colourless fractions of the protein, α-chymotrypsinogen and finally yellow-coloured fractions of potassium ferricyanide. Therefore, visual monitoring of coloured bands on a column helps students to understand the principle of gel chromatography involving separation of the molecules based on their size.

Figure 2 shows elution profiles (absorbance versus elution volume) of three components of the mixture, when monitored at 540, 280 and 420 nm for blue-coloured, colourless and yellow-coloured fractions, respectively. Peak A represents elution profile of blue dextran, monitored at 540 nm with an elution volume of 22 ml. Elution volume of the blue dextran represented void volume ($V_o$) of the column, as it was completely excluded by all the gel particles, resulting its elution with the interstitial volume (void volume) of the column. The protein, α-chymotrypsinogen eluted right after the blue dextran peak with an elution volume of 30 ml, when monitored at 280 nm (Peak B). This is understandable as its molecular weight (25, 000) lies between blue dextran and potassium ferricyanide. Therefore, some of the inner spaces and all interstitial spaces of the gel column would have been available to it. The last peak (Peak C) eluted from the column showed elution of potassium ferricyanide, when monitored for absorbance at 420 nm. Its elution volume (50 ml) was equal to the sum of the void volume ($V_o$) and the inner volume ($V_i$) of the column, as both the interstitial spaces and inner spaces of the gel column were available to it. Therefore, subtracting the void volume (22 ml) from the elution volume of potassium ferricyanide (50 ml) yielded the value of the inner volume (28 ml) of the column. Thus, all the three components of the mixture, differing in their molecular weights, were successfully separated by this column.

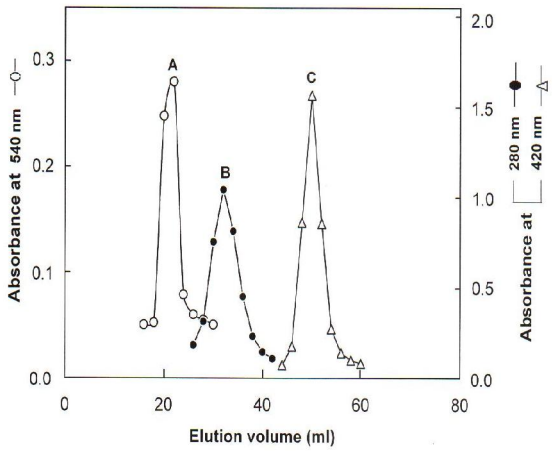


**Figure 2. Elution profile of the mixture containing blue dextran, α-chymotrypsinogen and potassium ferricyanide on Sephadex G-75 column (60 ×1.0 cm).** The column was monitored for blue dextran (fraction number 8-15) at 540 nm (A), α-chymotrypsinogen (fraction number 13-21) at 280 nm (B) and potassium ferricyanide (fraction number 22-30) at 420 nm (C).

To further check the elution behaviour of these components present in the mixture on Sephadex G-75 column (60 ×1.0 cm), these components were passed through the same column individually. The elution profiles of blue dextran, α-chymotrypsinogen and potassium ferricyanide are shown in Figure 3 A, B and C respectively. As can be seen from the figure, all these components eluted from the column in the form of a single symmetrical peak. Furthermore, their elution volumes (22 ml for blue dextran, 30 ml for α-chymotrypsinogen and 50 ml for potassium ferricyanide) were also found to be similar to those obtained from the mixture (Figure 2).

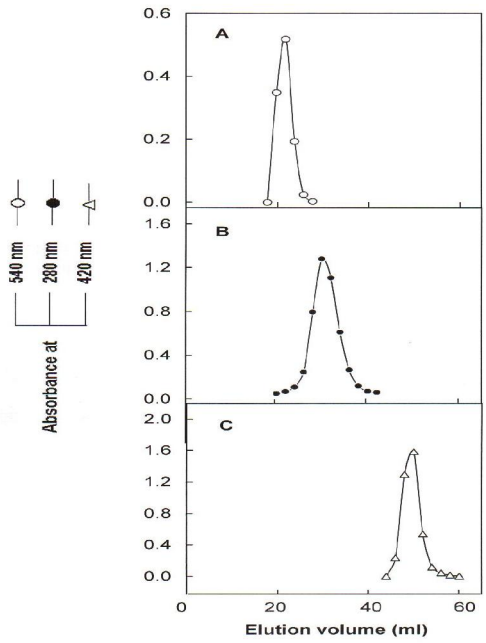


**Figure 3. Elution profiles of blue dextran (A), α-chymotrypsinogen (B) and potassium ferricyanide (C) on Sephadex G-75 column (60 ×1.0 cm).** Sample size and other conditions were the same as described in 'Materials and Methods' section.

In order to evaluate the impact of this exercise in the context of active learning, a questionnaire (Table 1) stating several queries about the exercise can be made and distributed among biology students. It was done with our biochemistry II year students and found that >95% students answered all queries in affirmative. This was indicative of getting the clear understanding and verification of the theoretical principle of a biochemical technique through this exercise.

In conclusion, the exercise described here is a simple visual method for the introduction of gel chromatographic technique for biology students at undergraduate level. The technique is useful in being simple, economical, independent of the use of any advanced instrumentation and interesting to visualize the separation of molecules. Similar kind of exercises can be developed to teach other chromatographic techniques to these students using coloured mixtures.

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**Table 1: Questionnaire about gel chromatographic technique**

| S. No. | Question |
|---|---|
| 1. | Were you eager to see the inclusion of this separation technique in your practical curriculum? |
| 2. | Were you excited to see the separation of different sized components on a column with your naked eyes? |
| 3. | Did you find it easy to repeat the experiment yourself without the help of anybody? |
| 4. | Did you find the exercise good enough to see the verification of theoretical knowledge about gel chromatography? |
| 5. | Did you feel more educative about the principle of gel chromatographic technique compared to your theoretical knowledge? |
| 6. | Can you teach your younger colleagues about gel chromatographic technique with greater confidence? |
| 7. | Would you like to include similar type of exercises in your practical curriculum, if you are appointed as a teacher in any university? |
| 8. | Do you think that this exercise has increased your interest and motivated you in this field of science? |
| 9. | Out of several exercises in your practical curriculum, would you like to place this exercise in the preferential pool? |

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