Sultan Qaboos University

College of Medicine & Health Science

Instrumentation and Biomedical Techniques

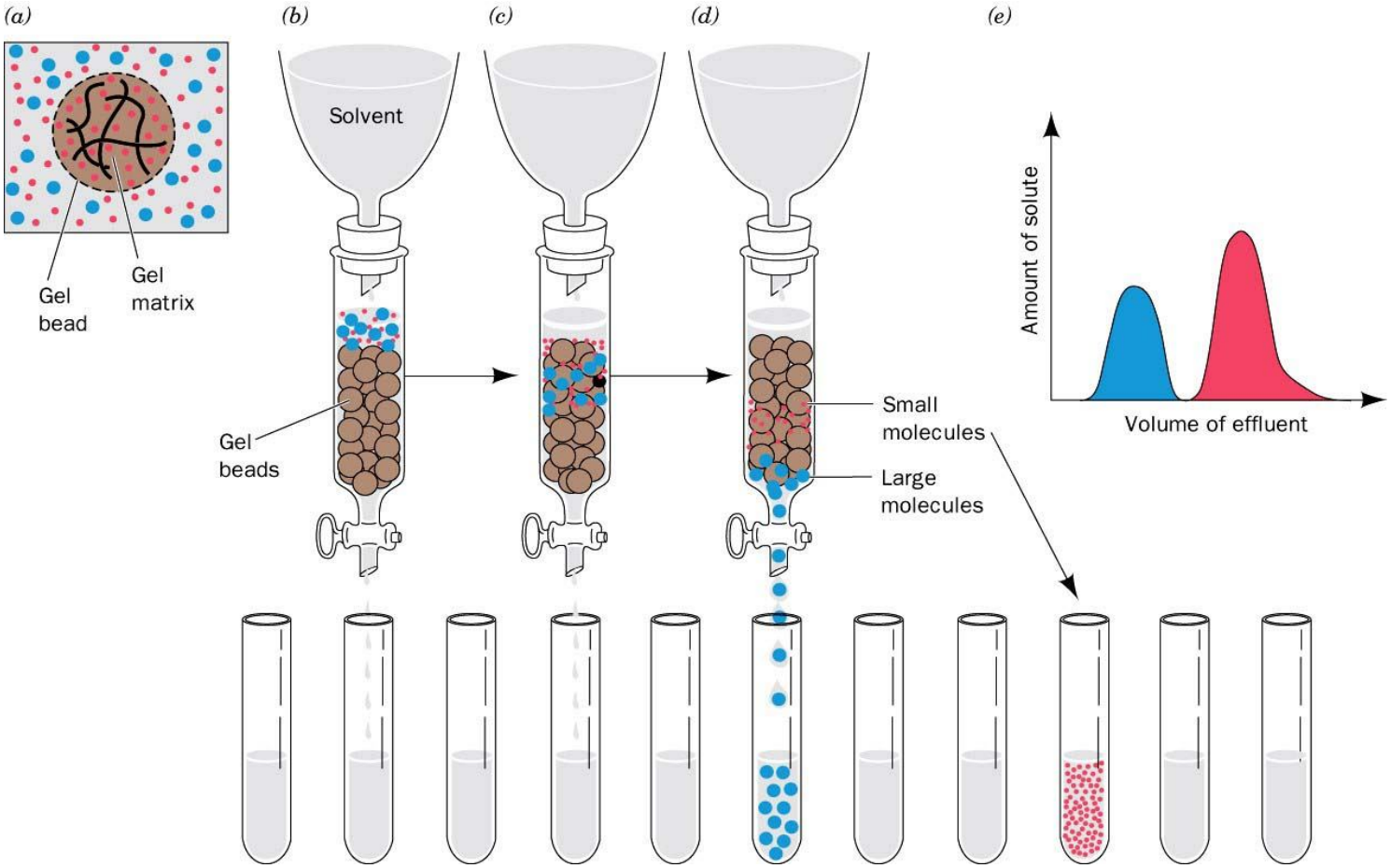
BIOC 6002

Laboratory Report

Practical No. (4+5): 16/10/2011 and 23/10/2011

Gel Filtration Chromatography

(Molecular Weight Determination)



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Gel permeation chromatography is also known as gel filtration and molecular weight size exclusion chromatography. The gel structure contains pores of varying diameter up to a maximum size. The test molecules are washed through a column of the gel and molecules larger than the largest pores in the gel are excluded from the gel structure. Smaller molecules, however, penetrate the gel to a varying extent depending upon their size and this retards their progress through the column. Elution, therefore, is in order of decreasing size. *See diagram below:*

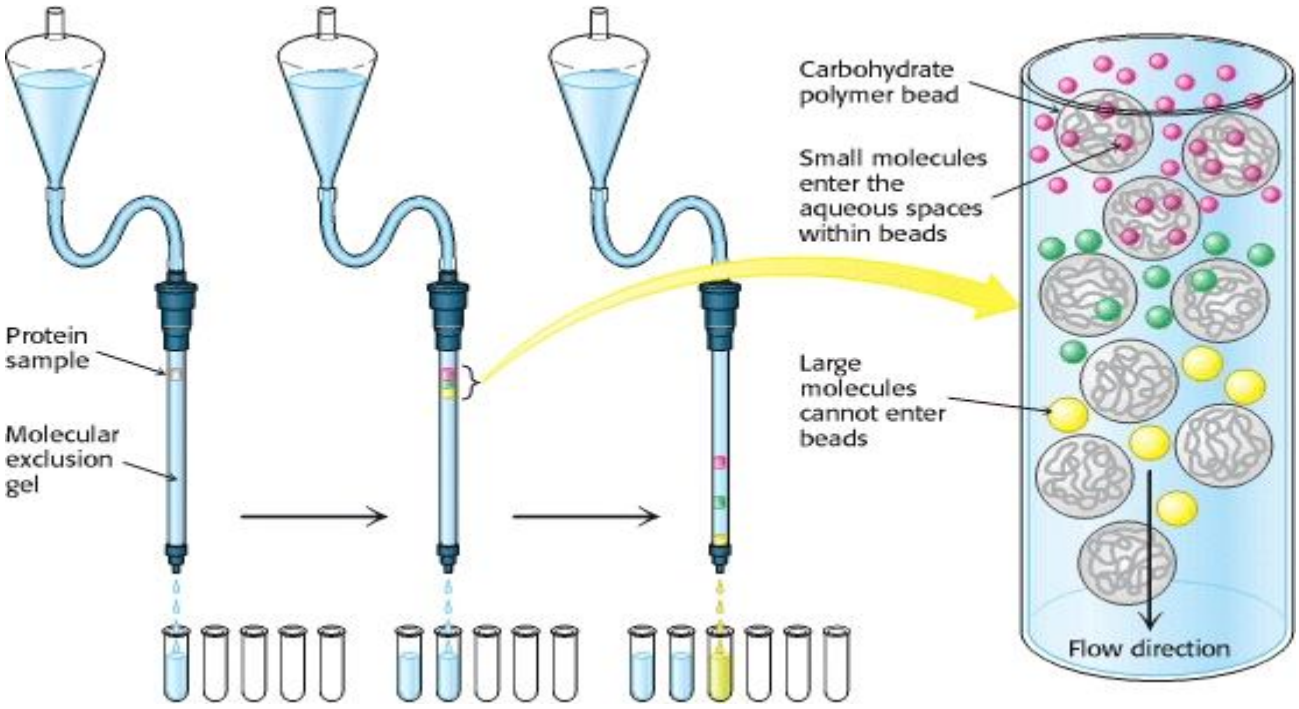


Diagram (1): Principle of Gel Filtration Chromatography

❖ **Aim:**

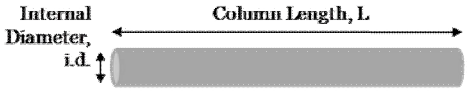
In this practical session, we have filtered out a color-dye dextran (sample size = 1-2% of total gel bed volume) through gel filtration chromatography and **void volume** of column been calculated. Moreover, we have collected **2.5mL fractions** of separated dye and then analyzed in spectrophotometer and absorbance been measured at 620nm. *V_0 is determined*.

After equilibration of the column, we have again applied a mixture of proteins into the gel filtration chromatography. **104 fractions** have been collected and analyzed by using UV-spectrophotometer at 280 nm wavelength. Result interpreted in the results section.

In second week we have separated our egg white isolated from our previous laboratory sessions **F_0(X50)** by gel filtration chromatography. Observed peaks activity in the fractions collected been measured using cell wall suspension with isolate lysozyme.

❖ **Materials:**

1. Gel filtration chromatography column
2. Elution buffer (0.05 M Sodium phosphate buffer, 0.15 M NaCl, pH 7.0) – ionic strength > 0.1 suggested, *already prepared and made by the staff.*
3. Sephacryl S-100 HR used by our colleagues and *we have used* ***Sephacryl S-300 HR***
4. 40 cm X 1.6 cm column been used by us. (What is total column volume?)



Formula to measure total column volume (bed volume) is = $\pi\, r^2 (l)$

Since the diameter (2r) = 1.6 cm then r =0.8 cm and r^2 = 0.64 cm^2

Length of the column measured by us = 26.8 cm + 3.5 cm

= 30.3 cm

Then the bed column volume (V_t) = π x (0.64 cm^2) x 30.3 cm

= 60.92 cm^3 = 60.92 mL (1L = 1 dm^3 = 10^3 mL = 10^3 cm^3)

5. Tubes, pumps, fraction collectors, tube-stopper, glassware, and plasticware
6. Spectrophotometer and UV- Spectrophotometer
7. Excel Microsoft Office 2007 program to plot the curves and calculators
8. Floppy disc (advised to change it into USB at least)
9. Ruler and pipettes
10. Dextran blue dye (2000) and mixture of Ribonuclease A (Mr =13700), Chymotrypsinogen A (Mr = 25000), Ovalbumin (Mr=43000) and Albumin (Mr=67000)

(Egg white lysozyme analysis)

1. GFC elution buffer as previously prepared
2. Lyophilized *M. leisodeikticus* cell wall fragments
3. 0.1 M sodium phosphate buffer pH =6.2
4. Commercial, purified lysozyme as standard (this was done by tutors)
5. 4 mL plastic cuvettes

❖ Methodology:

1. Sephacryl S-300-Column was ready to used and prepared with elution buffer sodium phosphate 0.15M. flow rate was adjusted to 1.6 mL/min (not 0.8 mL/min as suppose to be)

2. It will take about one day to settle the beads by gravity and to be equilibrated with desire buffer and follow rate. Be careful not formed air bubbles in the column rod and it should not be dried as well

3. We prepared solution of blue dextran 2000 dye (1.0 mg/mL) in the eluted buffer (1.2 mL of the dye is needed for application)

4. We stopped the flow rate by switching off the pump and then we opened the top valve cover of the column by removing all connected tubes

5. Elution buffer been sucked out with a pipette till we reach the top of the bead suspension. By carefully not to disrupt the surface and also not to cause column to be dried

6. Dextran solution been applied immediately (sample size = 2% of total gel bed volume) = 1.2 mL and we waited for 2 minutes then we fill up to the top of the column with elution buffer

7. Column been tighten out again with tubes, valves and pump started again. Flow rate been checked again to see wither its constant or not (1.6 mL/min).

8. Immediately when the pump switched ON, we have started to collect 12 fractions of dextran dye (2.5 mL/fraction) and we have seen the migration of dye through the column

9. Absorbance at 620 nm been measured for all fractions with spectrophotometer to determine (Ve) = (V0)

10. After we have calculated the void volume, we equilibrated our column to run with elution buffer for some time again

11. We have prepared our protein mixture (calibration kit protein in elution buffer of volume 1-2% of the total gel bed volume Vt)

12. Flow rate stopped and valve opened and excess buffer volume been sucked out carefully with pipette and mixture (1.2 mL) applied immediately

13. Tubes and valve been tighten again after filling the column again with buffer

14. Flow rate applied and check immediately. 104 Fractions been collected manually! Due to fractionation analyser was not working! tough duty – strong arm muscle required

15. Fractions been measured at 280 nm by using UV-spectrophotometer and column been wash out with elution buffer with 500 mL

(Egg white lysozyme analysis)

1. Previous F_0 (X50) sample fraction been used to run it in gel filtration chromatography. We have already equilibrate the column with buffer

2. Sodium phosphate buffer been prepared pH 6.2 and Molarity = 0.1M for cell suspension preparations

3. We adjusted this time the flow rate **at 0.8 mL/minute** (not 1.6 mL/minute)

4. We stopped the flow rate by switching off the pump and open the top valve cover of the column by removing tubes

5. Elution buffer been sucked out with a pipette till we reach the top of the bead suspension. By carefully not to disrupt the surface and also not to cause column to be dried

6. Then we applied 2.5 mL of our F_0 solution into GFC

7. Then we return the system its dynamic movement by eluting buffer at 0.8 mL/minute this time

8. Fractions been collected by using fractionation analyser which saved a lot of time and absorbance been recorded for all at 280 nm by using UV-spectrophotometer and graph plotted

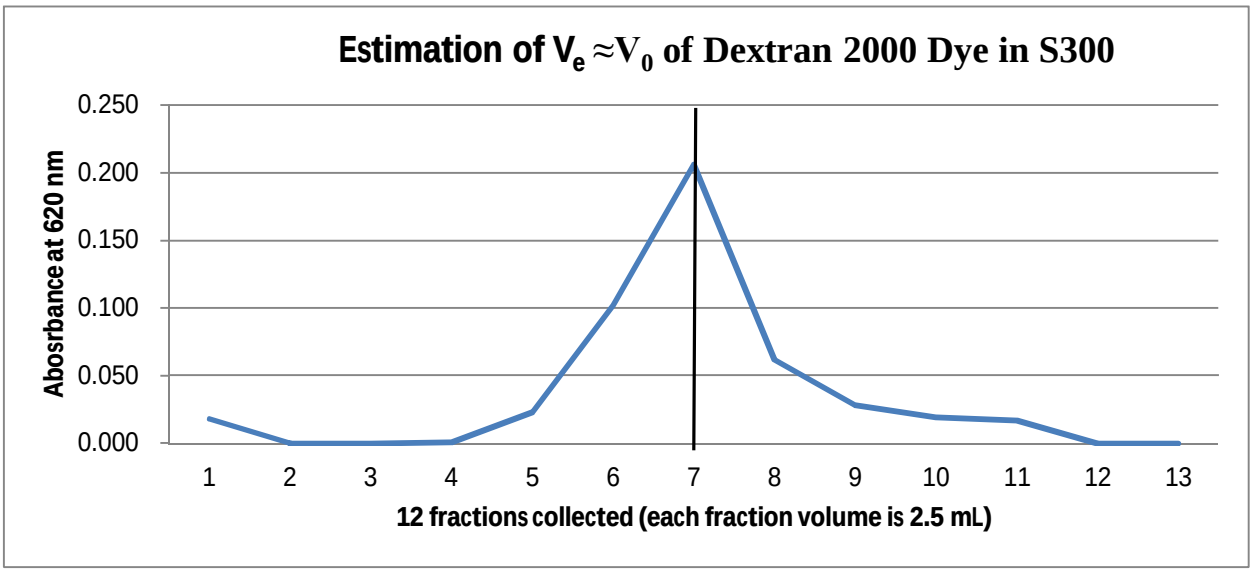
9. After we have identified the peaks we measured their cell wall lytic activity to see wither these fractions contain lysozyme

16. F_0 (X50)-Neat also been measured together with isolated peaks from GFC

❖ **Results, Calculations & Interpretation:**

Fraction Numbers	Volume contained (mL)	Absorbance at 620 nm
1	2.5 mL	0.018
2	2.5 mL	0.000
3	2.5 mL	0.000
4	2.5 mL	0.001
5	2.5 mL	0.023
6	2.5 mL	0.102
7	2.5 mL	0.206
8	2.5 mL	0.062
9	2.5 mL	0.028
10	2.5 mL	0.019
11	2.5 mL	0.017
12	2.5 mL	0.000

Table (1): Fractionation of Dextran 2000 Dye to estimate $V_e \approx V_0$



Graph (1): Shows that fraction number 7 has the highest peak value

Thus V_0 = 7 x 2.5 mL =**17.5 mL**

Then $V_{gel} = V_t - V_0$

$= 60.92 \text{ mL} - 17.5 \text{ mL}$

43.42 mL of gel bead suspension used in this practical

And thus (V_s or V_i) = V_t - V_0 - V_{gel}

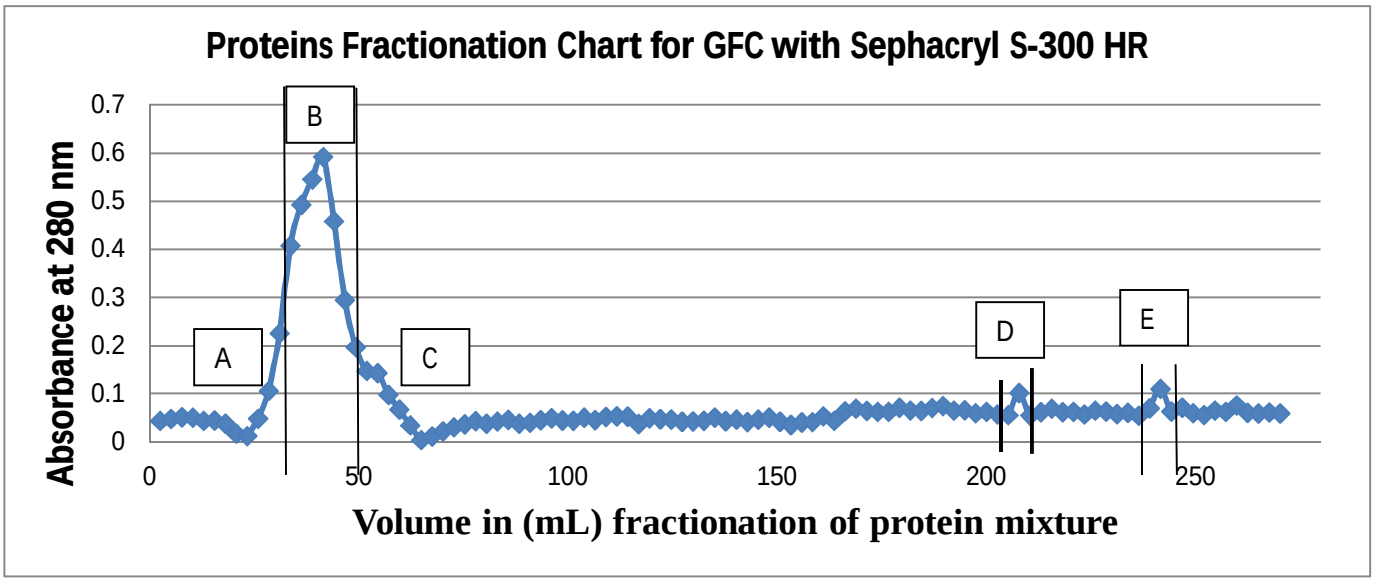
= 0.000 difficult to measure!

Serial Number	Each fraction Volume in mL	Cumulative volume (mL)	Absorbance
1	2.5	2.5	0.0448
2	2.5	5.1	0.0491
3	2.5	7.7	0.0526
4	2.5	10.3	0.0515
5	2.5	12.9	0.0448
6	2.5	15.5	0.0457
7	2.5	18.1	0.0392
8	2.5	20.7	0.0182
9	2.5	23.3	0.0134
10	2.5	25.9	0.0494
11	2.5	28.5	0.1074
12	2.5	31.1	0.2268
13	2.5	33.7	0.4084
14	2.5	36.3	0.4935
15	2.5	38.9	0.5465
16	2.5	41.5	0.5931
17	2.5	44.1	0.4596
18	2.5	46.7	0.2957
19	2.5	49.3	0.198
20	2.5	51.9	0.1486
21	2.5	54.5	0.1443
22	2.5	57.1	0.0991
23	2.5	59.7	0.0684
24	2.5	62.3	0.0356
25	2.5	64.9	0.005
26	2.5	67.5	0.0127
27	2.5	70.1	0.0234
28	2.5	72.7	0.0316
29	2.5	75.3	0.0378
30	2.5	77.9	0.0443
31	2.5	80.5	0.0393
32	2.5	83.1	0.0436
33	2.5	85.7	0.0472
34	2.5	88.3	0.0391
35	2.5	90.9	0.0411
36	2.5	93.5	0.0464
37	2.5	96.1	0.0504
38	2.5	98.7	0.0458
39	2.5	101.3	0.0455
40	2.5	103.9	0.0515
41	2.5	106.5	0.0468
42	2.5	109.1	0.0533
43	2.5	111.7	0.0544
44	2.5	114.3	0.054

45	2.5	116.9	0.0385
46	2.5	119.5	0.0507
47	2.5	122.1	0.0483
48	2.5	124.7	0.0477
49	2.5	127.3	0.0432
50	2.5	129.9	0.0441
51	2.5	132.5	0.0454
52	2.5	135.1	0.052
53	2.5	137.7	0.0447
54	2.5	140.3	0.0475
55	2.5	142.9	0.0427
56	2.5	145.5	0.0475
57	2.5	148.1	0.0519
58	2.5	150.7	0.0433
59	2.5	153.3	0.0364
60	2.5	155.9	0.042
61	2.5	158.5	0.0428
62	2.5	161.1	0.0543
63	2.5	163.7	0.0448
64	2.5	166.3	0.0651
65	2.5	168.9	0.0703
66	2.5	171.5	0.0658
67	2.5	174.1	0.0638
68	2.5	176.7	0.0636
69	2.5	179.3	0.0725
70	2.5	181.9	0.0667
71	2.5	184.5	0.0657
72	2.5	187.1	0.0721
73	2.5	189.7	0.076
74	2.5	192.3	0.0664
75	2.5	194.9	0.0669
76	2.5	197.5	0.0607
77	2.5	200.1	0.0636
78	2.5	202.7	0.0588
79	2.5	205.3	0.0569
80	2.5	207.9	0.1029
81	2.5	210.5	0.056
82	2.5	213.1	0.063
83	2.5	215.7	0.0702
84	2.5	218.3	0.0632
85	2.5	220.9	0.0638
86	2.5	223.5	0.0585
87	2.5	226.1	0.066
88	2.5	228.7	0.0646
89	2.5	231.3	0.0592
90	2.5	233.9	0.0618
91	2.5	236.5	0.0558
92	2.5	239.1	0.0712
93	2.5	241.7	0.1112
94	2.5	244.3	0.0646

95	2.5	246.9	0.0726
96	2.5	249.5	0.0608
97	2.5	252.1	0.0574
98	2.5	254.7	0.0664
99	2.5	257.3	0.0636
100	2.5	259.9	0.0774
101	2.5	262.5	0.0618
102	2.5	265.1	0.0602
103	2.5	267.7	0.0624
104	2.5	270.3	0.0602

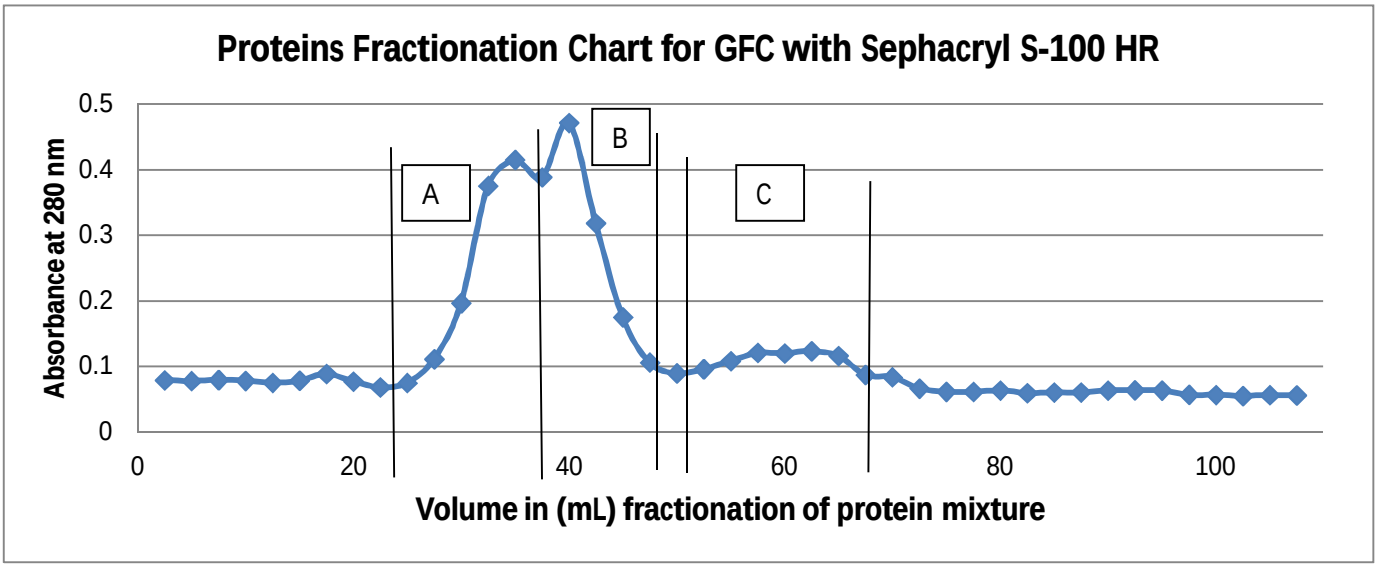
Table (2): Fractionation of protein mixture



Graph (2): Shows peaks for different proteins in GFC

Peaks Determination	V_e (in mL)	K_{av}	**Log_{10} (Mr)**
A-Albumin	36.3	0.49286	4.8
B-Ovalbumin	41.5	0.552741	4.6
C-Chymotrypsinogen A	51.9	0.792262	4.3
D-Ribonuclease A	207.9	4.385076	4.1
E? = Unknown Peak	241.7	5.163519	-

Table (3): K_{av} values for calibration proteins run on Sephacryl S 300 gel



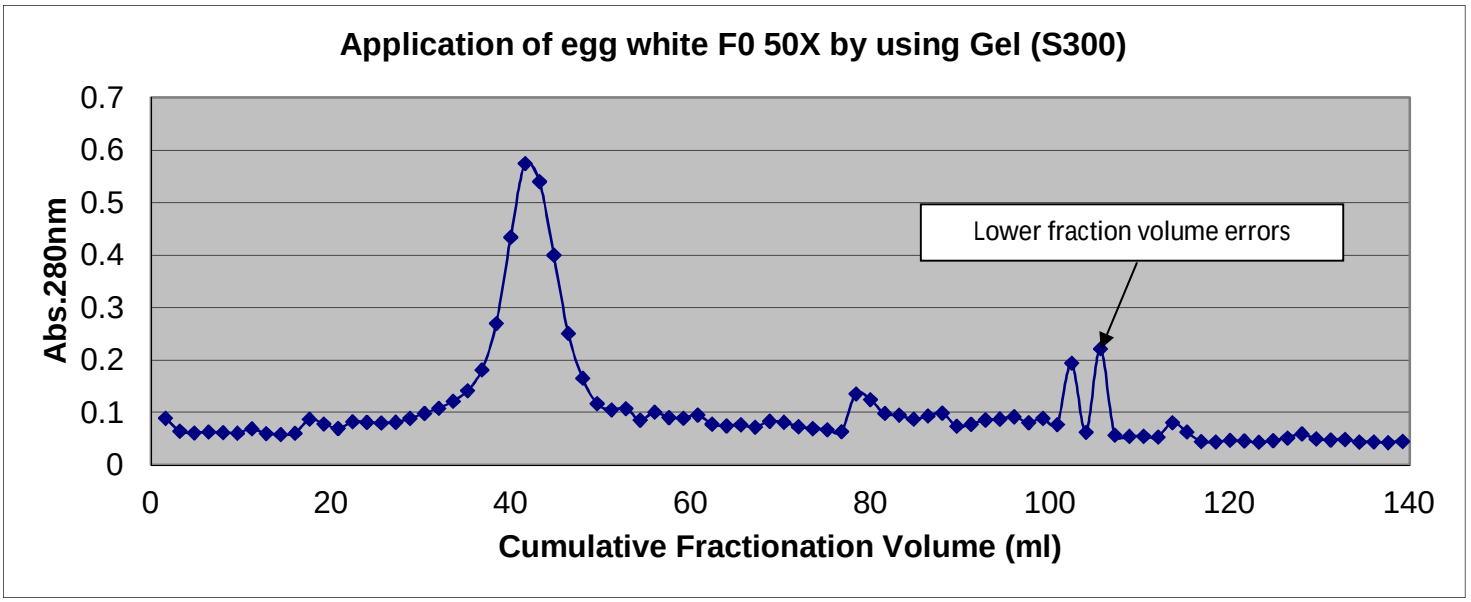
Graph (3): Shows peaks for different proteins in GFC

ID	Vol.(ml)	Abs.
1	1.6	0.0891
2	3.2	0.0645
3	4.8	0.0614
4	6.4	0.0632
5	8	0.0619
6	9.6	0.0608
7	11.2	0.069
8	12.8	0.0599
9	14.4	0.0582
10	16	0.0604
11	17.6	0.0874
12	19.2	0.0784
13	20.8	0.0698
14	22.4	0.0823
15	24	0.0813
16	25.6	0.0802
17	27.2	0.0815
18	28.8	0.0889
19	30.4	0.0984
20	32	0.1083
21	33.6	0.1216
22	35.2	0.1416
23	36.8	0.1813
24	38.4	0.2699
25	40	0.4338
26	41.6	0.5745
27	43.2	0.5399

28	44.8	0.3998
29	46.4	0.2509
30	48	0.1652
31	49.6	0.1169
32	51.2	0.1053
33	52.8	0.1075
34	54.4	0.0858
35	56	0.1006
36	57.6	0.0905
37	59.2	0.0895
38	60.8	0.0956
39	62.4	0.0781
40	64	0.0747
41	65.6	0.0768
42	67.2	0.0721
43	68.8	0.0835
44	70.4	0.0815
45	72	0.073
46	73.6	0.0695
47	75.2	0.0676
48	76.8	0.0637
49	78.4	0.1356
50	80	0.1245
51	81.6	0.0983
52	83.2	0.0952
53	84.8	0.0875
54	86.4	0.0935
55	88	0.099
56	89.6	0.0741
57	91.2	0.0778
58	92.8	0.0859
59	94.4	0.0876
60	96	0.092
61	97.6	0.0809
62	99.2	0.089
63	100.8	0.0773
64	102.4	0.194
65	104	0.0625
66	105.6	0.2213
67	107.2	0.0568
68	108.8	0.0547
69	110.4	0.0549
70	112	0.0532
71	113.6	0.0809
72	115.2	0.063
73	116.8	0.0449
74	118.4	0.0442
75	120	0.0471
76	121.6	0.0459
77	123.2	0.0433

78	124.8	0.0466
79	126.4	0.0516
80	128	0.0594
81	129.6	0.05
82	131.2	0.0479
83	132.8	0.0487
84	134.4	0.0438
85	136	0.0441
86	137.6	0.0428
87	139.2	0.0452

Table (4): Fractionation results of egg white (F0X50) proteins

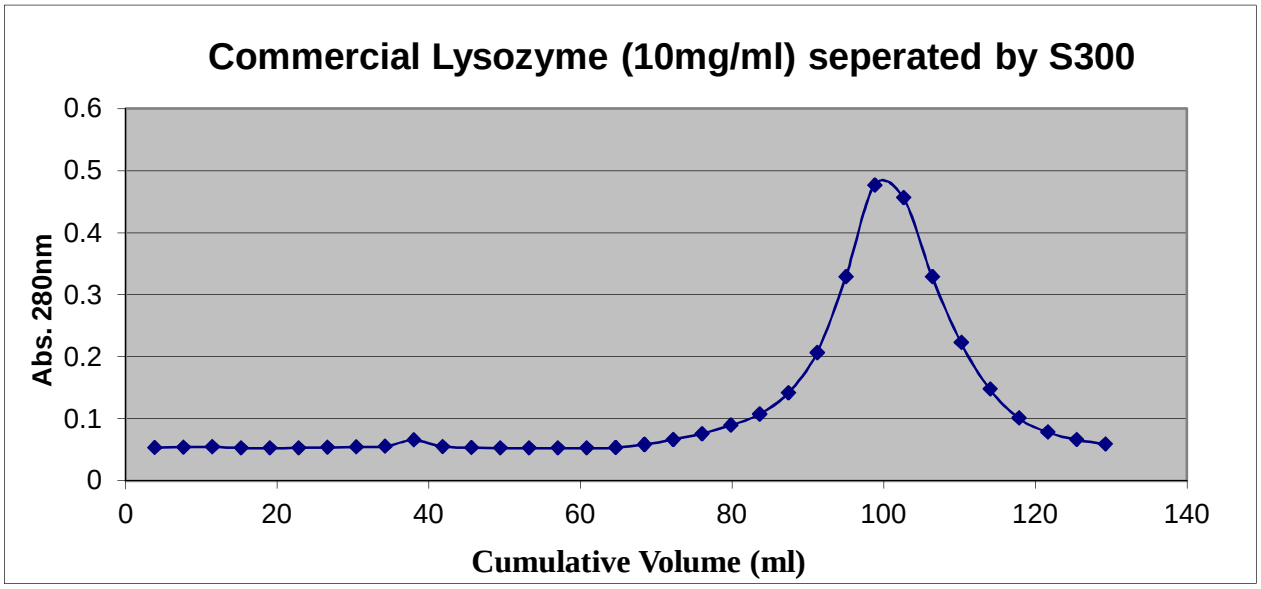


Graph (4): Shows peaks eluted from F0X50 in GFC

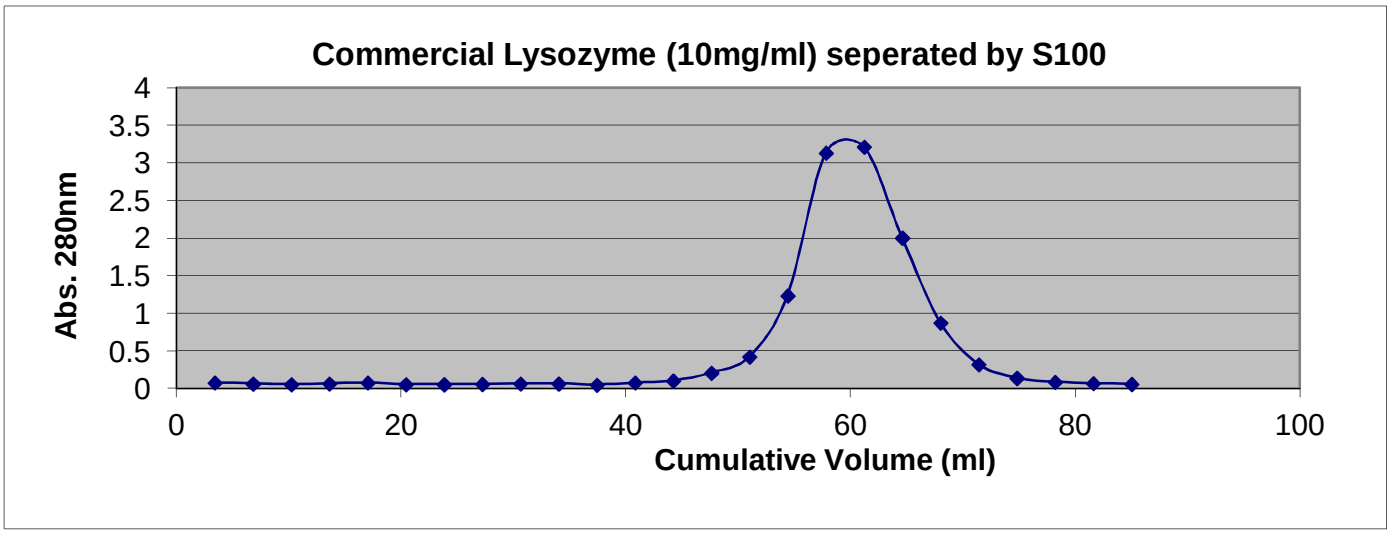
Egg fraction ID	Abs (T_0) in seconds	Abs (T_{120}) in seconds
47	***0.926***	***0.922***
48	***0.920***	***0.920***
49	***0.925***	***0.923***
50	***0.927***	***0.925***
51	***0.925***	***0.924***
52	**0.930**	**0.930**
53	**0.927**	**0.926**
54	**0.922**	**0.922**
62	**0.920**	**0.911**
63	**0.931**	**0.923**
64	**0.922**	**0.916**

65	**0.927**	**0.923**
66	**0.927**	**0.925**
23	**0.923**	**0.921**
27	**0.923**	**0.923**
70	**0.925**	**0.923**
71	**0.923**	**0.921**
72	**0.918**	**0.918**
78	**0.927**	**0.920**
80	**0.921**	**0.920**

Table (5): Shows no lytic activity in the V_e eluted for F_0 (X50)



Graph (5): Shows peaks eluted from commercially prepared lysozyme in GFC



Graph (6): Shows peaks eluted from commercially prepared lysozyme in GFC

❖ Observations & Conclusion:

As we know that void volume (V_0) is equal to 1/3 of total column volume (V_t or V_{bed}) which should be **60.92 mL** x 1/3 = 20.31 mL. What we have actually got from the graph (1) is **17.50 mL** by using blue dextrane dye. However, this was V_0 was for Sephacryl S-300 HR which we have used in the practical. Sephacryl S 300 fractionation rage is from 20,000 to 1,500,000.

This means our interested small size peptides will definitely elute after 17.5 mL from this chromatography. As we know only big molecules which have entered the pores inside the bead will be present in V_0. Since we know the V_0 and V_t so thus we can calculate V_{gel} which is **43.42 mL**.

As can be seen from graph no (2) our Gel calibration kit proteins have not separated very well by using 1.6 mL/min flow rate instead of 0.8 mL/minutes. Nearly two distinct peaks can be visualized only. (Bad resolutions)

By using Sephacryl S 100 resolution still was not as expected but high molecular weight proteins were little bit resolved better than if we compare graph 3 with graph 2. This all could be due to high flow rate application.

Previously prepared egg white F_0 (X50) been loaded into GFC and fractionations obtained V_e for each peak resulted after void volume peak. However; these resulted fraction showed **no or very little lysozyme activity** maybe due to lysozyme denaturation or we has to load new egg white extraction in this practical session instead of using 2 weeks old once. See table 5. It also shows last 2 peaks which are actually random error due low volume obtained during sample fractionation, which should not be counted.

For confirmation we have repeated next day the neat sample F_0 (X50) and examine its activity at 450 nm wavelength and found the same very little lytic activity! Which correlates with our findings by GFC? Change was from 0.889 Abs to 0.771 Abs in 2 minutes.

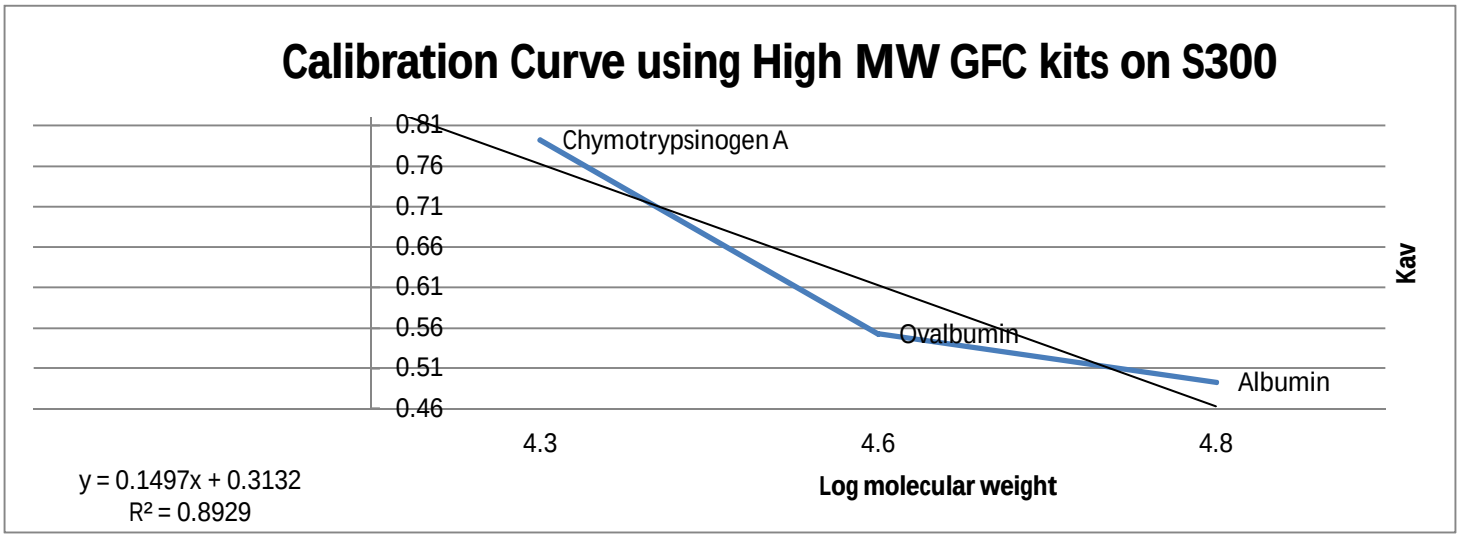
It can be easily seen that S100 are far better than S300 in our practical to separate mixture of proteins (calibration kit) and F_0. Resolution and curves are separated better in S100. In addition, V_e compared in S100 is lower than in S300 for the same peak see graph 2, 3, 5, and 6.

1. **For the calibration and sample run: Establish an elution profile plotting the absorbance of each fraction at 280 nm against the elution volume.**

   Nearly 4 peaks isolated from gel filtration chromatography over 3 peaks came out early and they were merged together. However, two small peaks elute lately! **See graph (2)** above which should be smaller molecular weight RNA

Number of Peaks	Elute Volume (mL)	Highest Absorbance	Comment
Peak 1	36.3	0.5465	3 peaks were merged together
Peak 2	41.5	0.5931	
Peak 3	51.9	0.1443	
Peak 4	207.9	0.1029	RNA
Peak 5	241.7	0.1112	Ghost peak

Table (6): Fractionations to estimate V_e



Graph (7): Calibration Curve using High MW GFC kits on S300 & RNA excluded

By using lysozyme MW ($14.6X10^3$) we cannot unfortunately use above calibration curve because of very poor resolution of calibration standard proteins which are not well separated. Thus very low MW protein (RNA) can be plotted in this graph so that our linearity will be lost.

2. **For the sample run: Establish an elution profile plotting the lysozyme activity in each fraction against the elution volume.**

   Unfortunately no activity found in all fractions due to protein denaturation may lose its lytic activity. See table no (5)

3. **Determine the molecular weight of lysozyme and compare your result with the theoretical value of 14.6 kDa.**

Lysozyme K_{av} = Log (14600) = **4.16** and cannot be determine exactly by using calibration curve due to bad resolution of all peaks. However, K_{av} for RNA = 4.1 which very close to lysozyme value more likely. Since MW of **RNA = 13.7 kDa**, can be use as indicator for where lysozyme fraction maybe located within the egg white sample. Again using of high flow rate may cause these curves to merge to each other.

4. **Compare and discuss the results obtained under different gel filtration conditions (gel matrix, flow rate).**

Resolution in GFC depends on the particle size, pore size, flow rate, column length and diameter, and sample volume. Generally, the highest resolution is obtained with low flow rates; long, narrow columns, small particle size gels, small sample volumes (1-5% of the total bed volume); a two-fold difference in molecular weight; and a sample viscosity that is the same as the eluent.

At 1.6 mL/minutes peaks eluted faster and did not resolved fully with S300 gel but using S100 gel were somehow better than S300. Using S100 gel peaks elution time and fractionation volume become lesser than S300 gel.

We have used 1.6 mL/minutes for separating our dye, calibration kit, and in lysozyme egg white samples flow rate adjusted to 0.8 mL/mintues. Commercially prepared lysozyme loaded into S100 and S300 gels as follows:

S100	
[Lysozyme] =	10 mg/ml
loaded vol. =	1.2 ml
flow rate =	0.8 ml/min
fraction vol. =	3.4 ml
bed vol. =	60ml

S300	
[Lysozyme] =	10mg/ml
loaded vol. =	1.2ml
flow rate =	0.8ml/min
Fraction vol. =	3.8ml
bed vol. =	60ml

If we compared our sample F0 run in GFC and commercially run lysozyme its seems the V_e for lysozyme in commercial sample is 98.8 mL with flow rate 1.2 mL/minutes in and if we see our sample for F0 which is run on flow rate 1.6 mL/minutes we got 2 peaks with V_e 80 to 84 mLs. This interval also showed little lytic activity.

5. **Explain the principle of separation of gel filtration chromatography. Give another example when it is used.**

See diagram (1) in the introduction.

Gel permeation chromatography is also known as gel filtration and molecular weight size exclusion chromatography. The gel structure contains pores of varying diameter up to a maximum size. The test molecules are washed through a column of the gel and molecules larger than the largest pores in the gel are excluded from the gel structure. Smaller molecules, however, penetrate the gel to a varying extent depending upon their size and this retards their progress through the column. Elution, therefore, is in order of decreasing size.

6. **What is the difference between Sephacryl S-100 HR and Sephacryl S-300 HR. which type of phase is more appropriate for isolating lysozyme?**

Characteristics	**Sephacryl S-100 HR**	**Sephacryl S-300 HR**
Uses	designed for separating peptides and small proteins	are for purifying antibodies, serum proteins, and mid-size proteins
Useful fractionation range (MW) for Mr	1×10^3–1×10^5	1×10^4–1.5×10^6
Bead form	Spherical, diameter 25–75 µm in wet form (mean 47 µm)	
Bead structure	Allyl dextran and N,N-methylene bisacrylamide	
pH stability	3–11	
Antimicrobial agent	20% ethanol	
Maximum operating pressure	0.2 MPa (2 bar, 28 psi)	
Maximum linear flow rate	60 cm/hour	
Recommended volumetric flow rate	0.3-1.2 ml/min	
Chemical stability	All commonly used buffers, 0.2 M NaOH, 0.2 M HCl, 1 M acetic acid, 8 M urea, 6 M guanidine HCl, 1% SDS, 24% ethanol, 30% propanol, 30% acetonitrile	

So S100 is more useful to separate lysozyme enzyme than to use S300

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