

**biochemical  
Practical course  
for  
advanced**

**Experiment number f-02/03:  
Isolation and examination  
of  $\alpha$ -Lactalbumin**

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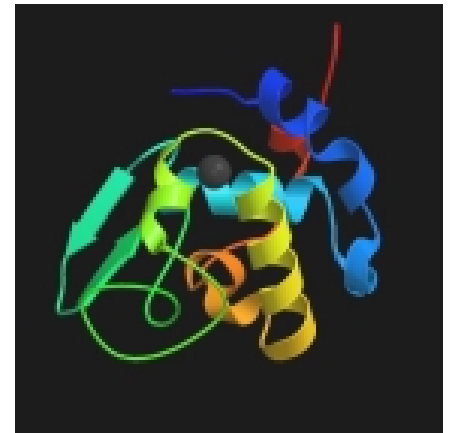
## I. Introduction

### $\alpha$ -lactalbumin

The protein to be examined in this experiment consists of 123 amino acids and its molecular weight is round about 14 kD.  $\alpha$ -lactalbumin occurs in the milk of mammals in different parts (in cow's milk 0.14%-0.33%)<sup>1</sup>.

Its primary structure is mostly conforming with lysozyme but the protein itself shows no enzymatic activity. However,  $\alpha$ -lactalbumin alters the specificity of the galactosyl transferase such that it utilizes glucose as an acceptor, rather than *N*-acetylglucosamine, to form lactose instead of *N*-acetyllactosamine. Nevertheless, lysozyme and  $\alpha$ -lactalbumin do not interfere in any way: Neither can lysozyme modify the activity of the galactosyl transferase nor can  $\alpha$ -lactalbumin hydrolyze glycosidic  $\beta(1\rightarrow4)$  bonds.

Because of  $\alpha$ -lactalbumin being an albumin it can be separated easily from other milk proteins. This kind of proteins precipitate at higher salt concentrations than other classes of proteins. The isoelectric point of the protein is about 4.6 because it has a great amount of negative charges (at physiological pH).



**Fig. I-1:** Ribbon structure of  $\alpha$ -lactalbumin from baboon milk.

## II. Isolation of $\alpha$ -Lactalbumin

### a) Theoretical background

Like mentioned before the separation of the protein is more or less uncomplicated. Problems can cause the high concentration of lipids (> 3.8%) because they interfere with dyes (see below), manipulate the extinctions at 280 nm (see ) and make the bands in the gel electrophoresis blur. Lipids are not perfectly removable from milk because they form small droplets which are kept in suspension even after long and fast centrifugation. For that reason you first have to remove the main part of them.

To precipitate most of the proteins not being albumins you denature by warming and simultaneously adding medium concentrations (20%) of ammonium sulfate. The heat causes deformations in some proteins and therefore causes (partial) unfolding. The precipitation by salting out is based on the ions competing for solvent molecules with other solutes. So the amount of bulk solvent becomes insufficient to dissolve other solutes like large proteins.

Another method is to change the pH to about the isoelectric point of the interesting protein. By doing so the negative charges (which most proteins excluding histones show at pH~7) diminish and the solubility decreases; polar and ionic molecules are better soluble than uncharged. The repetitive variation of pH can be very effective for separating proteins.

After having isolated most of the unwanted proteins the last step of protein purification often is a kind of chromatography. This is also one of the most powerful separating methods in biochemistry; the gel filtration chromatography is used in this experiment. The principle of this method is as follows:

<sup>1</sup> Source: CD-Römpp Chemie Lexikon

A column is filled with beads of a hydrated, spongelike material containing pores that span a relatively narrow size range of molecular dimensions (Fig. II-1). These beads build the stationary phase. The black lines represent the gel matrix (with surrounding internal solvent space). Small molecules (small red dots) can freely move into the matrix while molecules greater than the *exclusion limit* (blue dots) stay outside. The smaller the molecules are the longer they are kept within the bead for it is a matter of equilibrium. And, of course, the ability of penetrating the gel depends on the molecule's shape. In order to elute the samples one or more buffers have to be applied according to the desired effect. One can buy different types of gels with specific exclusion limits and bead diameters for nearly every purpose. The specificity of the gels we used are shown below (Tab. II-1, p. 4).

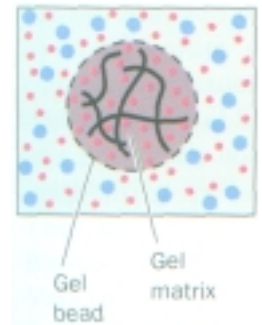


Fig. II-1: A gel bead.

The following spectroscopic analysis is based on the different wave length at which proteins and nucleic acids have a high absorption. At 260 nm the bases of DNA and RNA have a maximum and at 280 nm the residues tyrosine and tryptophane. The ratio of these extinctions are a measure for the included nucleic acids and therefore give a clue to the purity, but the amount of lipids has to be considered.

### b) Task and Aim

The aim of this experiment is to isolate  $\alpha$ -lactalbumin from cow's milk. This milk must neither have been homogenized (to be able to remove most of the lipids) nor been pasteurized (to have native proteins). The first step therefore is to separate the fat to get skim milk. Next, proteins are salted out by medium salt concentrations and denatured by heat. The supernatant contains the albumins and is treated with acid to precipitate these proteins. For higher purity the pellet is resolved in alkaline solution and again precipitated with acid. At last the solution is fractionated by gel chromatography.

In order to trace the efficiency of the used purification methods, a sample (~1 ml) is taken both from the pellet and the supernatant of every step.

### c) Realisation

#### First Step: Fat separation

The fresh milk is centrifuged for 15 min at 4000 rpm (3053  $\times g$ ) and the tube is punctured at the bottom to collect the milk. It is recommended to pour the milk into a graduated cylinder in order to determine the volume.

#### Second step: Combined Denaturing and Salting Out

The collected milk is poured into a 1 L beaker and heated to 40° C while shaking carefully. Slowly, 20% (m/v) dry ammonium sulfate are added (within 15 min). We got 93 ml of milk, e.g. we had to add 18.6 g salt. After adding all of the salt the shaking is continued for another quarter. For the precipitated proteins are numerous the solution first is filtered through about six layers of mull and afterwards sucked through filter paper. The filtrate should be much clearer than before. The residue is discarded.

#### Third step: Acid Precipitation

The filtrate is transferred into a 250 ml beaker and adjusted to a pH of 3.0 $\pm$ 0.1 by dropping in 1 M HCl (control with a glass electrode). To separate the precipitated proteins the solution is centrifuged at 11.500 rpm (10.318  $\times g$ ) by using rotor SS34 for 15 min.

#### Fourth step: Alkaline Extraction

The pellet is resuspended in 10 ml of distilled water. While stirring 1 M NaOH is added to achieve a pH between 8.5 and 9.0. Not dissolved material is separated by centrifugation for 10 min at 12.500 rpm (12.191  $\times g$ ). This time the supernatant is kept in a 25 ml beaker for further treatment.

### Fifth Step: Acid Precipitation

To precipitate the  $\alpha$ -lactalbumin the solution is brought to pH 4.0 by dropping in 1 M HCl. Afterwards the suspension is centrifuged by 12.500 rpm (12.191  $\times g$ ) for 10 min and the pellet resolved in 1 ml aqueous solution of ammonium hydrogen carbonate. If necessary a few  $\mu$ l of ammonia may be added to enhance the solubility.

### Sixth Step: Gel Filtration Chromatography

The used columns for the chromatography contained two different gels: One has been filled with Sephadex G 75 and the others with Sephadex G 100. These gels have different specifications:

	Name	fractionating range
G 75-100	G 75	3-80
G 100	G 100	4-150

**Tab. II-1:** Specifications of different Sephadex gel types.

The column is equilibrated with 0.05 M ammonium hydrogen carbonate solution. The solution acquired in the last step is carefully applied and eluted with maximum velocity using the same buffer. Fractions are collected in portions of 5 ml and their extinctions at 260 nm and 280 nm are measured. The extinctions at 280 nm and the ration 280/260 nm are plotted against the fraction number.

### d) Observations and Results

The first centrifugation separates most of the fat which can be observed by a solid plug above the liquid milk. The second step results in a large amount of precipitated proteins to be hold back by the following filtration. The solution is clearer and yellow in colour. In the next step the  $\alpha$ -lactalbumin is precipitated at pH  $\sim$ 3.6 while reeking of putrid milk (caused by denatured caseins). After centrifugation the pellet is white, the solution yellow. During the alkaline extraction the solution becomes cloudy. Because of being still muddy after the first centrifugation the solution has to be centrifuged once again. Afterwards the supernatant becomes clear and muddy again after adding the acid.

The values achieved by the gel chromatography are listed in the table below:

fraction	$E_{260}$	$E_{280}$	$E_{280}/E_{260}$	$c/\text{mg} \cdot \text{ml}^{-1}$	fraction	$E_{260}$	$E_{280}$	$E_{280}/E_{260}$	$c/\text{mg} \cdot \text{ml}^{-1}$
1	0	0	0.00		15	0.021	0.035	1.67	0.038
2	0	0	0.00		16	0.02	0.02	1.00	0.016
3	0.012	0.008	0.67	0.003	17	0.008	0.008	1.00	0.006
4	0.015	0.009	0.60	0.003	18	0.004	0.002	0.50	
5	1.076	1.178	1.09	1.001	19	0.002	0	0.00	
6	0.168	0.24	1.43	0.245	20	0	0	0.00	
7	0.069	0.104	1.51	0.109	21	0	0	0.00	
8	0.043	0.062	1.44	0.063	22	0	0	0.00	
9	0.038	0.042	1.11	0.036	23	0	0	0.00	
10	0.031	0.042	1.35	0.042	24	0.01	0	0.00	
11	0.068	0.058	0.85	0.038	25	0	0	0.00	
12	0.052	0.07	1.35	0.069	26	0.01	0	0.00	
13	0.045	0.071	1.58	0.077	27	0.014	0.005	0.36	
14	0.029	0.039	1.34	0.039	28	0.015	0.007	0.47	

fraction	E <sub>260</sub>	E <sub>280</sub>	E <sub>280</sub> /E <sub>260</sub>	c/ mg · ml <sup>-1</sup>	fraction	E <sub>260</sub>	E <sub>280</sub>	E <sub>280</sub> /E <sub>260</sub>
30	0	0	0.00		40	0.004	0	0.00
31	0	0	0.00		41	0	0	0.00
32	0	0	0.00		42	0.02	0	0.00
33	0.012	0	0.00		43	0	0	0.00
34	0.006	0	0.00		44	0.02	0	0.00
35	0.004	0.004	1.00	0.00324	45	0.006	0	0.00
36	0.003	0.008	2.67		46	1	1	0.00
37	0.006	0.004	0.67	0.00168	47	1	1	0.00
38	0.007	0.002	0.29		48	1	1	0.00
39	0	0	0.00		49	1	1	0.00
30	0	0	0.00		50	1	1	0.00

**Tab. II-2:** Absorptions of the fraction of the gel chromatography. The values are plotted in Fig. II-2 on the next page.

### e) Interpretation and Discussion

The observation made during the experiment are as expected. As shown in Fig. II-2 there are four peaks (related to the ratio): fractions 6, 12, 28 and 36. Although the absorption at 280 nm is higher in fraction 5 we chose the next fraction because our assistant told us that this is based on contained lipids. The last two peaks of the ratio are high in spite of showing little amount of proteins, because the matching absorptions of nucleic acids are even lower. The precision of the photometer limits the reliability of the values (the ratio has to be between 0.6 and 1.8).

More detailed information for example about the obtained purity can only be made after further analysis. The next tasks therefore deal with determination of protein amount and containing macromolecules but the results are mentioned right here (comp. the photos on page 12):

Step one: As expected the lipids have not been removed completely, what can be seen in the differences of the extinction between the LOWRY and the BRADFORD determination (see p. 9). The gel shows many bands that indicates the missing protein separation.

Step two: The most useful information is given by the proteins that precipitated so that the appliance on the gel was cancelled.

Step three: The sample of the supernatant shows bands in the gel above the one according to  $\alpha$ -lactalbumin. The pellet also contains bands not indicating lactalbumin but another that does. The other bands may be caused by caseins, the main protein component. That only small and few bands are visible speaks for an effective purification step.

Step four: The bands of the supernatant and pellet are nearly identical so that no great purification occurred. It can also be seen that much of the  $\alpha$ -lactalbumin remained in the precipitate.

Step five: The precipitated fraction contains barely other proteins than  $\alpha$ -lactalbumin while the supernatant contains a few amount of it. This step is therefore good but not sufficient.

Step six: This separation step is the most useful because one of the fractions contains only the wanted protein (F12 in our case). The protein eluted later with the Sephadex G 100 column, i.e. these columns have a higher degree of separation. The estimated concentration of  $\alpha$ -lactalbumin is the sum of its peak's concentrations:

$$c_{\text{lactalbumin}} = 0.038 + 0.069 + 0.077 = 0.184 \text{ mg} \cdot \text{ml}^{-1}$$

**Fig. II-2:** Elution profile of the gel chromatography. The blue areas represent the amount of proteins and the red line the ratio of proteins to nucleic acids.

### III. Protein Determination

#### a) Theoretical Background

There are at least three usual methods how to value the amount of protein in a solution. A simple but inaccurate one is the determination after **WARBURG** and **CHRISTIAN**: As described previously special residues of amino acids in a protein show high absorption at 280 nm. This is a rough measure for proteins. We already used this method in the former experiment. More interesting are the methods of **LOWRY** and **BRADFORD**.

**LOWRY**-method:

This method is an extension of the biuret-method. It bases on the reduction of the **FOLIN-CIOCALTEAU** reagent (phosphomolybdate/phosphowolframate) by a copper complex. The copper complex is formed by at least two peptide bonds and a bivalent copper ion in alkaline solution. The result of the reduction is an intensive blue colour. Beside the proof reaction all reducing agents like mercaptoethanol and dithiothreitol interfere with the **FOLIN**'s reagent. The detection is made by spectroscopy at 540 nm.

**BRADFORD**-method:

This time the reversible binding of a dye (Coomassie Brilliant Blue G250) is used for the determination. In acid solution this dye causes a bathochrome shift of the absorption maximum from 465 nm to 595 nm. The absorption at 595 nm is therefore proportional to the amount of protein. Because of being a lipophilic reagent Coomassie Blue lipids (and detergents like SDS and Triton X 100) can also bind and falsify the results.

For these methods are based on spectroscopic evaluation a calibration curve has to be recorded. By comparing results of both methods different sources of errors (caused by unwanted contents) can be pointed out.

#### b) Tasks and Aim

The amount of protein of the previously selected fractions is to be determined by using both the **LOWRY** and the **BRADFORD** method.

#### c) Realisation

**BRADFORD**-method:

For the calibration curve six tubes are equipped with aqua bidest., protein standard and the dye (CBB) as follows:

reagent (in ml)	tube number					
	1	2	3	4	5	6
aqua bidest.	2.3	2.2	2.1	1.9	1.7	1.5
protein standard (0.1% $\gamma$ -globulin)	--	0.1	0.2	0.4	0.6	0.8
CBB	1.0	1.0	1.0	1.0	1.0	1.0

The tubes are shaken and allowed to stand 10 min at room temperature. After that the absorption at 595 nm (against tube no. 1 as reference) is measured.

The preparation of the samples is similar: Add 0.5 ml of the fraction to 1.8 ml of aqua bidest. and then 1.0 ml of CBB. Shake well again and after 10 min measure the absorption at 595 nm. A new reference is made like before. If the values exceed the linear range the sample has to be diluted.

LOWRY-method:

At first some of the solutions have to be prepared because they are stable only for a limited time. Most important is the alkaline copper reagent; it contains three solution:

- solution a: 1% copper-II-sulfate in aqua bidest.
- solution b: 2% sodium carbonate in 0.1 M NaOH
- solution c: 2% sodium tartrate in aqua bidest.

1.0 ml of solution c is pipetted into a 150 ml Erlenmeyer flask and while stirring 1.0 ml of solution a is added. Afterwards the solution is filled up to 100.0 ml with solution b.

The samples for the calibration curve are prepared after the following scheme:

reagent (in ml)	tube number					
	1	2	3	4	5	6
aqua bidest.	1.0	0.9	0.8	0.6	0.4	0.2
protein standard (0.1% serum albumin)	--	0.1	0.2	0.4	0.6	0.8
alkaline copper reagent	5.0	5.0	5.0	5.0	5.0	5.0

The solutions are mixed well and allowed stand for 10 min at room temperature. Afterwards to each tube 0.25 ml of 2 N FOLIN's reagent are added and the tubes are immediately forcefully shaken. Then they are incubated at room temperature for half an hour. The following absorption measuring is made at 540 nm against tube 1 as reference.

The fractions are examined by adding 0.5 ml aqua bidest. and 5.0 ml of the alkaline copper reagent to 0.5 ml of the sample. The further treatment is the same as above (and a new standard has to be made).

#### d) Results

The following tables contain the obtained values of our spectroscopic measurements; the way of calculation is explained below. We had some problems with the calibration curve so that we took over the data from another group (see also discussion).

calibration curve	tube	Bradford		Lowry	
		m/mg	E <sub>595</sub>	m/mg	E <sub>540</sub>
	1	0	0	0	0
	2	0.01	0.056	0.01	0.013
	3	0.02	0.104	0.02	0.029
	4	0.04	0.213	0.04	0.062
	5	0.06	0.282	0.06	0.096
	6	0.08	0.341	0.08	0.124

samples	fraction	Bradford			Lowry	
		E <sub>595</sub>	c/ mg · ml <sup>-1</sup>	dilution	c/ mg · ml <sup>-1</sup>	E <sub>540</sub>
	F6	0.294	0.259	1:2	0.187	0.139
	F12	0.197	0.084		0.048	0.070
	F28	0.054	0.018		0.006	0.008
	F36	0.104	0.041		0.017	0.024
	supernatant	0.155	0.649	1:10	3.747	0.561
	pellet	0.085	0.325	1:10	2.461	0.368

The values are already those which have been fitted by dilution. The concentrations are calculated like that:

The equation of the regression line is

$$E = a \cdot m_{\text{protein}} + b$$

with  $\frac{\Delta E}{\Delta m_{\text{protein}}} \equiv \text{slope} \equiv a$  and  $b \equiv \text{intercept}$ . Rearranging leads to the amount (mass) of protein:

$$m_{\text{protein}} = \frac{E - b}{a}$$

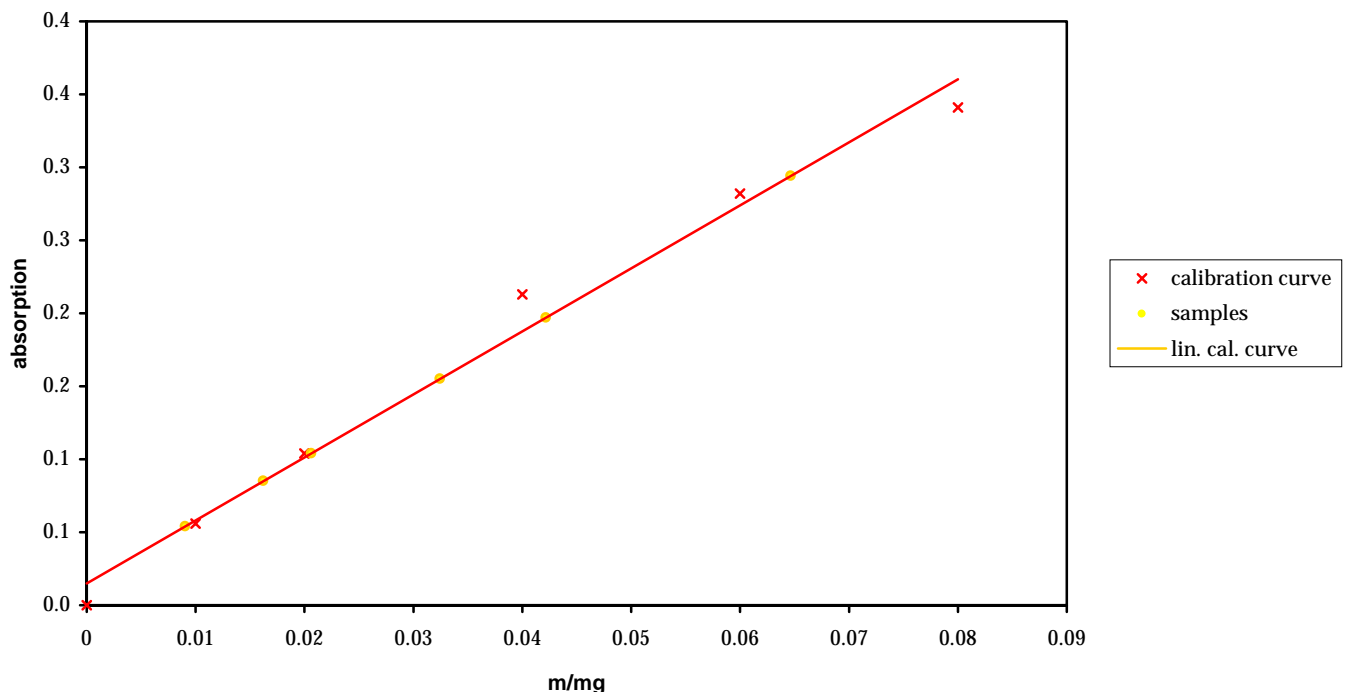
In order to obtain the concentration the added volume of protein solution (incl. dilution) has to be considered:

$$c_{\text{protein}} = \frac{E - b}{a \cdot V_{\text{protein}}} \cdot \text{dilution}$$

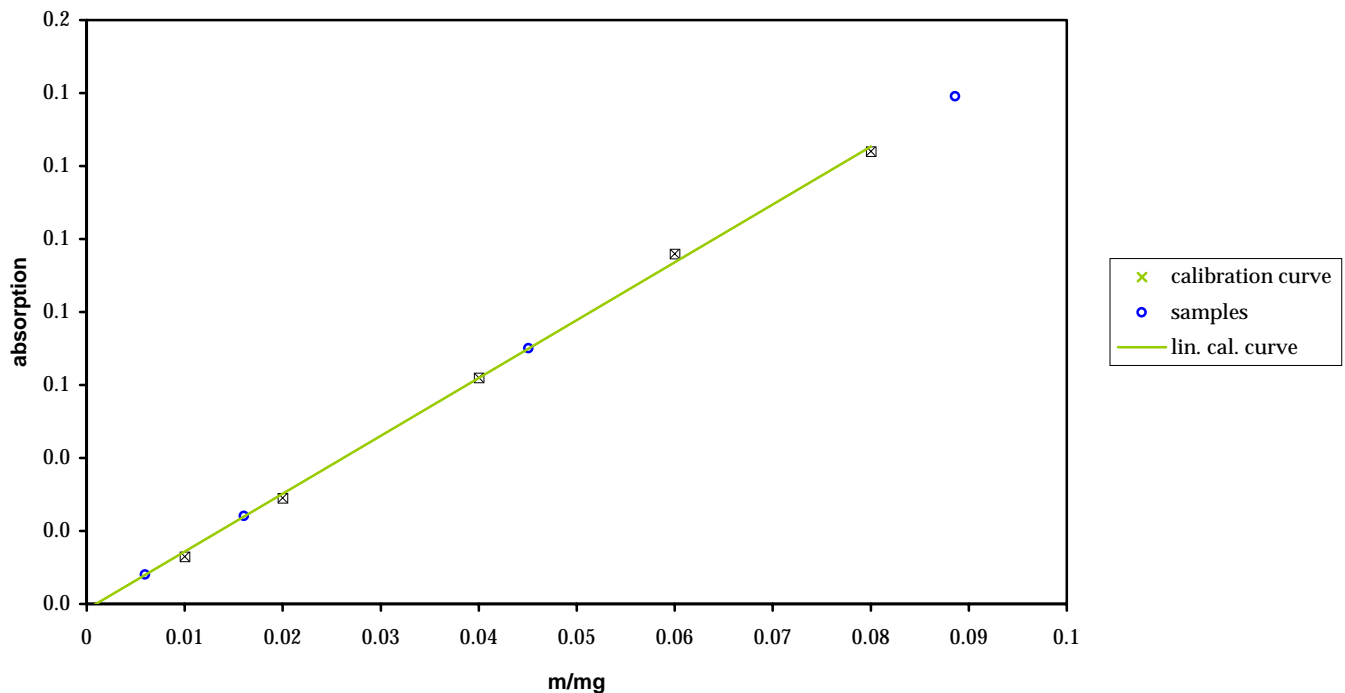
### e) Interpretation and Discussion

As you can see below and on the following page the calibration curves are good. Our first had to be rejected because we put in the cuvette in a wrong way. The displayed values are from another group. The amounts of protein measured by the BRADFORD method are higher than those measured with the other method. This can easily be explained by the lipids which interfere with the dye. This may even be the reason for the extraordinary high values of the supernatant and the pellet in the BRADFORD method. What kind of proteins the absorption caused will be examined in the next task.

Determination of proteins after Bradford



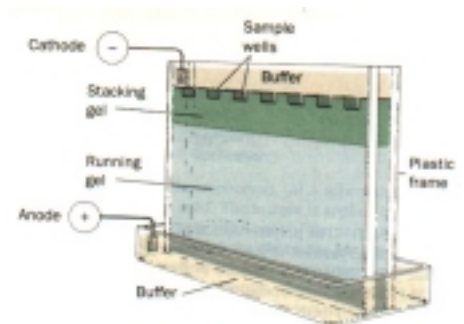
## Determination of proteins after Lowry



## IV. Gel Electrophoresis

### a) Theoretical Background

Gel electrophoresis is one of the most powerful methods of macromolecular separation. The gels are made of polyacrylamide or agarose with variable pore sizes. In contrast to the previously used gel filtration chromatography *smaller* molecules move faster than larger ones. This is because of the higher electrophoretic mobility, i.e. smaller molecules migrate faster through the gel. In our case we use a special modification of the polyacrylamide gel electrophoresis (PAGE). These gels are generally made by the free radical-induced polymerization of acrylamide and *N,N'*-methylenebisacrylamide in the buffer of choice. The modification is known as discontinuous pH or disc electrophoresis (Fig. IV-1). This technique requires a two-gel system and different buffers. The two gels are on the one hand the stacking gel which is large-pored and the running gel in which the separation takes place. The buffer in the stacking gel and the sample solution has a pH less than the buffer used for the running gel. Because of the different pH in the upper buffer and the stacking gel, the buffer ions which migrate into the gel become uncharged (e.g. zwitterionic form). This causes an increase in the electrical field (less charged molecules) and therefore makes the macromolecules move faster. The result is that the macromolecular ions approach the running gel as stacks of narrow bands (discs) that are ordered according to their mobilities. In the running gel the macromolecules are slowed down by the filtration effect so that the buffer ions can overtake them (they regained their charged form because of the higher pH) and thus the electrophoresis continues as normal.



**Fig. IV-1:** A diagram of a disc electrophoresis apparatus.

## b) Task and Aim

This is the most informative part of the experiment. From every peak one sample fraction is applied to the gel. Additionally the pellet and supernatant from every purifying step is applied (one step by one group). With the obtained bands after the gel electrophoresis an information about the containing proteins is given. The identification is realised with markers and standards.

## c) Realisation

At first the gels have to be prepared after the following scheme:

substance	running gel (15%)	stacking gel (3%)
aqua bidest.	3.4 ml	3.14 ml
lower Tris (pH 8.8)	3.8 ml	--
upper Tris (pH 6.8)	--	1.25 ml
10% SDS	7.5 $\mu$ l	0.57 ml
30% acrylamide solution, containing 0.8% bisacrylamide	150 $\mu$ l	50 $\mu$ l
20% ammoniumperoxidisulfate solution	150 $\mu$ l	50 $\mu$ l
TEMED	15 $\mu$ l	5 $\mu$ l

**Notes:** First the running gel is prepared then the stacking gel. The substances below the dotted line start the polymerisation and the gel have to be poured immediately. After the running gel polymerised completely the stacking gel can be applied (insert the comb while still liquid!).

From each sample 20  $\mu$ l has to be applied (except the marker solution with 14  $\mu$ l) and has to contain the same amount of protein. Thus, the fraction with the lowest (but still detectable) concentration is used as reference. The totally added volume of protein solution is 15  $\mu$ l; if necessary the fractions have to be diluted with aqua bidest. (see table below).

fraction	V/ $\mu$ l	V(H <sub>2</sub> O)
F6	3.87	11.13
F12	11.85	3.15
F28	15.00	--
F36	15.00	--
supernatant	1.54	13.46
pellet	3.08	11.92

Although our fractions F28 and F36 contained very low protein we applied them without diluting. The other fractions yielded samples with 1  $\mu$ g of protein, the lowest quantity to be detected. The remaining 5  $\mu$ l are LAEMMLI-buffer.

Before applying the samples they are heated to 95° C for 3 min, put on ice for some minutes and afterwards centrifuged shortly.

The electrophoresis starts with a voltage of 50 V until the visible bands reach the running gel. Then

the voltage is increased to 100 V. It is finished when the first bands nearly reached the end of the running gel.

The proper detection is the staining. After the current has been switched off the gel is separated and put into the staining solution (Coomassie Brilliant Blue R 250 in ethanol and ice acetic acid. It is kept in it for round two hours. Then it is destained overnight in ethanol and acetic acid. A photograph of the gel is made and evaluated.

## d) Results

The photos of each gel are stuck on the next page. A discussion about the found bands has already been made on page 5.

The first gel contains the samples of the first acid precipitation (supernatant 2<sup>nd</sup> from left, pellet 3<sup>rd</sup> from left). The second contains those of the alkaline extraction (pellet 2<sup>nd</sup> from left, supernatant 3<sup>rd</sup> from left). Thus, the last one shows the results of the second acid precipitation (pellet 2<sup>nd</sup> from left, supernatant 3<sup>rd</sup> from left).