Laboratory exercise 1 - Purification of histidine-tagged lactate dehydrogenase utilising Immobilised Metal ion Affinity Chromatography (IMAC)

Hugo Wahlström

Tobias Ellingsen

Jakob Vaktnäs

March 6, 2018

Introduction

The aim of this lab is to purify a protein through the use of *Immobilised Metal ion Chromatography* (*IMAC),* to determine the protein concentration and to measure enzyme activity through spectrophotometry. The efficiency of the purification will be determined by use of *Sodium Docedyl Sulphate-Polyacrylamide Gel Electrophoresis* (*SDS-PAGE*). The protein purified in this lab is a modified *Lactate dehydrogenase* (*LDH*) enzyme with a six histidine tail.

Methods and theory

IMAC

The chelating gel was washed, loaded with Zn^{2+}, incubated and then washed again to remove unbound Zn^{2+} ions, according to the lab instructions. Next, 1 ml of raw protein extract was added to the gel and then incubated on a rocking table for 20 minutes. The rest of the protein extract was saved and kept on ice as Sample 1**.** During the incubation, the histidine tail, attached to the LDH, bound to the Zn^{2+} ions in the chelating gel, separating it from the rest of the protein extract. After incubation, the sample was centrifuged and the supernatant was pipetted off, saved and put on ice as Sample 2. The gel was then washed as per lab instructions with the supernatants saved in between washes. Saving the supernatants from the washing was done in order to control eventual for errors when measuring the activity later in the lab. After washing, an MES-buffer with a pH of 6.5 and EDTA was added on to the gel and then incubated on a rocking table as per lab instructions, this is done in order to elute the LDH from the chelating gel. After incubation, the sample was

centrifuged and the supernatant (now containing purified protein) was pipetted of and saved on ice as Sample 3.

SDS-PAGE

The SDS-PAGE equipment was assembled and prepared by the lab instructor as per lab instructions. Sample 1,2 and 3**,** commercial LDH, and a molecular weight ladder was then prepared with a sample buffer containing buffer, SDS , β-mercapto ethanol and bromphenol blue, and then heated to 95°C for five minutes. The β-mercapto ethanol reduces sulphur bonds in the polypeptide chains giving rise to negative charges in the proteins. The SDS and boiling causes the proteins secondary and tertiary structure to collapse and makes them unfold, a process known as denaturation. The proteins are now unfolded and have a negative charge. Next, the weight marker solution and commercial LDH was added to wells in the SDS-PAGE equipment corresponding to MW and LDH as shown in Fig. 1. Samples 1, 2 and 3 were then added to wells corresponding to A-1, A-2 and A-3, as shown in Fig.1. Electrophoresis and staining was then carried out by the lab instructor. During the electrophoresis, the proteins travel through the wells and the gels due to the different electric potentials of the protein and the bottom of the gel. Small proteins travel faster through the gel due to the pore-size in the gel, and thus, the proteins are separated throughout the gel. Pictures were then taken, here shown as Fig.1.

Bradford determination

The samples were diluted according to the lab instructions and allowed to react with the Bradford reagent (Coomassie Brilliant Blue-G 250) for five minutes. The absorbance at 595nm was then measured.

A standard curve expressing the relation between protein concentration (C_c) and the absorbance at 595nm (A_{595}) was provided by the lab instructor. The following relation can be used to calculate the concentration in the cuvette from an observed absorbance:

$$A_{595} = C_c \cdot 0.0338 + 0.457 \Rightarrow C_c = \frac{A_{595} - 0.457}{0.03388} \tag{1}$$

The dilution factor (DF) is calculated by dividing the total volume of a given sample (V) by the volume of the diluted substance (V_s);

$$\text{DF} = \frac{V}{V_s} \tag{2}$$

The dilution factor can then be used to determine the concentration per unit of volume of a given

sample (C_s) by the following formula:

$$C_s = C_c \cdot DF \tag{3}$$

Activity measurements

The LDH activity of the samples was measured by measuring the rate of change of absorbance at 340nm of a mixture of the sample, MES buffer, NADH and pyruvate. This test builds upon that as pyruvate is transformed to lactate, catalysed by LDH, NADH is oxidised to NAD+. NADH is absorbant at 340nm while NAD+ is not and thus absorbance at this frequency is a measure of NADH. The mixture was prepared according to the lab instructions apart from that 580μg MES buffer and 300μg diluted sample were used per oral instructions from the lab assistant.

The Lambert-Beer's law can be used to express the rate of change of concentration ($\frac{dC}{dt}$) as a relationship between the rate of change in absorbance ($\frac{A_{340}}{\Delta t} = \frac{dA}{dt}$) , the length of the cuvette (l), and the molar absorptivity (ϵ):

$$\frac{dA}{dt} = \frac{dC}{dt} \cdot l \cdot \epsilon \Rightarrow \frac{dC}{dt} = \frac{dA}{dt} \cdot \frac{1}{\epsilon \cdot l} \tag{4}$$

For a given dilution factor, the activity per liter of sample (Act_s) can be expressed in terms of the activity per unit of volume of diluted sample ($\text{Act}_d = \frac{dC}{dt}$) as follows:

$$\text{Act}_s = \text{Act}_d \cdot \text{DF} \tag{5}$$

Finally, the specific activity (SA) of the pure sample, which is expressed in activity per microgram, can be calculated by dividing the activity per unit of volume of the sample (Act_s) by the mass concentration per unit of volume of the sample (C_s)

$$\text{SA} = \frac{Act_s}{C_s} \tag{6}$$

Results

SDS-PAGE

Results from SDS-PAGE can be found in Fig.1.

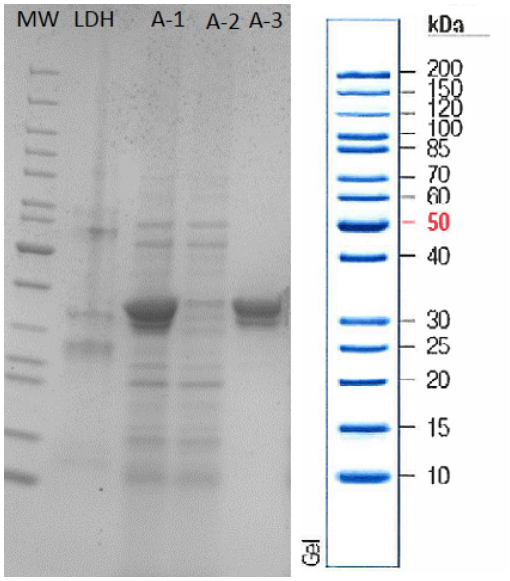

Figure 1: Results from SDS-PAGE (left). MW (left) corresponding to scale of molecular weight in kDA (right). LDH, A-1, A-2 corresponds to commercial LDH, Sample 1, Sample 2 and Sample 3 respectively.

Bradford determination

The Bradford absorption test results are shown in Table 1. The protein concentration in the cuvette (C_c) was calculated according to (1), the dilution factor (DF) was calculated according to (2) with V = 1000 µl and V_s = 2 µl , and the protein concentration in the original sample (C_s) was calculated according to (3).

Activity measurements

The results of the activity measurements are shown in Table 2. Samples 2a and 2b returned only noise, represented as 0 in the table. The activity per volume diluted sample in the cuvette (Act_d / V) is given by $\frac{dC}{dt}$in Lambert Beer's law (4) with $\frac{dA}{dt} = \frac{\Delta A_{340}}{\Delta t}\left[\text{min}^{-1}\right]$, l = 1cm and $\epsilon_{NADH,\,340} = 6200\text{M}^{-1}\text{cm}^{-1}$. The dilution factor was calculated according to (2) with V= 980 µl and V_s = 3 µl .

Table 1: Table showing results of Bradford absorption test at 595nm, dilution factor (DF) and calculated values for the protein concentration in the cuvette (C_c) and the corresponding sample (C_s).

Sample	$\mathbf{A_{595}}$	$\mathbf{C_c}$ / $\frac{\mu g}{mL}$	**DF**	$\mathbf{C_s}$/ $\frac{mg}{mL}$
1a	0.500			
1b	0.481			
1 mean	0.491	0.991	500	0.5
2a	0.485			
2b	0.458			
2 mean	0.472	0.429	500	0.21
3a	0.600			
3b	0.510			
3 mean	0.555	2.90	500	1.45

The activity per volume orignial sample (Act_s / V) was calculated according to (5) and the specific activity was calculated according to (6).

Table 2: Table showing the measured results of LDH activity measured as the maximum activity per time at 340nm (A_{340}), as well as calculated values for the dilution factor (DF), activity per volume for the cuvette (Act_d/V) and sample (Act_s/V) and the sample specific activity.

Sample	$\mathbf{\Delta A_{340}/\Delta t}$ / $\mathbf{min^{-1}}$	$\mathbf{Act_d/V}$ / $\frac{U}{l}$	**DF**	$\mathbf{Act_s/V}$ / $\frac{U}{ml}$	**Specific activity** / $\frac{U}{mg}$
1a	-0.270				
1b	-0.240				
1 mean	-0.255	41.1	327	13.4	27.1
2a	0				
2b	0				
2 mean	0	0	327	0	0
3a	-0.450				
3b	-0.660				
3 mean	-0.555	89.5	327	29.2	20.17

Discussion

SDS-PAGE

Results given by SDS-PAGE in Figure 1 indicates a relatively high efficiency of purification, as seen in Sample 3. Bands given by Sample 1 indicates a mixture of substances found in Sample 2 and Sample 3. Bands given by Sample 3 indicate that the sample is relatively pure, containing one protein with a molecular weight of approximately 35 kDa, corresponding to the weight of LDH [1]. It should be noted that the commercial LDH used was not properly prepared, as evidenced by the range of molecular weights found in the sample. However, it does showcase a band of what appears to be LDH with a slightly lower molecular weight than the LDH of Sample 3, which was to be expected as the commercial LDH lacks the histidine tag present in the LDH in Sample 3.

Bradford determination

The results of the Bradford test were unexpected; Sample 1 has a lower protein concentration than Sample 3, which is not to be expected. All of the protein present in Samples 2 and 3 are derived from Sample 1 and diluted to the same extent, meaning that the protein concentrations of Sample 2 and Sample 3 combined shouldn't exceed the concentration of Sample 1.

One possible explanation is that Sample 1 denatured due to not being stored on ice while Sample 3 was prepared and that this affected the Bradford test.

Activity measurements

The results in Table 2 show a significantly higher activity for Sample 3 than for sample 1. This should not be the case as sample 1 and 3 should contain approximately the same amount of LDH. This is likely due to that Sample 1 was denatured due to not being kept off ice for too long. This hypothesis is in accordance with the results from Bradford test. The errors in concentration calculations and activity measurement, likely due to denaturation of Sample 1, propagate to the calculations of specific activity yielding a higher specific activity for Sample 1 than for Sample 3. This should not be the case as Sample 3 should have a much higher fraction LDH than sample 1 and an approximately equal activity. It should therefore be expected that Sample 3 should have a higher specific activity than Sample 1. If we re-do the specific activity calculations for Sample 1 with assumed probable values for concentration ($C_1 = C_2 + C_3$) and activity ($Act_1/V = Act_3/V$) this is indeed the case. This is further evidence that the error likely lies in the denaturation of Sample 1.

References

1. Lactate Dehydrogenase - Worthington Enzyme Manual [Internet]. Retrieved from: http://www.worthington-biochem.com/ldh/default.html