Bioinformatics Practical Course 5

Isolation and characterization of *E. coli* β-galactosidase

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Introduction

There are many proteins that are of interest for medical or biotechnological applications. However, the organism in which such a protein is discovered seldomly produces it in the quantities that are needed. The common solution to this problem is boosting the expressing the protein of choice by genetic modification. This can be done in the original organism or the protein encoding DNA can be expressed in a foreign host which can be cheaply and easily grown in large cell numbers. In the next course we will construct such an overproducing strain for the proteins that we are using in this course.

An example of a biotechnologically interesting protein is β-galactosidase. This enzyme hydrolyses the disaccharide lactose into the two monosaccharides glucose and galactose. Lactose is present in cow milk at about 5 grams per litre. A large proportion of the world population is lactose intolerant and thus cannot consume cow milk or derived products. Hydrolysing the lactose relieves this intolerance.

We will extract, purify and analyse β-galactosidase from an *E. coli* strain harbouring an over-expression construct (Figure 1). Since (over)producing a protein can be harmful to the host cell, its expression usually is regulated by using an inducible promoter. In our case, this is the promoter from the arabinose operon of *E. coli*, which is only active if arabinose is added to the medium.

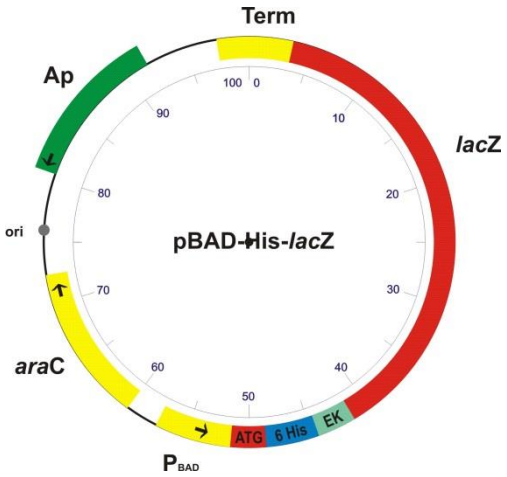

Figure 1: Simplified map of pBAD-His-*lacZ*

Ap	ampicillin resistance gene
ori	origin of replication
*ara*C	repressor of *ara* promoter
P_{BAD}	*ara* promoter
ATG	translation startcodon
6 x His	His-tag
EK	Enterokinase cleavage site
*lac*Z	β-galactosidase coding region
Term	transcription terminator

Protein purification is a challenge in itself. Imagine that a simple prokaryote like *E. coli* contains over 2000 different proteins! Purification involves separation of proteins on basis of size (gel filtration chromatography), charge (ion exchange chromatography), hydrophobicity (hydrophobic interaction chromatography) and/or affinity for a specific ligand (affinity chromatography). The latter is the most rapid and efficient, but can only be applied if such a ligand for the protein of interest is available and can be coupled to small beads. Of course, this will rarely be the case, but for many applications an artificial affinity 'tag' can be included in the construct that is made for producing the desired protein.

Several different affinity tags are available, but in our case we will make use of the so called His-tag. This affinity tag simply consists of 6 consecutive histidine residues, which are added to the N- or C-terminus of the protein. Histidine has affinity for nickel ions and the specific configuration of 6 His residues results in very tight binding (Figure 2a.).

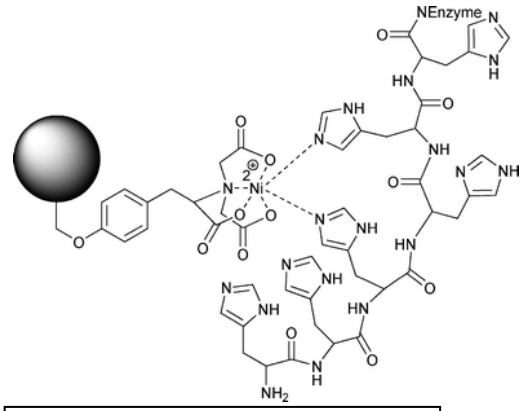

Figure 2a Binding of His-tag to Ni^{2+} beads

from Ulf Hanefeld et al,
Chem. Soc. Rev., 2009,38, 453-468

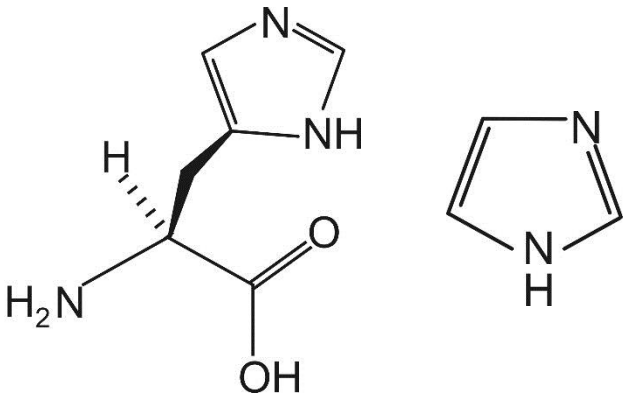

Figure 2b Structure of histidine (left) and imidazole (right)

By coupling Ni^{2+} to silica or agarose beads, a chromatography column can be made that binds the tagged protein with very high affinity and specificity. The bound protein can be eluted from the column with imidazole, a structural analogue of histidine (Figure 2b) which, when added in very high concentration, will displace the protein by competition.

We will purify and analyse a His-tagged version of the wild-type β-galactosidase gene. Mutants in which one amino acid has been altered (His-*lac*Z His_{540}Phe) are also available, but will not be measured during this practical. Such mutants are created during the last practical course in year 3.

Some questions that have to be addressed when producing a protein are:

- In which phase of growth of the host organism should expression of the recombinant protein (ß-galactosidase) be induced?
- What is the optimal concentration of the inducer and the optimal time span of induction?
- Is the target-protein produced intracellularly or extracellularly (or both)?
- What is the best method to extract (biologically active) recombinant protein from the cells?
- What is the biological activity of the protein?
- Which purification steps are needed to separate the recombinant protein from the many other cytoplasmic proteins?
- How can the (purified) recombinant protein best be preserved?
- How stable is the biological activity of the recombinant protein under different conditions?

Some of these questions will be dealt with in this course.

We will produce the β-galactosidase enzymes in the *E. coli* strain TOP10, which is specially adapted to optimise expression from the *ara*BAD promoter in the pBAD-His vectors.

The fusion proteins produced are released from *E. coli* by sonification: high frequency vibrations destruct the cells, freeing their contents. By spectrophotometry on the Nanodrop the efficiency of the sonification process is evaluated. On completion of cell disintegration, the insoluble cell debris is removed by centrifugation. Of the resulting crude extracts the protein concentrations are determined.

Next, the activity of the enzymes are determined at the physiological (37 °C) temperatures.

Meanwhile, part of the extract is used to purify the enzymes on affinity columns and the activity and protein concentration of the resulting fractions is again determined.

Materials

Cultures of *E. coli* TOP10 with pBAD-His-*lac*Z, induced with arabinose.

PMSF: 10 mM phenylmethylsulfonyl fluoride in isopropanol.

Protein extraction buffer: 50 mM $NaHPO_4$ (pH 8.0), 1 mM EDTA, 300 mM NaCl, 20 µM PMSF.
Add PMSF (2 µL/mL) just prior to use.

Heating blocks and/or water baths of 37 °C

Cuvettes.

Spectrophotometers for 420, 595 nm.

Centrifuge tubes 50 mL ('Bluecaps').

Polypropylene tubes (Greiner)

Ultra-sonicator.

Refrigerated centrifuge with swing-out rotor and 50 mL adapters.

Physiological salt: 0,9 % NaCl in demi water, autoclaved.

BSA (1 mg/mL)

BioRad Protein Assay Reagent

Z buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol.
Add β-mercaptoethanol (4 µL/mL) just prior to use.

ONPG substrate solution (20 mM) in sterile miliQ water.

1 M Na_2CO_3 (stop reagent). Dissolve 10.6 g Na_2CO_3 in 100 mL miliQ. Prepare freshly!

99% EtOH

Protino TED or Protino IDA columns.

Lysis-Equilibration-Wash (LEW) buffer: 50 mM $NaHPO_4$ (pH 8.0), 300 mM NaCl.

Elution buffer (EB): 50 mM $NaHPO_4$ (pH 8.0), 300 mM NaCl, 250 mM imidazole.

Points of attention

- Disinfect workspaces prior to AND after use with 70% ethanol and paper towel.
- Collect all bacterial waste in proper containers and deliver those to the autoclave room for destruction.
- Wash hands with disinfecting soap if you leave the laboratory.
- Clean contaminated equipment with 70 % ethanol.
- Note **ALL observations** and results (masses, volumes, temperatures, spectrophotometer readings, colour changes etc.) in your lab journal. **You will need these data for your report!**
- Label **all samples** clearly with your name or initials, contents, concentration etc. Use adhesive labels and wrap Scotch tape around for protection.

Procedure for preparing cultures

Action 1 day prior to the course. Start as early as possible.

- Prepare 'low salt' LB medium with 100 µg /mL ampicillin.
- Transfer 50 - 100 mL to one sterile 300 mL Erlenmeyer flask.
- Inoculate one flask with *E. coli* TOP10 harbouring pBAD-His-*lac*Z
- Incubate the flask for 6 - 7 hours at 37 °C and 250 rpm in a shaking incubator.
- Prepare 'low salt' LB medium with 100 µg/mL ampicillin and transfer 250 mL portion to sterile 1 L Erlenmeyer flasks: (1 per 2 groups of students).
- Inoculate each flask with 25 mL of one of the pre-cultures.
- Incubate for 30 minutes at 25 °C and 250 rpm in a shaking incubator.
- Add arabinose to each culture to obtain an 0.1 % end concentration (= 1.25 mL 20% stock solution).
- Note time of start of induction on the flasks.
- Continue incubation at 25 °C and 250 rpm overnight.
- Put cultures on ice until use.

Note

Cultivation and induction will be performed by one of the Lab Assistants.

Harvest of cells

Note:

Keep the cells and extracts on ice all the time! Elevated temperatures will lead to faster denaturation and/or degradation of proteins, including the β-galactosidase we want to study.

- Make a reservation for the centrifuge and pre-cool it with the proper adapters at 4 °C.
- Cool the contents of the flaks to 0 - 4 °C by putting them in ice water.
- Precool physiological salt (0.9 % NaCl) on ice.
- Mark and weigh two empty Bluecaps (50 ml) and note their mass.
- Collect the cells by centrifugation:
  Transfer 50 mL of the culture to each of the bluecaps.
  Place the buckets in the rotor. Make sure to balance the rotor!
- Centrifuge for 10 min. at 3500 rpm and 4 °C.
- Gently poor off the clear culture medium, collecting it in the original Erlenmeyer flask.
- Resuspend the cells in each tube in 25 mL ice-cold 0,9% NaCl and combine in one tube. Centrifuge again (washing to remove residual medium).
- Drain off all liquid and collect in the Erlenmeyer flasks with medium (biological waste !)

Extraction of proteins from *E. coli* cells

Let op: Een nieuw ultrasoon apparaat maakt dat mogelijk het volume van de celsuspensie en de te gebruiken buis moet worden aangepast. Bij twijfel, vraag aan docent.

- Add PMSF to protein extraction buffer.
- Resuspend each bacterial pellet in 10 mL protein extraction buffer per gram wet weight, with a minimum of 5 mL, keeping the suspension on ice all the time. Use a 50 mL Bluecap tube for this step.
- Mount the tube in a clamp beneath the sonicator, with the tip of the probe just below the middle of the suspension level. Place a container with ice and water on a lab jack so the bottom part of the tube is submerged.
- Program the sonicator to the desired amplitude (consult your teacher). Sonicate 3 times 60 seconds, with 60 second pauses in between (why?).
  To save time, another group can sonicate during the cooling pause of the first.

Determine the extent of cell lysis:

- Start the computer of the Nanodrop. Use the module 'protein' or create a custom program that measures at 260, 280 and 350 nm.
- Switch on and calibrate the Nanodrop; for the old model use miliQ on the pedestal, the new model does not require this. Clean both plates with tissue.
- Set the baseline by pipetting 1.5 µL miliQ. Click ‘Blank’.
- Clean both plates with tissue and pipette 1.5 µL protein extraction buffer. Click ‘Measure’ and note the reading in your lab journal (= blank).
- Transfer 50 µL sonicated cell suspension to an Eppendorf tube. Centrifuge 1 min at 14000 rpm and 4 °C. in a pre-cooled Eppendorf centrifuge.
  Clean Nanodrop plates again and mount 1.5 µL of the clear homogenate. Measure and note the reading.
- If the absorption is too high for an accurate measurement (in case of doubt consult your teacher) prepare and measure a 1 : 10 dilution of the clear extract with protein extraction buffer.

Realise that you extract both nucleic acids and protein by this method and that nucleic acids have a much higher absorption coefficient (= absorption / µg). This makes actual determination of the protein concentration in these types of extracts by UV spectrophotometry very difficult.
At 260 nm nucleic acids (DNA & RNA) have their absorption maximum, at 280 nm proteins. The 350 nm reading serves as background level.

- Sonicate the suspension again 3 times 60 seconds with 60 seconds pauses. Again measure on the Nanodrop as above.
- Repeat this procedure until the absorptions at 260 en 280 nm do not increase significantly any more. This means that almost all cells will have lysed.

- Clean the Nanodrop with milliQ and the sonicator with 70 % ethanol after use.
- Centrifuge the extract for 10 minutes at maximum speed, 4 °C in the precooled (and special) centrifuge, mounted with the right adapters! Make sure the rotor is balanced.
- Transfer the supernatant to a well-marked, clean 15 mL Bluecap tube. Make sure none of the solid matter is transferred! Filter the extract if necessary.
- Store the bluecaps at 0 °C until the β-galactosidase activity will be determined.
  For long term storage at 0 °C place the tubes in a rack in a Styrofoam container in ice. Put the box in the cold room. Regularly check the ice condition and refresh if needed!

Protein concentration with the Bio-Rad assay.

The BioRad assay is based on the method of Bradford and uses staining of proteins by Coomassie Brilliant Blue in acid environment. The advantage of this method towards the direct measurement as above is the elimination of interference by nucleic acids. A disadvantage is that not every protein stains with the same efficiency: CBB preferentially binds to basic and aromatic amino acids.

Summary of the samples to be measured:

BSA standard curve (8 samples).

Wild type raw extract (8 samples).

Column fractions of wild type (6!), 4 samples each, 24 in total.

All in all you will prepare and measure 40 samples.

Protocol

- From the standard BSA solution of 1 mg/mL, prepare 1 mL working solution of 100 µg/mL by diluting with miliQ!
- From the BSA working solution of 100 µg/mL, prepare a series of 8 standard dilutions by mixing in 1½ mL tubes:

miliQ (µL)	BSA (µL)		= protein (µg)
800	0		0
790	10		1
780	20		2
770	30		3
750	50		5
700	100		10
650	150		15
600	200		20

- In the same fashion, prepare 8 samples from the wild type extracts. Use 1, 2, 3 and 5 µL undiluted extract and 1, 2, 3 and 5 µL of a 1 : 10 dilution in protein extraction buffer (prepare 100 µl of the 1:10 dilution).
- Do the same with the fractions (six of each extract!) from the Protino affinity purification, this time using 1, 2, 3 and 5 µL of each fraction.
  Prepare the tubes with the right amount of miliQ (miliQ and sample must be 800µl) in advance, so you do not lose time.
- When ALL the samples have been prepared, add 200 µL Bio-Rad Protein Assay reagent to the first tube and mix immediately by shaking.
- With **10 second intervals**, add 200 µL Bio-Rad Protein Assay reagent to the other tubes, mixing each time immediately after the addition.
- Incubate for exactly 15 minutes at room temperature and measure the absorption at 595 nm:
  Poor the contents of the first tube in a disposable semi-micro cuvette (small ones), place in the right orientation in the spectrophotometer and measure.
- Note the reading in your lab journal. Empty the cuvette in a beaker and dispose of it.
- With 10 second intervals, measure all the other samples.

Make a standard curve of the BSA measurements and determine the protein concentrations of the extracts. They are needed together with the β-galactosidase activities to calculate the specific activities of the produced enzyme.

Determination of the β-galactosidase activity.

Summary of the measurements:

Activity of the wild type (crude) extract at 37 °C in triplicate.

Activity of the six fractions from the purification of the wt extract at 37 °C once.

Activity of wt extract at 25 °C in triplicate.

Protocol

- Add β-mercaptoethanol (4 µL/mL) to a portion Z-buffer in a 50 mL Bluecap.
- Prepare 100 mL 1 M Na_2CO_3 (stop reagent): dissolve 10.6 g Na_2CO_3 in 100 mL miliQ.
- Prepare disposable cuvettes (large ones) with 2 mL 1M Na_2CO_3.
- Take two Eppendorf tubes. Mix 100 µL ONPG substrate solution (20 mM) and 0,9 mL Z-buffer in both Eppendorf tubes. Pre-warm the tubes at 37 °C.
- Add 5 µL enzyme (crude) extract (the actual amount is dependent on the activity: see below) to the Eppendorf tube and shake to mix. Don't add anything to the other Eppendorf tube, this is the blank!
- Incubate **exactly** 10 min at 37°C. Check colour formation by eye after a few minutes: if the yellow colour is too intense, prepare a new reaction with less (or diluted) extract.
- Stop the reaction by transferring all of the contents by pipette to a cuvette filled with Na_2CO_3. Mix immediately.
- Measure the extinction at 420 nm with a spectrophotometer. Dispose of the cuvette.

  Once you have established the right amount of enzyme, repeat the reaction at least three times
- Also determine the activity of all the fractions (six of each extract) from the purification at 37 °C. Use 1 µL of the wild-type.

The difference in extinction between reaction and blank should be > 0,1 and < 0,6 for an accurate measurement. If this is not the case, repeat the reaction with more or less enzyme solution. For extracts with very low activity the incubation time can be extended to 30 minutes.

- Next, perform activity assays of the wt extract at 25 °C. Use the same dilutions as at 37 °C, but incubate twice as long. Again, perform in triplicate.

Calculation of the β-galactosidase activity in units/mL:

1 unit β-gal is defined as the amount of enzyme that hydrolyses 1 µmol ONPG to o-nitrophenol en D-galactose in 1 minute:

β-gal units = 1000 x OD_{420} /(t x V); in which

t	=	*incubation time (in min)*
V	=	*volume of extract in the assay (mL)*
OD_{420}	=	*absorption of sample - blank*

Calculate the β-galactosidase activity in units/mL of both extracts at both temperatures.

Do the same for all the fractions from the purification and the samples from the inactivation experiments.

Make graphs that show the thermal inactivation of each of the enzyme extracts.

*Calculate the **specific** β-galactosidase activity at 37 °C in units/mg protein for both extracts and all the fractions (6) from the purification.*

Purification of β-galactosidase

Purification will be performed on a prefab column, filled with Protino® Ni-agarose. The column should flow by gravity, but sometimes some gentle pressure has to be applied. This can be done with a small pipetting balloon. Take care, however, that the flow rate is lower than one drop per second!

Column procedure:

- Equilibrate the column with 2 mL LEW buffer. Empty the collection tube.
- Transfer 1.5 mL of the protein extracts to 2 mL microvials and spin for 5 minutes at maximum speed.
- Load the column with the clear supernatant of these protein extracts. Collect the flow-through and transfer 1 mL to a marked Eppendorf tube and store on ice. Empty the collection tube.
- Wash the column with 2 mL LEW buffer. Collect the flow through (= first wash) and transfer 1 mL to a marked Eppendorf tube and store on ice. Empty the collection tube.
- Wash the column again with 2 mL LEW buffer. Collect the flow through (= second wash) and transfer 1 mL to a marked Eppendorf tube and store on ice. Empty the collection tube.
- Elute the column with 1 mL Elution buffer (EB). Collect the flow through (= first eluate) and transfer 1 mL to a marked Eppendorf tube and store on ice. Empty the collection tube.
- Elute the column twice more with 1 mL Elution buffer (EB). Collect the flow through (= second and third eluate) and transfer 1 mL to a marked Eppendorf tubes and store on ice.
- Determine the β-galactosidase activity and protein concentration (BioRad assay) of all the collected fractions.

Put 1 mL of the crude extracts in Eppendorf tubes. You now should have 7 samples:

1. crude extract
2. flow through
3. 1st wash
4. 2nd wash
5. 1st eluate
6. 2nd eluate
7. 3rd eluate

Label all tubes!.

Storage, reuse, and regeneration of Protino® Ni Resin

Protino columns can be re-used, but need to be thorougly cleaned before. Consult your teacher if the columns need to be treated and in which way.

After use, wash resin with:

10 bed volumes of	LEW buffer
10 bed volumes of	deionised water
2 bed volumes of	20% ethanol

Store resin in 20% ethanol at 4°C. Seal top and bottom of the columns with Parafilm.

Depending on the nature of the sample Protino® Ni Resin can be reused 3-5 times. Reuse should only be performed with identical poly-histidine tagged proteins to avoid possible cross-contamination.

Complete regeneration

If a complete regeneration is mandatory, wash resin with the following solutions:

2 bed volumes of	6 M GuHCl, 0.2 M acetic acid
5 bed volumes of	deionised water
3 bed volumes of	2 % SDS
5 bed volumes of	deionised water
5 bed volumes of	100 % EtOH
5 bed volumes of	deionised water
5 bed volumes of	100 mM EDTA pH 8
5 bed volumes of	deionised water
5 bed volumes of	100 mM $NiSO_4$
10 bed volumes of	deionised water
2 bed volumes of	20% ethanol

Store resin in 20% ethanol at 4°C. Seal top and bottom of the columns with Parafilm.

Make sure you collect the flow-through of the $NiSO_4$ treatment. It should be disposed of as 'heavy metal' waste.

Assignments:

Prepare a short report of the complete experiment. At least the following topics should be dealt with:

- Aim of the experiment.
- Why the *E. coli* culture has to be induced with arabinose.
- Ultrasonication.
- How the purification works.
- Protein concentration: BioRad assay.
- Work out the data as indicated in this manual and use them to draw conclusions about:
  the (specific) activities of wild-type β-galactosidase in crude extract.
  the thermo-stability of wild-type β-galactosidase.
  the (specific) activity of wild-type after purification.

  Use Excel for these calculations.