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Sveučilište u Zagrebu
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Potraga za interakcijskim partnerima C-terminalne domene IleRS

Završni rad

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Isoleucyl-tRNA synthetase (IleRS) is an enzyme that binds isoleucine and its cognate tRNA molecule. The bacterium *Priestia megaterium* has two IleRS isoenzymes, PmIleRS1 and PmIleRS2. A prominent difference between these isoenzymes is a zinc finger domain at the C-terminus of PmIleRS1 that is not present in PmIleRS2. To determine if this domain has any protein interaction partners, PmIleRS1 was overexpressed in *Escherichia coli*, isolated, purified by Ni-NTA agarose affinity chromatography and concentrated with ultracentrifugation. The same was done with the PmIleRS1ΔZnF protein in which the zinc finger domain is missing. Then both proteins were used as bait in Ni-NTA pull down assays. As a negative control pull-down assays were done with leucyl-tRNA synthetase as well as without bait protein. The collected fractions were analysed by SDS-PAGE and the gels were stained with *Coomassie Brilliant Blue R-250*. To enhance sensitivity and potentially detect low amount of protein, the electrophoresis was repeated, only this time the gel was stained with silver. The results suggest that there is no specific interaction partner of the zinc finger domain of IleRS1 from *P. megaterium*.

Keywords: aminoacyl-tRNA synthetase, *Priestia megaterium*, zinc finger, *pull-down* assay
(26 pages, 9 figures, 0 tables, 22 references, original in: English)
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Mentor: Morana Dulić, PhD, assistant professor

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Izoleucil-tRNA sintetaza (IleRS) enzim je koji veže izoleucin i pripadnu tRNA molekulu. Bakterija *Priestia megaterium* ima dva IleRS izoenzima, PmIleRS1 i PmIleRS2. Značajna razlika između ovih izoenzima jest domena cinkovog prsta na C-terminalnom dijelu PmIleRS1 koja nije prisutna u PmIleRS2. Kako bi se odredilo ima li ta domena neke proteinske interakcijske partnere, PmIleRS1 prekomjerno je eksprimiran u bakteriji *Escherichia coli*, izoliran, pročišćen Ni-NTA agaroznom afinitetnom kromatografijom i ukoncentriran ultracentrifugiranjem. Isto je učinjeno s proteinom PmIleRS1ΔZnF kojem nedostaje domena cinkovog prsta. Oba su proteina korištena kao mamac u Ni-NTA metodi *pull-down*. Kao negativna kontrola napravljen je *pull-down* s leucil-tRNA sintetazom kao mamcem i još jedan bez proteina mamca. Skupljene frakcije analizirane su metodom SDS-PAGE i gelovi su obojani s *Coomassie Brilliant Blue R-250*. Kako bi se povećala osjetljivost i potencijalno uočili proteini u niskim koncentracijama elektroforeza je ponovljena, a gel je obojan srebrom. Rezultati ukazuju na to da ne postoji specifični interakcijski partner domene cinkovog prsta u proteinu IleRS1 iz bakterije *P. megaterium*.

Ključne riječi: aminoacil-tRNA sintetaze, *Priestia megaterium*, cinkov prst, metoda *pull-down*
(26 stranica, 9 slika, 0 tablica, 22 literaturnih navoda, jezik izvornika: engleski)
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Mentor: doc. dr. sc. Morana Dulić

Table of contents

1. Introduction

Aminoacyl-tRNA synthetases (aaRS) are enzymes that catalyse the reaction between a specific amino acid and its cognate tRNA molecule to form an aminoacyl-tRNA which is later used in protein synthesis. One of these enzymes is isoleucyl-tRNA synthetase (IleRS). In the Gram-positive bacteria *Priestia megaterium* there are two isoenzymes, enzymes that catalyse the same reaction but have different structures.

One of the IleRSs belongs to a group that is described as a bacterial type (PmIleRS1) and the other belongs to the eukaryotic type (PmIleRS2). (Cvetesic *et al.*, 2016) A very important structural difference between these two enzymes is the C-terminal Cys_4 cluster that is present in PmIleRS1, but not in PmIleRS2. This cluster consists of four cysteine amino acids that are positioned in a way in which they can interact with a Zn^{2+} ion to form a zinc finger motif. (Glasfeld *et al.*, 1996.) These motifs are known to interact with nucleic acids, but they can also mediate protein-protein interactions. (Brayer and Segal, 2008)

The goal of this thesis is to determine whether the C-terminal domain of PmIleRS1 containing the zinc finger motif interacts specifically with some protein in *P. megaterium*. For this purpose, electrocompetent *Escherichia coli* bacteria will be transformed to overexpress the PmIleRS1 protein, the overexpressed protein will be isolated, and then purified by Ni-NTA affinity chromatography. The same will be done with PmIleRS1ΔZnF, a protein in which the C-terminal zinc finger domain is missing. Afterwards the proteins will be used as bait in Ni-NTA pull-down assays to determine if the zinc finger domain has specific protein interaction partners. As a negative control, pull-down assays will be done with either leucyl-tRNA synthetase (LeuRS) or no bait protein. The obtained fractions will be analysed on polyacrylamide gels and visualized by *Coomassie Brilliant Blue R-250* staining and silver staining.

2. Literature review

2.1. Aminoacyl-tRNA synthetases

Proteins are biological macromolecules that have many different functions, from maintaining cell structure, to transportation, cell signalling and enzymatic activity. The first step to synthesise proteins in living organisms is to transcribe a DNA sequence into messenger RNA (mRNA) with RNA polymerases. Then this mRNA is translated into a polypeptide, a chain of amino acids. Ribosomes catalyse this reaction, but they are not directly responsible for determining which amino acid is added to the sequence. They use a transfer RNA (tRNA) molecule complementary to the mRNA codon in the ribosome. This tRNA molecule must be aminoacylated beforehand, meaning it must have an amino acid on its 3' end. The enzymes that catalyse this reaction are called aminoacyl-tRNA synthetases (aaRS).

AaRS are specific to one amino acid and its specific set of tRNA molecules. This is crucial for faithfulness of translation since ribosomes are not able to proofread whether the correct amino acid is bound to tRNA. AaRSs are divided into two main classes, class I and class II based on their structure and mechanism. Inside each class there are many similarities in their protein structures and domains, especially catalytic domains. (Gomez and Ibba, 2020)

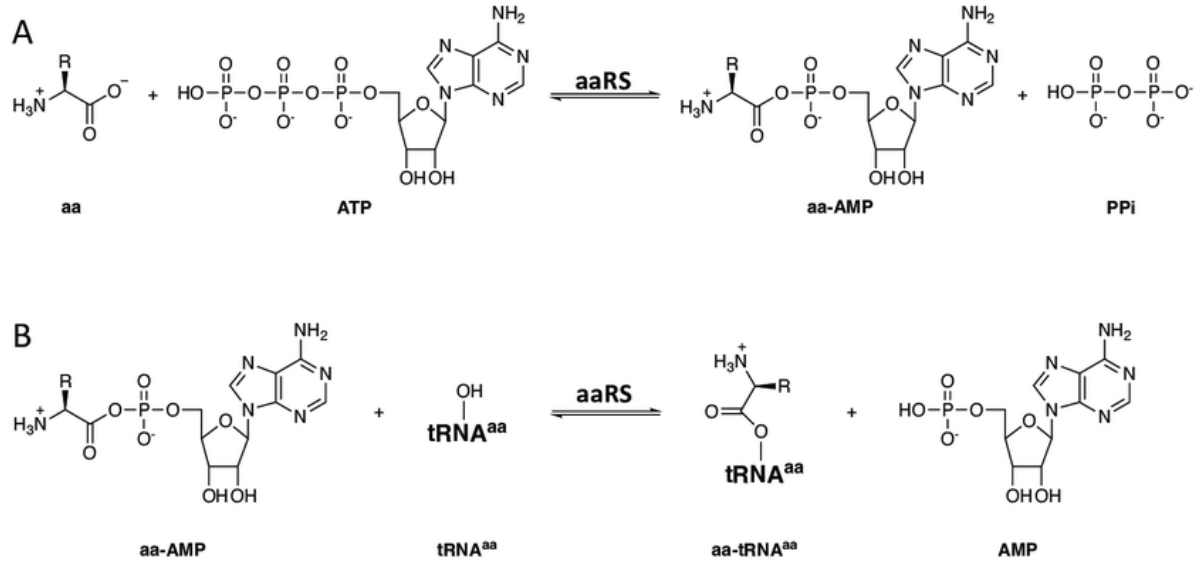

Figure 1. Synthesis of aminoacyl-tRNA. A) The amino acid (aa) is activated with an adenosine triphosphate molecule (ATP) that results in the formation of an aminoacyl adenylate intermediate (aa-AMP) and inorganic pyrophosphate (PP_i) B) The aa-AMP intermediate reacts with the free OH group of the tRNA forming an aminoacyl-tRNA molecule (aa-tRNA) and releasing adenosine monophosphate (AMP) (Hendrickson *et al.*, 2021)

2.2. *Priestia megaterium*

Until recently the genus *Bacillus* consisted of more than 280 species that were very different from each other. It contains some species that are important in everyday life and science, for example *Bacillus subtilis, B. anthracis, B. cereus* and until recently *P. megaterium.* Recently there was a restructuring of this genus in which new genera were added to split up the *Bacillus* genus. The change came as a result of phylogenomic and comparative genomic analysis of the genus. This is why the bacteria previously called *Bacillus megaterium* is placed in the *Priestia* genus with the new name *Priestia megaterium*. (Gupta *et al.*, 2020)

The *Priestia* genus is a monophyletic clade and consists of mostly aerobic Gram-positive species, but some Gram-negative and Gram-variable as well. They are rod shaped, can form endospores and they are motile. They can live in many different places such as in the atmosphere, soil, plant tissue, faeces, and sea sediment. Their temperature range is 5–48 °C, but the best conditions for growth are 28–37 °C. The genus can be distinguished from other genera by conserved specific indels (insertions or deletions) in an oligoribonuclease or cAMP/cGMP phosphodiesterase that are found exclusively in this genus. *Priestia megaterium* is the species on which its genus is based, the type species. (Gupta *et al.*, 2020)

Priestia megaterium is a species of bacteria first described in 1884 by de Bary, but it was known long before that. The cells are 1.5 μm thick and and 4 μm long making them around a hundred times larger than *E. coli*. (Bunk *et al.*, 2010) They are rod shaped, cylindrical and have rounded edges. They are motile, can form slightly curved chains, and can also form spores which makes them slightly shorter and up to half the thickens of regular cells. (de Bary, 1884) These properties allowed them to be a great model for researching Gram-positive bacteria. They were the main model organism before the use of *B. subtilis*. Some of the uses were the investigation of bacterial cell wall biosynthesis, cytoplasmic membrane formation and the spore formation, structure and organization. (Bunk *et al.*, 2010)

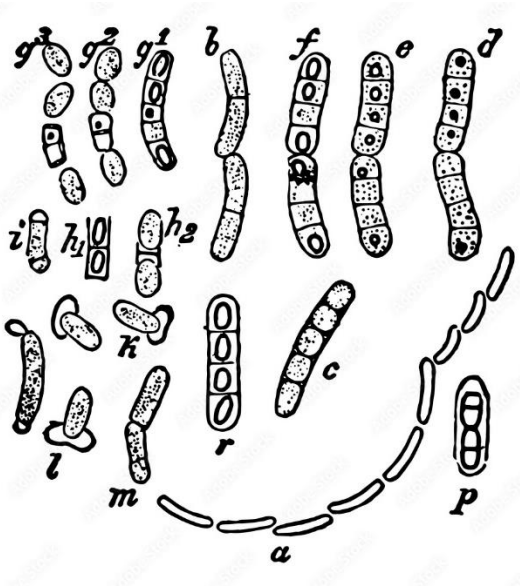

Figure 2. Drawings of *Priestia megaterium* showing among others a chain of cells at 250 × magnification (a), four cells after treatment with alcohol and iodine solution at 600 × magnification (p), and different stages of sporulation (c–f) at 600 × magnification (de Bary, 1884)

2.3. Isoleucyl-tRNA synthetase

The aaRS for isoleucine, isoleucyl-tRNA synthetase (IleRS), is a monomeric class I aaRS. Usually just one enzyme is used, but in the *P. megaterium* bacteria there are two enzymes that catalyse this reaction that are not structurally the same. One is a bacterial type (PmIleRS1) that is characteristic for bacteria as well as mitochondria and chloroplasts in eukaryotes and the other a eukaryotic type (PmIleRS2) mostly present in eukaryotic cytosol, but also in some bacteria. Although one is called bacterial and the other eukaryotic, both are of bacterial origin. Eukaryotes most likely acquired IleRS2 from bacteria by horizontal gene transfer. (Cvetesic *et al.*, 2016) Two main differences between these two isoenzymes are their different resistance to the antibiotic mupirocin and the presence or absence of a zinc finger domain at their C-terminus. (Zanki, 2022) Mupirocin is an antibiotic produced by *Pseudomonas fluorescens* that selectively blocks IleRS and that indirectly blocks protein synthesis. IleRS2 is much more resistant to this antibiotic, but the trade-off is its lower aminoacylation turnover. (Zanki *et al.*, 2022)

The structural difference is the zinc finger present at the C-terminus of PmIleRS1 that does not exist in PmIleRS2. Zinc finger is a protein motif with the main characteristic being

the coordination of a zinc ion (Zn^{2+}). These motifs can have a role in structural stability of the protein but are also able to interact with nucleic acids and can mediate protein-protein interactions as well. (Brayer and Segal, 2008) There are different ways the zinc ion can be coordinated and in the case of PmIleRS1 it is with 4 cysteine amino acids positioned to surround the ion, a Cys_4 cluster.

The presence of this motif does not seem to have a role in the synthesis of isoleucyl adenylate, the intermediate step in which the amino acid isoleucine is activated with ATP forming isoleucyl-tRNA. But it has an important role in the aminoacylation step where an activated isoleucyl adenylate is bound to the corresponding tRNA molecule. (Glasfeld *et al.*, 1996) It is not known if there are any other functions of this zinc finger domain in *P. megaterium*.

In general, it is not clear why *P. megaterium* has two variants of this enzyme when most organism have just one. The presence of PmIleRS2 can be explained with its higher mupirocin resistance. One possibility is that PmIleRS1 has an important protein-protein interaction that cannot be achieved with just PmIleRS2.

3. Materials and methods

3.1. Materials

3.1.1. Standard materials

Acetic acid (*LabExpert*), acrylamide-bisacrylamide (*Roth*), agar (*Liofilchem*), ammonium persulfate (APS) (*Sigma*), bromophenol blue (*Serva*), *Coomassie Brilliant Blue R-250* (*Sigma-Aldrich*), ethanol (*LabExpert*), formaldehyde (*T.T.T*), glycerol (*Kemika*), glycine (*Carlo Erba Reagents*), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (*Fisher Bioreagents*), imidazole (*Sigma-Aldrich*), β-mercaptoethanol (*Sigma*), magnesium chloride ($MgCl_2$) (*Kemika*), Ni-NTA agarose (*Macherey-Nagel*), nitric acid (*Kemika*), potassium dichromate ($K_2Cr_2O_7$) (*Kemika*), silver nitrate ($AgNO_3$) (*Sigma-Aldrich*), sodium carbonate (Na_2CO_3) (*Sigma-Aldrich*), sodium chloride (NaCl) (*Gram-mol*), sodium dodecyl sulphate (SDS) (*Roth*), N,N,N′,N′-tetramethylethylenediamine (TEMED) (*Sigma*), Tris (*Chemsolute*), tryptone (*Liofilchem*), yeast extract (*Biolife*).

3.1.2. Growth media

Liquid LB bacterial growth medium: yeast extract (γ = 5 g dm^{-3}), tryptone (γ = 10 g dm^{-3}), NaCl (γ = 5 g dm^{-3})

Solid LB bacterial growth medium: yeast extract (γ = 5 g dm^{-3}), tryptone (γ = 10 g dm^{-3}), NaCl (γ = 5 g dm^{-3}), agar (γ = 15 g dm^{-3})

3.1.3. Bacterial strains and plasmids

Escherichia coli BL21 (DE3) strain (F^- *ompT gal dcm lon hsdSB* (r_B^- m_B^-) λ (DE3 [*lacI lac*UV5-T7 gene *ind1 sam7 nin5*])) – used for transformation and protein overexpression. The *ompT* and *lon* genes code for proteases and mutating them allows for more protein expression. This strain has a λ prophage carrying T7 RNA polymerase under the strong *lac*UV5 promoter that can be induced with IPTG. It has a few more genes for regulation of the prophage, blocking galactose metabolism and blocking cytosine methylation. (Daegelen *et al.*, 2009)

Priestia megaterium DSM-32 strain – wild type.

pET28–PmIleRS1ΔZnF – plasmid coding for isoleucyl-tRNA synthetase 1 from *P. megaterium* without the C-terminal zinc finger domain.

3.2. Methods

3.2.1. Cultivation of bacterial cells

Escherichia coli and *Priestia megaterium* were routinely grown at 37 °C and 30 °C respectively. They were grown in LB medium (described in 3.1.2.) with shaking at 250 revolutions per minute (rpm). Overnight cultures were prepared in volumes of 5–50 mL with shaking at 100–150 rpm for 12–15 hours.

3.2.2. *E. coli* transformation

Electroporation is a method in which cells are exposed to high-voltage, electric pulses for a short duration. This causes changes in the cell plasma membranes, usually forming small pores, that allow transmembrane transport of molecules that cannot pass the membrane in normal circumstances. This is commonly used to transport DNA which leads to transformation of the bacterial cell. (Kotnik *et al.*, 2019)

Electroporation was done with a *MicroPulser* (*Bio-Rad*) electroporator. A pulse with a voltage of 2.5 kV, capacity of 25 μF and duration of 4–6 ms was used. The *Pulse Controller* (*Bio-Rad*) device for pulse control was used with resistance of 200 μΩ. Electroporation cuvettes and a holder for them was cooled to -20 °C for better efficiency. 50 μL of electrocompetent *E. coli* cells BL21 (DE3) was used and 1–2 ng of the plasmid pET28-PmIleRS1ΔZnF was added for transformation. The suspension was transferred to an electroporation cuvette and the pulse was applied. After electroporation the cells were resuspended in 1 mL of liquid LB medium and incubated for 60 minutes at 37 °C and 350 rpm. After incubation 50 μL of suspension was spread on a solid LB medium plate with the antibiotic kanamycin (γ = 30 μg mL^{-1}) and incubated overnight at 37 °C.

3.2.3. Lysis of bacterial cells and isolation of proteins

The bacterial protein extract was made with the use of a bacterial pellet made from centrifuging 0.5 L of bacterial culture and resuspending it in 5 mL of buffer A (c (HEPES) = 20 mmol dm^{-3} (pH = 7.5), c (NaCl) = 500 mmol dm^{-3}, c ($MgCl_2$) = 5 mmol dm^{-3}, c (imidazole) = 10 mmol dm^{-3}, c (β-mercaptoethanol) = 10 mmol dm^{-3}). This suspension was mixed with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (c = 0.1 mmol dm^{-3}), lysozyme, the enzyme used to break down bacterial cell walls (γ = 20 ng μL^{-1}), and the nuclease DNase I (γ = 3 ng μL^{-1}). The suspension was then sonicated on ice with the *High Intensity Ultrasonic*

Processor (*Bioblock Scientific*) sonicator 8 times for 45 seconds with cooling on ice in between sonication steps. After sonication the suspension was centrifuged for 30 minutes at 20 000 × *g* at 4 °C. The supernatant was used for protein purification by Ni-NTA affinity chromatography.

For the extraction of soluble proteins from *P. megaterium* a similar procedure was used. The difference is in the starting volume of the bacterial culture, 50 mL instead of 0.5 L, the concentration of lysozyme, γ = 2 mg μL^{-1}, the sonication time, 3 times 20 seconds, and the centrifugation time and speed, 20 minutes at 17 000 × *g*.

3.2.4. Protein purification by Ni-NTA agarose affinity chromatography

Chromatography is a biophysical method used for separation, identification, and purification of different molecules. It is based on the use of a stationary phase, which stays fixed and is often a solid, and a mobile phase which travels across the stationary phase and is often in liquid form. Affinity chromatography is a type of chromatography that separates the protein of interest from other molecules by using specific interactions between the protein of interest and a ligand which is tied to the stationary phase. (Coskun, 2016) Ni^{2+}-nitrilotriacetic acid (Ni-NTA) affinity chromatography is a method for protein purification that uses the ability of NTA to form a tetradentate chelate with the Ni^{2+} ion. NTA occupies only four of the six binding sites in the Ni^{2+} coordination sphere which means that two binding sites can freely interact with other molecules. To ensure that the protein of interest interacts with these binding sites, it is modified to have six histidines in a row forming a hexahistidine tag (His_6), which is most often added on the N- or C-terminus of the protein. More precisely, the histidine side chains have imidazole rings with electron donor groups that form coordinate bonds with the Ni-NTA complex. This interaction can be used in protein purification by using Ni-NTA coupled to agarose chromatography beads. (Khan *et al.*, 2006)

All the steps for this method were done in a cold room at 4 ˚C. Proteins were purified on 1 mL of Ni-NTA agarose beads. The column was washed with 15 CV of Mili-Q water and equilibrated with 15 CV of buffer A (*c* (HEPES) = 20 mmol dm^{-3} (pH = 7.5), *c* (NaCl) = 500 mmol dm^{-3}, *c* ($MgCl_2$) = 5 mmol dm^{-3}, *c* (imidazole) = 10 mmol dm^{-3}, *c* (β-mercaptoethanol) = 10 mmol dm^{-3}). The *E. coli* protein extract was loaded onto the column and the flow-through was reloaded twice after that to maximize yield. The column was washed with 30 CV of buffer A, then with 10 CV of buffer B (*c* (HEPES) = 20 mmol dm^{-3} (pH = 7.5), *c* (NaCl) = 500 mmol dm^{-3}, *c* ($MgCl_2$) = 5 mmol dm^{-3}, *c* (imidazole) = 20 mmol dm^{-3}, *c* (β-mercaptoethanol)

= 10 mmol dm^{-3}), then with 10 CV of buffer C (*c* (HEPES) = 20 mmol dm^{-3} (pH = 7.5), *c* (NaCl) = 500 mmol dm^{-3}, *c* ($MgCl_2$) = 5 mmol dm^{-3}, *c* (imidazole) = 30 mmol dm^{-3}, *c* (β-mercaptoethanol) = 10 mmol dm^{-3}) to remove nonspecific interactions with increasing imidazole concentration. Before the elution step the flow was stopped to incubate the column for 5 minutes with 5 CV of buffer D (*c* (HEPES) = 20 mmol dm^{-3} (pH = 7.5), *c* (NaCl) = 500 mmol dm^{-3}, *c* ($MgCl_2$) = 5 mmol dm^{-3}, *c* (imidazole) = 200 mmol dm^{-3}, *c* (β-mercaptoethanol) = 10 mmol dm^{-3}), the elution buffer. At the end there are a few more washing steps to clean the column, firstly with 2 CV of imidazole (*c* (imidazole) = 2 mol dm^{-3}), then 30 CV of Mili-Q water and finally with 10 CV of ethanol (φ = 20%) in which the agarose beads are kept at 4 ˚C for storage. This method was used to purify PmIleRS1 and PmIleRS1ΔZnF proteins.

3.2.5. Dialysis

Membrane dialysis is a biochemical method used during protein purification to remove substances with small molecular mass. (Purushothaman *et al.*, 2019)

This method was used to remove unwanted substances in the solution obtained from protein purification by Ni-NTA agarose affinity chromatography, mostly imidazole because a high concentration was used to elute the protein of interest and to transfer the protein into storage buffer. The solution with the protein of interest was dialysed in a cellulose tube (*Sigma*) in a dialysis solution (*c* (HEPES) = 20 mmol dm^{-3} (pH = 7.5), *c* (NaCl) = 50 mmol dm^{-3}, φ (glycerol) = 10%, *c* (β-mercaptoethanol) = 5 mmol dm^{-3}) for 3 hours, then overnight, then another 3 hours, each in 1 L of dialysis solution.

3.2.6. Protein concentrating

The fraction with the protein of interest obtained by Ni-NTA affinity chromatography was concentrated. This is achieved using the *Amicon Ultra Centrifugal Filters* (*Milipore*) with pores that let through smaller proteins with molecular masses less than 30 kDa. The solution with the protein of interest was loaded into the tube and centrifuged at 5 000 × *g* until the desired concentration was achieved. The concentration was measured using *NanoDrop One* (*Thermo Fisher Scientific*).

3.2.7. Pull-down assay

Novel protein-protein interactions can be determined using a pull-down assay, an *in vitro* technique used to detect physical interactions between two or more proteins. Usually, it involves the use of affinity purification followed by various washing and elution steps. The method is based on using a purified and tagged protein ("bait") to capture proteins that have specific interactions with it ("prey"). The tagged protein must be immobilized on an affinity ligand, an example is the interaction between a His_6 tag and Ni-NTA agarose beads which are also used in protein purification. After the bait is immobilized, the prey can be incubated together with the bait to form a complex. An example of prey is the whole lysate of a bacterial cell with all soluble proteins. Following the incubation there are different washing steps to remove nonspecific interactions and an elution step to collect the proteins with more specific interactions with the bait. To determine the validity of the results a negative control is very helpful. A negative control can be using a bait protein that is similar to the actual bait being tested and this is useful because it "takes up" the same space on the affinity ligand as the regular bait but doesn't have the same bait-prey interaction. Another negative control is not using a bait at all, just testing the interaction between prey proteins and the affinity column. (Louche *et al.*, 2017)

All the steps for this method were done in a cold room at 4 ˚C. To start, 100 μL of Ni-NTA agarose beads were used and the column was washed with 400 μL of Mili-Q water. 50 μg of the protein with a His_6 tag (bait) were mixed with 400 μL of buffer A (c (HEPES) = 50 mmol dm^{-3} (pH = 7.5), c (NaCl) = 10 mmol dm^{-3}, c ($MgCl_2$) = 10 mmol dm^{-3}, c (imidazole) = 10 mmol dm^{-3}, c (β-mercaptoethanol) = 10 mmol dm^{-3}), loaded onto the column and incubated for 10 minutes. The flow-through was reloaded onto the column twice and the final flow-through was kept for later analysis (fraction 1). 200 μL of *P. megaterium* cell lysate (prey) were mixed with 200 μL of buffer A and loaded onto the column followed by a 45-minute incubation time. The flow-through was kept for analysis (fraction 2). The washing steps were done by washing the column with 400 μL of buffer A and keeping the flow-through (fraction 3), then washing it three times with 400 μL of buffer B (c (HEPES) = 50 mmol dm^{-3} (pH = 7.5), c (NaCl) = 10 mmol dm^{-3}, c ($MgCl_2$) = 10 mmol dm^{-3}, c (imidazole) = 20 mmol dm^{-3}, c (β-mercaptoethanol) = 10 mmol dm^{-3}). The first wash with buffer B (fraction 4) and the last (fraction 5) were kept for analysis. Elution was done with a 10-minute incubation with 80 μL of buffer C (c (HEPES) = 50 mmol dm^{-3} (pH = 7.5), c (NaCl) = 10 mmol dm^{-3}, c ($MgCl_2$) = 10 mmol dm^{-3}, c (imidazole) = 200 mmol dm^{-3}, c (β-mercaptoethanol) = 10 mmol dm^{-3}). The

elution step was repeated and the flow-through was kept for analysis (fraction 6). At the end there are a few more washing steps to clean the column, firstly with 400 μL of imidazole (c (imidazole) = 2 mol dm^{-3}), then three times 400 μL of Mili-Q water and finally two times with 400 μL of ethanol (φ = 20%) in which the agarose beads are kept at 4 ˚C for storage.

3.2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a very commonly used biochemical method for protein analysis. The method is based on electrophoresis, the migration of charged particles under the influence of an electric field. When electrophoresis is applied to a porous matrix, made from for example polyacrylamide, it can be used to separate proteins based on their molecular mass. Usually there are two parts to a gel, the upper stacking gel used to concentrate proteins in a thin horizontal line to increase the final resolution, and the lower separating gel with more polyacrylamide to separate the polypeptides by molecular mass. (Koshkina *et al.*, 2023) SDS is a strong detergent, and its role is to ensure that all proteins dissociate into separate polypeptide subunits. This is achieved by the binding of approximately one SDS molecule per two amino acids which denatures the protein. Another useful property is the net negative charge of the polypeptide-SDS complex that is achieved by this interaction that leads to the masking of the intrinsic charge of the polypeptide. That is why proteins are separated by their molecular mass and the migration of the complexes is proportional to the relative size of the polypeptide chain. This can be used to estimate the molecular mass of an unknown protein by comparing it with proteins of known molecular mass. (Kielkopf *et al.*, 2021) The proteins can be visualized on the gel with *Coomassie Brilliant Blue* staining. This occurs because *Coomassie Brilliant Blue* has sulfonic groups that can interact with basic amino acids. (Tal *et al.*, 1985)

The lower separating gel was made with 8% (*w*/*V*) acrylamide-bisacrylamide (29:1), c (Tris-HCl) = 375 mmol dm^{-3} (pH = 8.8), γ (SDS) = 1 g dm^{-3}, γ (APS) = 0.7 μg mL^{-1}, 0.05% (*w*/*V*) TEMED. Ammonium persulfate (APS) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were added just before pouring out the gel because they are initiating and catalysing the polymerization reaction of acrylamide. Water was added at the top to make the surface even and to prevent contact with air. The gel was 0.75 mm thick, and it was left to polymerize for 45 minutes after which the water was removed. The upper stacking gel was made with 4% (*w*/*V*) acrylamide-bisacrylamide (29:1), c (Tris-HCl) = 125 mmol dm^{-3} (pH = 6.8), γ (SDS) =

1 g dm^{-3}, γ (APS) = 0.7 μg mL^{-1}, 0.05% (*w*/*V*) TEMED. APS and TEMED were again added just before pouring out the gel, the comb was added to make wells, and it was left to polymerize for 30 minutes.

All the fractions collected with the pull-down assay were mixed with a loading buffer (*c* (Tris-HCl) = 62.5 mmol dm^{-3} (pH = 6.8), *c* (β-mercaptoethanol) = 12.5 mmol dm^{-3}, φ (glycerol) = 6.25%, 1.25% (*w*/*V*) SDS, 0.002% (*w*/*V*) bromophenol blue). Bromophenol blue is a dye used to determine how far the front travelled in the gel. The samples are heated for 5 minutes at 95 ˚C before being loaded onto the gel to help with denaturation of the polypeptides. The devices used for electrophoresis were *Mini-PROTEAN Tetra Cell* (*Bio-Rad*) and *Power Pac 300* (*Bio-Rad*). The running buffer was made with γ (glycine) = 14.4 g dm^{-3}, γ (Tris-HCl) = 3.0 g dm^{-3} (pH = 8.3), γ (SDS) = 1.0 g dm^{-3}. As a molecular weight marker, 7 μL of *Precision Plus Protein Standards Unstained* (*Bio-Rad*) was used. All other wells were loaded with 20 μL of samples, except the wells with purified proteins after pull-down assays in which only 2 μL were added. The gel was run for 15 minutes at 120 V to stack the proteins and then for 45 minutes at 180 V to separate them. The gel was then stained for 15 minutes with gentle shaking in a solution of *Coomassie Brilliant Blue R-250* (φ (acetic acid) = 10%, φ (ethanol) = 50%, γ (*Coomassie Brilliant Blue R-250*) = 2.5 g dm^{-3}). After staining the unbounded dye was removed by destaining in boiling distilled water. Images of the gel were taken with *ChemiDoc MP Imaging System* (*Bio-Rad*).

3.2.9. Silver staining of polyacrylamide gels

Silver staining is a method to visualize proteins that is more sensitive than staining with *Coomassie Brilliant Blue*. A polyacrylamide gel is fixed with acetic acid and ethanol to remove interfering compounds, then sensitized to increase the sensitivity and final contrast. (Chevallet *et al.*, 2006) A solution containing soluble silver ions (Ag^+) is prepared and then a polyacrylamide gel is soaked in it for impregnation. Afterwards formaldehyde, a reductant, is applied which leads to development. The development leads to reduction of silver ions, they are turned into insoluble metallic silver (Ag^0) that is visible and detectable. This occurs where proteins are present in the gel because proteins promote silver deposition. The reaction is autocatalytic, metallic silver also promotes more silver to be deposited and this is why silver staining has such high sensitivity. The drawback is high susceptibility to contamination from other proteins. (Yan *et al.*, 2000) It is very important to stop the development at the right

moment otherwise there will be too much background interference. This is done by adding acetic acid. (Chevallet *et al.*, 2006)

Preparation of the polyacrylamide gel and electrophoresis is the same as described in 3.2.8. After electrophoresis the gel is moved to a glass dish. All incubations are done with gentle shaking and all solutions are removed by careful aspiration. The gel is fixed for 30 minutes with fixation solution A (φ (acetic acid) = 12%, φ (ethanol) = 50%), then 30 minutes with fixation solution B (φ (acetic acid) = 5%, φ (ethanol) = 10%). Sensitization is done for 5 minutes with a sensitization solution (c ($K_2Cr_2O_7$) = 3.4 mmol dm^{-3}, c (conc. nitric acid) = 3.2 mmol dm^{-3}). The gel is washed with Mili-Q water for 5 minutes three times in a row. Silver impregnation occurs for 30 minutes with an impregnation solution (c ($AgNO_3$) = 12 mmol dm^{-3}). The gel was then pre-developed (c (Na_2CO_3) = 280 mmol dm^{-3}, 0.01% (w/V) formaldehyde (37%)) and developed (c (Na_2CO_3) = 280 mmol dm^{-3}, 0.05% (w/V) formaldehyde (37%)). These steps were done one after another until a silver was visibly created. To stop the reaction when the contrast was optimal, a stop solution was added (φ (acetic acid) = 1%). Images of the gel were taken with *ChemiDoc MP Imaging System* (*Bio-Rad*).

4. Results

4.1. Protein purification by Ni-NTA agarose affinity chromatography

The proteins of interest, PmIleRS1 and PmIleRS1ΔZnF, were purified by Ni-NTA agarose affinity chromatography as described in 3.2.4. The proteins had a hexahistidine (His_6) tag on the C-terminus which could interact with the Ni-NTA agarose beads with high specificity. Cell lysate from transformed *E. coli* was centrifuged, and the supernatant contains a large amount of a protein at around 100 kDa as seen in fig. 3 and fig. 4 (SN). This is most likely the overexpressed protein. This suggests that the step preceding the protein purification, the transformation of *E. coli*, was done successfully. The pellet does not contain as much, or possibly any, of this protein (P) and this suggests that the protein of interest is soluble in water. Almost all proteins visible in these fractions are washed away with buffer A, B, and C, but these wash steps also removed some of the protein at around 100 kDa. They are washed away with higher imidazole concentrations because they do not form a stable complex with Ni^{2+} ions to keep them in the column. Most of the overexpressed protein was eluted with buffer D, a majority in fractions D1, D2 and D3. Only a small amount was washed away with the imidazole solution (I) because most of it was already eluted. Fractions D1–D5 were combined and concentrated as described in 3.2.6. The molecular masses and extinction coefficients were calculated using the *Protparam* (*Expasy*) tool (M (PmIleRS1) = 104960.84 Da, ε (PmIleRS1) = 172120 $dm^3\ mol^{-1}\ cm^{-1}$, M (PmIleRS1ΔZnF) = 100231.57 Da, ε (PmIleRS1ΔZnF) = 165130 $dm^3\ mol^{-1}\ cm^{-1}$). The final concentration was measured using *NanoDrop One* (*Thermo Fisher Scientific*) (γ (PmIleRS1) = 15.37 $g\ cm^{-3}$, γ (PmIleRS1ΔZnF) = 14.51 $g\ cm^{-3}$).

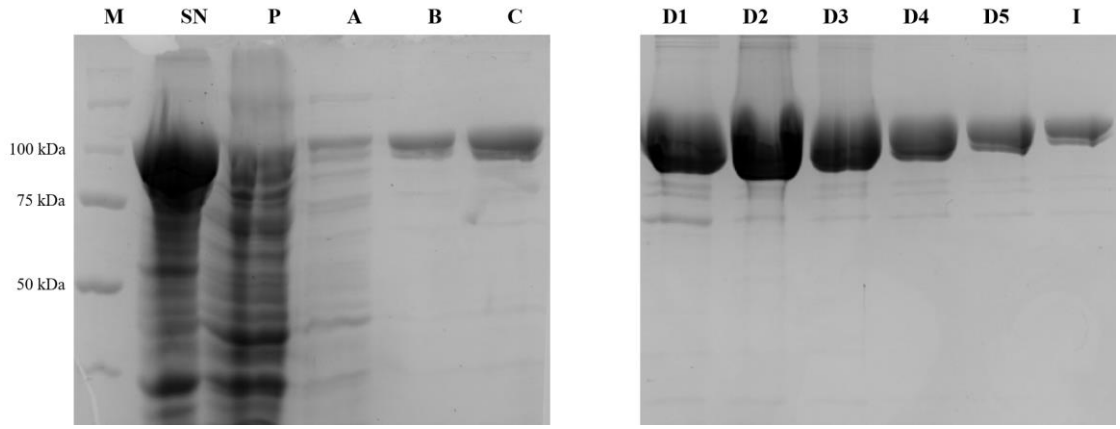

Figure 3. Analysis of PmIleRS1 protein purification by Ni-NTA agarose affinity chromatography on a 10% polyacrylamide gel after SDS-PAGE electrophoresis and *Coomassie Brilliant Blue R-250* staining. M – molecular weight marker *Precision Plus Protein Standards Unstained* (*Bio-Rad*), SN – supernatant after centrifugation of *E. coli* cell lysate containing soluble proteins, P – resuspension of the pellet in buffer A after centrifugation, A – fraction after wash with buffer A, B – fraction after wash with buffer B, C – fraction after wash with buffer C, D1–D5 – fractions after elution with buffer D, I – fraction after wash with imidazole solution.

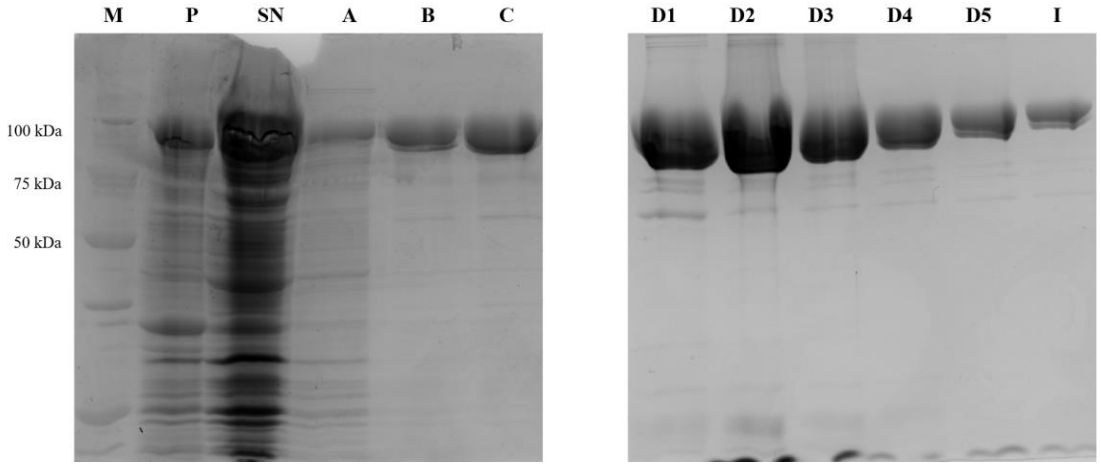

Figure 4. Analysis of PmIleRS1ΔZnF protein purification by Ni-NTA agarose affinity chromatography on a 10% polyacrylamide gel after SDS-PAGE electrophoresis and *Coomassie Brilliant Blue R-250* staining. M – molecular weight marker *Precision Plus Protein Standards Unstained* (*Bio-Rad*), P – resuspension of the pellet in buffer A after centrifugation, SN – supernatant after centrifugation of *E. coli* cell lysate containing soluble proteins, A – fraction after wash with buffer A, B – fraction after wash with buffer B, C – fraction after wash with buffer C, D1–D5 – fractions after elution with buffer D, I – fraction after wash with imidazole solution.

4.2. Pull down-assay and *Coomassie Brilliant Blue* staining

After the isolation and purification of proteins they were used as bait in Ni-NTA pull-down assays as described in 3.2.7. All fractions obtained by this method were loaded onto a gel and stained with *Coomassie Brilliant Blue* to analyse each step. In fraction 1 (flow-through (FT) after loading the bait protein) there is almost no protein in all gels which suggests that the bait protein was successfully bound to the Ni-NTA agarose beads. Fraction 2 (FT after loading cell extract) can be compared to the cell extract from *P. megaterium* bacteria (BE) to show that most proteins do not interact with the bait, they just pass through the column. Fractions 3 (FT after wash with buffer A) and 4 (FT after first wash with buffer B) and 5 (FT after third wash with buffer B) show that the wash steps are successfully removing proteins that do not interact specifically enough with the bait protein. Fraction 6 (FT after elution with buffer C) contains one band which is the purified bait protein, except in the assay where no bait protein is used. It also contains a fainter band at around 120 kDa that is present in all assays.

The presence of this band in all gels, even the one with LeuRS and no protein as bait suggests that this band is the result of an interaction with the agarose beads themselves, not the PmIleRS1 protein. If there was a band that is present when PmIleRS1 was used as bait and not present when bait was PmIleRS1ΔZnF, LeuRS or when no bait was used, that would most likely be a protein that interacts with the zinc finger domain of the PmIleRS1 protein. The results shown did not have such bands which suggests that no such protein exists.

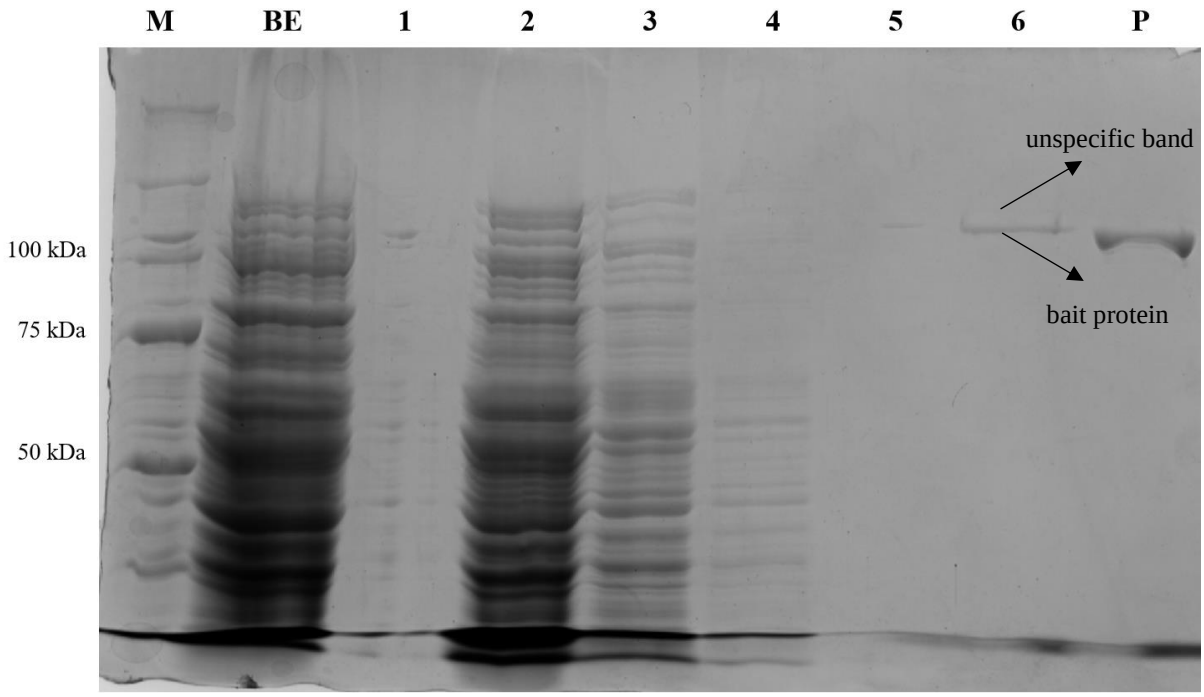

Figure 5. Analysis of a Ni-NTA pull-down assay with PmIleRS1 as bait and *P. megaterium* protein extract as prey on an 8% polyacrylamide gel after SDS-PAGE electrophoresis and *Coomassie Brilliant Blue R-250* staining. M – molecular weight marker *Precision Plus Protein Standards Unstained* (*Bio-Rad*), BE – bacterial protein extract of *P. megaterium* containing soluble proteins, 1 – flow-through (FT) of PmIleRS1 protein in buffer A, 2 – FT of bacterial cell extract in buffer A, 3 – FT after wash with buffer A, 4 – FT after first wash with buffer B, 5 – FT after third wash with buffer B, 6 – FT after elution with buffer C, P – purified PmIleRS1 protein.

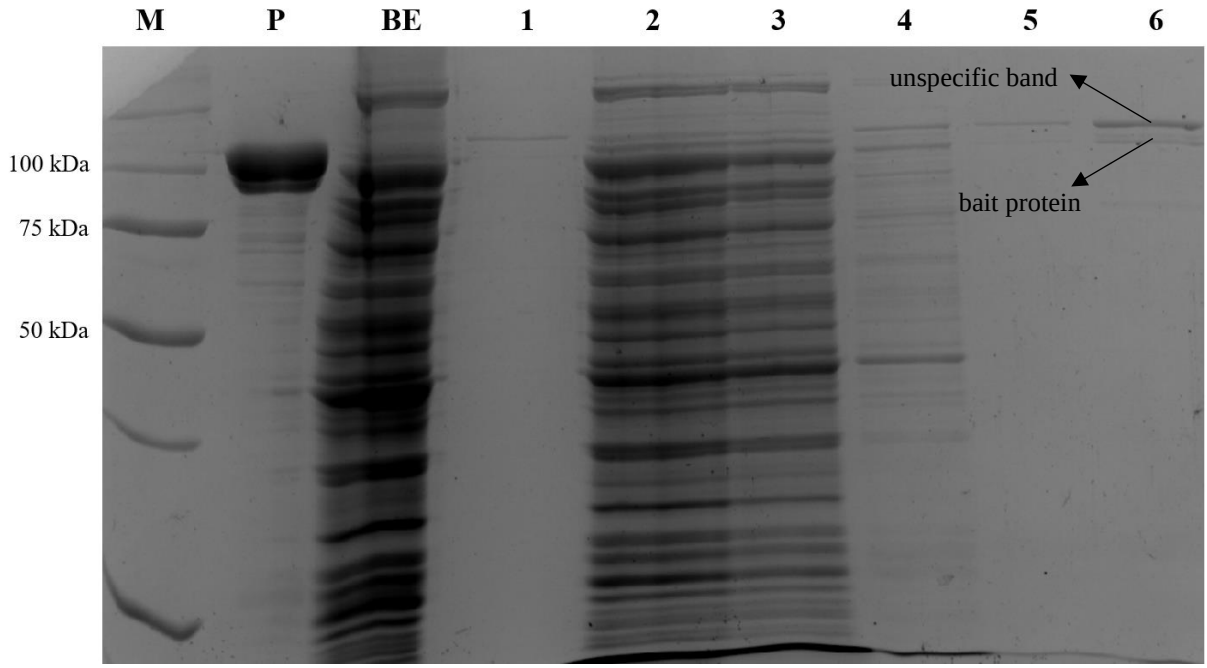

Figure 6. Analysis of a Ni-NTA pull-down assay with PmIleRS1ΔZnF as bait and *P. megaterium* protein extract as prey on an 8% polyacrylamide gel after SDS-PAGE electrophoresis and *Coomassie Brilliant Blue R-250* staining. M – molecular weight marker *Precision Plus Protein Standards Unstained* (*Bio-Rad*), P–purified PmIleRS1ΔZnF protein, BE – bacterial protein extract of *P. megaterium* containing soluble proteins, 1 – flow-through (FT) of PmIleRS1ΔZnF protein in buffer A, 2 – FT of bacterial cell extract in buffer A, 3 – FT after wash with buffer A, 4 – FT after first wash with buffer B, 5 – FT after third wash with buffer B, 6 – FT after elution with buffer C.

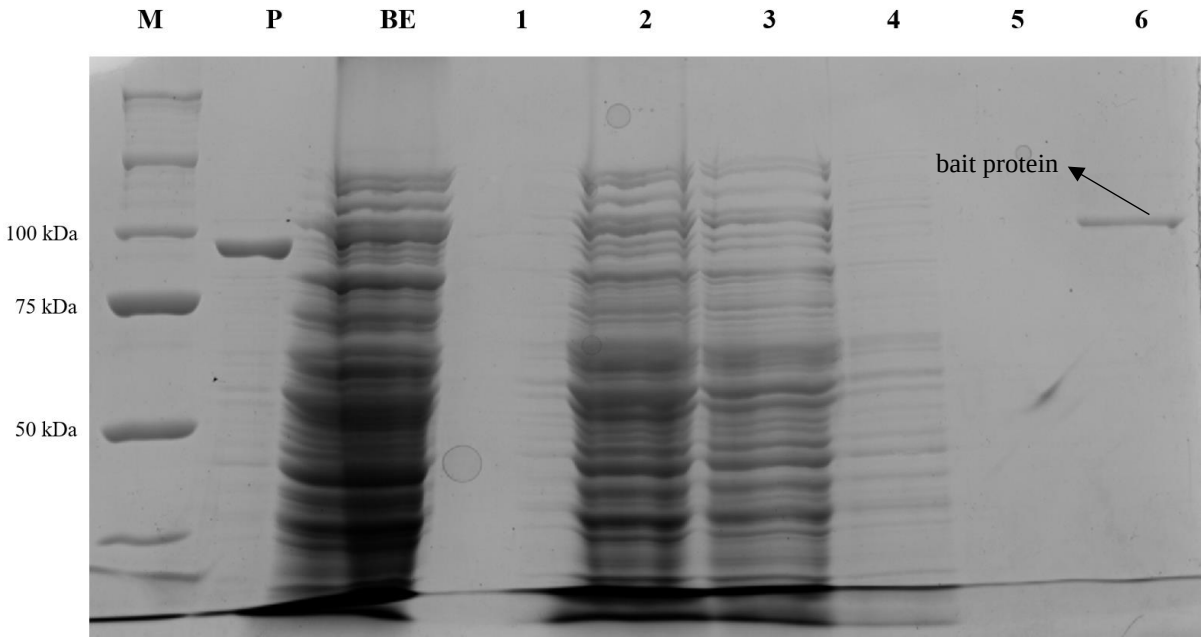

Figure 7. Analysis of a Ni-NTA pull-down assay with LeuRS as bait and *P. megaterium* protein extract as prey on an 8% polyacrylamide gel after SDS-PAGE electrophoresis and *Coomassie Brilliant Blue R-250* staining. M – molecular weight marker *Precision Plus Protein Standards Unstained* (*Bio-Rad*), P – purified LeuRS protein, BE – bacterial protein extract of *P. megaterium* containing soluble proteins, 1 – flow-through (FT) of LeuRS protein in buffer A, 2 – FT of bacterial cell extract in buffer A, 3 – FT after wash with buffer A, 4 – FT after first wash with buffer B, 5 – FT after third wash with buffer B, 6 – FT after elution with buffer C.

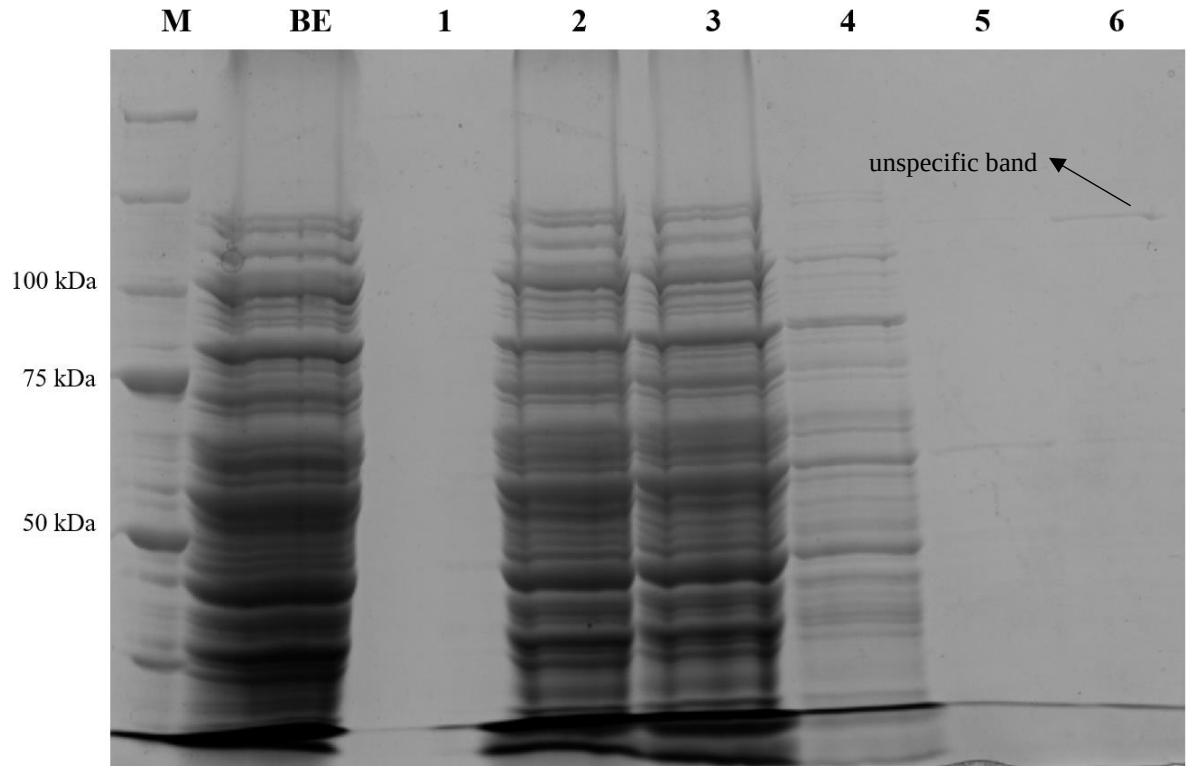

Figure 8. Analysis of a Ni-NTA pull-down assay with no protein as bait and *P. megaterium* protein extract as prey on an 8% polyacrylamide gel after SDS-PAGE electrophoresis and *Coomassie Brilliant Blue R-250* staining. M – molecular weight marker *Precision Plus Protein Standards Unstained* (*Bio-Rad*), BE–bacterial protein extract of *P. megaterium* containing soluble proteins, 1 – flow-through (FT) of buffer A, 2 – FT of bacterial cell extract in buffer A, 3 – FT after wash with buffer A, 4 – FT after first wash with buffer B, 5 – FT after third wash with buffer B, 6 – FT after elution with buffer C.

4.3. Silver staining

Eluted fractions obtained by Ni-NTA pull-down assays were loaded onto a gel and underwent silver staining as described in 3.2.9. Silver staining was done because it is more sensitive than *Coomassie Brilliant Blue* staining. The gel was loaded with fraction 6 (flow-through after elution with buffer C) from pull-down assays using PmIleRS1 as bait (IRS1), using LeuRS as bait (LRS) and using no protein as bait (NC). The gel was also loaded with purified PmIleRS1 and LeuRS proteins in two different concentrations. One was diluted 100 × from the original protein concentration (IRS-P1 and LRS-P1), and the other was diluted 20 × (IRS-P2 and LRS-P2). This staining also revealed the existence of a band at around 120 kDa as described in 4.2. This gel also shows many more proteins in the fraction 6 with no protein

as bait (NC). There are many smaller proteins present that were not very well visible with *Coomassie Brilliant Blue* staining. There is also more of the protein at 120 kDa than in the assays using PmIleRS1 and LeuRS as bait. This makes sense because there is no bait protein that "takes up space" around the Ni^{2+} ion which allows more proteins to interact and not be washed away in previous steps.

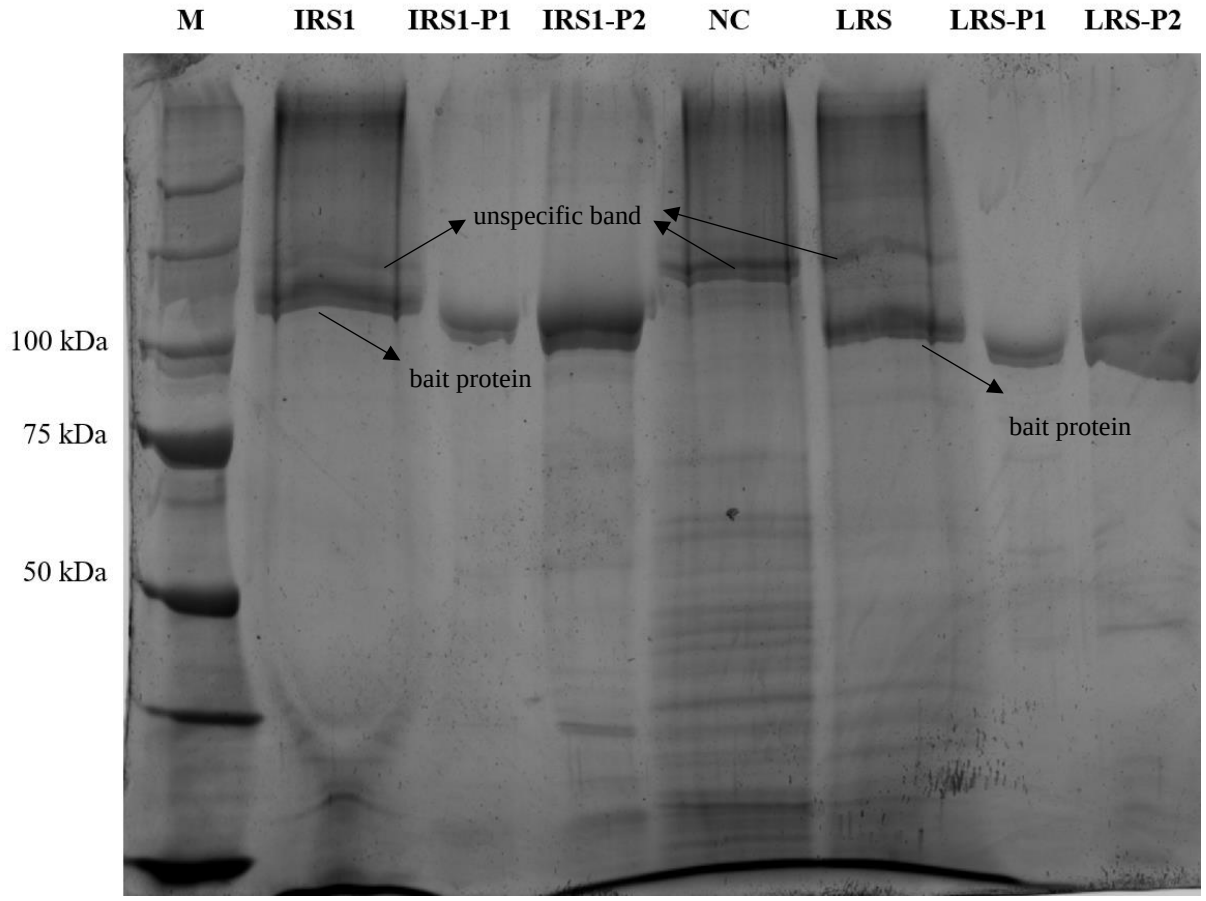

Figure 9. Analysis of a Ni-NTA pull-down assay with different proteins as bait and *P. megaterium* protein extract as prey on an 8% polyacrylamide gel after SDS-PAGE electrophoresis and silver staining. M – molecular weight marker *Precision Plus Protein Standards Unstained* (*Bio-Rad*), IRS1 – flow-through (FT) after elution with buffer C using PmIleRS1 as bait, IRS-P1 – purified PmIleRS1 protein, IRS-P2 –five times more concentrated purified PmIleRS1 protein, NC – (negative control) FT after elution with buffer C using no protein as bait, LRS – FT after elution with buffer C using LeuRS as bait, LRS-P1 – purified LeuRS protein, LRS-P2 – five times more concentrated purified LeuRS protein.

5. Discussion

The results of pull-down assays with PmIleRS1, PmIleRS1ΔZnF and negative controls in the form of LeuRS and no bait protein show no protein that interacts specifically with the PmIleRS1 zinc finger domain. This could be either due to technical reasons, where we could not detect interaction, or due to the fact that no such protein exists. Zinc finger domains have roles other than protein-protein interactions. As previously described, these can be interactions with DNA, interactions with RNA and structural stabilization and this zinc finger motif could have one of these roles. (Brayer and Segal, 2008) It is already shown that this domain has a role in the aminoacylation step of isoleucyl-tRNA synthesis, it could have a role in keeping the right conformation of the protein and positioning of tRNA. (Glasfeld *et al.*, 1996) Another explanation could be that there exists a protein that interacts with this domain, but the conditions are specific and were not replicated properly in the experiment. Maybe the conditions like pH, ionic strength or concentration of other proteins and solutes must be closer to those in the living *P. megaterium* cells to allow for interactions to happen. (Speer *et al.*, 2021) It is also possible that the proteins which interact with PmIleRS1 are not soluble enough. that they stay in the pellet after centrifugation of the cell lysate and are not in the pull-down assay. Another possibility is that there must be more than one protein or that a specific conformation is needed for interaction with the bait. It may be that energy in the form of ATP is needed for interactions to occur.

To determine if any of this is true more experiments are needed. A possibility is to try to mimic the conditions in the *P. megaterium* cells better. Another is to use a different method for determining protein-protein interaction such as co-immunoprecipitation (co-IP).

6. Conclusion

- Electrocompetent *E. coli* cell of the BL21 (DE3) strain were successfully transformed with the pET28-PmIleRS1ΔZnF plasmid.
- The PmIleRS1 and PmIleRS1ΔZnF proteins were overexpressed in *E. coli*, isolated, and purified by Ni-NTA agarose affinity chromatography. These proteins underwent dialysis to transfer them into storage buffer and successfully concentrated using ultracentrifugation.
- These proteins were used as bait in Ni-NTA pull-down assays, the obtained fractions were analysed with SDS-PAGE electrophoresis and stained with *Coomassie Brilliant Blue R-250* and some gels with silver for higher sensitivity. These results show one unspecific band but suggest that there is no specific interaction partner for the zinc finger domain of PmIleRS1.

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8. Resume

I was born in 2001 in Vinkovci. My primary school was OŠ Josipa Lovretića in Otok and my music school OGŠ Josipa Runjanica in Vinkovci. I went to high school in Gimnazija Matije Antuna Reljkovića in Vinkovci. In 2019 I took part in the 11th ISABS Conference on Forensic and Anthropologic Genetics and Mayo Clinic Lectures in Individualized Medicine in Split with a poster presentation. In 2020 I enrolled in a bachelor's degree of Molecular biology at the University of Zagreb. In the summer of 2022, I did an internship at the Institute of Biochemistry II at Goethe-Universität in Frankfurt am Main in the team of Professor Ivan Đikić where I researched ER-phagy and lipid droplets. There I also took part 3rd Frankfurt Conference on Quality Control in Life Processes. This summer I will do another internship in Frankfurt where I will research the effect of ubiquitination on the development of liver tumour for my master's thesis.