Characterization of FLAG-tagged *Vibrio* alkaline phosphatase

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Líf- og umhverfisvísindadeild

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Leiðbeinendur
Bjarni Ásgeirsson
Jens Guðmundur Hjörleifsson

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Útdráttur

Ensím eru prótein er virka sem hvatar fyrir hina ýmsu líffræðilegu ferla frumunnar. Í sínu virka formi eru þessi prótein gjarnan samsett af nokkrum undireiningum sem saman vinna verk ensímsins. Alkalískur fosfatasi úr kuldakæru *Vibrio splendidus* sjávarbakteríunni (VAP er tvíliða, þ.e. samsett úr tveimur eins undireiningum. Í verkefninu sem hér er lýst var unnið með tvö villigerðarafbriði af þessu ensími, annars vegar *Strep*-tag merkt en hins vegar 2x FLAG-tag merkt afbrigði. Í báðum tilfellum var ensímið lengt frá karboxýlenda með stuttri röð amínósýra til að auðvelda rannsóknir á samskiptum einiganna tveggja og jafnvægisástandinu þeirra á milli.

Verkefnið beindist að mestum hluta að því að hanna röð aðgerða til einangrunar á 2x FLAG afbrigðinu eftir tjáningu þess í örveru, sem og að finna leið til að fylgjast með mögulegum undireiningaskiptum þess við *Strep*-tag afbrigðið.

Hentug aðferð var þróuð fyrir hreinsun á 2x FLAG afbriðinu, en jafnframt komið auga á skref sem bæta má. Rannsóknin á samskiptum einiganna fól í sér að meta nokkrar aðferðir við að greina einliður frá tvíliðum, og tvíliður sem voru búnar að aðgreinast í einliður og endurparast aftur við nágranna. Auk notkunar á *Strep*-tag spunasúlum, voru til athugunar aðferðir til skoðunar á á undireiningaskiptum sem fólust annars vegar í rafdrætti (með eða án urea, eða "Coomassie Blue") en hins vegar jónaskiptaskiljun. Báðar aðferðirnar lofa góðu um að greina megi blending af *Strep*-tag og FLAG-tag merktum VAP undireiningum á hraðan og auðveldan hátt.

Abstract

Enzymes are proteins which act as catalysts for cellular biological processes. Enzymes are often composed of a few, or more subunits in their active form. Alkaline phosphatase from the cold-adapted *Vibrio splendidus* marine bacteria is a homodimer, i.e. it is composed of two identical monomers. In this project, two wild-type variants of the *Vibrio* alkaline phosphatase were used, one with a *Strep*-tag on it C-terminus, coupled with a 2-amino acid linker, and another variant with a 2x FLAG tag on its C-terminus. These tags are intended to simplify research based on the interaction of these variants and their subunits, as well as the equilibrium between the two.

The main objective of the project can be thought of as being twofold. First it was to design a purification protocol for the 2x FLAG variant (after expression in bacteria), and secondly to find a way to monitor the possible subunit exchanges between the two variants.

A simple and convenient protocol was developed for the purification of the 2x FLAG variant, but some elements of said protocol could be improved upon further. Regarding the subunit exchanges, a number of methods were tried and assessed on their ability to distinguish between monomers and dimers, as well as identifying dimers with had possibly unfolded into monomers and paired up with nearby monomers. *Strep*-tag spin columns, along with various electrophoresis techniques (with and without urea, or Coomassie Blue dye), and ion exchange chromatography were used for this purpose. Both electrophoresis and ion exchange techniques proved promising to be able to observe a hybrid of *Strep*-tag and 2x FLAG tagged variants, most notably urea gel electrophoresis and QFF ion-exchange chromatography.

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Abbreviations

AP	Alkaline phosphatase
ECAP	*E. coli* alkaline phosphatase
VAP	*Vibrio* alkaline phosphatase
PLAP	Placental alkaline phosphatase
TTR	Transthyretin
RT	Room temperature (20-25°C)
***p*NPP**	p-nitrophenyl phosphate
TMC	Tris-magnesium-chloride
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
VAP	*Vibrio* alkaline phosphatase
VAP-ST2	*Strep*-tagged wild-type alkaline phosphatase
2x FLAG	Double C-terminal FLAG-tagged wild type alkaline phosphatase
Caps	3-(Cyclohexylamino)-1-propanesulfonic acid
Mops	3-Morpholinopropane-1-sulfonic acid
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast protein liquid chromatography
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
DTT	Dithiothreitol
O.D.	Optical density

Acknowledgements

I would like to thank my instructors, Bjarni Ásgeirsson and Jens Guðmundur Hjörleifsson for their invaluable guidance and insight during the course of this project. A special mention of Kristinn Ragnar Óskarsson for his good company and counsel.

1 Introduction

1.1 Enzymes

Numerous studies have been reported on the properties of enzymes since their discovery more than a century ago (Kühne, 1877). They are known to be extremely efficient biological catalysts which are responsible for catalyzing most of the reactions essential for life. Being a catalyst, an enzyme does not affect the equilibrium constant of a reaction, i.e. it cannot make an unfavorable reaction favorable. What it does, is lower the activation energy needed for a reaction, often converting a one- or two-step process into smaller steps, each requiring less energy than the uncatalyzed reaction. Thus, an enzyme-catalyzed reaction, or *enzymatic reaction* can be up to 10^3-10^{17} times faster than the corresponding uncatalyzed reaction (Horton et al., 2002).

Unlike many of the catalysts that chemists may use in the laboratory, enzymes are usually highly specific in their action. Though their degree of specificity may vary. A highly specific enzyme can act on a single type of compound, while others may act on a variety of molecules. The compounds which enzymes are specific to are referred to as the enzyme's *substrate* (McMurry, 2011). A committee of the International Union of Biochemistry and Molecular Biology (IUBMB) maintains a classification scheme which, among other things, categorizes enzymes based on six main categories – oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (IUBMB, 2016). Hydrolases are a class of enzymes which catalyze hydrolysis. They catalyze the hydrolysis of esters, amides, and related substrates, and can be thought of as a special class of transferases, with H_2O serving as the acceptor of the group transferred (Horton et al., 2002). Alkaline phosphatase, the focal point of this research, is a hydrolase within the sub-subclass, "phosphoric monoester hydrolases", giving it the Enzyme Commission (EC) number, EC 3.1.3.1 (IUBMB, 2016).

1.2 Alkaline phosphatase

The family of alkaline phosphatases (APs) constitute a group of conserved enzymes which display a broad (non-specific) substrate specificity as catalysts for the hydrolysis or transesterification of phosphoryl esters (Kim & Wyckoff, 1991). The APs are widespread in nature and can be found in most organisms, from bacteria to vertebrates. They derive their name from their alkaline pH optimum for catalysis (k_{cat}), which is most often quite removed from the organisms' physiological pH. For example, the optimum pH of APs in animals has been shown to be above pH 10, but in bacterial variants it is closer to pH 9 (Helland et al., 2009).

The majority APs have been shown to be homodimers, where the active site of each monomer contains a nucleophilic serine residue along with three conserved metal ion binding sites, containing one Mg^{2+}, and two Zn^{2+} ions (Millán, 2006). Each monomer has a mixed α/β protein structure. A β-sheet can be found buried in the center of each monomer with α-helices on each side of said β-sheet (Kim & Wyckoff, 1991).

Comparison between APs from different organisms has shown the enzyme to display quite dissimilar structural details. Bacterial APs seem to span a wide size spectrum, from short polypeptide versions (TAB5, 375 a.a.) to longer polymer lengths (Atlantic *Vibrio* strain, 502 a.a.) similar to mammalian variants (Helland et al., 2009). Comparison between the surface residues of placental AP (PLAP) and *E. coli* AP found them only to have 8% of their residues in common. However, despite their dissimilarities, the core active-site structure between different organisms has been shown to be highly conserved. An analysis of the structure-function relationship between residues conserved in *E. coli* AP (ECAP) and PLAP indicates a conserved function in residues responsible for stabilizing the active sites of the Zn^{2+} and Mg^{2+} metal ions. (Hoylaerts et al., 2006).

Because of the highly conserved active sites, it has been suggested that the reaction mechanism for APs is similar to the mechanism of ECAP, first introduced by Kim and Wyckoff. The reaction mechanism has since been updated by Kantrowitz et al. (2000) as shown in Figure 1.

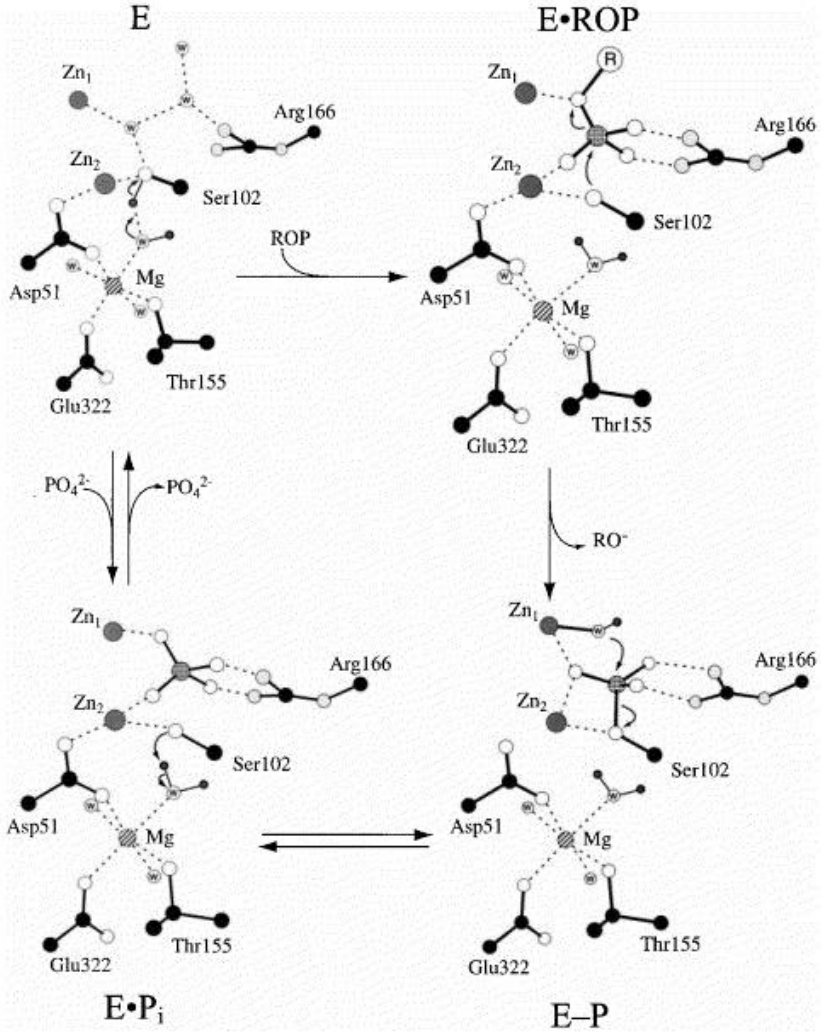

Figure 1. *In the free enzyme state (E), three water molecules fill the active site, and the hydroxyl group of the Ser102 residue participates in a hydrogen bond with the Mg-coordinated hydroxide ion. This leads to the forming of the enzyme-substrate complex (E·ROP), upon which the Ser102 becomes fully deprotonated for a nucleophilic attack following formation of Mg-coordinated water molecule through the concomitant transfer of the proton to the Mg-coordinated hydroxide group. The Zn2 stabilizes the Ser102 residue in its nucleophilic state, and the Ser102 hydroxyl group attacks the phosphorus center of the substrate in the E·ROP complex to form the covalent serine-phosphate intermediate (E-P). The Zn1 participates in this step by coordinating the bridging oxygen atom of the substrate and facilitating the departure of the leaving alcohol group (RO-). Next, a nucleophilic hydroxide ion coordinated to Zn1 attacks the phosphorus atom and hydrolyzing the covalent serine-phosphate intermediate to form the non-covalent enzyme-phosphate product complex (E·P_i) and regenerating the nucleophilic Ser102. The water molecule interacting with the Mg ion possibly plays a major role in the departure of the inorganic phosphate group by protonating the serine residue or the inorganic phosphate, thereby facilitating the leaving of the inorganic phosphate from the non-covalent enzyme-phosphate*

complex and the reproduction of the phosphate-free enzyme. Adopted from (Stec et al., 2000).

Although the ECAP crystallizes as a symmetric dimer, it seems to deviate from such a model, rather displaying a reaction characteristic of dimeric enzymes with unequal asymmetric subunits (half-of-sites reaction mechanism). It has been suggested that the asymmetry could be attributed to Mg^{2+} binding, but the magnesium ion is thought to enhance the APs activity by a mechanism that involves interactions between the two subunits (Orhanović & Pavela-Vrancic, 2003).

1.3 *Vibrio* alkaline phosphatase and cold adaptation

Cold oceans, such as the North Atlantic marine environment, can be thought of as a challenging ecosystem for native microorganisms. However, microbial life has evolved to proliferate in a broad spectrum of environmental temperatures. The organisms are at thermal equilibrium with their environment, and all components of their cells must be suitably adapted to the cold. The ability of these psychrophiles to proliferate in the cold is partly based on their ability to synthesize cold-adapted enzymes. These enzymes have evolved a range of features which confer a high level of flexibility compared to thermostable homologs. High flexibility (particularly around the active site) usually leads to low-activation enthalpy, low substrate affinity but high specific activity at low temperatures. This type of high flexibility is also accompanied by a trade-off in stability, resulting in the enzyme being heat labile (Siddiqui et al., 2006).

The *Vibrio* G15-21 AP (VAP) strain isolated from North Atlantic coastal waters near Reykjavík (Iceland) was originally isolated on the grounds that it produced a heat-labile alkaline phosphatase (Hauksson et al., 2000). Identical phosphatases have since been characterized from *Vibrio splendidus* through metagenomic data. VAP was found to be distinctly cold-adapted and quite heat labile with a half-life of 6 minutes at 40°C. Furthermore, the catalytic efficiency (k_{cat}/k_m) was found to be 2-3 times higher than that of ECAP at pH 8.0, which is to be expected of cold-adapted enzymes (Hauksson et al., 2000).

A study by Ásgeirsson and Andrésson (2001) on nucleotide gene sequences of the *Vibrio* AP showed that the residues involved in the catalytic mechanism were largely identical to those in other known APs. Compared to ECAP, the two zinc binding sites were identical, whereas two amino acid residues in the binding site for magnesium were found to be different between the two APs. Asp-153 and Lys-328 of ECAP are His-153 and Trp-328 in VAP. Furthermore, two additional stretches of amino acids not present in ECAP were found to be inserted close to the active site of the VAP (Ásgeirsson & Andrésson, 2001).

The 1.4 Å crystal structure of the *Vibrio* sp. alkaline phosphatase (VAP) was introduced by Helland et al. in 2009. The VAP structure was then compared to APs from various organisms, both bacterial and mammalian. The VAP was shown to be a dimer (Figure 2), where each monomer consisted of 502 amino acids but was without the long N-terminal helix which envelopes the other subunit in many other APs such as ECAP. However, a long insertion loop of the *Vibrio* AP was thought to serve a similar function before the crystal structure was obtained. The dimer interface was shown to contain similar number of non-covalent interactions as other APs, but its "crown" domain was found to be the largest in known APs, with a part of it sloping over the catalytic site (Helland et al., 2009).

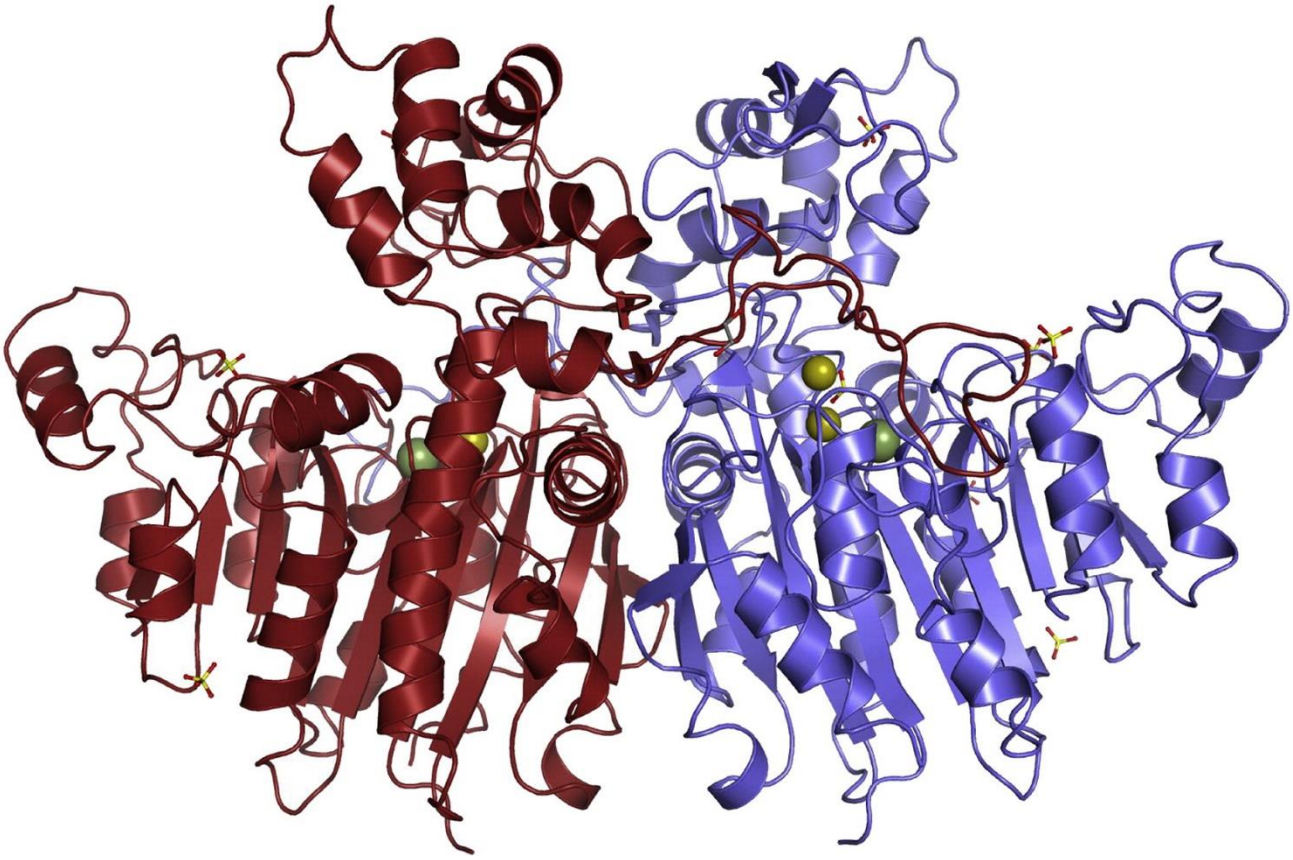

Figure 2. *A ribbon representation of VAP from Helland et al. (PDB: 3E2D). Subunit A is colored blue and subunit B is colored red. A conformation of the unique insert II of the B molecule (red) is seen extending along the surface of the monomer A. The "crown" domain is seen on the top of each monomer, and the catalytic zinc and magnesium ions in the active sites can be seen as gold and green spheres, respectively. Ball-and-sticks models on the figure represent sulfates and ethylene glycol molecules (Helland et al., 2009).*

As with other APs, the active site of each monomer contains three important metal ions, two zinc ions, and one magnesium ion. The VAP has its catalytic serine residue at position 65 and an important arginine residue in position 129 (Figure 3).

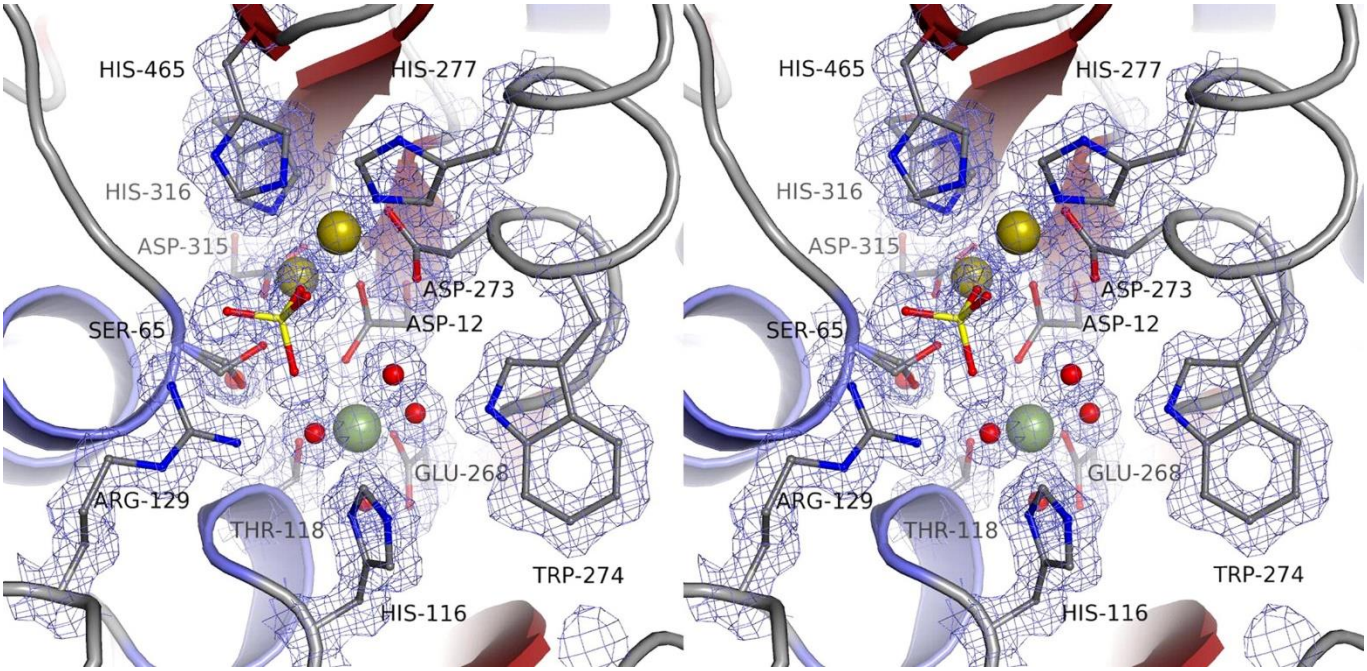

Figure 3. *A stereo figure of the VAP active site along with its electron density. The catalytic zinc (gold) and magnesium (green) ions can be seen along with sulfate molecules as red and yellow ball-and-stick models. The blue ball-and-stick models represent the residues involved in metal coordination. Water molecules are represented as red spheres, and the catalytic serine can be seen on the left side of each figure (SER-65) (Helland et al., 2009).*

1.4 Oligomers, monomers & subunit exchanges

A monomer is a single molecule or sub-unit that may bind to other molecules to form an oligomer or a polymer. A polymer is in principle not limited to the number of monomers it may consist of, but an oligomer normally consists of a few monomer units. For example, dimers, trimers, and tetramers are oligomers which consist of two, three and four monomers, respectively. As has been stated before, the alkaline phosphatase is a dimer consisting of two identical monomers, which can be thought of as its sub-units.

In 2001, Schneider et al. introduced a method to monitor the subunit exchanges in multimeric proteins using a recombinant 2x FLAG tag at the N-terminus, which did not seem to alter the stability or kinetics of the subunit exchange. Those experiments were done using transthyretin (TTR), a homotetrameric protein. Using ion exchange chromatography techniques

(Figure 4), the group managed to show that subunit exchanges can occur under native conditions, albeit slowly (Schneider et al., 2001).

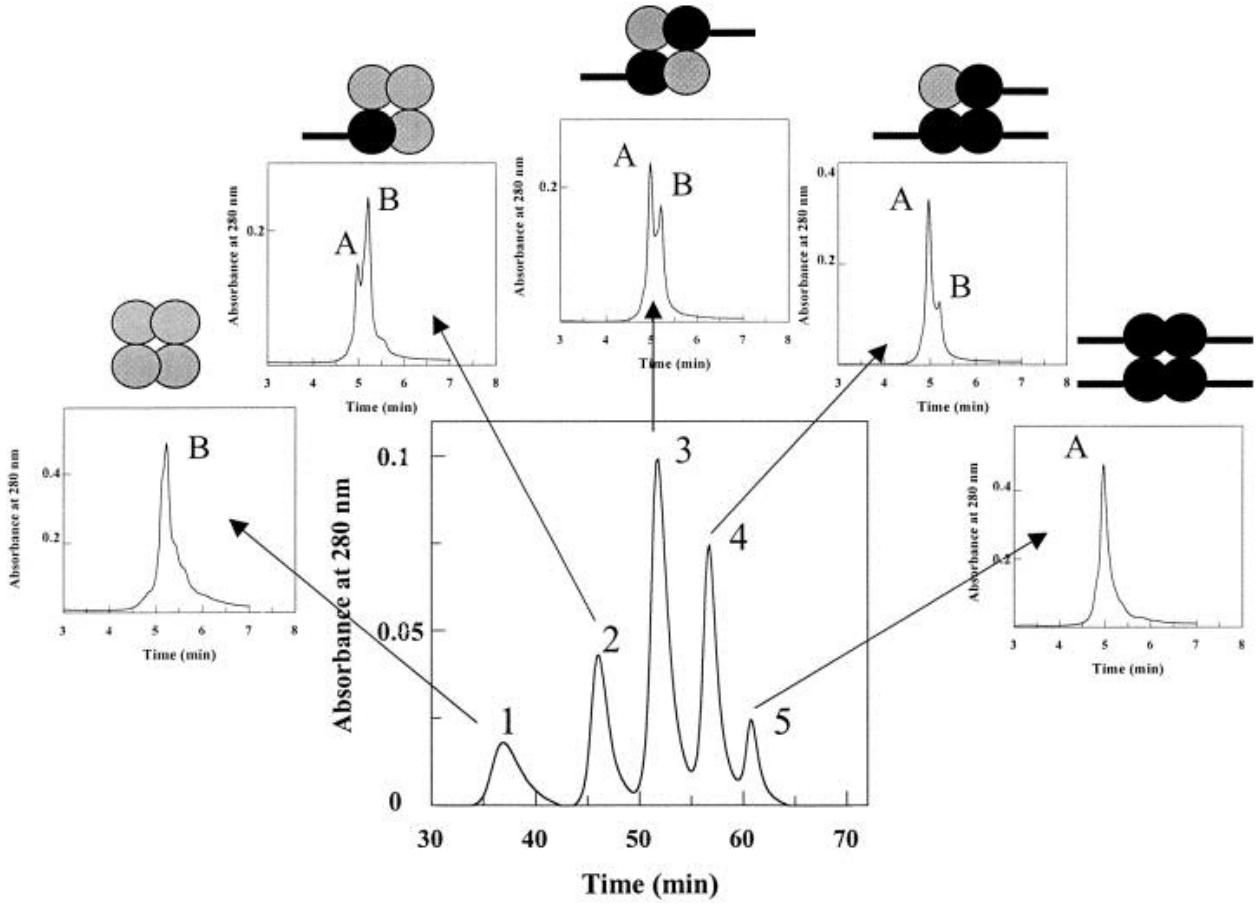

Figure 4. *Separation of hybrid transthyretin (TTR) tetramers with or without a FLAG-Tag on an ion-exchange column. The retention-time increased proportionally with the number of FLAG-Tag subunits, i.e. the more FLAG-tagged subunits in the tetramer, the longer it was retained on the column. Therefore, peak 1 contains no FLAG-tag subunits, while peak 5 contains four FLAG-tag subunits. The peak inset graphs show a RP-HPLC chromatogram of the collected peak. From Schneider et al. (2001).*

In 2014, Robinson and Reixach expanded on the idea of TTR subunit exchanges under physiological conditions, using FLAG-tagged TTR in tandem with an untagged wild-type. Using semi-native PAGE techniques (Figure 5), the group managed to characterize the different subunit variants (Robinson & Reixach, 2014).

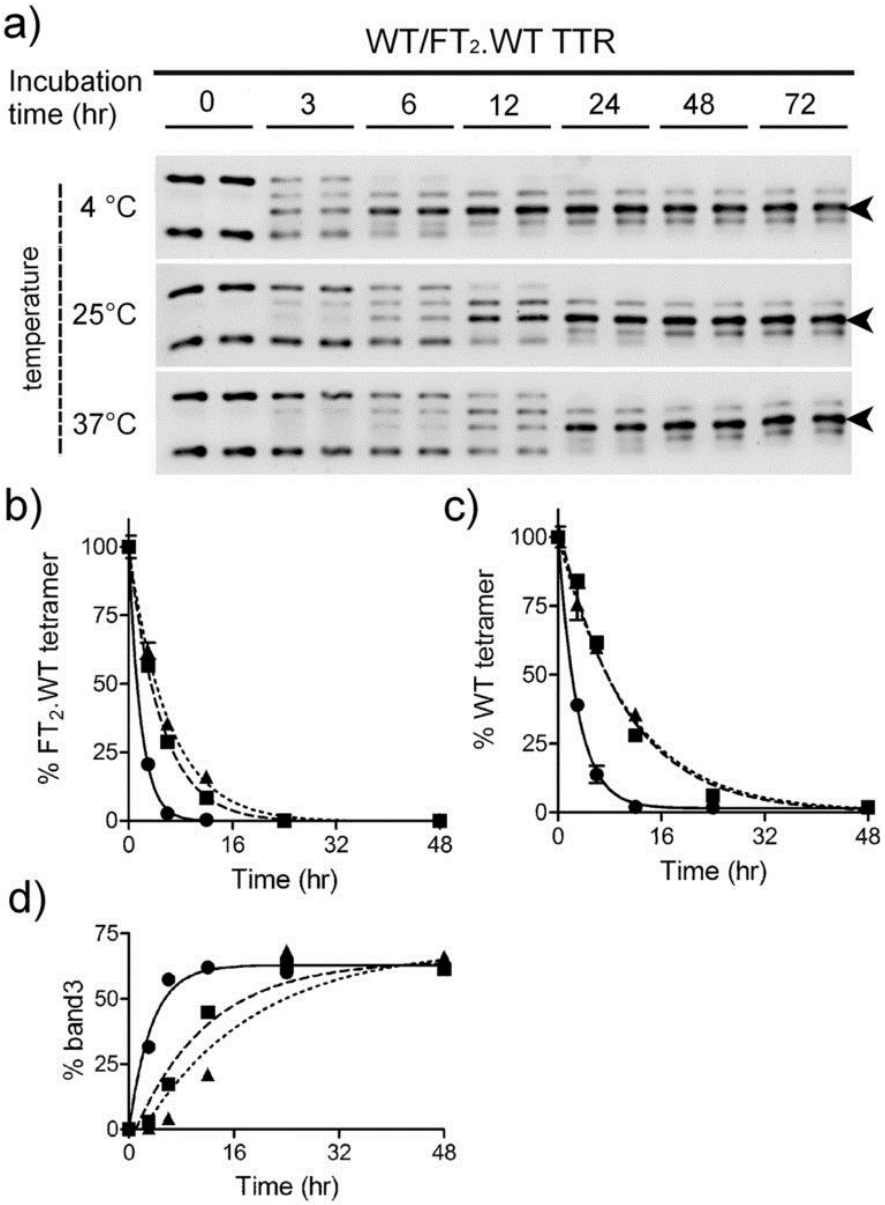

Figure 5. *Subunit analysis using semi-native PAGE between VAP-ST2 TTR and FLAG-tagged VAP-ST2 TTR incubated at different temperatures and for different periods of time (a). Parts b), c) and d) show the quantitation of bands corresponding to their respective variants and incubation temperatures. Circles represent 4°C, squares 25°C, and triangles 37°C. From Robinson & Reixach (2014).*

1.5 The aim of the project

1.5.1 Developing a purification protocol for 2x FLAG VAP protein variants

Using DNA recombinant techniques, the 2x FLAG VAP gene had previously been inserted into a pBluescript plasmid vector (Þorkelsson, 2015). The cell cultures in this research were then derived from *E. coli* LMG194 strain. However, developing a reliable purification protocol for the 2x FLAG VAP was one of the main challenges of the project. The purified protein samples were then to be used for further experiments.

FLAG-tag

The FLAG tag is a fusion part consisting of eight amino acids; Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, which were inserted at the C-end of the *Vibrio* alkaline phosphatase (Figure 6).

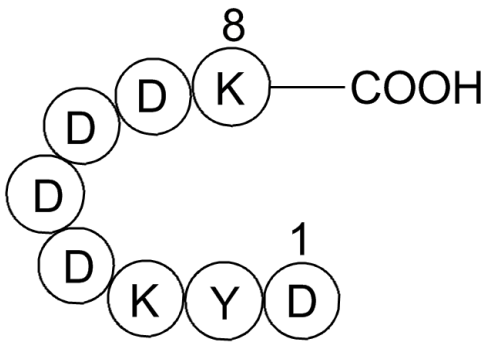

Figure 6. *The FLAG-tag and its connection to the C-terminus.*

Characterizing subunits and different protein variants

A key goal of this research was the differentiation of variants and the characterization of dimeric or monomeric forms of *Vibrio* alkaline phosphatase using various electrophoresis and chromatography techniques. Precedence for this has been briefly touched on above with the work of Schneider et al. (2001) and Robinson & Reixach (2014), where both chromatography and electrophoresis techniques were used to characterize the subunit exchanges of VAP-ST2-TTR and 2x FLAG-tagged VAP-ST2-TTR.

2 Materials

Bacto™ Yeast Extract and Bacto™ Tryptone purchased from BD Biosciences (New Jersey, USA). *Strep*-tactin® spin columns from IBA GmbH (Goettingen, Germany). CNBr-activated agarose purchased from Pharmacia Fine Chemicals (Stockholm, Sweden), which merged with Upjohn, later acquired by Pfizer. Magnesium chloride-hexahydrate ($MgCl_2 \cdot 6 H_2O$) and glycerine were purchased from Merck (Darmstadt, Germany). Magnesium sulfate ($MgSO_4$) and lysozyme from chicken egg white were purchased from Fluka Chemika-BioChemika (Buchs, Switzerland), later acquired by Sigma-Aldrich. USB® 5-bromo-4-chloro-3-indolyl phosphate, disodium salt for activity staining purchased from Affymetrix (Santa Clara, USA). Triton X-100 (isooctylphenoxypolyethoxyethanol) obtained from BDH chemicals Ltd. (Poole, England). Bromophenol blue purchased from Pierce chemical (Dallas, USA). NuPAGE™ 4-12% Bis-Tris electrophoresis gel purchased from Invitrogen (Carlsbad, USA), later acquired by Thermo-Fisher. Sodium chloride (NaCl), Ampicillin, L-histidine, ammonium sulfate ($(NH_4)_2SO_4$), glycine, tetramethylethylenediamine (TEMED), acrylamide, phosphonic acid (85%), CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), urea, MOPS; 3-(N-Morpholino) propane sulfonic acid ($C_7H_{15}NO_4S$), coomassie brilliant blue, deoxyribonuclease I (DNAase) and Trizma® base were obtained from Sigma-Aldrich (St. Louis, USA). HiTrap Q Sepharose FF column from GE Healthcare Life Sciences (Chicago, USA).

3 Methods

3.1 Cultivation of *E. coli strain* LMG194, containing the 2x FLAG-VAP.

LMG194 bacteria culture containing the 2x FLAG-VAP variant of *Vibrio* sp. G15-21 alkaline phosphatase was added, directly from -80 °C, to a petri dish containing Lamp medium and incubated in a incubator at 37°C overnight. Lamp medium is Luria-Bertani (LB) broth containing ampicillin (0.1 mg/ml).

A single colony was then transferred from the petri dish with a sterile loop and added to about 25 ml of Lamp medium and cultivated 3-5 hours at 37°C. While this preliminary culture was being incubated, 4 L of LB (Luria-Bertani) medium (see appendix) was prepared and divided into nine Erlenmeyer flasks (each containing about 440 ml) and sterilized in an autoclave at about 140°C for 10 minutes. After a couple of hours, 2 mL of the preliminary culture was added to each flask along with ampicillin (about 0.1 mg/mL). The Erlenmeyer flasks (with rag caps) containing the cell culture and ampicillin were then placed on an orbital shaker (170 rpm) and incubated at 18°C. There was no need to induce the recombinant protein production with IPTG, since the LMG194 strain lacks the lacI gene. Therefore, the lac operon is never occupied and the target gene never regulated. VAP has been successfully produced before with this method (Guðjónsdóttir & Ásgeirsson, 2007).

Two days later, 1.5 mL sample was taken from one flask (in a sterile manner) and the optical density measured at 600 nm. The target density should be about 1.7-1.8 (max density) and can be considered as the point of diminishing returns. Furthermore, 1 mL was taken from the cuvette, transferred to an Eppendorf tube and spun down at 10,000 xg for 5 minutes. The supernatant was removed and the remaining precipitate dissolved in 100 µl of MilliQ water. Ten µl of the dissolved precipitate was then taken and its enzyme activity measured at 405 nm with

990 µl of *p*-NPP (p-nitrophenyl phosphate). Measurements of about 1-2 U/mL should indicate a successful culture.

3.2 Protein Extraction

3.2.1 First culture

When the cell culture had reached adequate density and enzyme activity, it was centrifuged at 10.000 × g for 15 minutes at 4°C (Avanti-J-26 × P centrifuge with a JLA-16.250 fixed angle rotor from Beckman Coulter, California). The cell pellets were redissolved in 100 mL of TMC buffer (20 mM Tris, 10 mM $MgCl_2$, pH 8.0), with 1 mL 10% (w/v) Triton and about 50 mg of lysozyme (0.5 mg/mL) added. This was then kept in a refrigerator (at about 4°C) for just over an hour and then frozen in liquid nitrogen. All this was done to lyse, or break down, the membrane of the cell and expose the cells cytosol. The lysate containing sample was then thawed over a waterbath and 5 µg of DNAase (2000 u/mg) added and stored in a refrigerator overnight. After which the lysate + DNAase mixture was spun down at 10.000 xg for 15 minutes (4°C).

3.2.2 Second culture

In the second cell culture, the cells were centrifuged at 15.000 × g for 10 minutes at 4°C, the pellet then redissolved in 100 mL TMC buffer, with added 1 mL of 10% Triton and about 50 mg of lysozyme, just like before. The pellet was then kept in a refrigerator for 3 hours and then put in a -20°C freezer overnight.

The next day the pellet was thawed, and 15 µl of DNAase added to the lysate. This was kept at RT for about 30 minutes and the supernatant then centrifuged at 18.000 × g for 15 minutes at 4°C. The supernatant was then kept in a 2 M ammonium sulfate solution overnight in a refrigerator.

3.3 Protein purification

3.3.1 Purification with *Strep*-Tactin® spin columns

A VAP variant had previously been generated with a *Strep*-tag purification tag at the C-terminus (Heiðarsson et al., 2009). The *Strep*-tag itself is a short peptide sequence comprised of eight amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) which binds to streptavidin in a reversible manner. The *Strep*-tactin columns contain an immobilized streptavidin variant which has been optimized for the purification of *Strep*-tag proteins. The binding affinity of *Strep*-tag to *Strep*Tactin (K_d = 1 µl) is said to be nearly 100 times higher than to streptavidin (IBA GmbH, 2012)

At first, a buffer was prepared according to the *Strep*-tactin spin column protocol, "Buffer W". However, for our purposes, the buffer was made without EDTA, and $MgCl_2$ added instead. The spin column was then prepared by washing with "Buffer W" (100 mM Tris/HCl, 150 mM NaCl, 1mM $MgCl_2$, pH 8.0) for 30 seconds at 700 x g. This step was done twice. Supernatant (500 µl) was then loaded onto the equilibrated column and centrifuged for 30 seconds at 700 x g (this was also done twice). The flow-through was then collected and its enzyme activity measured or protein concentration at A280 nm. If the measurements indicated that the desired protein had been bound to the column, it should be washed four times with 100 µl of Buffer W (for 30 seconds at around 13.000 rpm) and the flow through collected. These purification steps were all done in accordance with the manufacturer's protocol (GmbH, *Strep*-Tactin Spin Column: Purification Protocol, 2012).

3.3.2 L-histidyldiazobenzylphosphonic acid affinity column preparation with – cyanogen bromide activated agarose beads.

An attempt was made at making an affinity column with cyanogen bromide coupled agarose beads (Landt et al., 1978), which could then be used as a step in the purification of the 2x FLAG proteins.

4-Aminobenzylphosphonic acid (95%) was first dissolved in 1.5 M HCl and kept on ice. 0.5 M $NaNO_2$ was dissolved in 3 mL ddH_2O and also kept on ice. 1 g of CNBr- activated agarose

was dissolved in 1 mM HCl solution and put on a cinder filter which had been washed extensively with MilliQ water. The agarose and filter were then washed with a coupling buffer (1% ammonium carbonate, 0,10 M, pH 9.17) before 5 millimoles of L-histidine (dissolved in 80 mL of the coupling buffer) were added onto the filter. The material was then removed from the filter and dissolved in 5 mL of MilliQ water, 0.5 M $NaNO_2$ and 4-aminobenzylphosphonic acid solutions mixed together and added to the agarose material, which was then titrated at pH 9.5 (with HCl and NaOH) and left in a refrigerator (at 4°C) overnight.

Later the same recipe was made fivefold to get more column material.

3.3.3 Purification with Q-Sepharose ion exchange column

The sample was loaded onto the column attached to a BioLogic™ Low-Pressure Liquid Chromatography System, with a flow rate of 1 mL/min. Two buffers were used to carry out the gradient; Buffer A (20 mM Tris, 2 mM $MgSO_4$, pH 8.0) and buffer B (same as buffer A, but with 0.7 M NaCl).

The column was first washed with buffer B and then equilibrated with buffer A. The sample was then loaded onto the column at a pace of 1 mL per minute. The program went from 0 to 100% 0.7 NaCl molarity in 60 minutes with a flow rate of 1 mL/min. A fraction collector was connected to the station and was configured to collect 2 mL into each test tube.

After collection, the enzyme activity of the samples was measured, and the fractions with the most activity were pooled together for further purification.

Second cell culture

During the purification of the enzyme from the second cell culture, the ion-exchange step came after the phenyl-Sepharose purification. It was performed the same as before with 0.7 M NaCl gradient.

3.3.4 Purification with phenyl-Sepharose column

This type of chromatography technique utilizes hydrophobic interactions to bind and ultimately release proteins. The sample had been kept in 1 M ammonium sulfate (AMSO)

overnight. The column was equilibrated with 1 M AMSO buffer (20 mM Tris, 2 mM $MgSO_4$, 1 M $(NH_4)_2SO_4$, pH 8.0). Before the gradient could be started, it appeared that most of the protein had been released from the resin during the equilibration. The test tubes containing enzyme activity were then pooled together. Another column was then packed with fresh resin (Sigma), to exclude the possibility of a faulty column. During the second try, the enzyme seemed to bind to the column, but came unbound when the column was washed with 1 M AMSO before the gradient was started.

Second cell culture

With the second cell culture, it was decided that the first chromatography step should be the phenyl-Sepharose, followed by an ion exchange step.

First, the supernatant was kept in 2 M AMSO (molarity was doubled) and centrifuged at 15.000 x g for 10 minutes. The supernatant of that was then collected and the enzyme activity of the precipitation measured. The activity remained in the soluble fraction. This might prove a useful purification step due to its simplicity and effectiveness.

The phenyl-Sepharose column was then equilibrated with 2 M AMSO buffer, the sample loaded on (1 mL/min) and the column equilibrated again with 2M AMSO buffer. This time, the gradient ($2 \rightarrow 0$ AMSO molarity) was performed with a stand-alone gradient mixer and a Pharmacia peristaltic pump. The flow-through was collected in fractions and their enzyme activity measured at 405 nm (chapter 3.6). The elute peaks were then pooled together and put in a dialysis bag with 15 kDa cutoff so that they could be loaded onto an ion exchange column later.

3.3.5 Mono-Q purification with fast protein liquid chromatography (FPLC)

This step was only performed on the first cell culture. The sample was first dialyzed in 1 liter of 10 mM Tris and 1 mM $MgSO_4$ buffer, and after about 3 hours, the buffer was switched out for a fresh one and left overnight. This was done to filter out the ammonium sulfate in the sample before loading (the sample) onto the ion-exchange column.

The Mono-Q column was first washed with buffer B (20 mM Tris, 2 mM $MgSO_4$, 0.8 M NaCl) and then equilibrated with buffer A (without NaCl). The sample was then loaded onto the column at a rate of 1 mL/min., but had to be injected into the column loop in two steps, half of the sample at a time. The first half was eluted continuously with buffer B at a rate of 1 mL/min. and the flow through collected using a fraction collector. The second half of the sample received the same treatment, but was eluted with a 1.6 M NaCl buffer B. The collected fractions were then measured for enzyme activity and the eluate peaks collected and eventually pooled together into two separate containers for each half of the original sample.

3.4 Determination of protein concentration

The protein concentration of the purified samples was estimated with the Zaman and Verwilghen method (Zaman & Verwilghen, 1979), a variant of the Bradford method (Bradford, 1976), which uses the same dye but uses perchloric acid instead of phosphoric acid. Both methods are based on the binding of proteins to Coomassie Brilliant Blue G250, and the subsequent light absorption of dye bound to proteins. The measurements were performed with a spectrometer (Evolution™ 220, ThermoScientific) measuring light absorption at 620 nm. The sample was measured in 3 mL plastic cuvettes, with 100 µl of sample, 150 µl of MilliQ water and 2.75 mL of Coomassie dye. The spectrometer was "blanked" using 2.75 mL of dye and 250 µl of distilled water.

A conversion factor had previously been determined from a standard curve using BSA as a standard and stated that 1 µg of protein should equal 0.007 in absorbance.

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3.5 Enzyme assay (activity measurements)

The activity of the *Vibrio* alkaline phosphatase was measured with 5 mM *p*-nitrophenyl phosphate (*p*NPP) in a 1.0 M diethanolamine buffer (1.0 mM $MgCl_2$, pH 9.8 at 25°C). Diethanolamine acts as a phosphate acceptor, and the alkaline phosphatase catalyzes the

transphosphorylation of *p*NPP to *p*-nitrophenol and diethanolamine phosphate. The color of the *p*-nitrophenol is distinctly yellow, and its maximum absorbance is at 405 nm.

The enzymatic measurements were performed with 990 µl of substrate solution (kept in a water bath at 25°C) and 10 µl of sample. If 10 µl of sample was too active to measure, the sample was diluted with MilliQ water or buffer and dilution accounted for in calculations. The sample was first placed on the inner wall of the cuvette, which was then put in place in the spectrometer and the substrate solution added to the cuvette. The absorbance was then measured for 30 seconds at 405 nm. The spectrometer calculated the activity (using the factor 5.405) as enzyme units per milliliter (U/mL) according to Beer's law:

$$A = \varepsilon * l * c$$

Where A is the absorbance, epsilon is the molar absorptivity (L mol^{-1} cm^{-1}), l is the pathlength of the cuvette (in which the sample is contained), and c is the concentration of the solution (mol L^{-1}). The 5.405 factor was calculated using the specific absorption coefficient of *p*NP (18500 M^{-1} cm^{-1} at pH 9.8) and taking into account a 100 fold dilution of the sample (10 µl sample / 1000 µl total volume).

3.6 Kinetics

The steady state kinetics constants (K_m and k_{cat}) were determined for both VAP-ST2 and the 2x FLAG variant of *Vibrio* alkaline phosphatase. The activity was measured at 9 different substrate concentrations (*p*NPP) in 0.1 M Caps hydrolysis buffer (100 mM CAPS, 1 mM $MgCl_2$, 500 mM NaCl, pH 9,8 (10°C)). This was done three times for the 2x FLAG mutant and two times for the wild type. The measurements were performed at 10°C and a pH of 9.8.

The solutions were made by accurately mixing 5 mM of a stock substrate solution with the amount of Caps buffer needed to dilute the substrate to the desired concentration. For example, to achieve the starting concentration of 0.025 mM of *p*NPP, 20 µl of 5 mM substrate stock solution was mixed with 3.98 mL of buffer.

The enzyme samples were diluted based on their light absorption at 280 nm (measured in a quartz cuvette). The sample was then diluted to about 0.020 absorption at 280 nm. For example, one sample of 2x FLAG had a light absorption of 0.375 at 280 nm. It was then diluted by mixing 53 µl of enzyme with 947 µl of CAPS buffer, and the resulting light absorption ended up at 0.018.

The activity measurements themselves were performed by adding 10 µl of diluted enzyme sample to a 1 cm plastic cuvette and the substrate mixture (*p*NPP + Caps buffer) added. The absorbance was measured at 405 nm for 30 seconds. Keep in mind that the measurements were all done at 10°C, with the substrate mixtures kept in a water bath, the empty cuvettes were kept on ice and a peltier cooling unit was attached to the spectrometer to maintain the temperature steady at 10°C.

Three samples were measured at each concentration, and one of them was put aside (sample + substrate) and left overnight for total hydrolysis to *p*-nitrophenol and phosphate. The absorbance of the fully hydrolyzed substrate + sample mixtures was then measured at 405 nm in a glass cuvette (blanked with Caps buffer). This was done to determine the actual concentration of the substrate mixture so they could be accurately taken into account during the data processing.

Microsoft Excel was used to determine the actual substrate concentration by using the measurements (A405 nm) from the fully hydrolyzed mixture and dividing it by the extinction coefficient of its product (18.500 M^{-1} cm^{-1}), in accordance with Beer's law. GraphPad Prism was then used to calculate the kinetic constants K_m and V_{max} with a non-linear fit to the Michaelis-Menten equation. Microsoft Excel was then again used to calculate the first order rate constant (k_{cat}) from the V_{max} and A280 data.

3.7 Electrophoresis

3.7.1 SDS-PAGE protein electrophoresis

The sodium dodecyl sulfate (SDS) polyacrylamide gel (PAGE) electrophoresis was used to determine the purity of the final samples. Pre-made gels from Invitrogen (Thermo-Fisher), the NuPage 4-12% Bis-Tris 12 track gels, were used here. The gels were washed with distilled water and placed in a cassette which was then put into place in the cathode chamber of the electrophoresis station (Novex minicell) and buffer 1xMES buffer added (see appendix). The sample was then added to a sample-buffer which included dye and DTT (NuPage LDS sample buffer) in a 1:3 ratio (3x sample) and boiled at 95°C for two minutes. About 20 µl of the dye + sample mixture were added to each pocket of the gel with a Hamilton syringe, furthermore, 10 µl of protein ladder (SpectraColor multirange) was added to one well. An electric current of about 120 volts was run through the gel until the dye had migrated to the end of the gel.

3.7.2 Native gel electrophoresis

Native gel refers to the gel being run under non-denaturing conditions, without SDS added, so that the enzyme or other analyte's natural structure is maintained. Under these circumstances, the charge of the protein also affects its mobility through the gel, along with its size. Two attempts were made at separating samples using these native gels.

The first native electrophoresis was performed with pre-made 4-12% Nu-Page Bis-Tris gels. The samples were treated with different concentrations of urea in 25 mM MOPS, 1 mM $MgSO_4$, pH 8.0. The samples were then left for about 3 hours before a Hamilton syringe was used to transfer about 20 µl of sample to each gel pocket. Both *Strep*-tag and 2x FLAG variants were used here. Two identical gels were run, one to be stained with Blue-silver and another to be activity stained.

After electrophoresis, the gel that was to be stained with Blue-silver dye method (Candiano, et al., 2004) was fixed by placing it in 50/50 mixture of ethanol and distilled water with 3% added phosphoric acid (85% H_3PO_4) for 20 minutes on a shaker. The gel was then washed twice with distilled water, 20 minutes at a time on a shaker. After fixing and washing

the gel was then stained with Blue-silver dye, a very sensitive colloidal Coomassie G-250 dye variant.

Before activity staining, the second gel was submerged in activity staining buffer (1 M diethanolamine, 1 mM $MgCl_2$, pH 9,8) for about 20 minutes (on a shaker) before it was placed in 2 mg of 5-bromo-4-chloro-3-indoyl phosphate disodium salt, dissolved in 20 mL of activity staining buffer.

The second native electrophoresis was performed with native gels made in-house. Two gels were prepared, one for Blue-silver staining and one for activity staining, same as with the 4-12% Bis-tris pre-made gels. The stacking gel was made to be 4% acrylamide and the separating gel was made to be 12% acrylamide. Detailed description can be found in the appendix.

The running buffer (5x = 0.25 M Tris, 1.9 M glycine, pH 9.11 at RT) was placed both in the electrode chamber and the tank. About 20 µl of sample was placed in each well (with a Hamilton syringe). The gel was then left overnight at constant 20 volts and 5 mA (divided by two gels).

The gels were then fixed and stained just as the 4-12% Nu-Page pre-made gels.

3.7.3 Blue-native Polyacrylamide Gel Electrophoresis (Blue-native PAGE)

The Blue-native PAGE was first demonstrated by Schagger and von Jagow (1991) as a technique for separating enzymatically active membrane protein complexes. In this gel electrophoresis variation, Coomassie Brilliant Blue, an anionic dye, was added to the cathode buffer (inner chamber), so it would bind to the sample before separation. A detailed description of the anode- and cathode buffer can be found in the appendix.

The samples were treated with different concentrations of urea in 25 mM MOPS buffer and 1 mM $MgSO_4$, pH 8.0 for about 3 hours. The electrophoresis was performed with a pre-made Nu-Page 4-12% Bis-Tris gel and was ran for about 4 ½ hours at about 25 mA. After

electrophoresis, the gel was treated with 20% acetic acid and then destained overnight in a 30% methanol solution.

3.7.4 Urea gel electrophoresis

Urea gels were made in house, in both 1 M and 2 M concentrations, and with 14% and 10% separation gels (for more details, see appendix). This type of electrophoresis technique was used to try and combat the possible reforming of dimers due to stacking.

3.7.5 Semi-native SDS gel electrophoresis

For the semi-native SDS, 1% SDS and 0.6 M DTT was added to the loading buffer (appendix) and the sample loaded onto the gel without boiling, similar to what was done by Robinson and Reixach (Robinson & Reixach, 2014). A pre-made Nu-Page 4-12% Bis-Tris gel was used. The semi-native SDS gel was run for about 5 hours at steady 15 mA. It was then fixed with Blue-silver dye like other gels.

3.8 Separation of *Strep*-Tag and 2x FLAG variants on Quaternary ammonium Fast Flow analytical column.

To be able to differentiate between the two alkaline phosphatase variants, it was decided to use a HiTrap Q FF column (Q-Sepharose Fast Flow; QFF) on an FPLC apparatus to evaluate if separation of the VAP-ST2 and 2x FLAG variants was feasible. The column was first equlibrated with TMC buffer and the sample injected onto the column via 2 ml sample loop. Then, a 35 minute gradient from 0 → 0.8 M NaCl was initiated. No fractions were collected, but instead, the data from the FPLC apparatus was used to plot up the process visually in Microsoft Excel.

4 Results

4.1 Binding of *Strep*-tag proteins on *Strep*-tactin spin columns

The binding and elution the enzyme to the spin columns was done according to the manufacturers' protocol. This process is explained in further detail in the methods chapter.

The first attempt was made with *E. coli* alkaline phosphatase which had a *Strep*-tag at its C-terminus. However, the supernatant had almost the same enzymatic activity as the sample before it was loaded onto the column, i.e. the sample did not seem to bind to the column. The reason was that the sample was kept in 2.5 mM desthiobiotin, and was thus unable to bind.

During the second attempt, it was decided to monitor the binding more closely. At first, the light absorption of 1 mL of sample dialized in 1 L TMC buffer (25 mM Tris, 1 mM $MgCl_2$, pH 8.0) was measured in a quartz cuvette at 280 nm. The absorbance was measured at 0.238. The sample was then diluted and activity measured. The activity was around 12.25 U/mL. Keeping in mind that the *Strep*-tactin protocol states that the maximum binding to a column is around 4 nanomoles of protein where the binding constant (K_d) is approximately 1 µM for the *Strep*-tactin resin. Using Beer's law, the required volume of sample to bind 4 nmoles of sample to the column was estimated.

$$A = \varepsilon * l * c$$

The protein concentration, c, was estimated by dividing the previously measured light absorption at 280 nm by its extinction coefficient, 38600 $M^{-1} * cm^{-1}$, which equals around 6.166 $* 10^{-6} M^{-1}$.

$$\frac{4 \times 10^{-9}\, moles}{6.166 \times 10^{-6} \frac{moles}{L}} = 6.49 \times 10^{-4} L \times \frac{1000\, mL}{L} = 0.649\, mL$$

According to these calculations, about 0.649 mL of sample equals 4 nanomoles of enzyme.

However, the calculations were performed using the extinction coefficient for VAP, resulting in 1 mL of sample being loaded onto the *Strep*-tactin column, 500 µl at a time. The enzymatic activity of the supernatant measured in at around 7 U/mL.

Considering the µg / mL of protein:

$$0.238 * \left(\frac{48260\ g/mol}{38600\ M^{-1} \times cm^{-1}}\right) = 0.298 \frac{\mu g}{mL}$$

$$\frac{12.25 \frac{U}{mL}}{0.298 \frac{\mu g}{mL}} = 41{,}1 \frac{U}{\mu g}$$

If 7 U/mL was present in the supernatant, then around 5.25 U/mL was left on the column.

$$\frac{5.25\ U}{41.1 \frac{U}{\mu g}} = 0.128\ \mu g$$

Given the maximum binding of 4 nanomoles of protein, let's consider how many moles were bound:

$$\frac{1.28 * 10^{-7} g}{48260 \frac{g}{mol}} = 2.652 * 10^{-12} moles$$

According to these calculations, only 2.6 * 10^{-12} moles, or 0.002652 nanomoles of protein were bound to the resin. This would mean that the relative binding of protein to the column was around 0.066%, seemingly non-existent.

During the third attempt, the washing was done with Buffer W, containing 1 mM EDTA. The protein concentration was measured before loading as A280 = 0.101. The absorbance of the supernatant was measured at 0.052 at 280 nm. The supernatant was then loaded onto the column

again, but with similar results. To elute bound proteins, 15 mM desthiobiotin buffer was used. Protein concentration measurements revealed that no protein seem to be eluting.

The fourth attempt was made both with and without EDTA in the buffer. The protein concentration of the samples was measured at 280 nm before loading onto the spin columns. The concentration was then deliberately lowered to be closer to the given K_d (1 µM), and were calculated to equal about 1.5 nanomoles. Sample with 1 mM EDTA, A280 = 0.217, sample without EDTA, A280 = 0.212. Around 250 µl (ca. 1.5 nmoles) were then loaded onto the column in accordance with the manufacturer's protocol. However, when the protein concentration was measured again, very little protein seemed to have been bound to the column. Because the K_d constant is relatively low, and very little resin is in the column, getting the right protein concentration might be important for adequate binding.

All in all, it seems that the *Strep*-tactin spin columns did not bind the desired amount of protein.

4.2 Cultivation of LMG194 strain of *E.coli* containing 2x FLAG-VAP.

The cultivation of the LMG194 *E. coli* with 2x FLAG was performed three times in a way that was described in the methods chapter above. It is worth noting that the orbital shaker on which the cultivating flasks rested on, tended to shut down at various times, which can affect the bacterial growth of VAP-ST2. Of the three cultures that were started, only two underwent adequate purification steps to make a purification table out of the data. (The single culture which was deemed not good enough had significantly less enzymatic activity than the other two, and it was determined not to continue with purification.)

As was stated in the methods chapter above, the OD600 of the undiluted culture was around 1.7-1.8 (about maximum density) and enzymatic activity was about 1-2 U/mL (1 mL sample was centrifuged and the precipitate dissolved in 100 µl of MilliQ water. Ten µl of said sample was then added to 990 µl of substrate solution), which was considered adequate to stop the culture and proceed to purify the sample.

The first culture was stopped after three days at 18°C, and it should be noted that a power outage occurred during the first night of cultivation, in which both the orbital shaker and the cooling system lost power for a few hours. On the third day, the optical density at 600 nm of the culture was measured at 1.530, which is under the desired density, but the enzymatic activity was measured at 1.95 U/mL which was deemed satisfactory to undergo purification.

The OD600 of the second culture was measured at 1.750 after two days. The enzymatic activity was not measured before lysing the cells, and after the precipitate had been collected and dissolved, it was kept in 2 M ammonium sulfate solution overnight. It was then centrifuged and the enzymatic activity of the supernatant and precipitate measured. The supernatant measured in at 3.5 U/mL with a total volume of 128 mL. The activity of the dissolved precipitate was measured at only 0.1 U/mL with a total volume of 38 mL. According to these measurements, the total enzyme units of the supernatant should equal about 448 units. This was determined to be inadequate, and another culture was started. It should be noted that the sample was kept in 2 M ammonium-sulfate, where sulfate acts as a competitive inhibitor for APs.

The third culture was then started, however, the orbital shaker stopped overnight the first night. The same thing happened again the second night. A sample was then taken and the optical density at 600 nm measured in at 1.87. The enzymatic activity was also measured at 2.8 U/mL. This was deemed adequate for further purifying.

4.3 Purification steps for the first cell culture

The first step in the purification of the first cell culture was to be with the in-house made L-histidyldiazobenzylphosphonic acid CNBr- activated agarose affinity column. The sample (about 100 mL) was loaded onto the column overnight at a temperature of 4°C in a cold room. A sample was taken from the flow through and its enzymatic activity measured after being diluted tenfold with distilled water. The average activity of four different measurements was 160.4 U/mL when corrected for dilution or around 16.000 enzyme units in total. Considering this, it was determined that the affinity column was not working as expected and it was removed as a possible step in the purification protocol.

After little to no binding was observed on the phosphonic acid affinity column, it was decided to use a Mono-Q (Q-Sepharose) column with a Bio-Rad station as the next purification step. The sample was loaded onto the column, and a salt gradient (0 - 0.7 M NaCl in TMC, pH 8.0) was used to elute the bound proteins from the Q-Sepharose resin. A more detailed description of this process can be found in the methods chapter above.

The Bio-Rad station was connected to a computer which monitored the process, measuring both the absorbance at 280 nm and the (electrical) conductivity in mS/cm. The column was then also attached to a fraction collector. The absorbance peaks were measured for enzymatic activity, and the most active fractions were pooled together where the volume was 27 mL in total (Figure 7).

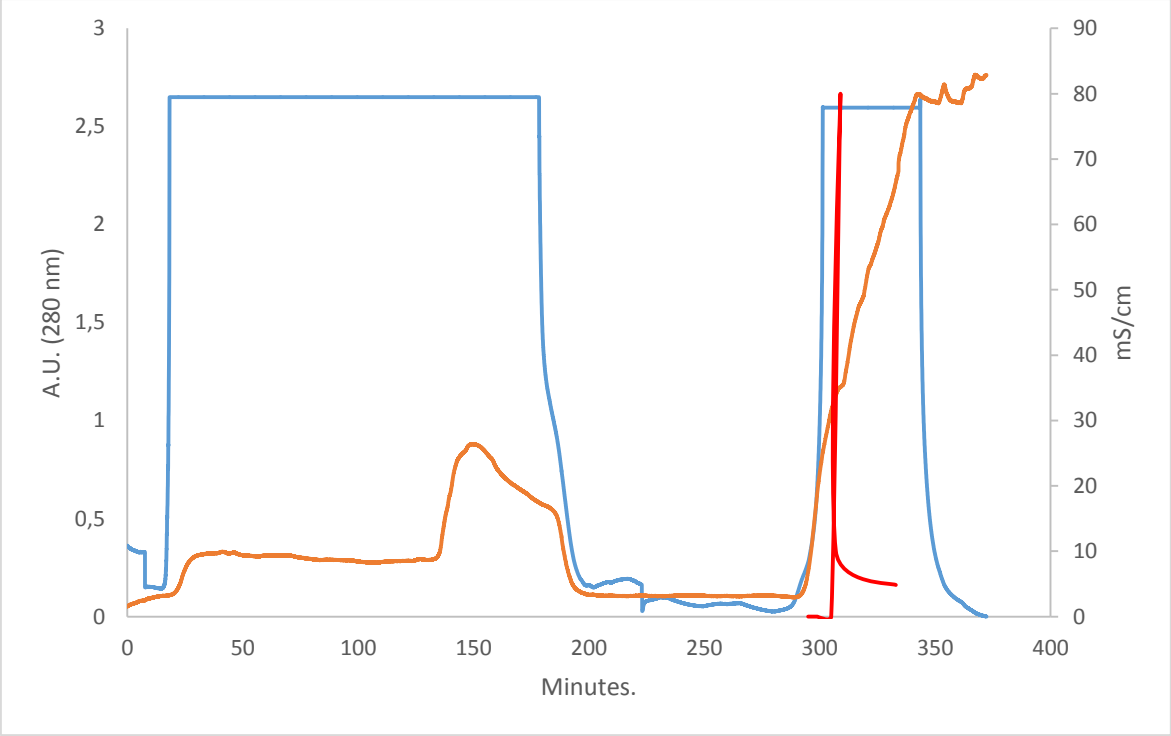

Figure 7. *Q-Sepharose purification step of FLAG-VAP from the first cell culture. The x-axis represents time in minutes while the left y-axis shows absorbance at 280 nm and the right y-axis displays electrical conductivity of the solution in mS/cm. The salt-gradient program was started at around 223 minutes. A red line has been inserted to represent the measured activity peak. The line has been roughly superimposed and altered to fit the graph, so nothing definitive should be concluded about the activity by looking at either y-axis.*

A sample was taken from the pool and its enzymatic activity measured. The average of three measurements indicated activity of about 595.8 U/mL or about 16086 enzyme units in total, similar to total units coming off the phosphonic acid affinity column. The sample was then kept in 1 M ammonium sulfate overnight. Note that the sample was eluted from Q-Sepharose in roughly 0.5 M NaCl, which also contributed to the ionic strength.

The sample was then centrifuged at 1730 x g in a swinging bucket centrifuge. Average enzymatic activity was measured at 508.9 U/mL given that the total volume of the sample was around 27 mL, the total enzyme units of the sample would equal about 13739 units. This is however an inaccurate representation of the total enzyme units because the ammonium sulfate binds to the active site, and acts as a competitive inhibitor and can thus cause the enzymatic activity measurements to be inaccurate, even if the sample is diluted >100 fold (1M/100 = 10 mM).

Next, it was decided to purify the sample further by loading it onto a phenyl-Sepharose column, where proteins bind based on hydrophobic interactions. Usually, this step is performed earlier in purification processess, preferably before using ionic exchange columns, but here it was decided to try and perform the ion exchange purification first due to high charge of the FLAG-tag (Figure 8).

A more detailed description of this process can be found in the methods chapter above. However, before the gradient was initated, it was clear that the sample was not binding to the column.

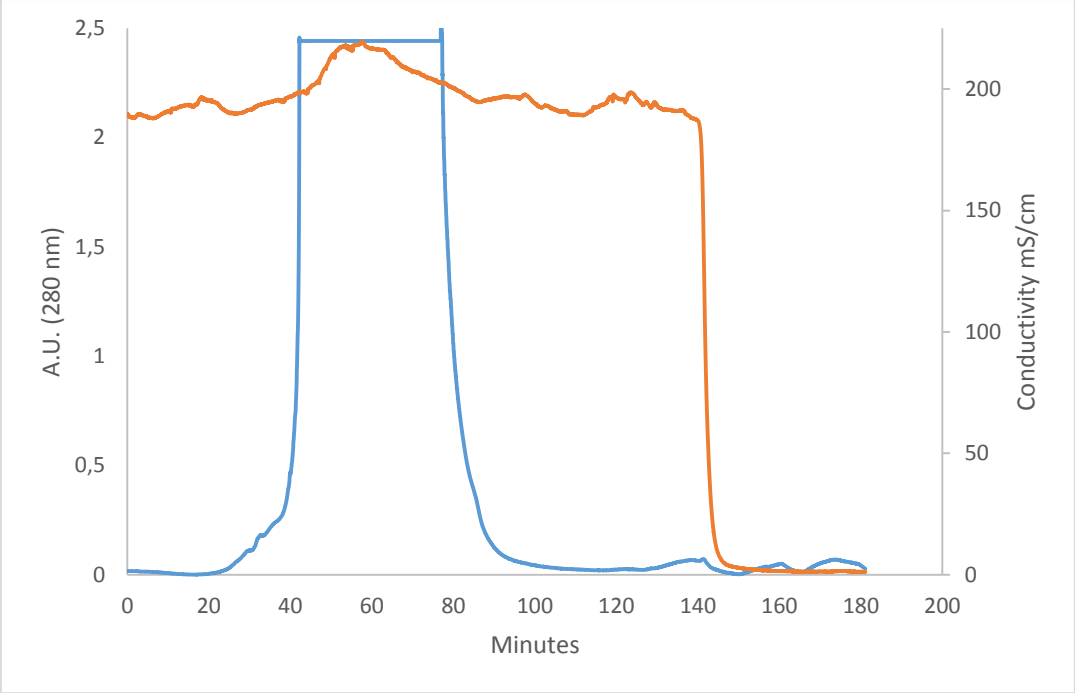

Figure 8. *Phenyl-Sepharose purification step for FLAG-VAP from the first cell culture. Left y-axis shows the light absorbance at 280 nm., the right y-axis represents electrical conductivity in mS/cm. The time in minutes is displayed on the x-axis.*

The fractions containing the samples from the absorbance peak (flow-through) were measured for enzymatic activity, and the active fractions pooled together. The total volume of the pooled glasses proved to be around 34 mL. The average enzymatic activity was 288 U/mL and the total enzyme units thus around 9792 U. This is only around 71% of the previously observed total units, that is before the sample was loaded onto the column.

A fresh phe-Sepharose column was packed and the process repeated.. The sample was once again loaded onto the column and equilibrated with 1 M ammonium sulfate buffer (Figure 9).

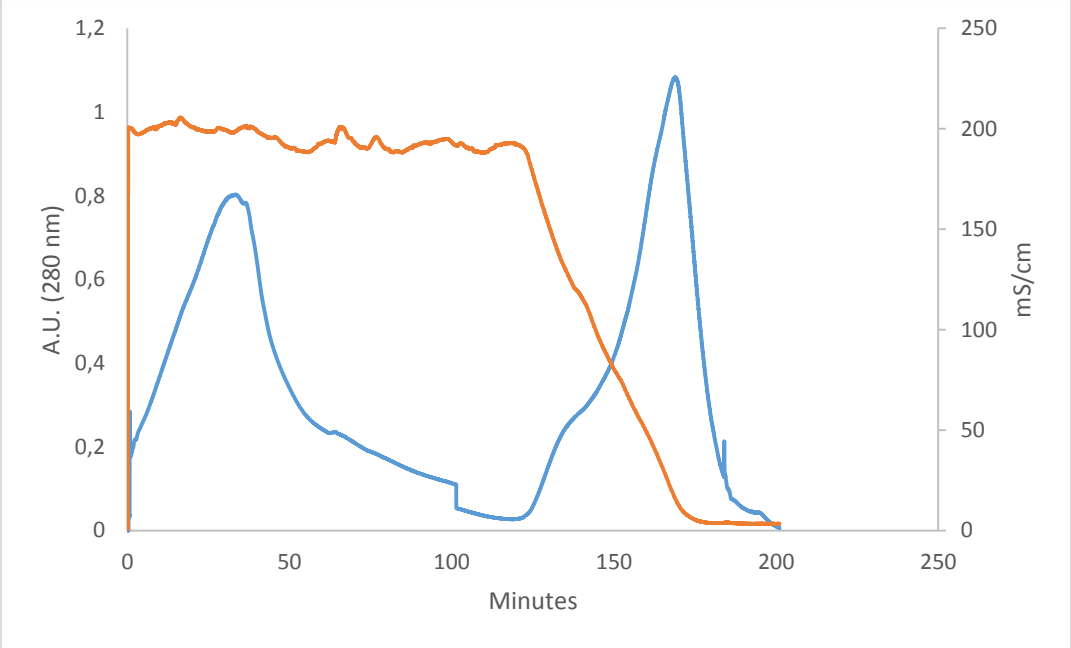

Figure 9. *Second attempt for phenyl-Sepharose purification of FLAG-VAP from the first culture. On the x-axis, time can be seen in minutes, the left y-axis displays the light absorbance at 280 nm, and the right y-axis shows electricity conductivity in mS/cm. Two absorbance peaks can be seen. The first one contained most of the enzymatic activity while the second one showed little to no activity. It should be noted that the enzymatic active peak is only a portion of the absorbance peak. The peak activity was in the very beginning of the process, and glasses 1-12 were pooled (2 mL per glass).*

Again, the protein was released in the loading phase. The FLAG-VAP protein is clearly extremely polar since it did not bind to the column at 1 M ammonium sulfate. The collected fractions were measured for enzymatic activity (same as the absorbance peaks), and the active fractions pooled together. The final volume was around 43 mL and the average enzymatic activity 177.3 U/mL. This should equal about 7627 total enzyme units contained within the sample. According to this, it can be assumed that over the course of those two phenyl-Sepharose purification steps the yield was roughly 60%.

Next, 15% ethylene glycol was added to the pooled sample, and it was snap frozen in liquid nitrogen.

In an attempt to achieve greater purification of the sample, the sample was loaded onto a Mono-Q column fitted to a fast protein liquid chromatography (FPLC) station to separate the

proteins based on ion exchange. But first, the ammonium sulfate had to be filtered out of the sample. This was done in a dialysis bag placed in dialysis buffer (see appendix) overnight.

When the FPLC apparatus had been equilibrated, the sample had to be injected into the system via a syringe. Due to the volume of the sample, this had to be done in two runs. The first half of the sample was loaded onto the column and released from the resin with a 0 → 0.8 M NaCl gradient (Figure 10).

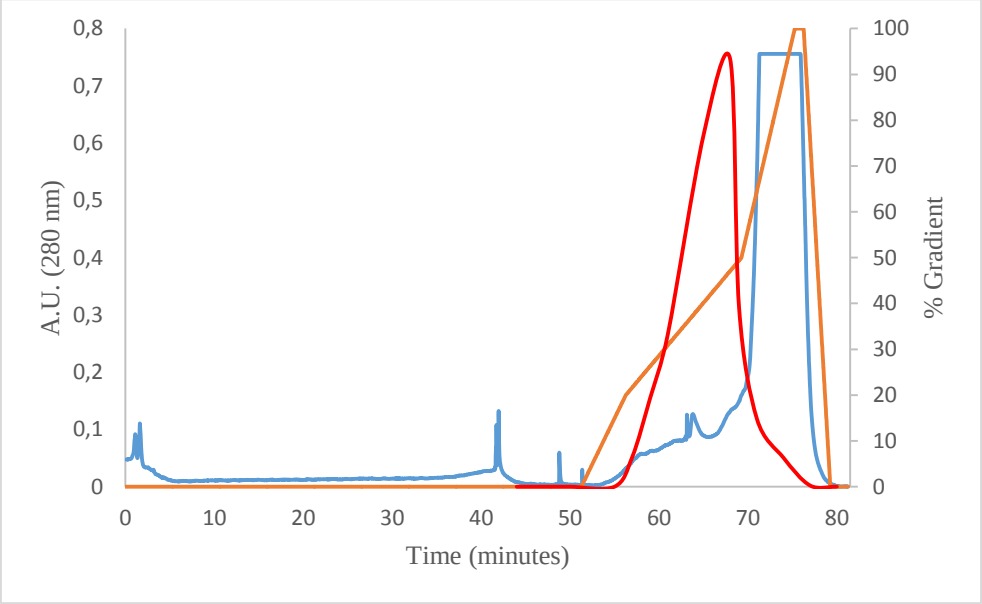

Figure 10. *FPLC-MonoQ chromatography purification of the FLAG-VAP from the first culture. The x-axis represents time in minutes while the primary y-axis (left) shows the light absorbance at 280 nm and the secondary axis (right) displays the 0.8 M NaCl gradient in percentages. Data from activity measurements have been superimposed and fitted to the graph (red line)*

The fraction collected samples were then measured for enzymatic activity and the most active test tubes pooled together for a total volume of 16.5 mL, and the average activity was 137.1 U/mL.

Due to the fact that a large absorbance peak appeared late in the gradient process, it was decided to repeat the run with a gradient of 0 → 1.6 M NaCl on the pooled sample (Figure 11).

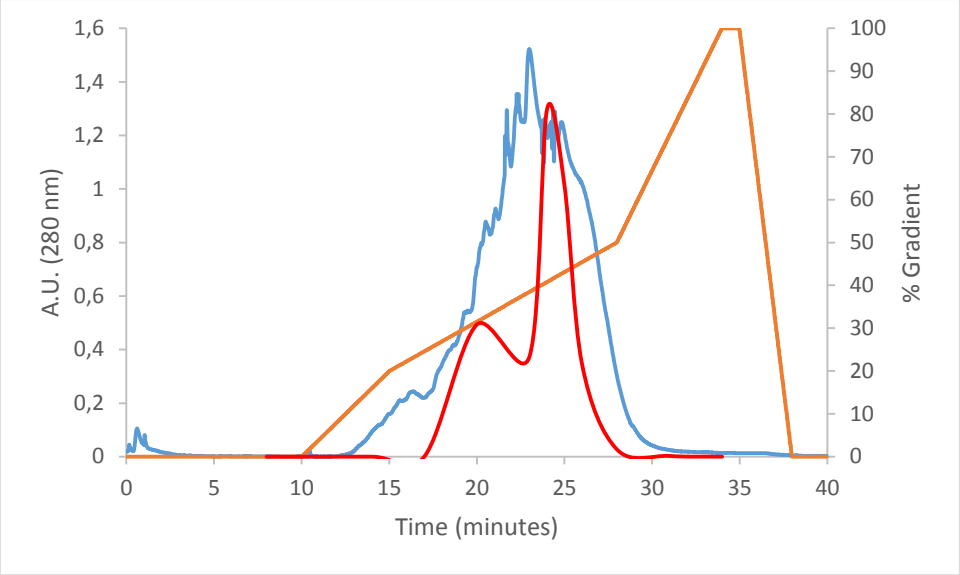

Figure 11. *The FPLC (Mono-Q) process for purifying FLAG-VAP as performed in the second run. A primary axis (left), showing the light absorption at 280 nm and a secondary axis (right) showing the gradient completion in percentages have been plotted against the time in minutes (x-axis). Data from activity measurements have been superimposed and fitted to the graph (red line)*

The test tubes containing the fraction of collected samples were then measured for enzymatic activity and the most active samples pooled together for a total volume of 8 mL. The average activity was around 272.9 U/mL.

4.4 Purification steps for the third cell culture

Considering the results from the purifying process of the first cell culture, it was decided to start with a phenyl-Sepharose column separation (based on hydrophobic interactions). Before any column separation was performed, the sample was kept in 2 M ammonium sulfate (AMSO) solution at 4°C (in a refrigerator) overnight. The 2 M AMSO sample was then centrifuged at 15.000 × g for 10 minutes (longer period might prove more effective). The 110 mL of the supernatant were collected and measured for enzymatic activity, which was 54.7 U/mL. It should be noted again that this is likely an underestimation due to ammonium sulfate inhibition. Furthermore, little to no activity was found in the precipitate, indicating this as a useful purification step.

This time around the phenyl-Sepharose chromatography was performed with a stand-alone gradient mixer and a peristaltic pump, as was described in the methods chapter above. The gradient went from 2 → 0 M concentration of AMSO (see appendix for buffers). A fraction collector was used to collect the samples in test tubes. The enzymatic activity was measured at 405 nm and the absorbance at 280 nm. A visual representation of this can be seen in Figure 12.

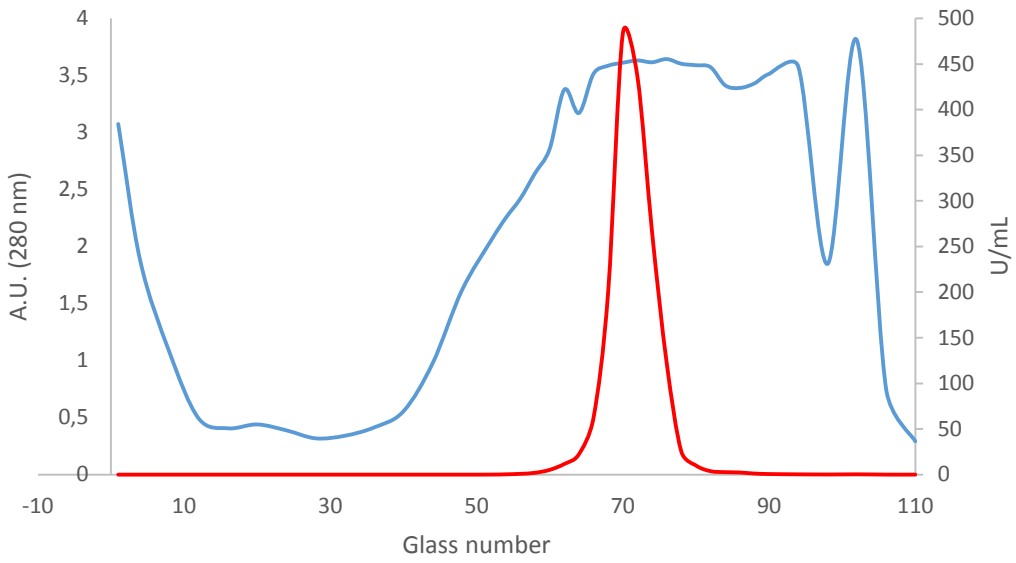

Figure 12. *Phenyl-Sepharose purification of the FLAG-VAP variant from the third culture**.** As can be observed, a large enzymatic activity peak (orange) can be seen around the 70th test tube.*

Test tubes containing enzymatic activity were pooled together (18.5 mL total volume) and put in a dialysis bag and filtered overnight in 2 L of dialysis buffer (see appendix). After dialysis, the sample proved to be around 28 mL in total volume.

Next, it was decided to further separate the sample with an ion exchange process. For this, a Q-Sepharose column was used on a Bio-Rad Biologic station. After the column had been washed with 1 M NaCl and starting buffer, the sample was loaded onto it. Ionic concentration was used to release the proteins from the column using a NaCl (0 - 0.7 M) gradient (Figure 13).

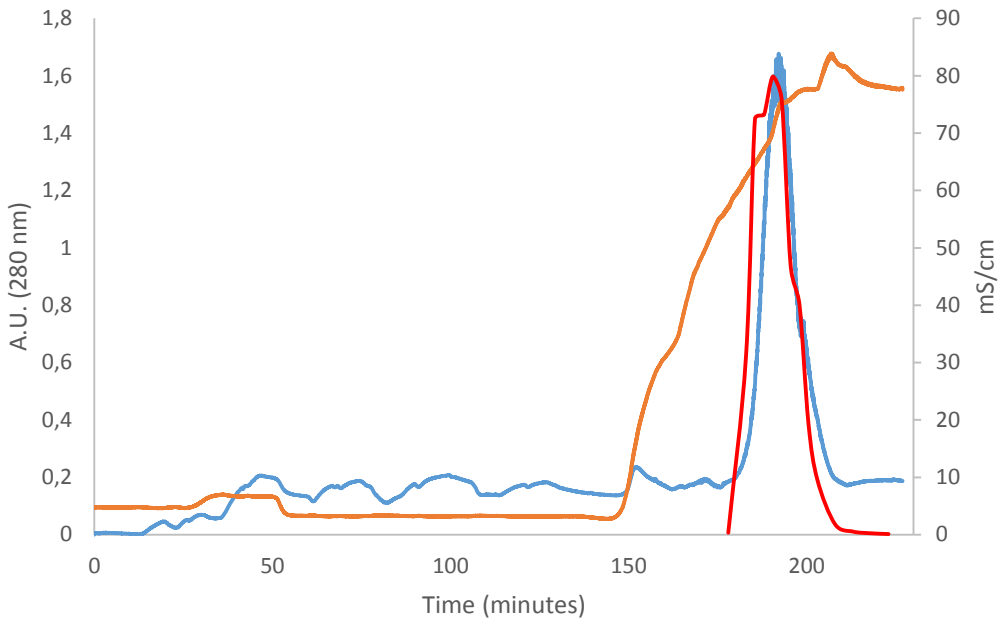

Figure 13. *Q-sepharose purification of FLAG-VAP from the third culture**.** The absorbance at 280 nm (left y-axis) and the electrical conductivity (right y-axis) have been plotted against the time in minutes (x-axis). Data from enzymatic activity measurements have been roughly superimposed and adjusted to fit the graph (red line).*

Two sample pools were collected. One out of the fractions which displayed the most enzymatic activity and another pool out of the samples which displayed considerably less, but still adequate activity. The more active pool was measured at 712 U/mL with a total volume of 5.5 mL. The absorbance at 280 nm for this sample was 0.375. The less active pool measured at 164 U/mL and contained 7 mL of sample, and had a light absorbance (at 280 nm) of 0.359.

4.5 Determining purity of sample with SDS-PAGE electrophoresis

When samples from both cultures had undergone their purification steps, they were subsequently subjected to SDS-PAGE electrophoresis to get a sense of their purity.

4.5.1 First cell culture

Two pooled samples were obtained after the last purification step, the ion exchange column on an FPLC station. This was because the sample was injected into the FPLC station via a syringe and only has a limited capacity. Therefore, the procedure had to be repeated twice to fully purify the sample, resulting in two final samples. Samples were taken from each of these pools and loaded onto pre-made 4-12% Nu-Page Bis-tris gels. Two gels were used, each for one pool (Figure 14).

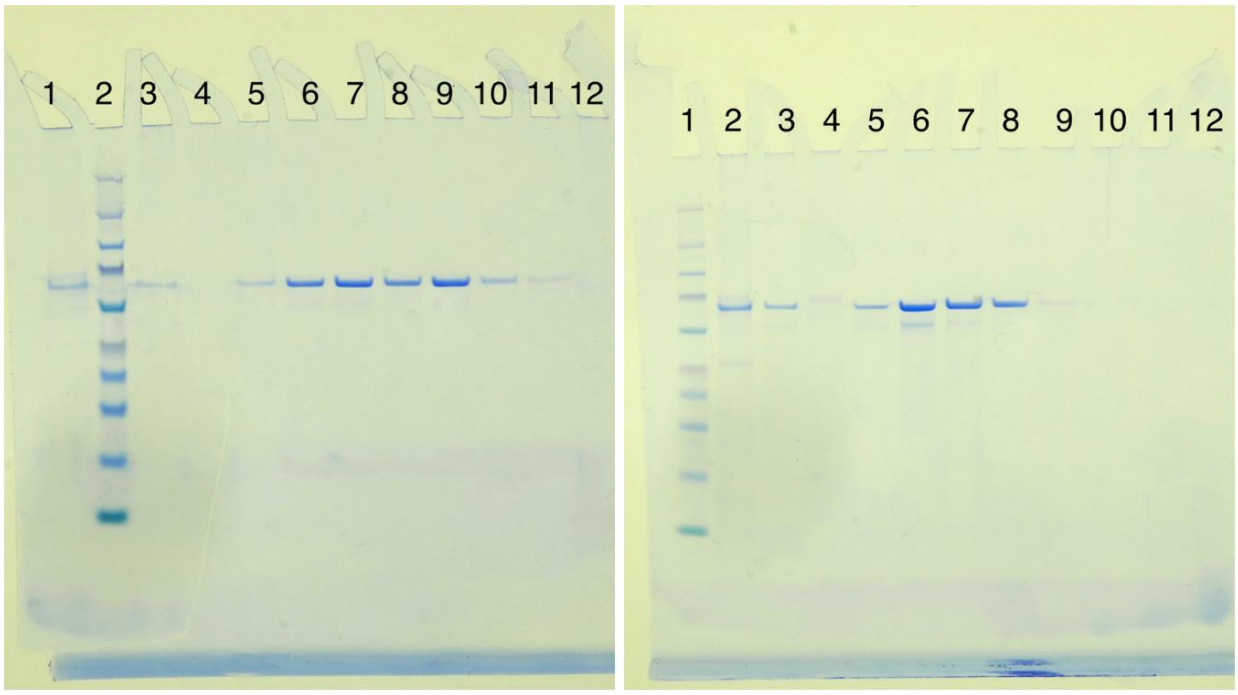

Figure 14. *A composite image of two Blue-silver stained SDS gels. On the left are samples from the first FPLC separation, with a protein size ladder (Spectra multicolor broad range ladder) in the second well of the gel. The FPLC sample is located in the first well, and a Pre-FPLC sample in the third well. On the right is then an image of the electrophoresis gel from the second FPLC separation, as can be seen, the ladder is located in the first well of the gel. Here the FPLC sample is in the second well, and the Pre-FPLC sample in the third well. Other ladders for both gels (i.e. not wells 1, 2 or 3) should be ignored.*

4.5.2 Third cell culture

The purification of the third cell culture ended with an ion exchange column on a Bio-Rad Biologic station. Two samples were pooled from the last separation step, one from fractions with highest activity band and another from the peaks tail.

At first, too much protein was in each well of the electrophoresis gel (see appendix), so the samples were diluted to about 1 µg of protein in each well. The SDS-PAGE electrophoresis itself was performed with a pre-made 4-12% Nu-Page, Bis-Tris gel (Figure 15).

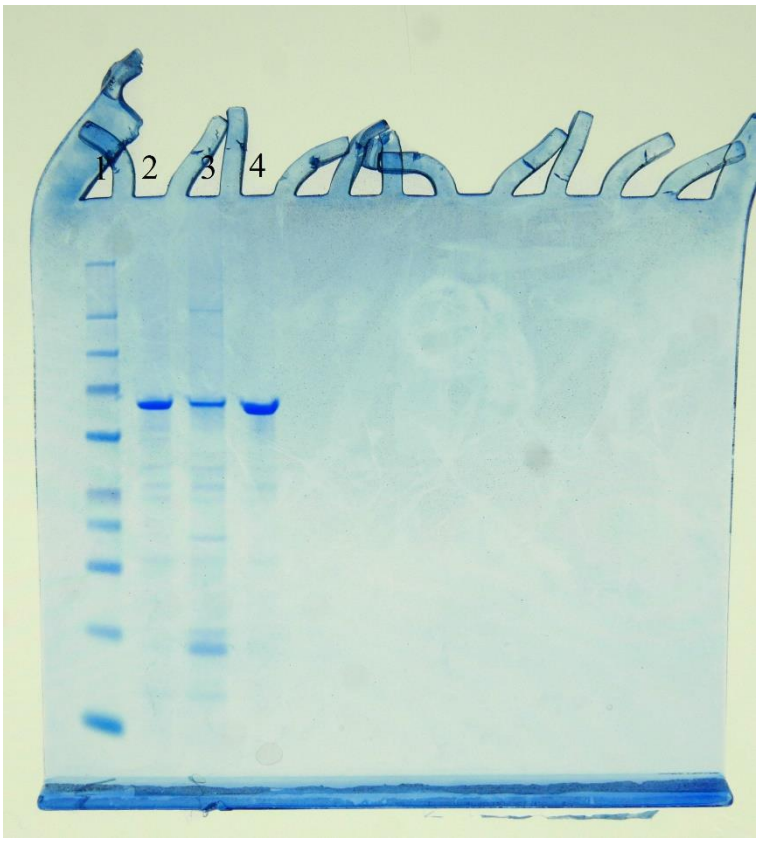

Figure 15. *SDS-PAGE gel stained with Blue-silver method for the FLAG-VAP from the third cell culture. A protein ladder can be seen in the first well, followed by a sample from the "strong" activity peak from the final purification step. The third well shows the "weak" pooled samples from the elution peaks periphery (from the final purification step, Mono-Q) and a 2x FLAG-VAP and VAP-ST2 (Strep-tag) Vibrio alkaline phosphatase mixture (50/50) can be found in well 4.*

As can be seen in the figure above, the sample from the Mono-Q activity peak looks to be purer than the one from its periphery. This sample was then used to perform kinetic measurements and various urea-PAGE experiments.

4.6 Assessment of purification with purification tables

After the samples had undergone their respective purification steps and concentration measurements, a purification table was made to summarize the process and assess the purification as a whole (Tables 1, 2 and 3).

Table 1 shows and overview of the purification process for the first cell culture. As can be seen, the culture underwent 3 main column purification steps, Q-Sepharose, then two phenyl-Sepharose steps, followed by a QFF ion exchange step on a FPLC apparatus. The enzyme yield was about 15% purification with a specific activity of 2668 U/mg.

Table 1. *Purification table for the first cell culture. The activity was measured with p-nitrophenyl phosphate under transphosphorylation conditions as decribed in the Methods section.*

Sample	Volume (mL)	Activity (U/mL)	Protein-conc. (mg/mL)	Enzyme-units	Total Protein (mg)	Specific-activity (U/mg)	Enzyme yield (%)	Purification factor (X)
Supernatant	97	160	2.22	15559	215	72	100	1
Q-Sepharose	27	596	2.03	16087	55	293	103	4
Phe-Sepharose	34	288	1.77	9792	60	163	63	2
Phe-Seph. (2)	43	177	0.06	7627	3	2803	49	39
Q-FF #1	16,5	137	0.06	2262	1	2442	15	34
Q-FF #2	8	273	0.09	2183	1	2894	14	40

Table 2 shows that the purification process for the third cell culture. An AMSO precipitation was utilized as a first step, followed by a phenyl-Sepharose step (not shown). The last step was a Q-sepharose step. The end yield was around 23%, with a specific activity of 3370 U/mg.

Table 2. *Purification table for FLAG-VAP from the third cell culture. The sample was initially treated with 2 M ammonium sulfate (AMSO) which does not precipitate the VAP. Followed by dialysis, this was applied to a phenyl-Sepharose column (not shown) and the active fractions further purified on a Q-Sepharose column. Activity was measured at 25°C with p-nitrophenyl phosphate and protein concentration assessed using coomassie blue as described in the Methods section.*

Sample	Volume (mL)	Activity (U/mL)	Protein-conc. (mg/mL)	Enzyme-unitsunits	Total protein (mg)Protein (mg)	Specific-activity (U/mg)activity(U/mg)	Enzyme yield (%)	Purification factor (X)factor(X)
Pre-AMSO	96	177	3,02	16952	290,2	58	100	1.0
Post-AMSO	110	55	2,57	6019	282,9	21	36	0.4
Q-Sepharose #1	5.5	712	0,21	3918	1,2	3370	23	57.7
Q-Sepharose #2	7	164	0,07	1147	0,5	2207	7	37.8

4.7Michaelis-Menten Enzyme Kinetics

An enzyme's affinity for its substrate is often expressed with the Michaelis-Menten constant, K_m, which is equal to the substrate concentration when the initial velocity (v_0) is half of the maximum velocity (V_{max}).

The k_{cat} value, or turnover number, represents the number of substrate molecules each saturated enzyme is able to convert to product per unit of time. The s^{-1} value is equal to the number of converted substrate molecules to product per second.

The kinetic constants K_m and k_{cat}, were determined for both 2x FLAG (from the third cell culture) and VAP-ST2, which had been previously purified. The kinetic measurements were performed in 100 mM Caps, 1 mM $MgCl_2$, 500 mM NaCl at 10°C with *p*NPP ranging from 0-2 mM.

In the table 3, the k_{cat} (s^{-1}) values can be seen for both AP variants and their respective measurements.

Table 3. Comparision of the kinetic properties of FLAG-VAP and Strep-tag VAP. *K_m and k_{cat} values derived from their respective sample measurements at 10°C under hydrolysing condition with 500 mM NaCl are shown. The substrate was p-nitrophenyl phosphate and the buffer 0.1 M Caps with 1 mM $MgCl_2$. The different lines are repeats with each enzyme variant.*

Sample	K_m (mM)	k_{cat} (s^{-1})
2x FLAG #1	0.235	76.5
2x FLAG #2	0.282	109.2
2x FLAG #3	0.177	60.4
VAP-ST2 #1	0.326	199
VAP-ST2 #2	0.244	304

Figures 16 and 17 show Michaelis-Menten graphs from samples; 2x FLAG #3 and VAP-ST2 #1.

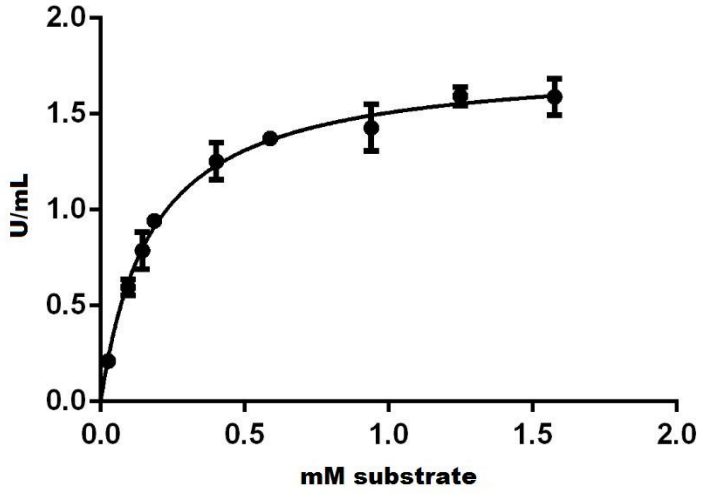

Figure 16. *Michaelis-Menten graph of 2x FLAG-VAP #3 measurements. The y-axis represents the enzymatic activity, which has been plotted against the substrate concentration (mM) at the x-axis.*

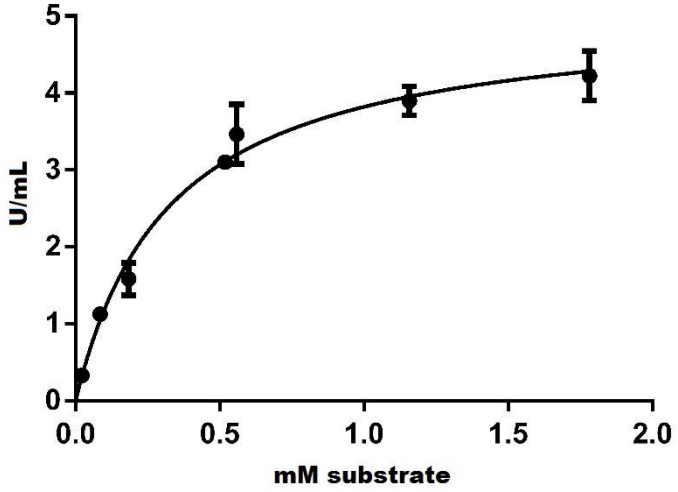

Figure 17. *Michaelis-Menten graph derived from measurements of sample VAP-ST2 #1. Enzymatic activity (y-axis) has been plotted against the substrate concentration (x-axis).*

More graphs and further statistics can be found in the appendix section.

4.8 Monomer-dimer separation of samples using various gel electrophoresis techniques

To be able to differentiate between 2x FLAG and *Strep*-tag variants of the *Vibrio* alkaline phosphatase, and to further recognize the difference between a monomer and dimeric state of these two variants, it was decided to use various gel electrophoresis techniques to accomplish this.

4.8.1 Native gel electrophoresis

As was explained in the methods chapter, the native gel is run under non-denaturing conditions so that the enzymes' native structure is maintained. This way, the physical size of the protein and its charge at the respective pH in the gel should be the determining factors for its mobility through the gel.

Two attempts were made. Each time two identical gels were run, which were then fixed and stained differently. One with Blue-silver dye and the other with activity staining. The first attempt was made with a pre-made 4-12% Nu-Page, bis-tris gel, the second with a native gel made in house (Figure 18). (see appendix for further details).

The samples were treated with different levels of urea concentration. This was done in an attempt to distinguish between a dimeric- and monomeric state (Figure 18).

Native gel electrophoresis with a pre-made Nu-Page gel

Loading the samples onto the gels proved quite challenging, and much glycerol had to be added to the sample for it not to diffuse from its respective well. This was due to the running buffer not being properly diluted, causing it to be too dense for the glycerol to have its desired effect. The gels were then run overnight at 5 mA and constant 20 volts.

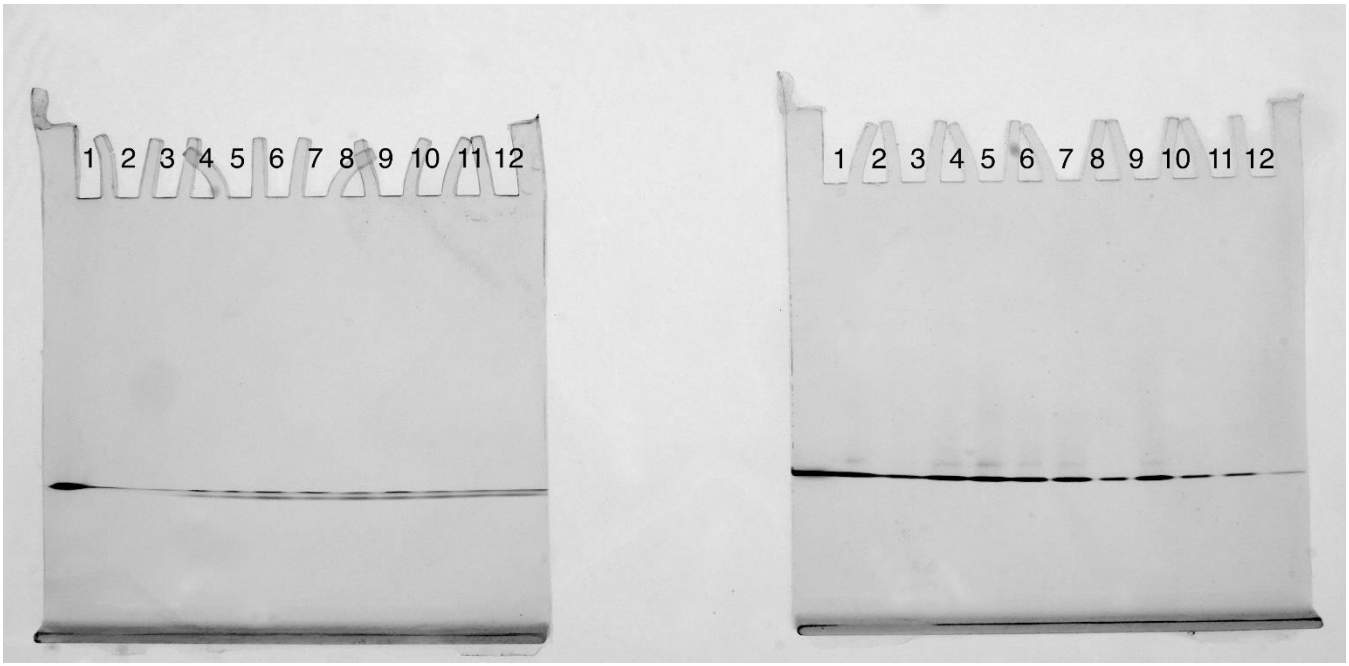

Figure 18. *Native gel electrophoresis of Strep-tag (ST2) and 2x FLAG variants. On the left can be seen the Blue-silver stained gel, on the right, the activity stained gel. Both gels have the following well scheme, with brackets representing the molar concentration of urea. Well 1: pI ladder. Well 2: ST2 [0]. Well 3: ST2 [5]. Well 4: 2x FLAG [0]. Well 5: 2x FLAG [0.3]. Well 6: 2x FLAG [0.5]. Well 7: 2x FLAG [1]. Well 8: 2x FLAG [1.5]. Well 9: 2x FLAG [2]. Well 10: 2x FLAG [3]. Well 11: 2x FLAG [4]. Well 12: 2x FLAG [5].*

No significant difference could be seen between samples (Figure 18). However, in the activity stained gel, a weak band couldbe seen above the stronger front. These might possibly be monomers, however VAP monomers should be inactive.

Furthermore, the native gel electrophoresis with an in-house made native gel proved no better. No pictures of the in-house made gels are available. Since the pI ladder did not even separate, it was deemed that these electrophoresis conditions were not adequate.

4.8.2 Blue-native polyacrylamide gel electrophoresis (Blue-native-PAGE)

The Blue-native PAGE is similar to the native gel electrophoresis, as no SDS detergent is present in the gel or electrophoresis buffers, proteins should remain native when loaded onto the gel. The Blue-native technique differs from the Native one in that it uses a cathode buffer which

contains anionic Coomassie Blue dye, which binds to proteins during electrophoresis. That way the gel is stained during the electrophoresis.

Strep-tag (ST2) and 2x FLAG VAP variants were loaded onto a pre-made, Nu-Page, 4-12% Bis-tris gel (Figure 19). The samples were treated with different concentrations of urea.

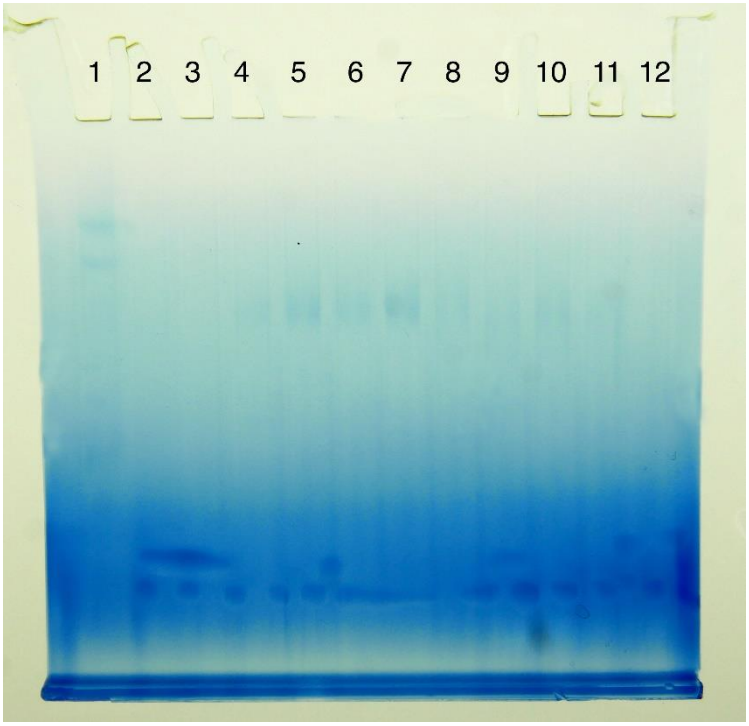

Figure 19. *Shows the Blue-Native Urea gel. Wells have been numbered. Numbers in brackets represent the urea molar concentration. Well 1: pI ladder. Well 2: ST2 [0]. Well 3: ST2 [5]. Well 4: 2x FLAG [0]. Well 5: 2x FLAG [0.3]. Well 6: 2x FLAG [0.5]. Well 7: 2x FLAG [1]. Well 8: 2x FLAG [2]. Well 9: 2x FLAG [3]. Well 10: 2x FLAG [4]. Well 11: 2x FLAG [5]. Well 12: empty.*

Very little can be seen from the picture above (Figure 19). It is likely that the gel was allowed to stay in the dye cathode buffer for too long, leading to overstaining. It was difficult to destain the gel, but some bands seem to form, most notably in the ladder well (1) and wells 4 through 11, which could possibly be dimers, but it is difficult to conclude anything with clarity because of the overstaining. Some bubbles seem to form at the lower end of the gel. These are most likely not related to the sample. The Blue-native gel technique was then abandoned based on these results but this method might be improved by optimizing the concentration of the dye and the destaining method.

4.8.3 Urea gel electrophoresis

In both the native and the Blue-native electrophoresis gels, no difference seemed to be in the separation between the different concentrations of urea with which the samples were treated. The urea should dissociate the enzyme to monomers at some point, causing a mixture of dimeric and monomeric states in the sample. Evidence of this has previously been shown, where the VAP was still in its dimeric form at 1 M urea concentration, but completely monomeric at 2 M urea (Hjörleifsson & Ásgeirsson, 2016). It was speculated that due to the stacking effect in the upper gel before the samples enter the separation gel, the equilibrium would heavily favor the reforming of dimers. Also as soon as the enzyme enters the gel, the concentration of urea decreases to zero. To combat this, it was decided to add the desired urea concentration to the electrophoresis gel before polymerization. However, this would mean that the concentration had to be fixed to one urea concentration per gel (urea gradient gels are also a possibility but not trivial to cast). Both 1 M and 2 M urea gel were made. Furthermore, gels were made with both 10% and 14% polyacrylamide concentrations in the separation gel. Further details on the ingredients of the gels can be found in the appendix section.

Initially, four urea gels were made, two 1 M (Figure 20) and two 2 M (Figure 21). One of each gel was then stained with Blue-silver while the other two were activity stained. The scheme (placement of samples in wells) of the Blue-silver gels was the same as the scheme for the activity stained ones. A pI ladder was added to the first well of both gels which were to be stained with Blue-silver, the second and third wells were loaded with 2x FLAG-VAP sample and the fourth and fifth wells with VAP-ST2 samples.

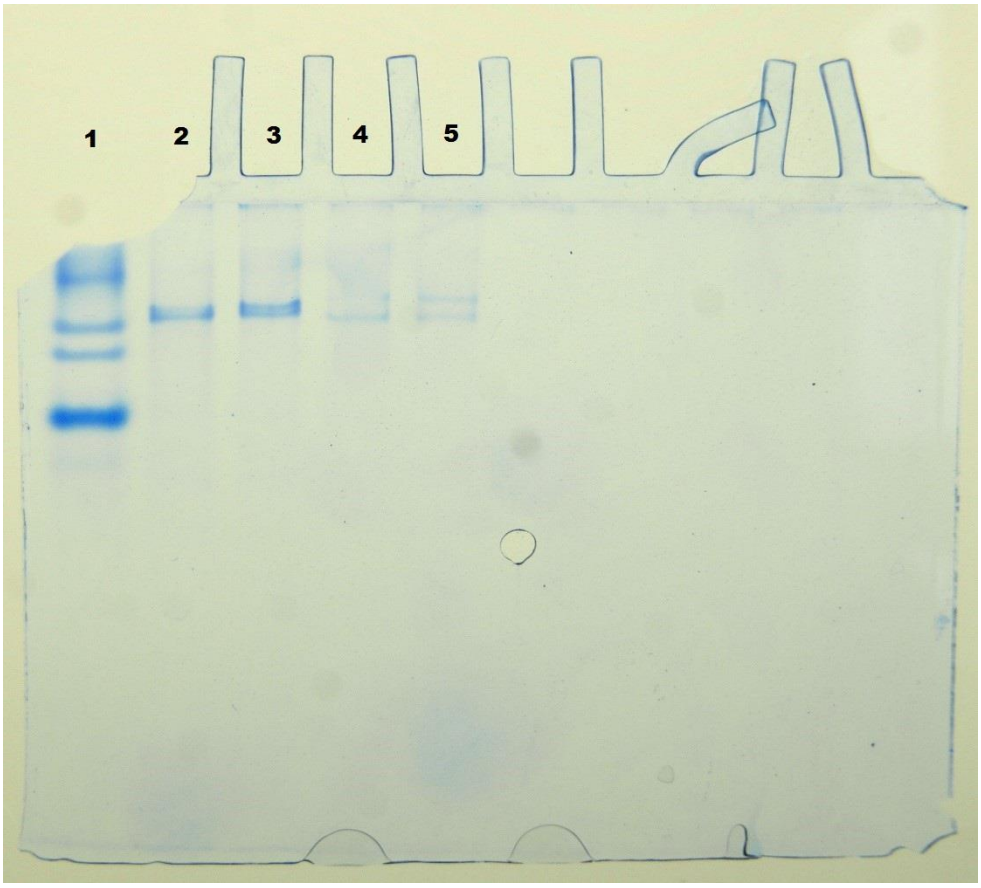

Figure 20. *VAP variants analysed on a Blue-silver stained 1 M urea electrophoresis gel. A pI ladder is present in the first well, followed by two 2x FLAG VAP samples (wells 2 & 3) and two VAP-ST2 samples in wells 4 and 5. Gel was run at about 25 mA (per gel) at 4°C for 3 hours.*

As can be seen in Figure 20, the 2x FLAG samples produced two closely spaced bands, indicating that they might be separating loosely into monomeric- and dimeric states. Wells 4 and 5, containing the VAP-ST2 variant, also produced two, though more dinstinctly separated bands, but not quite as distinctly stained as samples in wells 1 & 2. These bands indicate that a mixture of monomeric- and dimeric forms are present in those two wells. However at this point it cannot be confirmed which band is the monomer and which is the dimer (activity stained gels were then used to determine this).

Figure 21 here below shows a gel run in 2 M urea conditions for comparison.

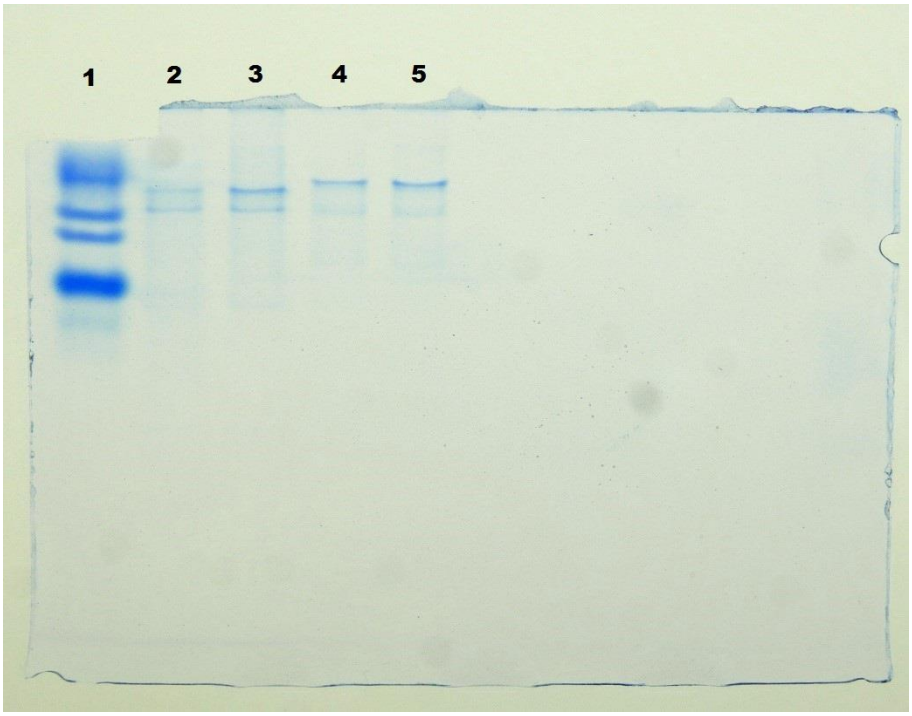

Figure 21. *VAP variants analysed on a Blue-silver stained 2 M urea gel after electrophoresis. A pI ladder can be seen in well 1, followed by two 2x FLAG samples in wells 2 and 3. Samples of VAP-ST2 can be found in wells 4 and 5. Gel was run at about 25 mA (per gel) at 4°C for 3 hours.*

Congruent to what could be seen with the 1 M gel, the VAP-ST2 was separated into two bands. However, this time around, the upper band in both wells appears to be much more intense. This could indicate that more monomeric forms of the enzyme are present relative to the dimeric form. Interestingly, the 2x FLAG-VAP samples which formed closely positioned bands in the 1 M gel, now separated into more widely spaced dimeric- and monomeric forms when treated with 2 M concentration of urea.

Considering the physics of this electrophoresis technique, the mobility of the sample through the gel is based both on its charge and size. Due to the 2x FLAG-VAP being more negatively charged, it should move further into the gel than its *Strep*-tagged counterpart. Furthermore, the dimeric form, being twice the size of the monomeric form, should have more trouble moving through the polyacrylamide maze, resulting in it moving a shorter distance into the gel than a smaller molecule would. When combined, these conditions contradict each other and make it quite hard to differentiate between the bands. Thus, the monomer should move

further into the gel based solely on its smaller size, but the dimeric form, containing double the charge, should move further into the gel based on its negative charge.

This is where the activity staining comes in handy, as the enzyme is active in its dimeric form. This way, the dimer should only be stained, and the monomers should remain invisible. However, the pictures of the activity staining were not as good as one would have hoped, and only the 1 M gel was deemed clear enough to be taken with any significance (Figure 22).

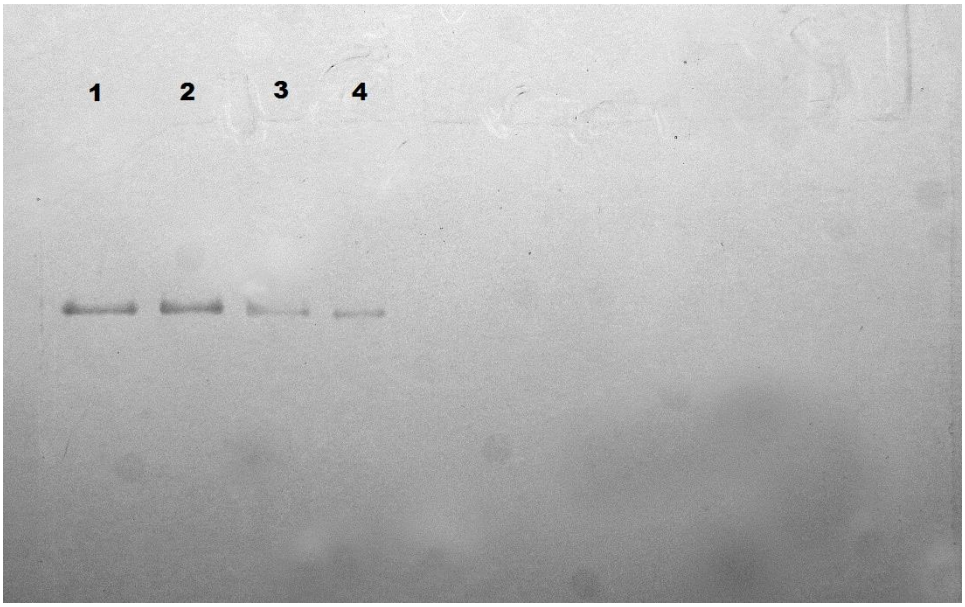

Figure 22. *Activity staining of the 1 M urea gel analysing VAP variants**.** The image has been cropped and contrast-edited with ImageJ for improved clarity. The first two bands (wells 1 & 2) are 2x FLAG-VAP samples while the samples in wells 3 and 4 are of the VAP-ST2 (Strep-tag). Gel was run at about 25 mA (per gel) at 4°C for 3 hours.*

Figure 23 below shows a composite image of Figures 20 & 22, where the gels have been cropped and placed beside each other for comparison.

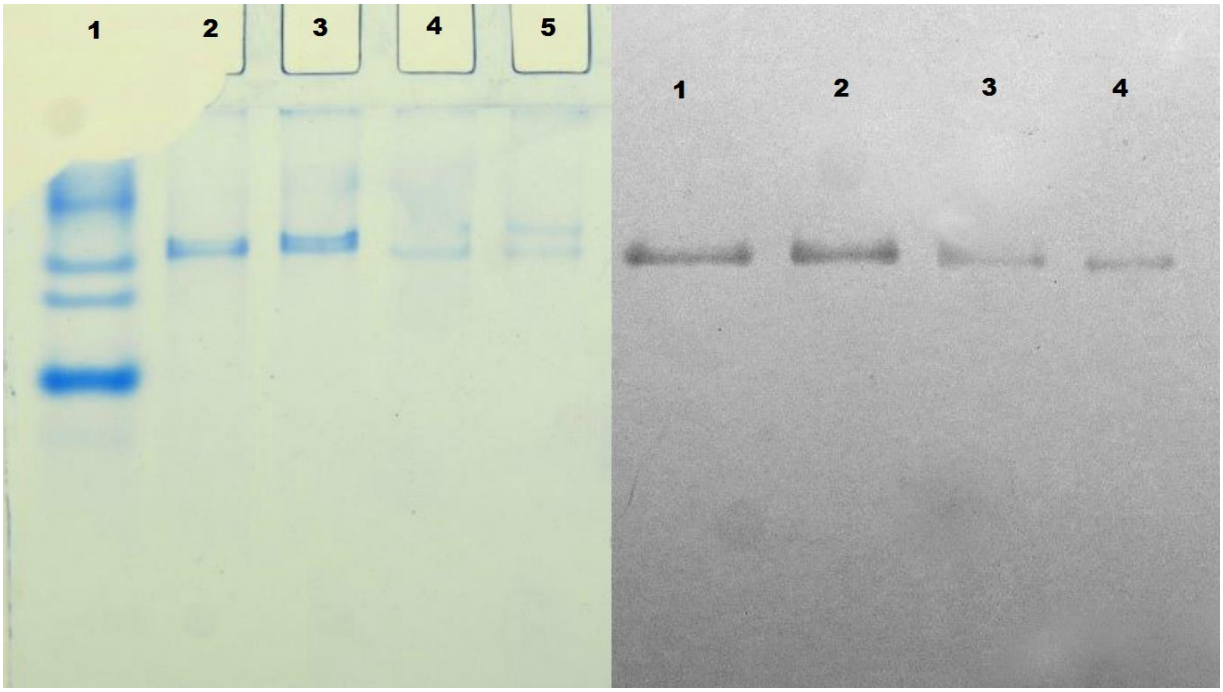

Figure 23. *Composite image of figures 20 & 22, where the figures have been cropped and placed beside each other. Both are 1 M urea gels. The first gel (left), is stained with Blue-silver and has a pI ladder in well 1, followed by two 2x FLAG samples in wells 2 & 3. ST2 VAP samples are in wells 4 and 5. Gel on the right is activity stained and contrast edited with ImageJ. It has 2x FLAG samples in wells 1 & 2, followed by two ST2 VAP samples in wells 3 & 4. Gels were run at 25 mA (per gel) for about 3 hours at 4°C.*

Considering Figure 23, it was decided that the lower bands on the gels above represent the enzymes dimeric form. Furthermore, this would indicate that charge might play a larger role in the molecules mobility through the gel than size, since the larger, but more charged dimers seem to travel farther into the gel than the smaller, but lesser charged monomers. The fact that the upper bands (monomers) of the 2x FLAG-VAP samples seem to travel farther into the gel than the upper bands of the VAP-ST2 samples only gives credence to the assumption that charge is the leading contributor to the enzymes' mobility through the native urea electrophoresis gels.

Subsequently, two 2 M urea gels were made, one with a 10% acrylamide separation gel and another with 14% concentration. These gels were then stained with Blue-silver dye. However, nothing was visible on these gels after electrophoresis. This might be caused by an unknown error somewhere in the process. Pictures of these gels and the other activity stained gel can be seen in the appendix section.

4.8.4 Semi-native SDS gel electrophoresis

As was discussed in the methods chapter, a semi-native SDS gel electrophoresis was performed, where the SDS detergent was added to the sample buffer, but the samples were not boiled. The boiling step is usually thought as an important step for SDS electrophoresis as the high temperature promotes the denaturing by SDS and can also promote disulfide bond reduction and a reaction with redox reagents like dithiothreitol (DTT). This follows the previous work of Robinson and Reixach (2014).

Six samples were loaded onto the bis-tris gel along with a Spectra broad range protein size ladder (Figure 24).

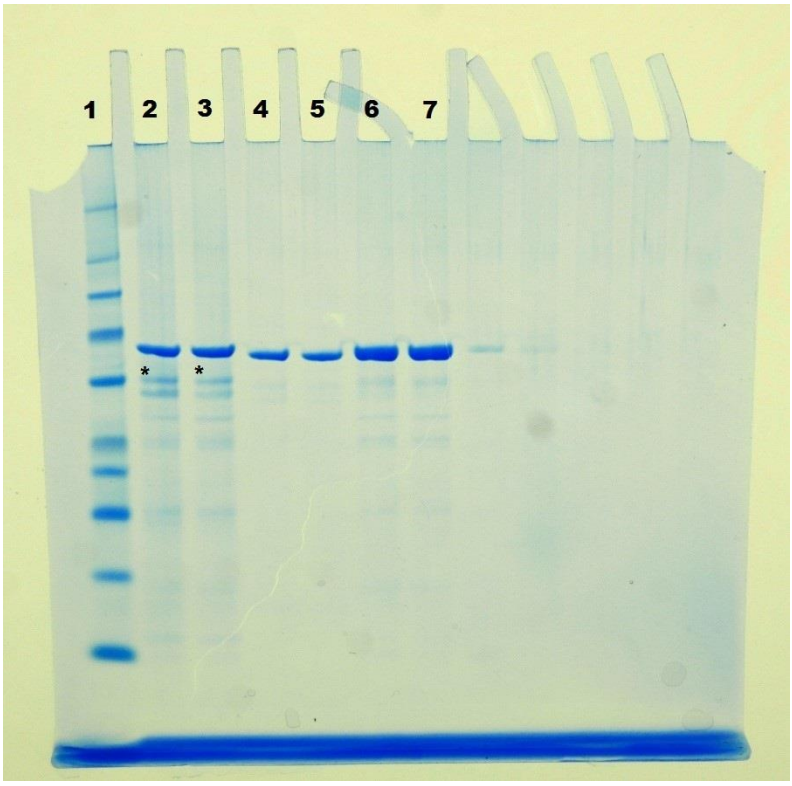

Figure 24. *Semi-native SDS gel of VAP variants. A ladder can be seen in the first well, followed by two 2x FLAG samples in wells 2 & 3. Wells 4 & 5 contain VAP-ST2 samples and lastly, wells 6 & 7 contain 2x FLAG + VAP-ST2 50/50 mixture samples. Gel was ran for 5 hours at 15 mA (constant). Asterisk symbol represents where two bands can be seen in rapid succession.*

On Figure 24, two bands can be seen in rapid succession (marked with an asterisk), especially in wells containing the 2x FLAG-VAP sample. This might indicate a contamination or impurity in the sample.

4.9 Timing of Strep-Tag and 2x FLAG elution on QFF column.

We wanted to find out if *Strep*-tag and 2x FLAG variant dimers could be separated on a ion exchange column as Schneider et al. (2001) had previously done. It was decided to use a Q-Sepharose Fast Flow (QFF; GE Healthcare) column on an FPLC apparatus. The samples were loaded onto the column and released with a 0-0.8 M NaCl gradient (Figure 25).

About 500 µl of sample (0.1 – 1.0 mg / mL) was loaded onto the QFF column after it had been desalinized with an Amicon centrifugal filter (30 kDa cut-off).

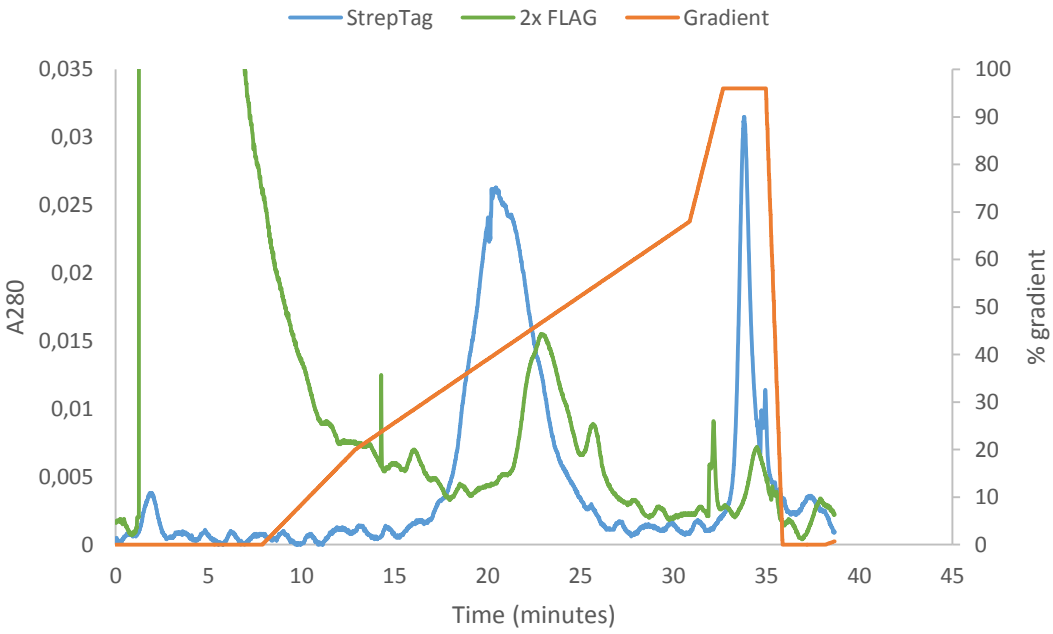

Figure 25. *Strep-tag (VAP-ST2) and 2x FLAG separation on a Q-Sepharose column. The chromatograms are superimposed onto each other. The light absorption at 280 nm can be seen on the left y-axis, while the gradient completion is represented on the right y-axis. The time in minutes can be seen on the x-axis. A legend is displayed on the top of the figure, showing what data corresponds to which color.*

As can be seen in Figure 25, the 2x FLAG-VAP is released with a little more NaCl concentration than the *Strep*-tag VAP (ST2). The elution volume at the peak height was 20.5 min and 22.5 min for VAP-ST2 and 2x FLAG-VAP, respectively. The high absorption in the beginning of the 2x FLAG-VAP sample at 280 nm is due to air bubbles reaching the detector during the injection phase of the sample. Another air peak can be seen at about 32 min. Also, a shoulder can be seen in the 2x FLAG-VAP peak which could be a contamination. At the end of the gradient a peak was eluted which is also possibly a contamination. Figure 26 shows the elution of the VAP variants more closely.

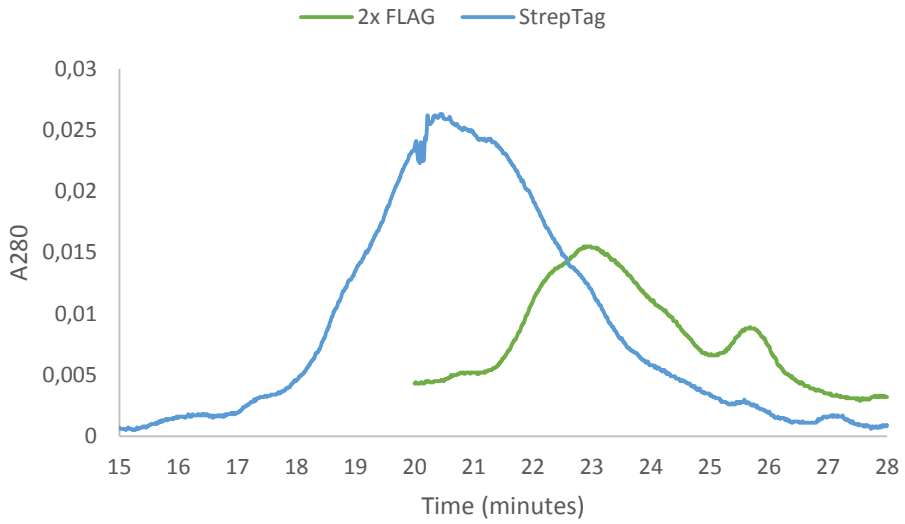

Figure 26. *Comparison of the elution volume for the 2x FLAG-VAP and Strep-Tag VAP variants on a Q-Sepharose FF column. The y-axis represents the light absorption at 280 nm, while the x-axis displays the time in minutes. A legend is displayed at the top of the chart, matching the curves to their respective variant and color scheme.*

The absorption peak of the *Strep*-Tag sample started at around the 18th minute, while the 2x FLAG-VAP peak starts somewhere between the 21st – 22nd minute (Figure 26). The *Strep*-tag peak appears to be wider, but the absolute absorbance peaks of the two samples are also different, with the *Strep*-tag peak around 20th and a half minute, and the 2x FLAG-VAP around the 23rd minute.

5 Discussion

The aim of project was to produce a new purification protocol for the 2x FLAG VAP variant and test a quick and easy way to isolate *Strep*-tag bound VAP variants using *Strep*-tactin spin columns. Furthermore, the project ventured into somewhat uncharted territory, trying to differentiate between the different VAP variants and their respective mono- and dimeric states, with one of the primary goals of the project being to observe and identify possible subunit exchanges of the VAP variants.

Initially, an in-house made affinity column was tested as a possible purification step in the 2x FLAG VAP protocol, but it did unfortunately not bind any of the desired protein. It is worth noting that the CNBr-activated agarose that was used in the making of the column was intended to be used before 1984. Also, the *Strep*-tactin spin columns did not bind the desired amount of *Strep*-tagged proteins (either *E. coli* AP or *Vibrio* AP), and after a few tries and approaches, the *Strep*-tactin purification method was not pursued further.

Two purification processes for the newly produced FLAG-VAP variant going through a column series were seen through to the end, with the first one involving some trial and error during the handling of the sample. The second purification process was then performed with a more meticulous approach. An easy and effective first step is worth noting, the protein precipitation step using 2 M ammonium sulfate and subsequent centrifuging left the FLAG-VAP in the supernatant where it was collected and subjected to further purification using a phenyl-Sepharose column. This was quite convenient because the gradient by which the hydrophobic column separation was made started with a 2 M ammonium sulfate solution. Thus, no additional steps were needed between the ammonium sulfate centrifugation and phenyl-Sepharose column loading steps. The second column step was an ion-exchange. Using a Mono-Q column and a Bio-Rad station, the sample was loaded onto the column and subsequently released during a 0 → 0-7 M NaCl gradient.

Using the ammonium sulfate precipitation step and the two column separation steps, a quick and relatively easy method was developed to purify the 2x FLAG-VAP variant. However, this protocol can certainly be improved and expanded upon since the sample was not deemed pure enough by electrophoresis.

As was mentioned before, one of the main goals of the project was to identify the possible subunits exchanges between VAP variants using similar methods to those employed by others. By mixing two differently labelled VAP dimers, one should see the emergence of hybrids, in addition to the two homodimers. One could mix wild-type VAP without a tag with either 2x FLAG-VAP or *Strep*-tag VAP, or mix the latter two together. Then, a method to observe the mono- and dimeric states for both variants is needed that relies on different charge or binding properties. Precedence for using an ion-exchange column had already been established with transthyretin (TTR) by Schneider et al. (2001), and Robinson & Rexiach (2014), utilizing semi-native PAGE techniques.

The initial idea was to use strepavidin-spin columns to isolate the *Strep*-tag-containing dimers (either homodimers or heterodimers with one *Strep*-tagged subunit) leaving unlabelled dimers to flow through. The amount should decrease as time goes by from half of the total protein to one-quarter, if there is an active monomer exchange. Also, the bound enzyme could be released off the column and analysed by electrophoresis under condition where the *Strep*-tag and FLAG variants would migrate differently and might be quantified. As there was no prior example in the literature, and the columns were not working well, this apporach was abandoned.

We decided to start by testing electrophoresis for the analysis of hybrid dimers containing the FLAG-VAP variant. A number of different PAGE techniques were tried, with both pre-made and in-house made electrophoresis gels. Native PAGE, Blue native PAGE, semi-native PAGE and urea PAGE were the most notable techniques used in this endeavour. The most promising results came about using an in-house made urea gel. This was done to try and counter the effects of possible stacking and subsequent reforming of the dimers in the stacking gel. Using this method, a clear differentiation was observed between the monomeric- and dimeric states in the sample, most notably at 2 M urea concentration. Furthermore, the different VAP variants seemed to display some difference with regards to their position within the gel, with

the 2x FLAG-VAP monomers seemingly traveling further into the gel than its *Strep*-tag (VAP-ST2) counterpart. This is a promising technique which could be expanded upon and used in future projects, possibly by altering the electrophoresis duration - letting it run for a longer period of time.

At the end of the project, it was decided to try and use a quaternary ammonium ion exchange column (Q-FF) on an FPLC apparatus to separate the two VAP variants (FLAG vs. *Strep*-tag). The samples were eluted from the column using a 0 - 0.8 M NaCl gradient. The results indicated that the 2x FLAG-VAP variant required more salt concentration to come off the column. This should maybe not come as a surprise, as the variant carries a more negative net charge than its *Strep*-tagged counterpart.

Due to time constrictions, no follow-up experiments were done for this technique, but it appears to be very suitable to differentiate between the two variants in future projects.

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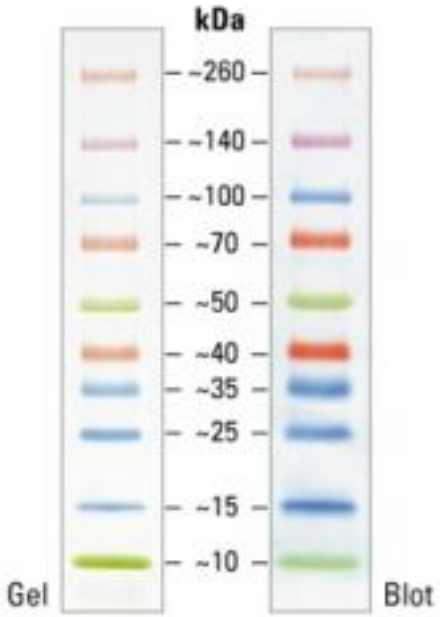
Appendix

Spectra™ Multicolor Broad Range Protein Ladder



Luria-Bertani (LB) medium

4 L recipe of LB :

- 40 g – Tryptone (10 g/L)
- 20 g - Bacto™ Yeast Extract (5 g/L)
- 40 g – NaCl (10 g/L)

Buffers

Dialysis buffer/TMC (used at 4°C)
20 mM Tris
10 mM $MgCl_2$
pH 8 (RT)

Dialysis buffer used for AMSO samples
10 mM Tris
1 mM $MgSO_4$

1x MES buffer
50 mM MES
50 mM Tris
0.1% w/v SDS
1 mM EDTA
pH 7.3

Mono-Q gradient buffers (A & B) :

Buffer A	Buffer B
20 mM Tris	20 mM Tris
2 mM $MgSO_4$	2 mM $MgSO_4$
pH 8	pH 8

-	Desired NaCl concentration (e.g. 0,7 M)

Ammonium sulfate (AMSO) buffer
20 mM Tris
2 mM $MgSO_4$
Desired AMSO concentration (e.g. 1 M)
pH (at 4°C)

Native-Page buffers :

Running buffer
0.25 M Tris
1.9 M Glycine (99%, for electrophoresis)
pH 9.11 (at RT)

Sample buffer (5x)
15.5 mL –1 M Tris
2.5 mL – 1% Bromophenol blue
7.0 mL – dH_2O
25 mL – Glycerol

Coomassie Native Gel buffers :

Cathode buffer	Anode buffer
15 mM - Tris	12,5 mM - Tris
50 mM - Tricine	96 mM - Tricine
0,01% - Coomassie G250	-
pH 7.0 (4°C)	pH 8.3 (4°C)

CAPS (Hydrolysis) buffer :

CAPS – Hydrolysis buffer
100 mM CAPS
1 mM $MgCl_2$
500 mM NaCl
pH 9.56 (RT)

SDS Loading Buffer :

SDS Loading Buffer (5x)
350 mM Tris (pH 6,8)
30% glycerol
1% SDS
0.6 DTT
0.012% Bromophenol blue

Electrophoresis gels

The gels are all made with the same components. However, measurements of individual ingredients may vary between different gels.

It is important to note that all ingredients are mixed, save for the ammonium persulfate, which is added last because it is the agent which catalyzes the polymerization of the gel.

Native-gel (stacking and running gels) :

Ingredients	12% (running/lower) gel	4% (stacking/upper) gel
30% acrylamide, 2.6% bis-acrylamide	6 mL	1.98 mL
Tris	3.75 mL (1,5 M)	0.5 mL (0.5 M)
MilliQ H_2O	5 mL	9 mL
TEMED	15 µl	15 µl
10% APS	75 µl	75 µl

1 M Urea gel :

Ingredients	14% (running/lower) gel	4% (stacking/upper) gel
30% acrylamide, 2.6% bis-acrylamide	7 mL	1.98 mL
Tris	3.75 mL (1,5 M)	0.5 mL (0.5 M)
MilliQ H_2O	2.48 mL	7.48 mL
TEMED	15 µl	15 µl
10% APS	75 µl	75 µl
9.89 M Urea	1.52 mL	1.516 mL

2 M Urea gel :

Ingredients	14% (running/lower) gel	4% (stacking/upper) gel
30% acrylamide, 2.6% bis-acrylamide	7 mL	2 mL
Tris	3,75 mL (1,5 M)	0,5 mL (0,5 M)
dH_2O	1.15 mL	9.4 mL
TEMED	15 µl	15 µl
10% APS	75 µl	75 µl
9,89 M Urea	3 mL	3 mL

2 M, 10% running gel :

Ingredients	10% (running/lower) gel
30% acrylamide, 2.6% bis-acrylamide	5 mL
Tris	3.75 mL (1.5 M)
MilliQ water	3.15 mL
TEMED	15 µl
10% APS	75 µl
9.89 M Urea	3 mL

Figures not included in the results chapter

Electrophoresis gels:

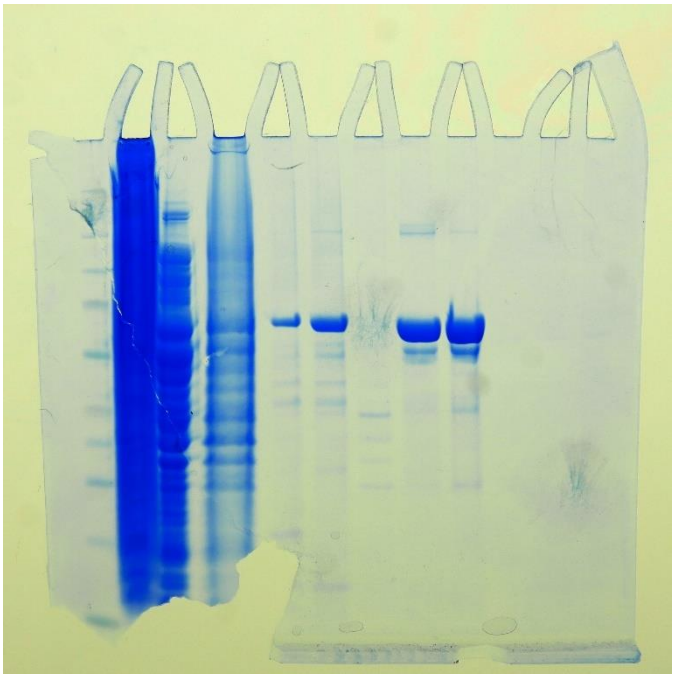


Figure 27. *SDS electrophoresis gel for FLAG-VAP purified in the third cell culture. Too much protein was put into some wells. Wells 2, 3, and 4 contain samples from the original supernatant, pre-AMSO centrifuging and post-AMSO centrifuging, respectively. Well 5 contains the "weak" pooled sample, and well 6 contains the "strong" pooled sample.*

Figure 28. *Shows the activity stained gel from the 2 M Urea electrophoresis. As can be seen, not much can be concluded from this figure.*

Figure 29. *A composite image of 10% and 14%, 2 M urea gels where FLAG-VAP and Strep-tag VAP variants were analysed. However, nothing seems to be stained on the gel. Reasons for this are not clear.*

Additional Kinetic graphs:

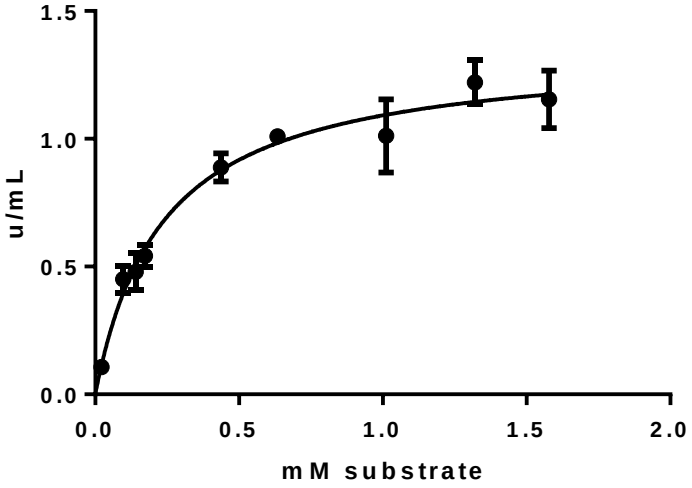


Figure 30. *Shows a graph representing the first Michaelis-Menten kinetic measurements of 2x FLAG-VAP sample.*

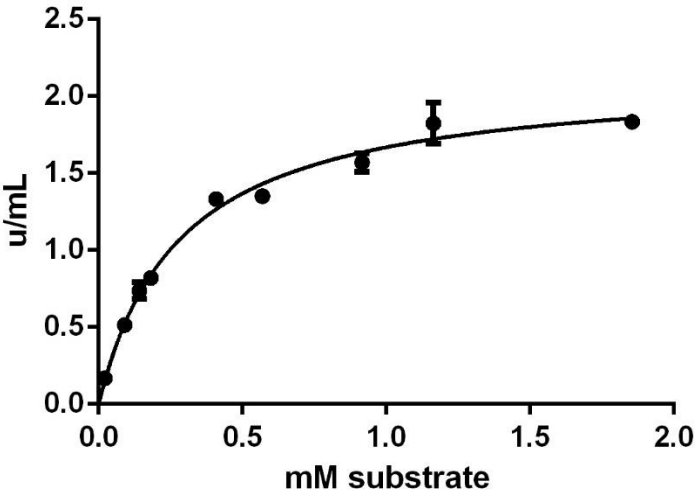


Figure 31. *Shows a graph representing the second Michaelis-Menten kinetic measurements of 2x FLAG-VAP sample.*

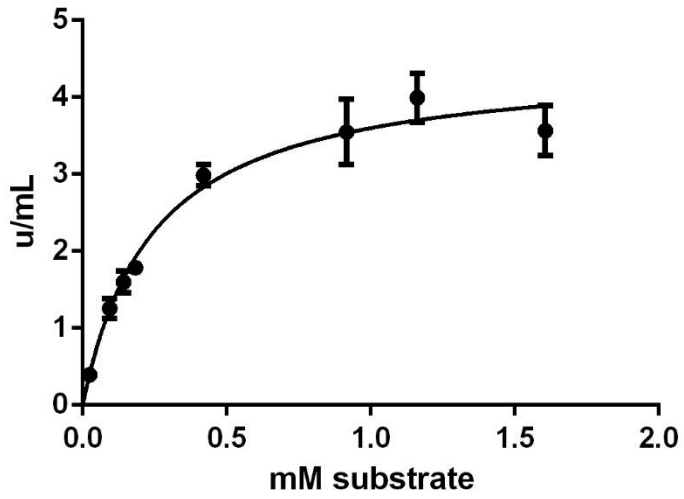


Figure 32. *Shows a graph representing the second Michaelis-Menten kinetic measurements of a Strep-tagged VAP wild-type sample.*