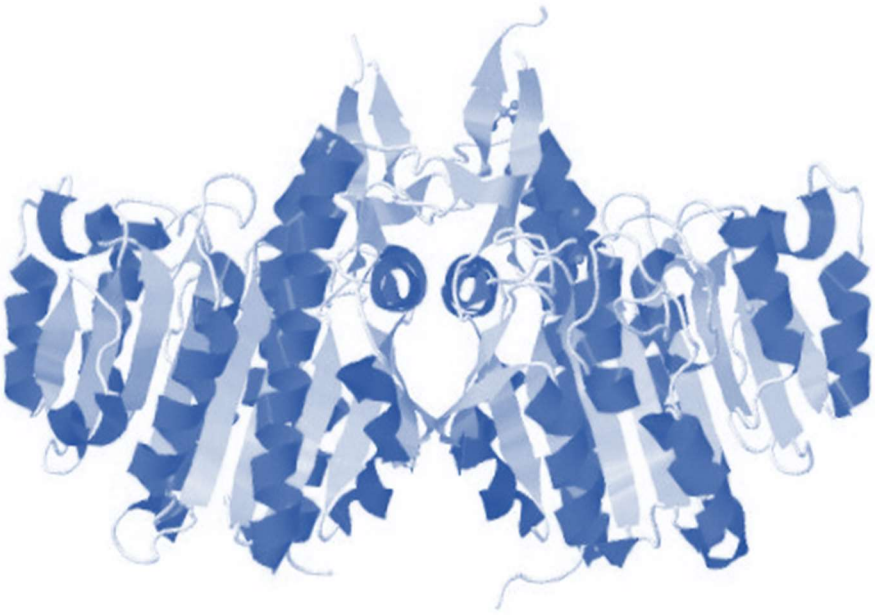
University of Crete – Biology Department

Foundation for Research and Technology
Institute of Molecular Biology & Biotechnology

Graduate Programme in Molecular Biology & Biomedicine

Master Thesis

Characterization and putative biotechnological applications of a psychrophilic alkaline phosphatase from the bacterial strain TAB110a



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Cover picture:

Three dimensional structure of the Antarctic bacterium TAB5 (PDB ID: 2IUC) alkaline phosphatase as depicted by JSmol viewer

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Περίληψη

Η Γη αποτελείται από ποικίλα φυσικά περιβάλλοντα, τα οποία χαρακτηρίζονται στην πλειοψηφία τους από ακραίες περιβαλλοντικές συνθήκες. Παρά αυτό το γεγονός, αναρίθμητοι οργανισμοί έχουν βρεθεί να αποικίζουν και να ευδοκιμούν σε αυτά τα σκληρά και αφιλόξενα περιβάλλοντα. Μεταξύ αυτών, οι πιο διαδεδομένοι, ονομάζονται ψυχρόφιλοι, καθώς έχουν προσαρμοστεί για επιβίωση σε πολύ χαμηλές θερμοκρασίες. Αυτοί οι ψυχρόφιλοι οργανισμοί παρουσιάζουν ιδιαίτερο βιοτεχνολογικό ενδιαφέρον, το οποίο επικεντρώνεται τα ένζυμα που κωδικοποιούν. Τέτοιου είδους προσαρμοσμένα στο κρύο ένζυμα χαρακτηρίζονται από υψηλότερη καταλυτική ενεργότητα σε χαμηλές θερμοκρασίες και μικρότερη θερμική σταθερότητα συγκριτικά με ομόλογα τους από μεσόφιλους οργανισμούς. Τέτοιου είδους χαρακτηριστικά θεωρούνται ως πλεονέκτημα σε ποικίλες βιοτεχνολογικές εφαρμογές.

Σε προηγούμενη ερευνητική εργασία του εργαστηρίου MINOTECH biotechnology, μια βακτηριακή συλλογή αποτελούμενη από 262 στελέχη, τα οποία συλλέχθηκαν από θαλάσσια ύδατα στην Ανταρκτική, ελέγχθηκαν για την έκφραση ενεργότητας αλκαλικής φωσφατάσης. Εννέα από αυτά τα στελέχη επιλέχθηκαν για αλληλούχιση του γονιδιώματος τους, καθώς παρουσίασαν υψηλή ενεργότητα αλκαλικής φωσφατάσης αλλά και μείωση αυτής έπειτα από θερμική επεξεργασία. Το γονίδιο APT110α από το στέλεχος TAP110α επιλέχθηκε και μετέπειτα απομονώθηκε, βάση της μεγάλης ομολογίας της αλληλουχίας του και της συντήρησης των καταλοίπων του ενεργού κέντρου συγκριτικά με άλλες χαρακτηρισμένες αλκαλικές φωσφατάσες. Το γονίδιο αυτό, κλωνοποιήθηκε σε κύτταρα *E. coli* προκειμένου να επαληθευτεί η ενεργότητα αλκαλικής φωσφατάσης.

Στην παρούσα εργασία, η παραπάνω έρευνα συνεχίστηκε, με σκοπό τον βιοχημικό και βιοφυσικό χαρακτηρισμό της TAP110α αλκαλικής φωσφατάσης, αλλά και τον ανασχεδιασμό των ιδιοτήτων της μέσω εισαγωγής στοχευμένων μεταλλαγών στην αλληλουχία της. Αναλυτικότερα, παρουσιάζεται ένα ολοκληρωμένο πρωτόκολλο καθαρισμού για την απομόνωση της TAP110α αλκαλικής φωσφατάσης. Επιπροσθέτως, υπολογίστηκαν οι κινητικές παράμετροι του ενζύμου, ενώ η πρωτεΐνη κρυσταλλώθηκε επιτυχώς και δεδομένα περίθλασης από την ακτινοβόληση τους συλλέχθηκαν, με απώτερο σκοπό την ανάλυση της τριτοταγούς δομής του ενζύμου. Η τεχνική της φασματομετρίας κυκλικού διχρωισμού χρησιμοποιήθηκε για την ανάλυση της θερμικής σταθερότητας αλλά και της δευτεροταγούς δομής του ενζύμου. Τέλος, με βάση τα αρχικά αποτελέσματα από τον χαρακτηρισμό της TAP110α αλκαλικής φωσφατάσης, σχεδιάστηκαν αμινοξικές μεταλλαγές με σκοπό την αύξηση της καταλυτικής ενεργότητας του ενζύμου αλλά και την μείωση της θερμικής σταθερότητας του.

Με βάση τα αποτελέσματα από τα παραπάνω πειράματα, η TAP110α αλκαλική φωσφατάση παρουσιάζει αξιοσημείωτη θερμική σταθερότητα συγκριτικά με ομόλογα ψυχρόφιλα ένζυμα. Επιπροσθέτως, οι μεταλλαγές D179H και D179E έδειξαν ότι οδηγούν σε θερμική αστάθεια του ενζύμου, με την μεταλλαγή D179E να αυξάνει και την ενεργότητα του ενζύμου. Ο χαρακτηρισμός και των άλλων σχεδιασμένων μεταλλαγών ίσως να οδηγήσει σε περαιτέρω βελτίωση του ενζύμου. Τέλος, η αποσαφήνιση της τρισδιάστατης δομής του ενζύμου θα μπορούσε να χρησιμοποιηθεί για την διερεύνηση των μηχανισμών θερμικής προσαρμογής του ενζύμου, αλλά και για τον σχηματισμό περισσότερων κατευθυνόμενων μεταλλαγών.

Summary

The majority of Earth's environments are characterized by extreme biological conditions, which facilitates their characterization as inhospitable by humans. Despite this, life has adapted to these harsh habitats, as many organisms have been found to colonize and thrive in them, characterized as extremophiles. The most widespread in this group are psychrophiles, meaning organisms that have adapted to life in cold environments. This doesn't come as a surprise, as most of Earth's oceans and around one fifth of Earth's soils are permanently at temperatures below 5° C or covered by permafrost, respectively. Psychrophilic organisms possess a special biotechnological interest, in terms of their biocatalysts. Cold-adapted enzymes are, in general, characterized by higher catalytic activity at low temperatures and heat- lability compared with their mesophilic counterparts. Both of this characteristics are considered advantageous in many biotechnological applications.

In a previous study at MINOTECH biotechnology, a bacterial collection of 262 strains, collected from the Antarctic Ocean, was screened for the expression of alkaline phosphatases activity, based on a chromogenic-based high-throughput assay. The genome of nine strains with high and temperature-depended expression of alkaline phosphatase activity were sequenced and, based on sequence similarity and the preservation of the active site residues with other characterized alkaline phosphatases, the gene APT110α from the strain TAP110α was isolated. The corresponding gene encoding for a putative psychrophilic alkaline phosphatase was cloned in *E.coli* for further characterization.

In this thesis, the above research initiative was continued, with the biochemical and biophysical characterization of TAP110α alkaline phosphatase, together with the rational redesigning of its properties. In detail, a full purification scheme is standardized and presented for the isolation of TAP110α. Furthermore, the kinetic parameters for the enzyme were calculated, while crystallization of the protein resulted in the acquisition of diffraction data, in order to elucidate its three-dimensional structure. Additionally, circular dichroism spectrometry was used to assess the thermal stability and secondary structure of the enzyme. Lastly, based on the resulting information, amino acid substitutions were designed and performed, in order to enhance both the catalytic activity and the heat- lability of the enzyme. Similar characterization steps were followed for the resulting mutants.

Based on the results of these experiments, TAP110α alkaline phosphatase shows a striking thermostability, resembling that of mesophilic homologous. Furthermore, the designed mutations D179H and D179E result to a more heat-labile enzyme, while the mutant D179E shows also an increase in activity. Characterization of all the designed mutants may further improve the TAP110α alkaline phosphatase. Lastly, elucidation of its three-dimensional structure will give insights of its temperature adaptation, and provide means for a more insightful and targeted redesigning of its properties.

1. Introduction

1.1. Life in the cold

From an anthropocentric point of view, most of Earth's environments are considered as inhospitable. Major limitations for any life form in these habitats are the extreme variations in temperature, pH, pressure and salt concentration. Despite these restraining conditions, life has an admirable ability to adapt to such harsh habitats and, as a consequence, various life forms have been found colonizing almost every place on the planet. Among the challenging conditions in which life had to prevail, low temperature may be considered as the most widespread on the planet. Indeed, 90 % of the oceans on Earth have permanently an average temperature of 5 °C or less (Rodrigues and Tiedje, 2008), while approximately 20 % of terrestrial soils are covered by permafrost (Feller, 2013).

Organisms thriving in such low temperatures span the domains of Bacteria, Archaea and Eukarya, with dominant representatives being microorganisms, such as bacteria, fungi and microalgae (Feller, 2003). These cold-adapted organisms can be categorized to psychrophilic or psychrotolerant, with the latter forming the majority of this group of extremophiles (Russell and Cowan, 2006). According to the definition set by Morita (1975), as psychrophiles are considered organisms that grow optimally at a temperature of ≤15 °C, have a maximum growth limit of about 20 °C and a minimum temperature of growth of 0 °C or below. On the other hand, psychrotolerant organisms exhibit a more extended growth temperature range, with the upper limit reaching temperatures similar to mesophilic optima, while still retaining the ability for growth at temperatures close to 0 °C. Such a classification in many cases is considered arbitrary, as cold-adapted organisms that do not abide to such definitions are often encountered. For example, Antarctic soil bacteria have been characterized with an optima growth below 15 °C, but a maximum growth limit surpassing the 20 °C range limit (Russell and Cowan, 2006).

Despite the ambiguity in their definition, cold-adapted organisms have evolved several features, both genotypic and phenotypic, in order to facilitate their capacity to survive and thrive in low temperatures, thus, distinguishing themselves from their mesophilic and thermophilic counterparts. The two main psychical challenges associated with cold stress is the high viscosity and low thermal energy. Both of these parameters influence paramount functions in the cell, like membrane fluidity, solute diffusion, enzyme kinetics and macromolecular interactions. The array of adaptation mechanisms in psychrophilic and psychrotolerant organisms are numerous, but not universal, with each organism presenting its own, unique adaptation strategy (Casanueva et al., 2010).

Relative examples of such adaptation mechanisms is the increase in membrane fluidity, promoted by alterations in lipid composition, increase in membrane proteins and carotenoid pigment integration. Furthermore, anti-freeze proteins and numerous cryoprotectans (e.g. glycine, sorbitol) prevent the growth of ice crystals and lower the cytoplasmic freeze point, respectively. Cold- adaptation also leads to altered transcriptional levels, with up-regulation of antioxidant proteins, like catalase and peroxidases, for protection against increased levels of reaction oxygen species (ROS) due to increased oxygen solubility, expression of cold-shock protein and RNA helicases, and, lastly, expression of numerous chaperones to compact difficulties in protein folding. Last but most importantly, concerning

the biochemical reactions taking place in a cell, various adaptation strategies have been invented to alleviate the reduction in reaction rates due to low temperature by altering the properties of the enzymes that facilitate them. (reviewed in Casanueva et al., 2010, De Maayer et al., 2014, D' Amico et al., 2006).

1.2. Cold adapted enzymes

Temperature dependence of enzymatic reactions

Perhaps, one the most severe restrictions that organisms have to surpass in order to be able to survive in the widespread cold environments of Earth, is the significant reduction in the rates of enzyme catalyzed reactions. The activity of enzymes is directly dependent on the temperature of the environment in which they act. In detail, according to the collision theory and the Arrhenius equation, the temperature in which an enzymatic reaction occurs directly affects its catalytic constant (k_{cat}). Increase in the absolute temperature or decrease in the activation energy (Eα) results in increase in k_{cat} (Equation 1). The absolute temperature exerts its effect on k_{cat} by altering the proportion of collisions with sufficient kinetic energy to surpass the activation energy barrier, set by the activation energy of the reaction. When a biochemical reaction, catalyzed by an enzyme, suffers a drop in temperature, for example from 37 °C to 0 °C, this results in a 20-80 times lower activity due to insufficient kinetic energy in the system (Struvay and Feller, 2012). It is not surprising then, that the main obstacle that prevents non-adapted to low temperatures organisms from colonizing cold environments, is the inability of their enzymes to cope with the reduction in their activity.

$$k_{cat} = \mathrm{A}\kappa e^{-E_a/\mathrm{R}T}$$

Equation 1. *Arrhenius Equation on the temperature dependency of reaction rates. A is the preexponential factor, k is the dynamic transmission coefficient and R the universal gas constant.*

The fact that psychrophilic organisms have adapted to survival even to sub-zero temperatures means that they have evolved strategies to compensate for the reduction in all their metabolic reactions. Examples are the increased production of an enzyme, resulting in higher concentration in order to compensate for its low activity in the cold (Devos et al., 1998), or the evolution of enzymes for which the activity becomes independent of the temperature due to a near-zero activation energy (Georlette et al., 2004). Both of these adaptation approaches are not widespread, as increase in enzyme production is considered energetically taxing, while temperature-independent enzymes are rare. Instead, most psychrophilic enzymes have resulted in the evolution of enzymes with enhanced kcat and catalytic efficiency (k_{cat}/K_m) (Feller and Gerday, 1997)

Kinetic optimization of psychrophilic enzymes

In order to increase their catalytic activity in low temperatures, and according to equation 2, which introduces thermodynamic parameters in the calculation of kcat based to the transition state theory, cold-adapted enzymes reduce the free energy of activation $\Delta G^{\#}$.

$$k_{cat} = k\frac{k_B T}{h} e^{-\frac{\Delta G^{\#}}{RT}}$$

Equation 2. *Arrhenius equation on the temperature dependency of reaction rates, according to the transition state theory, where k is the transmission coefficient, k_B is the Bolzmann constant, h, the Plank constant and, R, the universal gas constant.*

Cold-active enzymes have adapted two strategies to reduce the energy barrier of $\Delta G^{\#}$. The first approach depends on the increase of Km, which results in lower affinity of the enzyme towards the substrate. According to the transition state theory, the enzyme (E) and substrate (S) forms a complex (ES) which enters into a thermodynamic energy pit due to strong substrate binding. In order for the reaction to continue, this complex must reach the activated transition state and produce the enzyme and resulting product. The free energy of activation $\Delta G^{\#}$ represents this energy barrier between the ground state ES and the transition state $ES^{\#}$. When a cold-adapted enzyme increases its Km, this results to lower affinity towards its substrate, which subsequently makes the energy pit for the ground complex ES less deep, resulting to lower $\Delta G^{\#}$ and , subsequently, a higher reaction rate (Figure 1) (Feller and Gerday, 1997).

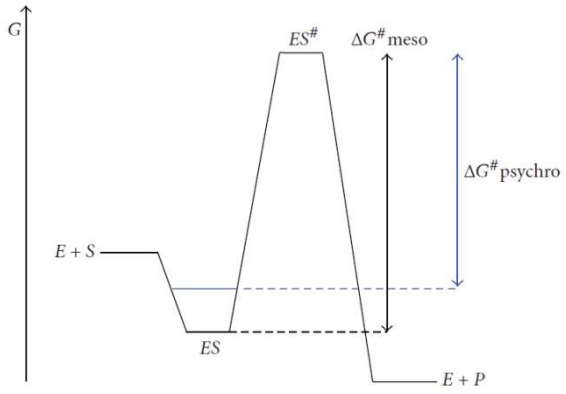

Figure 1. *Profile of a reaction catalyzed by a mesophilic and a psychrophilic enzyme with increased Km. For the psychrophilic enzyme, weak affinity for the substrate results in a decreased free energy of activation ($\Delta G^{\#}$ psychrp), which increases the reaction rate. Adapted from Feller and Gerday, 1997.*

The second approach, which is also more widespread and considered the main adaptation strategy in psychrophilic enzymes is based on a lower ΔH#, which represents the enthalpy of activation. According to the Gibbs- Helmholtz relation, $\Delta G^{\#}$ equals to $\Delta H^{\#}$-$T\Delta S^{\#}$, so the needed reduction in the free energy of activation ΔG# can occur by alteration either in ΔH# or $\Delta S^{\#}$. A lower enthalpy of activation ΔH# has been found for almost all psychrophilic enzymes, which results to a decreased temperature dependence of their activity. This means that for psychrophilic enzymes, a lowering in temperature results in smaller reduction in reaction rates compared with other enzymes (Struvay and Feller, 2012).

The decrease in activation enthalpy $\Delta H^{\#}$ is achieved by an enzyme, by decreasing enthalpy driven interactions that have to be broken during the formation of the transition state $ES^{\#}$. This, consequently, leads to an enzyme with reduced structural stability and increased flexibility (Feller, 2010 and 2013), a characteristic of many psychrophilic enzymes.

Structural characteristics of psychrophilic enzymes

Consequently, psychrophilic enzymes achieve their characteristic higher catalytic reaction rates by destabilizing their structure, leading to an increased flexibility of the molecule. The resulting flexibility is

correlated with the heat lability observed in psychrophilic enzymes. In most cases, the higher activity is a consequence of localized alterations in flexibility and stability, which, primarily, are targeted in the active site of the enzyme. This is illustrated in Figure 2, which shows activity and unfolding relationship between a psychrophilic α-amylase and its mesophilic counterpart. Specifically, the psychrophilic enzyme is inactivated by temperature increase before complete unfolding of the enzyme. In contrast, for the mesophilic enzyme these events are coupled, as inactivation occurs upon unfolding. These results suggested that, for psychrophilic enzymes, in which loss of activity precedes complete loss of structure, main site of reduced stability and increased heat lability is the active site. Comparative analysis between psychrophilic and mesophilic crystal structures has indicated that these alterations in the active site of the former occur without altering the catalytic residues, suggesting conversation of catalytic mechanisms. Despite that, general alterations in the structure of the active site have been observed, as for example is the enlargement of the catalytic cleft in order to facilitate better accessibility of the substrate or easier release of the product. Additionally, in some cases, the active site of psychrophilic enzymes is characterized by alterations in electrostatic potentials resulting from substitution of non-conserved amino acids. Such alterations are thought to correlate with ligand interactions. (Struvay and Feller, 2012, D' Amico et al., 2003).

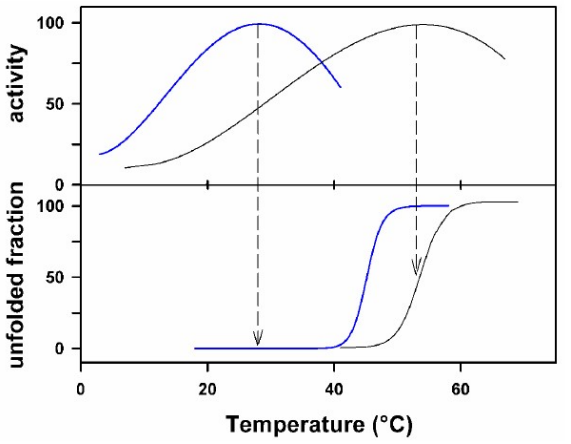

Figure 2 *Relationship between activity and unfolding in psychrophilic enzymes. For the psychrophilic enzyme (blue) inactivation begins before unfolding of the overall structure, while for the mesophilic homologue, (black) loss in activity coincide with loss in protein overall structure. Adapted from Struvay and Feller, 2012)*

Concerning the overall structural stability of psychrophilic enzymes, differential scanning calorimetric studies have been used to probe the adaptation of psychrophilic enzymes. Enzymes such as α-amylases and DNA ligases have been studied in order to elucidate the thermal denaturation of psychrophilic, mesophilic and thermophilic homologues (Figure 3) (D' Amico et al., 2001, Georlette et al., 2003). According to such studies, psychrophilic enzymes are characterized by lower Tm values in which protein unfolding occurs, while ΔHcal, the calorimetric enthalpy, which reflects the enthalpy of the disruption of bonds necessary to maintain the protein structure, is lower. Furthermore, the transition from the folded to the unfolded state is sharper for psychrophilic enzymes which indicates a cooperativity during unfolding. For psychrophilic enzymes, this transition is also characterized by a lack of intermediate steps, suggesting an overall and uniform low stability of their structure, compared to their mesophilic and thermophilic homologues, which are

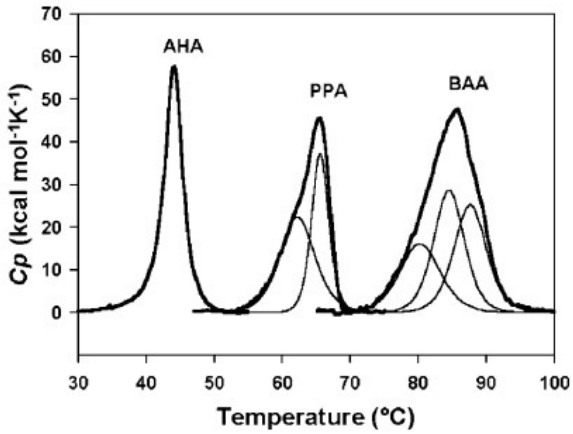

Figure 3 *Thermal unfolding of psychrophilic (AHA), mesophilic (PPA), and thermophilic (BAA) α-amylases from different organisms as recorded by differential scanning calorymetry. Adapted from D'Amico et al.,*

characterized by several transition step, indicative of patches of different structural stability with independent unfolding (Feller, 2010). Although the lower stability is considered to be a characteristic of cold-adapted enzymes, the overall structural instability isn't a prerequisite. Examples of enzymes with the flexibility localized only in a small area of the overall structure exists. Such enzymes are characterized by a thermolabile domain, while the overall structure of the enzyme is heat-stable. Such enzymes indicate that cold-adaptation in terms of increased flexibility can be also localized in only some areas of the structure of the protein, instead of being a widespread phenomenon (Bentahir et al., 2000, Lonhienne et al., 2001).

At the molecular level, many diverse adaptations have been characterized, which explain the increased flexibility observed in psychrophilic enzymes. This information results from comparative studies in sequences and structures between psychrophilic and mesophilic or thermophilic homologues. In general, adaptation to low temperature correlates with reduction in stabilizing features. Stability can be reduced by increases in glycine residues and clusters, or reduction of proline and arginine residues. Glycines, due to lack of side chains, offer increased degree of freedom for the polypeptide backbone, while proline residues, which have only one dihedral angle decrease this freedom and, thus, are substituted or deleted, primarily in loops. The reduction in arginine residues is correlated with reduction of hydrogen bonds and salt bridges, as this residue possesses the ability to form multiple such interactions. Cold-adapted enzymes are characterized also by lower levels of salt bridges, hydrogen and disulfide bonds, as well as, fewer aromatic interactions between side chains. Furthermore, in psychrophilic enzymes, the N- and C- caps of α-helicases can be altered by weakening of the charge-dipole interaction, leading to destabilization of the secondary structure, while binding of ions, such as calcium, which can have stabilizing effects, can be weaker. Additionally, psychrophilic proteins tend to have fewer hydrophobic residues within the protein core, which usually interact with each other rather than with water. Such an event can lead to lower compactness and, thus, stability of the protein structure. Insertions and deletions have also been associated with lowered structural stability, as acquisition of extracellular charges and weakening of subunit interactions have been reported, respectively. Lastly, in some psychrophilic enzymes, altered solvent interactions have been observed, primarily through the increase in hydrophobic residues in the surface of the protein and decrease in charged residues. Such a tactic leads to destabilization due to reorganization of water molecules around hydrophobic side chains. (reviewed in Feller et al., 1997,Russell, 2000, Siddiqui and Cavicchioli, 2006, Feller, 2013). From these numerous adaptation tactics each enzyme adopts its own, unique combination in order to improve its local or global flexibility.

It becomes apparent that the two most important altered functions characterizing psychrophilic enzymes are their activity and stability, which increases and decreases, respectively. Concerning the relationship between stability and activity, the most widely accepted view suggest that psychrophilic enzymes are subjected to strong evolutionary

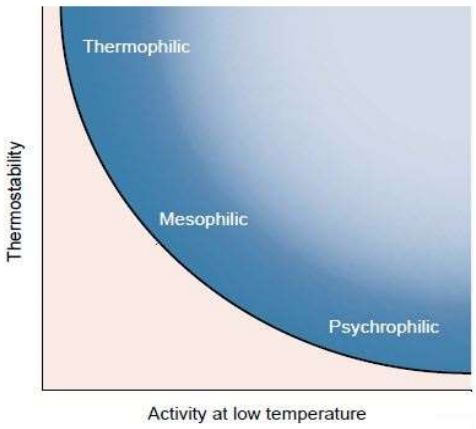

Figure 4 *Activity- stability in homologous enzymes adapted to different temperatures. Although not correlated, there is an apparent trade-off between activity at low temperatures and stability, as psychrophilic enzymes are characterized by high activity by low stability, while the reverse occurs for thermostable enzymes. These naturally occurring enzymes balance between the minimum stability and activity which is*

pressure to increase their activity due to low temperature, while such conditions result in absence for stability selection. So, these two functions, activity and stability, are not physically linked, but tend to appear simultaneously, due to genetic drift from lack of selective pressure for stability in cold-adapted proteins (Figure 4) (Feller, 2013). Although, higher activity and lower stability tend to coexist, maybe due to lack of selective pressure for both thermostable enzymes in low temperatures, several studies, primarily through directed evolution have showed that it is possible to simultaneously improve the activity and thermostability of an enzyme (Koutsioulis et al., 2008). Characterization of naturally occurring enzymes that do not fit to the "high activity-low stability" profile of psychrophilic enzymes, such as isocitrate dehydrogenase isolated from a psychrophilic bacterium, which retains cold activity but is thermostable, further support the notion that activity and stability are not necessarily connected (Fedøy et al., 2007)

Psychrophilic enzymes in biotechnology

Overall, cold-adapted enzymes display apparent optimum temperatures towards lower temperatures, while having a much higher catalytic activity than their thermophilic counterparts in temperatures up to their apparent optimum. Furthermore, the vast majority of cold-adapted enzymes rapidly inactivate in low temperatures (Gerday, 2013). The ability for cold activity and heat-lability is considered as a positive trait in many biotechnological applications involving enzymes. In detail, the ability of catalysis in low temperature means that biological reactions could be performed in ambient temperatures. As result of their high catalytic activity, enzyme concentrations could be reduced, thus reducing the amount of enzyme needed for the desired activity. Lastly, their heat lability, could be desirable in some applications as they can be efficiently and selectively inactivated by a heat treatment step (Feller, 2013, Margesin and Feller, 2010). The biotechnological applications in which psychrophilic enzymes have been adopted range from uses in molecular biology, where heat-lability is the most desirable trait, up to industry level, where they have been incorporated in tap water-active detergents, or used in food processing.

1.3. Alkaline phosphatases

Enzymatic activity and distribution

Alkaline phosphatases are homodimeric metalloenzymes that catalyze the hydrolysis of phosphomonoesters to an alcohol and inorganic phosphate (P_i) or their transphosphorylation in the presence of a phosphate acceptor. The kinetic scheme for this reaction involves the transient phosphorylation of a conserved active site nucleophilic serine, resulting in the formation of a covalent enzyme-phosphoseryl intermediate (E-P). Next, this covalent intermediate is hydrolyzed, leading to the formation of a non-covalent enzyme-phosphate complex (E●P_i). If a phosphate acceptor is present (e.g. Tris), the enzyme transfers the phosphate to the alcohol, forming another phosphomonoester (Holtz and Kantrowitz, 1999) (Figure 5). These enzymes are not specific, reacting with a variety of phosphate-containing compounds, from which of special interest are nucleic acids and proteins.

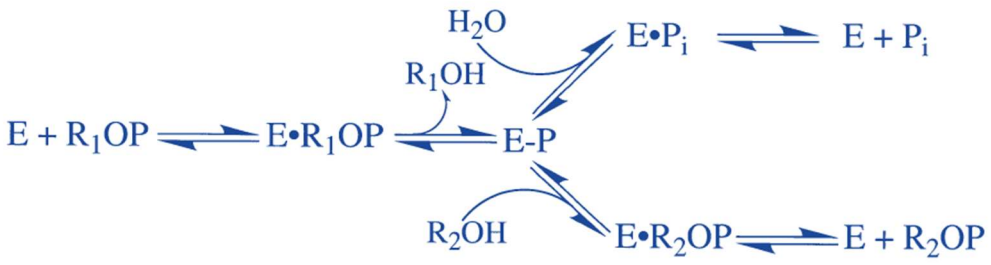

Figure 5 *Scheme of the reaction catalyzed by alkaline phosphatase. Adapted from Holtz and Kantrowitz, 1999.*

These enzymes are widely distributed in nature, with various alkaline phosphatases identified from bacteria to mammals. Regardless of their origin, and in stark contrast to their extensive enzymatic study, the role and action of alkaline phosphatases in the cellular level are not well understood. In bacteria, alkaline phosphatases are possibly involved in phosphate metabolism, and more specifically in phosphate starvation response systems, as P_i elimination in the environment has been shown to induce their expression, while excess phosphate repress their activity (Torriani, 1990, Oh et al., 2007). On the other hand, in humans, alkaline phosphatases are classified in at least four tissue-specific isoforms, and their expression levels in serum are correlated with various bone and liver diseases, with most prominent example being hypophosphatasia, an illness characterized by abnormal bone mineralization caused by deficiency in a specific alkaline phosphatase isoform (reviewed in Sharma et al., 2013).

Structure and mechanism of catalysis

Several alkaline phosphatase structures have been solved by X-rays crystallography. The most thoroughly characterized, both biochemically and biophysically, alkaline phosphatase is the *Escherichia coli* homologue (ECAP), which was the first to have its structure analyzed (Kim and Wyckoff, 1991). Each monomer has 3 metal-binding sites located in its active site, occupied by two zinc and one magnesium ion. The overall structure of ECAP is that of extended β-sheets stretching through the two monomers, with α-helicases of various length flanking them (Figure 6). Furthermore, a crown domain is located at the subunit interface, formed by strands from both monomers. The crystal structures of other alkaline phosphatases have also been solved, with notable examples being the human placental AP (Le Du et al., 1991), the tissue non-specific AP from arctic

Figure 6 *Side- view of the secondary structures of Escherichia coli (ECAP) (PDB ID: 1ALK) and Antarctic bacterium TAB5 (PDB ID: 2IUC) as depicted by JSmol viewer in a resolution of 2 Å and 1.95 Å, respectively.*

shrimp *Pandalus borealis* (SAP) (de Backer et al., 2002), the Antarctic bacterium TAB5 (Wang et al., 2007), the cold-active Vibrio sp. G15-21 AP (Helland et al., 2009) and the psychrophilic Shewanella sp. AP (Tsuruta et al., 2010). All these examples show general structural similarities with ECAP, in terms to the α/β core structure and the conserved active site metal binding sites, which is also reflected in their sequence homology.

In regards to the detailed catalytic mechanism of alkaline phosphatases, ECAP has been used as a prototype for its elucidation and the model proposed is generally accepted for all the homologues identified thus far. The mechanism of ECAP is described as a double-in line displacement, three metal ion assisted catalysis. In detail, the Mg ion is involved in the deprotonation of the catalytic serine (Ser) residue upon binding of the phosphomonoester substrate (R-OP) and formation of the enzyme-substrate complex (E-ROP). The deprotonated Ser, stabilized from coordination of Zn2 and in the first in-line displacement, performs a nucleophilic attack on the phosphorous atom P_i, resulting in a covalent enzyme- phosphate intermediate (E-P). Next, a nucleophilic hydroxide ion coordinated to the Zn1 metal ion attacks the phosphorous Pi, resulting in the formation of a non-covalent enzyme-phosphate complex (E●P_i) by hydrolysis in the second in-line displacement, and the regeneration of the nucleophilic catalytic Ser residue. Lastly, a Mg-coordinated water molecule either reprotonates the catalytic Ser or protonates the inorganic Pi, facilitating the disassociation of the phosphate product from the non-covalent E●P_i complex (Kim and Wyckoff, 1991, Stec et al., 2000).

Applications in medicine and molecular biology

In medicine, the measure of serum alkaline phosphatase levels is considered a routine application and used for diagnosis of health problems associated with the liver or bones. For example, altered levels of AP can indicate hepatitis or cirrhosis, both linked to liver malfunction, or vitamin D deficiency which may cause reduction in bone density.

In molecular biology, alkaline phosphatases are used for a variety of applications. The most common use is for the dephosphorylation of DNA vectors before cloning to prevent recircularization. Furthermore, they are utilized for the dephosphorylation of 5'-nucleic acid termini before 5'-end labeling by kinases, for the dephosphorylation of proteins, and for the removal of dNTPs from a PCR reaction, thus eliminating the need for subsequent purification (Struvay and Feller, 2012). Lastly, they have found applications in enzyme-linked immunosorbent assays (ELISA), where they are used as conjugates with antibodies for colorimetric detection.

The first alkaline phosphatases used in the above applications were the calf intestinal AP and ECAP, which were heat-stable and required complex treatment for elimination of their activity after the intended use. For the first time in 1984, Kobori et al., proposed the use of a heat labile alkaline phosphatase, isolated from the Antarctic bacterium strain HK47, for rabid inactivation by heat treatment after its applications, minimizing loss of nucleic acid due to purification steps or the possibility or contamination. Nowadays, almost all commercially available alkaline phosphatases are heat-labile, with prominent examples being the alkaline phosphatase from Antarctic bacterium TAB5 and arctic shrimp *Pandalus borealis.*

1.4. TAB110α alkaline phosphatase

Library Screening and Evaluation

Considering the application potentials of psychrophilic alkaline phosphatases in molecular biology, and as part of a previous master thesis in our laboratory, a high-throughput approach was adopted for identification of novel homologous enzymes from a bacterial collection. Specifically, a library consisting of 262 bacterial strains isolated from Antarctic seawater was screened for alkaline phosphatase activity based on the hydrolysis of pNPP. Cell lysates from the above library were subjected to heat treatment and subsequently screened for remaining alkaline phosphatase activity to access the heat-lability of the putative alkaline phosphatases, too. Based on the above criteria nine strains were chosen for further analysis for the identification of putative heat labile alkaline phosphatases.

The selected strains were sequenced using the Ion Torrent platform and after *de novo* annotation due to the absence of reference genomes, the coding regions where determined. The resulting sequences where screened against the bacterial non redundant database of NCBI, in order to estimate the closest neighbors, showing that all strains most likely belong to genus containing psychrophilic or psychrotolerant species (Table 1).

Sample No	*Sample name*	*No. of reads Proton*	*Read lenght*	*No of contigs (De novo assembly)*	*N50 (contigs)*	*Average coverage*	*Closest neighbors*	*Similarity*
1	TAB108α	9.398.514	195 bp	269	208666	300	*Pseudomonas fluorescens PfO-1*	72.89%
2	TAB110α	9.773.174	197 bp	297	413040	355	*Serratia proteamaculans 568*	82.18%
3	TAE23	6.999.366	204 bp	3980	9681	136	*Pseudomonas psychrophila HA-4*	-
4	TAE5	6.618.998	197 bp	2097	18047	136	*Pseudomonas psychrophila HA-4*	-
5	TAE122	7.736.367	186 bp	181	167353	242	*Pseudomonas fluorescens PfO-1*	69.53%
6	TAE125	9.100.994	197 bp	294	164792	300	*Pseudomonas fluorescens PfO-1*	70.31%
7	TAE126	7.939.833	200 bp	371	188169	261	*Pseudomonas fluorescens PfO-1*	70.32%
8	TAD1	10.410.090	190 bp	1831	437054	314	*Rahnella sp. Y9602*	71.23%
9	TAD7	11.109.249	194 bp	5219	19282	182	*Camobacterium sp. AT7*	-

Table 1 *Results of the de novo assembly of the nine selected strains and the estimated closest neighbor.*

TAB110α strain selection and cloning

In order to identify putative sequences encoding for alkaline phosphatases, the resulting coding regions were screened for similarity with other well characterized alkaline phosphatases (SAP, ECAP and TAB5). The gene No. 2624 (codes for a 475 amino acids protein, APT110α) from the strain TAB110α displayed the best results, as depicted in Table 2. The aforementioned protein had a striking sequence homology with the mesophilic ECAP, which is uncommon between psychrophilic and mesophilic homologous, while it displayed approximately 30 % homology with the psychrophilic TAB5 and SAP, although the first is of bacterial origin, like TAB110α, while the latter is eukaryotic.

Subject Sequence	% Identity	% Similarity	E-value	Query Cover
ECAP	79	88	0	99 %
TAB5	30	44	$6e^{-41}$	84 %
SAP	32	45	$1e^{-36}$	72 %

Table 2 *Alignment of the gene No. 2624 from the bacterial strain TAP110α against the characterized alkaline phosphatases from Escherichia coli (ECAP, Uniprot ID: P00634), the Antarctic bacterium TAB5 (Uniprot ID: Q9KWY4) and the arctic shrimp Pandalus borealis (SAP, Uniprot ID: Q9BHT8), representing the best results of all the identified genes form the nine sequenced bacterial strains*.

Furthermore, based on the same sequence alignments, APT110α exhibits significant conservation of the amino acid residues forming the active site and the metal- binding sites (Table 3).

Active site & metal binding sites	TAB5	ECAP	TAB110α
	D83	D101	D127
	W260	**K328**	**K354**
	R148	R166	R192
	Y325	Y402	Y428
M1 (Zn^{2+})	D259	D327	D353
	H263	H331	H357
	H337	H412	H438
M2 (Zn^{2+})	D43	D51	D77
	S84	**S102**	**S128**
	D301	D369	D395
	H302	H370	H396
M3 (Mg^{2+})	D43	D51	D77
	H135	**D153**	**D179**
	T137	T155	T181
	E254	E322	E348

Table 3 *Amino acid residues of the active site and metal binding sites in TAB5 and ECAP and predicted residues in APT110α based on sequence alignment (Clustal Omega). Sequences: TAB5 (Uniprot ID: Q9KWY4), ECAP (Uniprot ID: P00634). In blue in depicted the catalytic serine residue and in red the varying amino acid residues.*

Interestingly, the two residues which are shown to be different between TAB5 and TAB110α (W260 and H135 in TAB5) have been studied in the former enzyme, through directed mutagenesis (Tsigos et al., 2001). In detail, the substitution Trp260 by Lys in the mutant W260K showed that Trp260 is important for the catalytic activity of TAB5 in low temperatures. Also, the mutation H135D in TAB5 highlighted the importance of the H135 residue in the heat-lability of the enzyme, as the mutant showed considerable stabilization. The results that TAB110α has a high sequence homology with the mesophilic ECAP, in combination with the presence of two residues which have been shown in TAB5 to correlate with a mesophilic phenotype, made TAB110α an interesting enzyme for further analysis, when considering that it has been isolated by an Antarctic bacterial strain.

Based on these results, and the knowledge that psychrophilicity of an enzyme can be directed by subtle structural changes, the putative gene APT110α was chosen as the best candidate for the expression of an alkaline phosphatase with psychrophilic characteristics. Consequently, the APT110α nugleotide sequence, together with its predicted signal peptide was cloned in the pRSET-B vector under the NdeI and Bgl II restriction sites, in order to be expressed in *E.coli* and biochemically characterized.

2. Scientific significance- Aims

Enzymes isolated from psychrophilic organisms are considered advantageous for certain applications in biotechnology, as they are characterized by lower thermal stability and higher catalytic activity compared to their mesophilic counterparts. These characteristics contribute to easy termination of activity upon heat treatment, which is advantageous in certain applications, while higher catalytic activity, especially in ambient temperatures, facilitates cost reduction associated with lower enzyme amounts used and elimination of external heating. Among these enzymes, of special interest are alkaline phosphatases, as they are used in various molecular biotechnology techniques. With that in mind, a strain collection of seawater bacteria isolated in Antarctica was screened for novel putative psychrophilic alkaline phosphatases. The alkaline phosphatase expressed by the strain TAB110α was selected based on its high activity and heat- lability and, subsequently, was cloned.

Concerning the present master thesis, principal intention is the kinetic and biophysical characterization of TAB110α alkaline phosphatase, in order to access its potential for application in molecular biology. Specific mutations were designed and applied to enhance or alleviate its native properties. Furthermore, crystallographic studies were initiated in order to elucidate the three dimensional structure of the enzyme, which could offer insights into the adaptation of enzymes for activity in low temperatures, and offer the possibility for targeted redesigning of its properties.

3. Materials & Methods

3.1. Site directed mutagenesis

Site directed mutagenesis (SDM) was performed by overlap extension using the polymerase chain reaction (PCR) (Ho et al., 1989, Horton et al., 1990). The method involves the generation of DNA fragments in two independent PCR reactions, which based on complementarity can be fused together in a second round of PCR to generate the full length gene with the desired mutation (Figure 7).

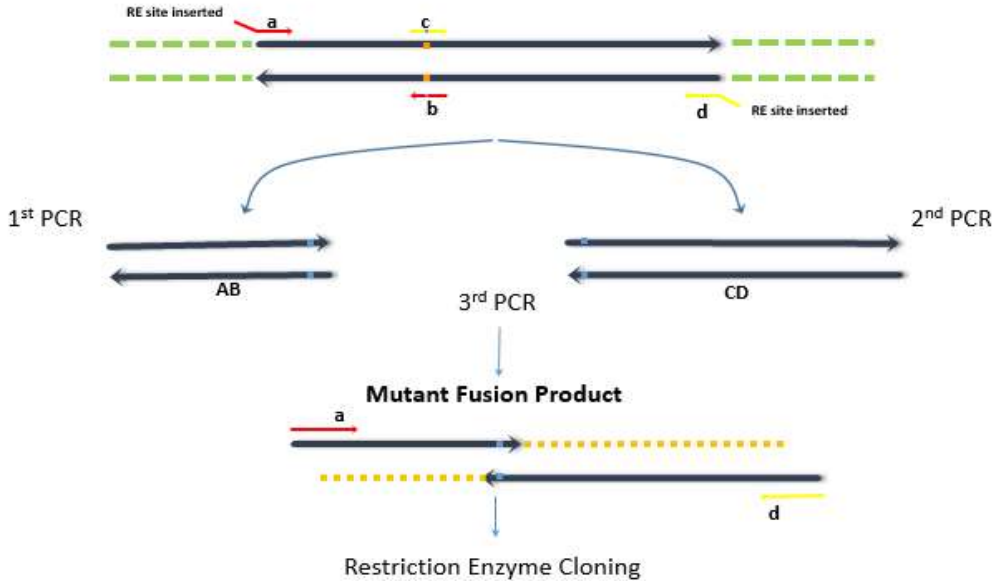

Figure 7 *Site directed mutagenesis by overlap extension. Insertion of the desired mutation is performed in 2 PCR rounds. In the first round, the DNA fragments from the start of the target gene up to the mutation site (AB) and the end of the gene up to the mutation site (CD) are amplified independently in two RCR reactions, using one flanking primer (a or d) and one internal primer (c or b) which hybridizes at the site of the mutation and contains mismatched bases. Because the two internal primers overlap, the resulting fragments AB and CD can be fused by denaturing and annealing in a second PCR round, in which each fragment acts as a primer for the other. Use of the flanking primers in this PCR facilitates further amplification of the mutated gene.*

The primers used to construct the designed mutations are presented in Table 4 below:

	Name	Sequence
Flanking Primers		
Forward	FAP1.1	5'-CAAAACA**CATATG**TTGCAGCCTGTTTCCC-3'
Reverse	RAP1.1	5'-GGA**AGATCT**TTACTTGATCGCCATGGCG-3'
Internal Primers		
D179H Forward	D179HF	5'-GCAGAGTTGCAA**CAC**GCCACACCTGCGGCG-3'
D179H Reverse	D179HR	5'-CAGGTGTGGC**GTG**TTGCAACTCTGCGGTG-3'
D179E Forward	D179EF	5'-GCAGAGTTGCAA**GAA**GCCACACCTGCGGCG-3'
D179E Reverse	D179ER	5'-CAGGTGTGGC**TTC**TTGCAACTCTGCGGTG-3'
K193G Forward	K193GF	5'-CACCTCGCGT**GAT**TGTTACGGCCCGGAAG-3'
K193G Reverse	K93GR	5'-GGCCGTAACA**ATC**ACGCGAGGTGACGTG-3'
A130S/S131G Forward	A130S/S131GF	5'-TGACTCCGCC**TCCGGC**GCGACTGCCTGGTCC-3'
A130S/S131G Reverse	A130S/ S131GR	5'-AGGCAGTCGC**GCCGGA**GGCGGAGTCAGTGAC-3'

Table 4. *Sequences for Site Directed Mutagenesis. In blue are depicted the Nde I and Bgl II restriction sites, subsequently. In red are depicted the targeted codon.*

The PCR reaction were performed as follows (Table 5):

Reaction Components	Reaction Conditions
5 µl 5x Phusion HF buffer	1. 98 ° C for 30 seconds
0.2 mM dNTPs	2. 98 ° C for 10 seconds
0.5 µM Forward Primer	3. 67 ° C for 30 seconds
0.5 µM Reverse Primer	4. 72 ° C for 15-30 seconds
10-20 ng DNA Template	Repeat steps 2-4 for 30 times
0.5 Unit Minopol Polymerase	5. 72 ° C for 10 minutes
ddH_2O to a final volume of 25 µl	6. Retention at 4 ° C

Table 5 *PCR reaction components and conditions. For the first round of PCR 10 ng of DNA template were used and an extension time of 15 seconds, while 20 ng of DNA template were used in the second PCR round, together with a 30 second extension.*

After each PCR reaction, the subsequent product was visualized in 1 % agarose gel with ethidium bromide straining, gel extracted using the NucleoSpin Gel and PCR Clean- up Kit from Macherey- Nagel and quantified by a Nanodrop spectrophotometer.

3.2. Cloning of mutated genes

The wild type TAB110α alkaline phosphatase gene with its signal peptide was cloned in the pRSET-B vector (name of construct: pRSETB+APT110α(w)) as part of a previous master thesis. This vector was used as a template for the first round of PCR in the site directed mutagenesis procedure, in order to produce the mutated, overlapping fragments. After the overlapping extension PCR and gel purification, the resulting amplified, mutated genes were digested with Nde I (New England Biolabs) and Bgl II (Minotech Biotechnology) restriction enzymes, simultaneously. The same digestion was performed for the ampicillin-resistance conferring pRSET- B vector. A ligation reaction was performed, in order to incorporate the mutated AP genes in the pRSET-B vector, using the Minotech Biotechnology ligase. After transformation to cloning DH5α cells and selection of positive colonies (through colony PCR slection), plasmid purification was performed using the Plasmid DNA Purification kit from Macherey-Nagel, and the resulting plasmid constructs were sent for sequencing to Macrogen for proper mutation insertion verification. The sequencing results were analyzed using the Vector NTI software package.

3.3. Overexpression of TAB110α alkaline phosphatase (wild type or mutant variants) in E. coli cells

BL21 Star (DE3) pLysS *E.coli* competent cells were transformed with the appropriate pRSET-B plasmid construct, carrying either the wild type or the mutated sequences of TAP110α alkaline phosphatase. A heat- shock transformation protocol was performed and the transformed cells were plated on LB-agar plates with 100 µg/ml Ampicillin and 34 µg/ml Chloramphenicol. For each construct, a fresh colony was chosen for the inoculation of a LB pre-culture, in the presence of the above- mentioned concentrations of the two antibiotics and incubated overnight (16 hours) at 37 °C under vigorous shaking.

The pre-culture was used to inoculate 1 liter of LB in a ration of 1:40, in the presence of Ampicillin and Chloramphenicol. The inoculated cultures were grown at 30 °C until the OD_{600} reached 0.5-0.6 and, subsequently induced with 0.5 mM IPTG (IsoPropyl- beta-D-thioGalactopyranoside), 10 mM $MgSO_4$, 0.4 mM $ZnSO_4$ and 10 mM KCL. Cultivation continued for another 4 hours at 25 ° C under shaking. The cells

were collected by centrifugation of the cultures at 5000 g, 4°C for 12 minutes and stored at -20 °C until use.

3.4. Purification of TAB110α alkaline phosphatase (wild type or mutant variants)

Cell disruption

Frozen cell paste (4-7.5 gr) expressing the wild type or mutants variants of APT110α was thawed on ice and resuspended in a solution containing 50 Mm Tris-HCl pH: 7.6, 100 mM NaCl, 1 mM DTT (Dithiothreitol), 0.3 mg/ml lysozyme and 1 mM PMSF (PhentlMethylSulfonylFluoride) in a ration 1:3 at 4° C. The cells were disrupted by sonication (10x30 seconds sonication steps, with resting intervals on a dry ice- ethanol bath) and the lysate was centrifuged at 15000 g, 4 °C for 30 minutes. Subsequently, the cytoplasmic supernatant was collected and diluted in a ration 1:5 with 25 mM Tris-HCl pH: 7.6 chilled buffer solution, in order to reduce the NaCl concentration to approximately 20 mM before loading to the chromatography column.

Chromatography with Q Sepharose Fast Flow matrix

A chromatography column containing 15 ml (1 column volume) Q Sepharose Fast Flow beads (GE Healthcare) was packed and equilibrated with buffer A (25 mM Tris-HCl pH: 7.6, 5 mM NaCl). Next, the diluted supernatant was loaded in the column using a peristaltic pump (flow rate: 3 ml/min). After the sample was loaded, the column was washed using buffer A (flow rate: 4 ml/min), until the protein absorbance was zero. The bonded proteins were eluted by a linear gradient of NaCl in Buffer A, starting from 5 mM and reaching 250 mM (15 column volumes) and collected in 30 fractions of 7.5 ml each (flow rate: 4 ml/min). Next, the fractions were analyzed by SDS-PAGE in order to assess their relative concentration in alkaline phosphatase, and choose which ones will be further purified.

Elution Step	Volume of Elution Step	Composition of Elution Step Buffer
1	15 ml	25 Mm Tris-HCl pH: 7.6, 40 mM NaCl
2	15 ml	25 Mm Tris-HCl pH: 7.6, 50 mM NaCl
3	45 ml	25 Mm Tris-HCl pH: 7.6, 60 mM NaCl
4	52.5 ml	25 Mm Tris-HCl pH: 7.6, 70 mM NaCl
5	45 ml	25 Mm Tris-HCl pH: 7.6, 80 mM NaCl
6	30 ml	25 Mm Tris-HCl pH: 7.6, 90 mM NaCl
7	15 ml	25 Mm Tris-HCl pH: 7.6, 100 mM NaCl
8	15 ml	25 Mm Tris-HCl pH: 7.6, 150 mM NaCl
9	15 ml	25 Mm Tris-HCl pH: 7.6, 200 mM NaCl

Table 6 *Step elution of TAP110α from the second Q Sepharose Fast Flow chromatographic column.*

The same chromatographic column was used again to perform a purification with step elutions of varying NaCl concentration. The fraction with substantial alkaline phosphatase concentration from the previous chromatographic column were dialyzed overnight against a solution containing 25 mM Tris-HCl pH: 7.6, 5 mM NaCl (2 liters). The sample was loaded onto the 15 ml Q Sepharose Fast Flow column

(flow rate:3 ml/min), after it was regenerated (4 volumes of Buffer B: 25 Mm Tris-HCl pH: 7.6, 1 M NaCl, 30 minutes contact time with NaOH, equilibration with Buffer A, 10 column volumes with a flow rate of 5 ml/min). After wash with Buffer A until a zero absorbance (flow rate: 4 ml/min), the bonded proteins were eluted with sequential steps of varying NaCl concentration and volume, as seen in table 6. The eluted proteins were collected in 33 fractions, 7.5 ml each, and analyzed by SDS-PAGE electrophoresis.

Chromatography with Sephacryl S200 HR matrix

The fractions chosen for further purification from the second chromatography column were concentrated using Amicon Ultra-15 Centrifugal Filter Units (Merck) in a final volume of 1.5-3 ml and loaded onto a Sephacryl S200 HR column (360 ml), previously equilibrated with a buffer containing 25 mM Tris-HCl pH: 7.6, 200 mM NaCl and 1 mM $MgCl_2$ (flow rate: 1 ml/min). Elution of proteins was performed by 1 column volume of the same buffer and collected in 36 fractions, 10ml each at the same flow rate. The respective fractions were analyzed be SDS-PAGE electrophoresis and the ones with detected alkaline phosphatase were concentrated using Amicon Ultra-15 Centrifugal Units to a final protein concentration of 2 mg/ ml, as quantified by a Nanodrop spectrophotometer. The purified alkaline phosphatase was stored in a buffer containing 12.5 mM Tris-HCl pH: 7.6, 100 mM NaCl, 0.5 mM $MgCl_2$ and 50 % v/v glycerol and preserved at -20° C.

3.5. Crystallization of wild type TAB110α alkaline phosphatase

For the crystallization of wild type TAB110α AP the purified protein sample was further concentrated using Amicon Ultra-15 Centrifugal Units, as described above, at a final concentration of 7 mg/ml, prior to the addition of glycerol. Concentration was determined spectrophotometrically by a Nanodrop instrument, using a molecular extinction coefficient of 40.000 M^{-1} x cm $^{-1}$ at 280 nm, as calculated by ExPASy ProtParam tool using the TAB110α amino acid sequence. Crystals were grown by the hanging-drop, vapor diffusion method (McPherson, 1985) at 4 °C after mixing of the protein sample, in a ration 1:1, with 20-22 % PEG 3350+0 and 1 M ammonium acetate. Crystals appeared after two to three days, possessing a thin plate-like shape. Crystals were collected and stored in 35 % PEG 400 cryoprotectant until data collection

3.6. Diffraction data collection

X-ray diffraction data were collected using synchrotron radiation on the BL14 beamlines operated by the Helmholtz-Zentrum Berlin (HZB) at the BESSY II electron storage ring (Berlin-Adlershof, Germany). The crystals were cooled in a nitrogen gas stream and data were collected at a wavelength of 0.9184 Å.

3.7. Calculation of kinetic parameters

The steady-state enzyme kinetics were measured at a temperature of 30 °C. The reaction mixtures for wild type and mutant variants of TAP110α AP contained 1x SH buffer (Minotech Biotechnology), while for shrimp alkaline phosphatase SAP (New England Biolabs) 1x CutSmart buffer was used. The reactions were monitored every 38 seconds in varying concentrations (0.1- 4 mM) of p- Nitrophenyl Phosphate (Sigma), using Fluostar Galaxy microplate reader (BMG Labtech), with measurements taken at 405 nm. The program HYPER version 1.1 was used to calculate the Vmax and Km constants, while k_{cat} was determined by using a molecular mass of 49.7 kDa for the enzyme, as calculated by the ExPASy ProtParam tool. For SAP, the used molecular weight was 54 kDa, as indicated by the manufacturer. The

final enzyme concentrations in the reaction were 118,5 nM for SAP, 400 nM for wild type TAB110α AP, 0.270 nM for Asp179Glu TAB110α AP and 800 nM for Asp179His TAB110α AP.

3.8. Heat inactivation of wild type TAB110α alkaline phosphatase and mutants

In order to test their thermal stability, the enzymes were subjected to heat treatment. Specifically, 0.5 μg of each purified protein were incubated at 80° or 90° in water for 5, 15 or 30 minutes. After resting on ice for 15-20 minutes, 10x SH buffer was added in a final dilution of 1x, and remaining activity was calculated at 30°C in the presence of 5 mM pNNP by measuring the absorbance at 405 nm by Fluostar Galaxy (BMG LABORATORIES) spectrometer. Reported values are the average of at least two independent measurements.

4. Results

4.1. Purification of TAP110α alkaline phosphatase and mutant variants

The overall purification scheme for TAP110α alkaline phosphatase is presented in Figure 8.

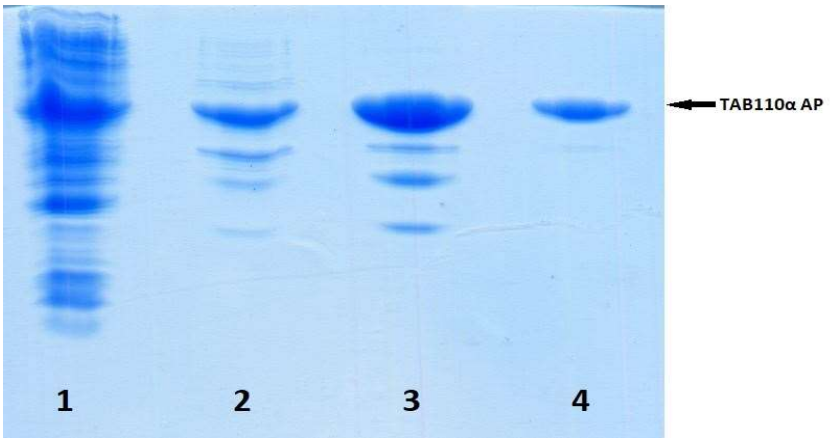

Figure 8 *Purification scheme for wild type TAP110α alkaline phosphatase and mutants D179 H and D179E in steps as depicted in a 12.5 % SDS-PAGE gel stained with Coomasie Blue R-250.*

In detail, in lane 1 is depicted the cytoplasmic fraction after induction, sonication and centrifugation of the cell lysate. TAB110α alkaline phosphatase was adequately overexpressed in *E.coli* cells, with the majority of the enzyme present in the cytoplasmic fraction and only a small percentage in the membrane fraction. Consequently, purification was undertaken from the cytoplasmic fraction. Lane 2 presents the resulting pooled fractions from the first Q-Sepharose Fast Flow chromatography column. Elution of TAB110α AP occurred between 80-150 mM NaCl. Next, in lane 3 is depicted the overall purity of TAB110α AP after the second Q-Sepharose Fast Flow column, were elution was carried out with step increases in NaCl concentration. Elution of the desired protein was observed between 60 and 80 mM of NaCl. Finally, in lane 4 is presented the final TAB110α AP protein solution after polishing purification with the Sephacryl S200 HR size exclusion column, were elution occurred between fractions 16 and 19, which correspond to 150-200 ml from the 360 ml of the total elution volume.

The resulting purification scheme leads to the isolation of approximately 15 mg of protein from 7 grams of frozen cell paste. The resulting TAP110α protein sample is characterized from a high purity level, above 95 %, which is necessary for functional and structural studies.

The same purification scheme was applied for all TAP110α variants, as the targeted mutations were located in or near the active site, and thus, not expected to change neither the overall surface charge of the protein, which is important in anion exchange chromatography, neither its molecular weight or size, which are the principles by which proteins elute in size exclusion chromatography.

4.2. Thermal stability of TAB110α alkaline phosphatase and mutant design

As mentioned before, one of the main characteristics by which TAP110α alkaline phosphatase was selected for further characterization was its potential thermo-lability, as detected by heat treatment of cell lysates at 65°C (Symeonidou, master Thesis). Specifically, an incubation of ten minutes was enough to reduce the absorbance resulting from the hydrolysis of pNPP by approximately 50 %. For several

applications in molecular biology, an easy inactivation of the enzyme, after it has exerted its action, by a brief heat treatment is considered advantageous.

After the purification of TAB110α alkaline phosphatase, its heat-lability was re-evaluated by incubating the enzyme in temperatures up to 90°C and, subsequently assessing its activity based on the hydrolyses of pNPP and measurement of the absorbance at 405 nm in the presence of 1x SH buffer. Incubation of the wild type TAB110α AP at 80°C for up to 30 minutes had no effect in activity, indicating a very thermostable enzyme (Figure 9). Such a result was unexpected, as enzymes isolated by psychrophilic organisms, in their majority are heat-labile, probably as a consequence of lack of selective pressure for stability in cold environments.

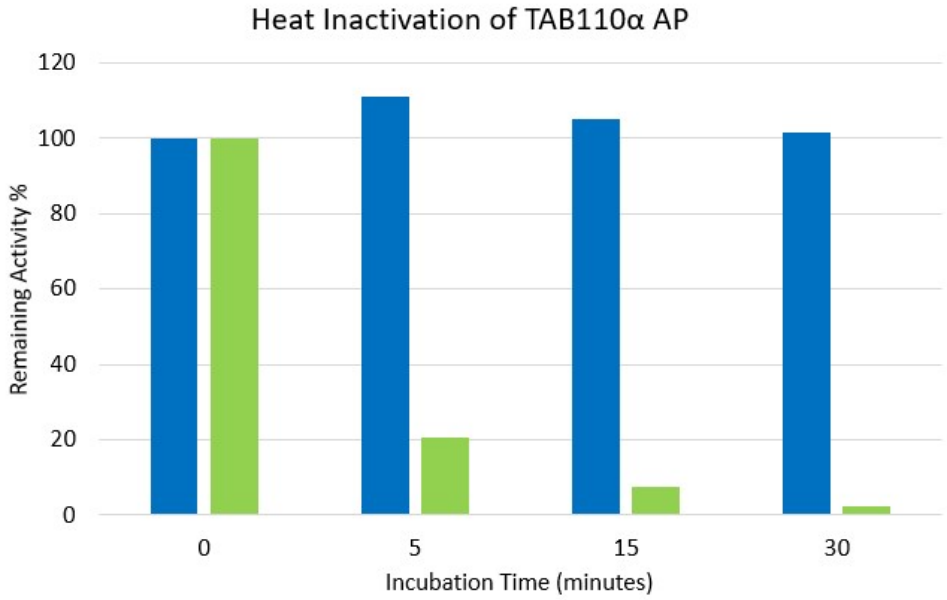

Figure 9 *Heat inactivation of wild type TAP110α alkaline phosphatase by incubation at 80°C (blue bars) or 90°C (green bars) for 0, 5, 15, and 30 minutes, respectively.*

Due to the observed high thermal stability of TAB110α alkaline phosphatase, we introduced specific amino acid mutations, in order to increase its heat- lability and activity. Alkaline phosphatases is a group of enzymes that has been studied extensively. Specifically, ECAP has been used as a model enzyme, with numerous works studying its properties and catalytic mechanism by amino acid substitutions. Also, the alkaline phosphatase from the Antarctic bacterium TAB5 has been studied, in regards to its thermo-lability. TAB110α alkaline phosphatase has a 79 % percent sequence identity with ECAP and 30 % with TAB5. Based on the sequence homology between the enzymes and the fact that different alkaline phosphatases have almost identical main structural elements, redesigning of TAP110α based on previous knowledge on ECAP and TAB5 was further investigated.

In table 7 and 8 are presented single amino acid substitutions or combinations of them that have been studied in ECAP or TAB5 APs, respectively, with their observed effect on the activity or stability of the enzyme.

Mutation in ECAP	TAP110α	TAB5	Activity	Stability	Reference
Asp153His	Asp179	His135	lower or higher, dependent on pH and Mg	lower	Janeway et al., 1992
Asp153His/Lys328 His	Asp179/Lys354	His135/Trp260	lower or higher, dependent on pH and Mg	lower	
Lys328His	Lys354	Trp260	higher	no change	
Asp369Asn	Asp395	Asp301	lower	-	Tibbitts et al., 1994
Asp369Ala	Asp395	Asp301	lower	-	
Asp51Glu	Asp77	Asp43	lower	-	Wojciechowski et al., 2003
Asp153Glu	Asp179	His135	higher	-	
His331Glu	His357	His263	lower	-	
His412Glu	His438	His337	lower	-	
His412Asn	His438	His337	lower	-	Ma et al., 1994
His412Ala	His438	His337	lower	-	
Asp330Asn	Asp356	Gly262	higher		Le Du et al., 2002
Asp153His/Asp330 Asn	Asp179/Asp356	His135/Gly262	higher	-	
Asp153Gly/Asp330 Asn	Asp179/Asp356	His135/Gly262	higher	-	
Asp153His/Asp330 Asn	Asp179/Asp356	His135/Gly262	higher	lower	Mullet at al., 2001
Asp153Gly/Asp330 Asn	Asp179/Asp356	His135/Gly262	higher	lower	
Asp153Gly	Asp79	His135	higher	lower	
Asp101Ser	Asp127	Asp83	higher	higher	Mandecki et al., 1991
Thr107Val	Thr133	Thr89	higher	higher	
Asp51Asn	Asp77	Asp43	lower	-	Tibbitts et al., 1996
Asp51Ala	Asp77	Asp43	inactive	-	
Thr59Arg	Thr85	Ser51	lower	lower	Boulanger et al., 2003
Thr59Ala	Thr85	Ser51	no change	lower	
Arg166Ser	Arg192	Arg148	lower	-	O'Brien et al., 2003
Arg166Lys	Arg192	Arg148	lower	-	
Glu322Asp	Glu348	Glu254	lower	-	Xu et al., 1993
Glu322Ala	Glu348	Glu254	lower	-	
Ala161Thr	Ser187	Ala143	no change	-	Chaidaroglou et al., 1993
Asp153Ala	Asp179	His135	higher	-	Matlin et al., 1992
Asp153Asn	Asp179	His135	no change	-	
Asp101Ala	Asp127	Asp83	higher	lower	Chaidaroglou et al.,1989

Table 7 *Amino acid substitutions studied in E.coli AP and their effect on the activity and stability of the enzyme. The symbol – indicates that no information on the stability of the enzyme was available in the respective study.*

Mutation in TAB	TAP110α	ECAP	Activity	Stability	Reference
Trp260Lys	Lys354	Lys328	lower	No change	Tsigos et al., 2001
Trp260Lys/Ala29Asn	Lys354/Asn289	Lys328/Asn263	higher or lower, depended on temperature	lower	
His135Asp	Asp179	Asp153	lower	higher	
Trp260Lys/Ala29Asn/ His135Asp	Lys354/Asn289/ Asp176	Lys328/Asn263/ Asp153	lower	higher	
Gly261Ala	Gln355	Gln329	higher	lower	Mavromatis et al.,2002
Gly262Ala	Asp356	Asp330	inactive		
Gly261Ala/Tyr269Ala	Gln355/Gly363	Gln3329/Gly337	lower	higher	
Ser86Ala	Ala130	Ala104	lower	higher	Koutsioulis et al., 2008
Gly87Ala	Ser131	Ser105	lower	higher	
Gly149Asp	Lys193	Lys167	lower	higher	
Ser86Ala/ Gly87Ala	Ala130/Ser131	Ala104/Ser105	lower	higher	
His135Glu/Gly149Asp	Asp179/Lys193	Asp153/Lys167	higher	lower	
Ser42Gly	Gly76	Gly50	lower	lower	
His135Glu	Asp179	Asp153	higher	lower	
Ser338Thr	Thr439	Thr413	lower	lower	
Ser42Gly/His135Glu	Gly76/Asp179	Gly50/Asp153	-	lower	
Ser42Gly/ S338Thr	Gly76/Thr439	Gly50/Thr413	lower	lower	
His135Asp	Asp179	Asp153	higher	higher	Koutsioulis et al., 2010

Table 8 *Amino acid substitutions studied in Antarctic bacterium TAB5 AP and their effect on the activity and stability of the enzyme.*

Based on the above tables, 3 single amino acid and one double amino acid mutations were chosen for introduction in TAB110α AP. The Asp179 in ECAP or His135 in TAB5 are involved in Mg $^{2+}$ binding in the active site (Janeway et al., 1993, Koutsioulis et al., 2008). As is evident from table 7, several substitutions of Asp179 have been studied in ECAP. Specifically, the Asp179His mutant resulted in an enzyme with decreased Mg binding affinity at the M3 site and increased activity compared to the wild type in the presence of the same ion (Murphy and Kantrowitz, 1994), while its thermal stability was decreased (Janeway et al., 1993). Accordingly, the Asp179Glu mutant showed a similar increased activity, which is thought to occur due to a broken salt link with Lys328, absence of which may cause faster release of phosphate (Wojciechowski et al, 2003). Based on the effect of these mutations on ECAP activity and stability, the analogous mutations Asp179His and Asp179Glu were designed for TAB110α alkaline phosphatase. These mutations were also chosen because they reside in the active site and in conserved regions between the sequences of TAB110α AP and ECAP (Figure 10).

On the other hand, mutations Lys193Gly and Ala130Ser/Ser131Gly where designed based on the effects that mutations Gly149Asp and Ser86Ala/Gly87Ala had on TAB5. Analytically, Gly149 affects the flexibility of Arg148 and thus the mutation Gly149Asp restricts its movement, so it cannot assume

proper conformational changes during catalysis. This restriction results in a less active and more stable enzyme, due to reduced flexibility (Koutsioulis et al., 2008). In TAB110α AP, the equivalent of Gly149 is Lys193, while the residue Arg148 is maintained (Arg192 in TAB110α AP). This suggested that a similar restriction may occur in TAB110α AP, as Lys193 has a long and charges side chain, which could affect the mobility of Arg192. With this in mind, the mutation Lys193Gly was designed in order to increase the local flexibility at the site, which could lead to higher activity and reduction in stability.

In TAB5, residues Ser86 and Gly87 are located in a α-helix after the catalytic Ser84 and are involved in its proper positioning. Ser86 interacts with Thr82 and Asn99 in order to hold in position the Ser84 containing loop, while Gly87 is involved in stabilization of Arg148 by interacting with Asp83. The two alanine substitutions in Ser86Ala/Gly87Ala prevent the Ser86 interactions, while altering the flexibility of Gly87 (Koutsioulis et al., 2008). In TAB110α AP, all these residues are conserved, except for the two mutated ones (Figure 10). In fact, those residues are the only two that differ in TAB5 in a large conserved region between ECAP, TAB110α AP and SAP. This double mutation in TAB5 leads to decreased activity, but elevated stability. The equivalent reverse mutation in TAB110α AP, Ala130Ser/Ser131Gly may have an opposite phenotype, which is the desired type of alteration.

Overall, based on a literature search for studied mutations introduced in ECAP or TAB5, three single and one double amino acid substitutions were designed in TAB110α AP in order to reduce its significant thermostability, but also increase its catalytic activity (Table 9).

Model Protein	Model Mutations	Designed Mutations
ECAP	Asp153His	Asp179His
ECAP	Asp153Glu	Asp179Glu
TAB5	Gly149Asp	Lys193Gly
TAB5	Ser86Ala/Gly87Ala	Ala130Ser/Ser131Gly

Table 9 *Designed mutations for TAB110A alkaline phosphatase based in model proteins ECAP and TAB5.*

Despite the design of four mutants, in the duration of this master thesis only two of them (Asp179His and Asp179Glu) were expressed, purified and characterized in terms of their kinetic parameters and thermal stability.

4.3. Kinetic characterization of TAB110α alkaline phosphatase and its mutants Asp179His/Glu

In table 10 are presented the calculated kinetic data for wild type TAB110α alkaline phosphatase and its mutant variants. Additionally, the kinetic parameters of commercially available SAP were also calculated.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
SAP	0.022	3.23108	1.47×10^5
TAB110α	n.d.	0.18	-
Asp179His TAB110α	0.01758	1.19403	6.79×10^4
Asp179Glu TAB110α	0.024927	2.38806	9.58×10^4

Table 10 *Kinetic parameters for SAP, wild type TAB110α and its two mutants.*

According to these values, both mutations Asp179His and Asp179Glu resulted to a higher k_{cat}, while a K_m comparison couldn't be made, as a calculation of the K_m value for wild type TAB110α AP wasn't

possible from the data and further measurements and analyses will have to be performed. The mutation Asp179Glu results in a 2-fold higher k_{cat} compared to the Asp179His mutation, while the effect on K_m is a 1.5- fold increase, respectively. Comparison of the wild type TAB110α AP and its two mutants with SAP shows that the latter has a higher k_{cat}, while Km is similar with that of Asp179Glu. Due to the higher value of k_{cat}, SAP shows the higher catalytic efficiency, as indicated by the k_{cat}/K_m value.

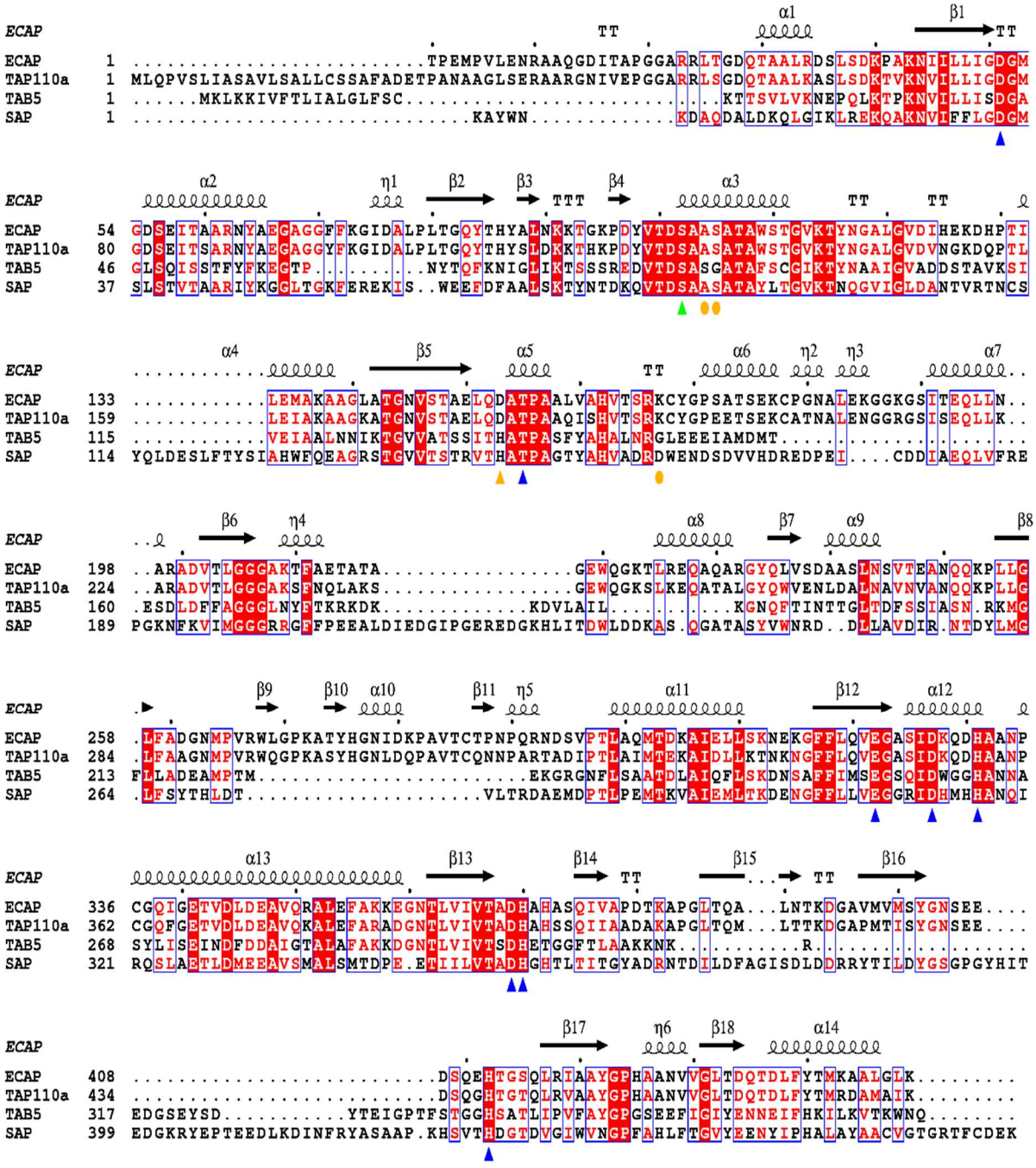

Figure 10 *Sequence alignment of ECAP (Uniprot ID: P00634), TAP110α, TAB5 (Uniprot ID: Q9KWY4) and SAP (Uniprot ID: Q9BHT8) with secondary structure elements of ECAP (PDB ID: 1ALK) depicted. Triangle: metal sites- associated residues, orange: mutated residues, green: catalytic serine. (EsPript version 3.0)*

4.4. Thermal stability of mutants Asp179His/Glu compared to wild type TAB110α alkaline phosphatase

In order to access the effect of the designed amino acid substitution had on the thermal stability of TAB110α AP, two different approaches were utilized. First, and as already partially presented in figure 9, the effect of temperature on the enzyme was studied by incubating at 80°C or 90°C and calculating the remaining activity. On the other hand, circular dichroism spectroscopy was applied in order to calculate the melting temperature Tm of the proteins, by observing the change on the CD signal as the temperature increased.

Based on the remaining activity after heat treatment, the wild type TAB110α AP retains its full catalytic activity for at least 30 minutes, when incubated at 80°C, while incubation at 90° has a more radical effect on its activity, with a 5 minute incubation resulting in an 80 % reduction in activity, which reaches approximately 98% after 30 minutes. On the contrary, both mutants Asp179His and Asp179Glu show a considerable decrease in activity after 5 minutes incubation at 80°C, reaching approximately 80 %, while a 30 minutes incubation reduces activity by 95 %. When both mutant enzymes were incubated at 90°C for 30 minutes, the remaining activity is less than 1 % for Asp179Glu and less than 3 % for Asp179His (Figure 11).

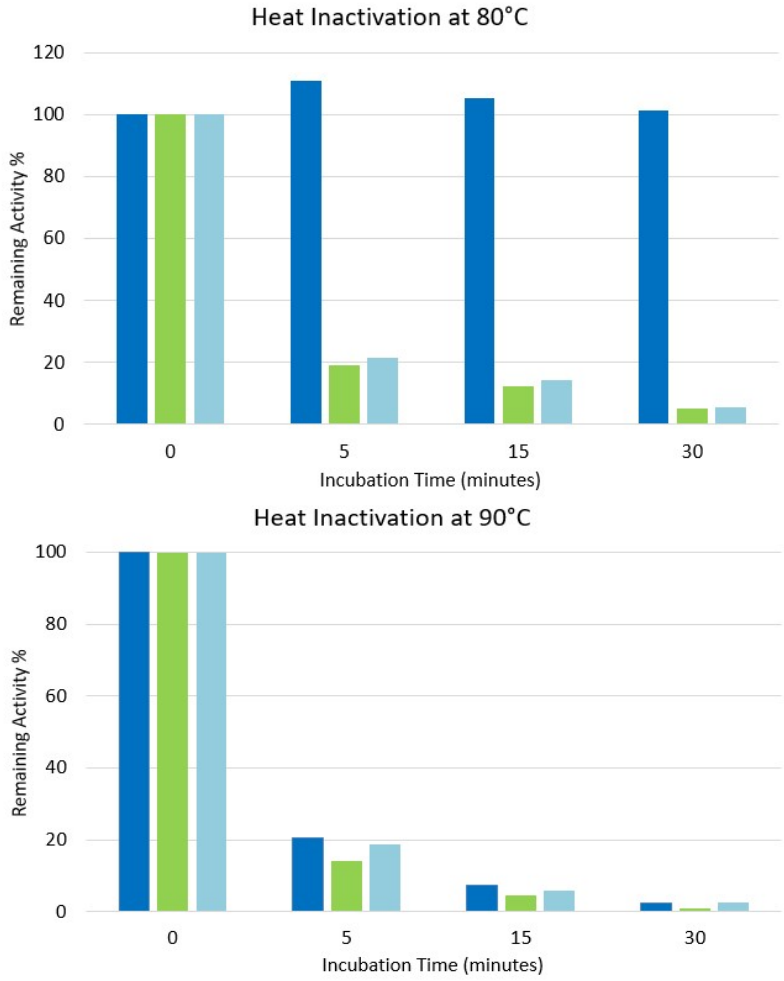

Figure 11 *Effect of heat treatment on the activity of wild type TAB110α AP and mutants Asp179Glu and Asp179His. Dark blue: wild type TAB110α, green: mutant Asp179Glu, light blue: mutant Asp179His.*

The above results are in agreement with preliminary circular dichroism (CD) experiments performed for the wild type enzyme and the two mutants (data not shown). Specifically, the enzymes were

subjected to thermal denaturation in a temperature range of 10°C up to 90°C and the change of the typical α-helical minima was observed. These results indicated that wild type TAB110α alkaline phosphatase has a higher melting temperature that the two mutants by approximately 10°C, while the latter had a similar melting profile. The accordance of the above experimetns indicates that the loss of activity of the enzyme is due to structure denaturation. Analytical CD results in the form of melting curves are no presented in this thesis due to the need of further analyses of the obtained spectra.

4.5. Diffraction data analyses of wild type TAB110α alkaline phosphatase

Crystallization experiments were performed on wild type TAB110α alkaline phosphatase in order to elucidate its three-dimensional structure. Knowledge of its structure could facilitate a more targeted design of amino acid mutations in order to improve the desired characteristics of the enzyme, which is a high catalytic activity and a thermal instability. Furthermore, based on structural analysis, the adaptation mechanisms which result in an enzyme capable for activity in low temperatures but of high stability could be elucidated.

X-ray diffraction patterns of TAB110a AP were successfully acquired (Figure 12). According to the analyses of the diffraction data, it was concluded that the protein crystals which were used for their acquisition were twinned. Twinning is not uncommon in crystallography, but it causes obstacles for structure determination. Briefly, this phenomenon is observed when a single crystal is formed by smaller, individual crystals, which join together to form an aggregate, when they share similar orientation. This result to a twinned crystal with multiple domains, which are related by one or more symmetry elements. Each domain is defined from its own unique diffraction pattern. Subsequently, the resulting diffraction pattern obtained from a twinned crystal is a superposition of all the domain diffraction patterns (Parsons, 2003). As a consequence, it is difficult to elucidate the space group of the protein crystal.

In order to overcome the problem of twinning in data analyses, specialized software can be used if the overlapping between the domain diffraction patters isn't severe. Alternatively, crystallization of TAP110α alkaline phosphatase will have to be repeated, with different crystallization conditions screened, varying at pH, ionic strength or precipitation agent. In Figure 12 is depicted one of the X-ray diffraction patterns of the twinned TAB110α alkaline phosphatase crystals. After overpassing of the crystal twinning problem, diffraction data will have to be indexed, integrated and scaled. Molecular replacement and refinement have to follow, in order to elucidate the structure of TAB110α AP.

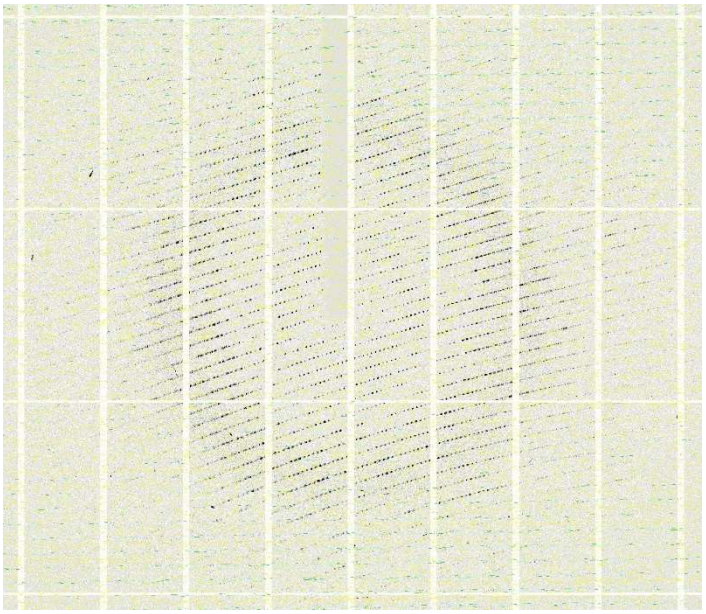

Figure 12 *X-ray diffraction pattern of TAB110α alkaline phosphatase*

5. Discussion

5.1. Stability and activity of TAB110α alkaline phosphatase

Overall, based on the results of the present study, TAB110α alkaline phosphatase presents exceptional thermal stability, despite being isolated from a bacterial strain able to grow at the low temperatures of the Antarctic seawaters. In detail, TAB110α alkaline phosphatase has a relatively stable activity profile at 80°C, with even small increases in activity up to 10 % when incubated for a short time (e.g. 5 minutes). On the other hand, substantial decrease in activity was observed at 90°C, as indicated by the remaining activity.

This result led to the re-examination of the TAB110α strain. Based on the genomic data that were obtained from the de novo sequencing of TAB110a stain 16S rRNA sequence analysis identified TAB110α as *Serratia liquefaciens,* a psychrotrophic bacterium. Specifically, *Serratia liquefaciens* is found in diverse ecosystems, ranging from fresh or processed foods to drinking water. Is has been described as an opportunistic pathogen too, found in fish, plants and humans. Interestingly, this species has been found in drinking water on the International Space Station, suggesting the presence of adaptation mechanisms for survival in extreme conditions found in space. *Serratia liquefaciens* was shown to be able for growth in conditions such as 7 mbar of pressure, 0°C and CO_2 enriched anoxic atmospheres, which create an extreme hypobaric, psychrophilic and anoxic environment. Such results indicate a generalist species, capable for broad ecological adaptation and ability to thrive in new conditions without having the specialization of extremophiles (), which have evolved for growth under precise strict conditions (Schuerger et al., 2013). The ability of *Serratia liquefaciens* to grow in such diverse conditions, may explain the presence of a thermostable alkaline phosphatase, as, in contrast to strict psychrophiles, evolutionary pressures may have facilitated both high activity in low temperatures and stability for its enzymes.

To confirm the idea of an organism able for survival in temperatures as low as 0°C, while retaining considerable stability in protein structure, a heat-resistance protease was identified in *Serratia liquefaciens.* The organism was found to contaminate milk during cold storage, which led to the isolation of the extracellular protease Ser2, which exerts in activity even after pasteurization (Machado et al., 2016, Bagliniere et al., 2017(1)). The protease showed remarkable stability, able to retained 87-100 % of its activity after treatment in 140°c in milk (Bagliniere et al., 2017 (2)). Furthermore, from a psychrotrophic *Serratia sp.* which is closely related to *Serratia proteamaculans,* a proteinase K-like enzyme was isolated and characterized. This enzyme showed thermal stability identical to that of a mesophilic homologue, while having higher k_{cat} and catalytic efficiency in temperatures between 12°C and 37°C (Larsen et al., 2006).

In order to reduce the thermal stability of TAB110α alkaline phosphatase, 4 mutations were designed, based on sequence similarity and conservation between its sequence and those of E.coli and TAB5 alkaline phosphatases. From those, only two were characterized during the time of this research, Asp179His and Asp179Glu. Both mutants showed decreased thermal stability to the wild type enzyme, by approximately 10°C, as showed by the reduced remaining activity after heat treatment at 80°C. The effect of the elevated temperature on their catalytic activity, may by correlated to the effect that they have on metal coordination of Mg^{2+}. For example, in TAB5 AP, the mutations His135Asp and His135Glu had a stabilizing or destabilizing effect, respectively, which were attributed to changes on the M3 metal

binding site (Koutsioulis et al., 2010). Similarily, for the mutant Asp153His in ECAP, a reduced affinity for Mg^{2+} has been observed (Janeway et al., 1993).

In respects to their activity, the two mutants showed a considerable increase in k_{cat} compared to the wild type enzyme, which indicates a faster release of the product during catalysis. The mutant Asp179Glu also had an increased Km of approximately 1.5-fold compared to the Asp179His mutant, corresponding with a lower binding affinity for the substrate. These results indicate that the designed mutations had a positive effect on the catalytic activity of TAB110α alkaline phosphatase, although further analyses is necessary in order to precisely calculate the effect on K_m. Finally, compared to SAP, both mutants show a lower catalytic efficiency, which is primarily exerted by the higher k_{cat} of this enzyme. Based on this measurements, Asp179Glu is better suited for the indented biotechnological applications compared to Asp179His, as in such setting, in which the substrate is usually present in excess, the k_{ca}t value is of higher relevance compared to the K_m.

5.2. Structural aspects of TAB110α alkaline phosphatase's adaptation

As analyzed in greater detail above, psychrophilic enzymes increase their catalytic activity in low temperatures in expense to their stability. The thermal lability of psychrophilic enzymes is thought to occur due to absence of evolutionary pressure of stability in cold environments. Despite this general rule, there are instances of psychrophilic enzymes with unusual high thermal stability. Examples are the isocitrate dehydrogenase from the psychrophilic bacterium *Desulfotalea psychrophila,* which has higher Tm than its mesophilic counterparts (Fedøy et al., 2007), the L-haloacid dehalogenase from the marine psychrophiles *Psychromonas ingrahamii* (Novak et al., 2013) or the aspartase (Kazuoka et al.,2003(1)), alcohol dehydrogenase (Kazuoka et al., 2007) and aldehyde dehydrogenase (Yamanaka et al., 2002) from the Antarctic seawater psychrotolerant *Flavobacterium frigimaris KUC-1*. Interestingly, from the organism *Flavobacterium frigimaris* thermolabile enzymes have also been isolated (Oikawa et al., 2005, Oikawa et al., 2001, Kazuoka et al., 2003(2)). TAP110α alkaline phosphatase shows a similar pattern, with preliminary results (not presented) showing activity in temperatures as low as 10°C. Thus, study of the TAB110α alkaline phosphatase's structure and comparison with structures of mesophilic and cold adapted Aps could shed light to how the enzyme has balanced the opposite adaptation forces for high catalytic activity in low temperatures and increased thermal stability. To our knowledge, a thermostable psychrophilic alkaline phosphatase has not been reported before, while only one crystal structure of a naturally occurring psychrophilic and thermostable enzyme has been published, which has attributed the catalytic activity of the enzyme in low temperatures on local flexibility, rather that global (Fedøy et al., 2007).

Although in the time course of this study the structure of TAP10α wasn't resolved, its elucidation could be exploited in order to study the adaptation mechanisms of the enzyme for catalysis in the cold. Similar studies have been undertaken for other psychrophilic alkaline phosphatases, like TAB5 (Wang et al., 2007), SAP (de Backer et al., 2002) or the one isolated from the *Vibrio* strain G15-21 (Helland et al., 2009). Such studies provide valuable information for the design of better biocatalysts, with improved catalytic efficiency, for use in biotechnology and industry.

6. Perspectives

In continuation of the present research, of foremost importance would be the purification of the two additional mutants designed (Lys193Gly and Ala130Ser/Ser131Gly) and subsequent evaluation of their kinetic and biophysical characteristics. For both wild type TAB110α and its mutants additional parameters can be studied, as for example the dependence of their activity in various pH or in the presence of different metals.

Furthermore, as one of the adjective for the application of TAB110α in molecular biology applications is its high catalytic activity in ambient temperatures, the activity profile of the enzyme in various temperatures should be identified. Similar characterization should also follow for the mutant variants. Additionally, comparing the temperature-activity profile of TAB110α alkaline phosphatase with that of a bacterial mesophilic and strictly psychrophilic alkaline phosphatase (e.g. ECAP and TAB, respectively) could elucidate if TAB110α has higher catalytic activity than its mesophilic counterparts in low temperatures, and if this level of activity is comparable with a well characterized psychrophilic alkaline phosphatase.

In addition, the potentials of TAB110α as a putative enzyme candidate for application in various molecular biology techniques should be evaluated. For example, one of the most common applications of alkaline phosphatases is for the dephosphorylation of DNA ends in order to prevent their circularization during cloning. In order to evaluate their potential in such an application, a digested plasmid (with blunt, 3'- and 5'- overhang ends) could be dephosphorylated with TAB110α AP and tested for ligation. The specificity of TAB110α for other common types of substrates, such as nucleotides, RNA and proteins should also be tested. Since TAB110α alkaline phosphatase exhibits increased thermostability, its potential application as a conjugate to secondary antibodies for immunodetection could be explored as well. Conjugation of enzymes usually involves harsh conditions, including high temperatures, in which TAB110α could considered advantageous.

In respects to the adaptation of TAB110α, its structure will have to be solved. Such knowledge could elucidate how the enzyme has adapted to both high catalytic activity in low temperatures and high stability. Furthermore, the structural alterations caused by the designed mutations of TAB110α alkaline phosphatase could be analyzed and, thus, provide an understanding regarding their effect on the activity and thermal stability of the enzyme. Lastly, knowledge of three dimensional structure of the enzyme will provide the advantage of targeted re-design by introducing novel mutations, with primary objective the reduction of its thermal stability. On the other hand, study of TAB110α alkaline phosphate by differential scanning calorimetry, could help elucidate if the enzyme shares the usual sharp, without intermediates, unfolding of psychrophilic enzymes.

Collectively, further experiments will have to be performed in order to evaluate the performance of TAB110α in molecular biology applications, while intensive studies on its structure could provide insights to how enzymes can simultaneously increase their activity in low temperatures while retaining considerable thermostability. Such enzymes are not common in nature, and thus TAB110α provides a unique opportunity in this area.

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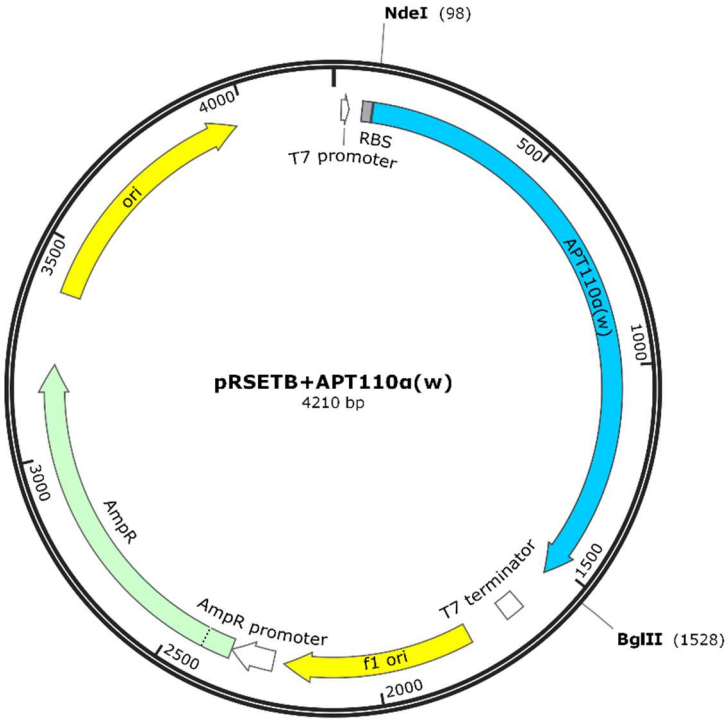
8. Appendix

8.1. Plasmid map of pRSETB+APT110α(w) construct



8.2. Nucleotide sequence of TAB110α alkaline phosphatase

ATGTTGCAGCCTGTTTCCCTGATCGCCAGTGCCGTGTTATCCGCCTTATTATGCTCAAGCGCCTTCGCCGATGAGAC

GCCGGCCAACGCTGCCGGACTTTCCGAGCGTGCGGCTCGCGGTAATATCGTTGAACCCGGCGGAGCACGTCGCCT

GAGTGGCGATCAAACCGCGGCATTGAAAGCTTCGTTGTCTGATAAAACGGTCAAGAATGTGATTTTGCTGATTGG

CGACGGCATGGGCGATTCGGAAATCACTTCGGCACGCAACTATGCCGAAGGCGCAGGCGGCTATTTTAAAGGCAT

CGATGCCCTGCCGTTAACCGGCCAATACACCCATTATTCGCTGGACAAGAAAACCCACAAGCCGGATTACGTCACT

GACTCCGCCGCCTCCGCGACTGCCTGGTCCACCGGCGTCAAAACCTATAACGGCGCGCTGGGCGTTGACGTTAAT

GGCAAAGACCAACCGACCTTATTGGAAATTGCCAAAGCGGCGGGTAAAGCCACCGGCAACGTTTCCACCGCAGAG

TTGCAAGACGCCACACCTGCGGCGCAGATTTCCCACGTCACCTCGCGTAAATGTTACGGCCCGGAAGAGACCAGT

GAAAAATGCGCAACCAACGCGCTGGAAAATGGCGGCCGCGGCTCGATCAGTGAACAGTTACTCAAAGCCCGCGC

CGACGTCACCTTAGGCGGCGGAGCAAAATCCTTTAATCAGTTAGCCAAAAGCGGCGAGTGGCAGGGAAAATCACT

GAAAGAGCAGGCAACTGCATTAGGTTATCAATGGGTGGAAAACCTCGATGCCCTGAACGCCGTTAACGTCGCCAA

TCAGCAAAAGCCGCTGTTGGGGCTGTTTGCCGCCGGCAATATGCCGGTACGCTGGCAGGGGCCGAAGGCCAGCT

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CGATCATGACCGAGAAAGCCATCGACCTGCTGAAAACCAACAAAAATGGTTTCTTCCTGCAGGTTGAAGGCGCAT

CGATCGACAAACAGGATCATGCCGCCAACCCGTGCGGCCAGTTCGGGGAAACTGTCGATCTGGATGAAGCCGTGC

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CAGCTACGGCAACTCGGAAGAGGATTCGCAGGGCCACACCGGCACCCAGCTGCGTGTTGCCGCCTACGGCCCGCA

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