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Faculty of Science

Crystallization and modelling studies of human oxydosqualene cyclase hOSC wild type and 580W mutant variant

Bachelor´s Thesis

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ANNOTATION

The aim of this study was to learn the basic and advanced crystallization techniques on the model protein Lysozyme, to crystalize the monotopic integral membrane protein human oxidosqualene cyclase hOSC-WT and its mutant variant hOSC-580W and to optimize the initial crystallization experiments. Moreover, the 3D hOSC protein structure was modelled and compared with already known structure of oxidosqualene cyclase.

DECLARATION

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

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České Budějovice, 6.5.2019 …….

Isabella Guger

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ABSTRACT

The low molecular weight protein Lysozyme, which is produced by animals, is the perfect protein for studying the different crystallization techniques due to its already world-famous structure. In comparison to other proteins, Lysozyme rapidly forms some well-shaped crystals in different crystallization conditions.

We were able to crystallize and model the secondary structure of the monotopic integral membrane protein human oxidosqualene cyclase and its mutant variant is particularly similar to its prokaryotic counterpart squalene-hopene cyclase. The amounts of α-helices, loops and ß-sheets are almost identical and can nearly be found at the same positions. Moreover, both have an active-site cavity in the centre of the molecule which allows linkage to some inhibitors.

LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
APS	Ammonium Persulfate
bOG	Octyl-beta-Glucoside
ExPASy	Expert Protein Analysis System
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
hOSC	human oxidosqualene cyclase
hOSC-WT	human oxidosqualene cyclase-wild type
hOSC-580W	human oxidosqualene cyclase-580W (point mutation)
IEF	isoelectric focusing
IMAC	Immobilized Metal Ion Affinity Chromatography
JBS True Blue	Jena Bioscience True Blue
LDL	low density lipoprotein
MPD	2-Methyl-2,4-pentanediol
MW	molecular weight
Ni-NTA	Nickel-Nitrilotriacetic acid
OSC	oxidosqualene cyclase
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
Phyre2	Protein Homology/AnalogY Recognition Engine
pI	isoelectric point
PyMOL	Python Molecular Modelling Computer software
RCSB – PDB	Research Collaboratory for Structural Bioinformatics - Protein Data Bank
Rh	hydrodynamic radius
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SHC	squalene-hopene cyclase
SIB	Swiss Institute of Bioinformatics
Tris (THAM)	Tris(hydroxymethyl)-aminomethan
Tris-HCl	Tris(hydroxymethyl)-aminomethan hydrochloric acid

TABLE OF CONTENT

1. Introduction

1.1 Protein Crystallization

Crystallization, especially protein crystallization, is an extensive and complex process which can take several months. One has to take into account that crystallization is influenced by a lot of parameters like solvent, precipitant, temperature and buffers, moreover the crystallization sample has to be well prepared. Before starting some crystallization experiments a first characterization, homogenisation and solubilisation should be done.

1.1.1 Protein Characterization

A protein can be characterized in several ways; the most common techniques are to do some protein electrophoresis like a SDS-PAGE / native PAGE or dynamic light scattering. More over one can do a IEF gel and mass spectroscopy (Bergfors, 1999).

1.1.1.1 Electrophoresis

In electrophoresis the proteins and macromolecules are separated according to their size. The macromolecules move through a gel because of an applied electric field, which results in the separation of the proteins (Bio-Rad Laboratories Inc., n.d.).

1.1.1.1.1 Native PAGE and SDS-PAGE

Normally there are some polyacrylamide or agarose gels used in electrophoresis which act like a sieve and separate the proteins according to their size in the presence of an electric field. Smaller proteins are allowed to move faster through the gel than bigger ones.

In native PAGE systems the buffers are non-reducing and non-denaturing and also the electrophoresis is done without denaturing and reducing agents.

Native PAGE cannot be used for molecular weight determination because it yields some unpredictable results. This is due to the fact that the native charge-to-mass ration of the proteins is maintained. It can be used to elucidate proteins of the same molecular weight and to separate proteins in their active state.

SDS-PAGE (sodium dodecyl sulphate-PAGE) is the most used form of electrophoresis. The proteins are separated in presence of SDS and denaturing agents and because of this they are fully denatured and divided from each other. The SDS binds to the protein in a constant rate which results in a separation according to the size only and so the molecular weight can be determined (Bio-Rad Laboratories Inc., n.d.).

1.1.1.1.2 Isoelectric Focusing (IEF)

In isoelectric focusing the proteins are separated according to their pI due to the combination of the electric field with a pH gradient. During the separation the net charge changes, it is responsible of the pH and if an electric field is applied the protein moves to the pH where its net charge is zero (Bio-Rad Laboratories Inc., n.d.).

1.1.1.2 Dynamic Light Scattering

In dynamic light scattering the size and size distribution – size homogeneity – of macromolecules in suspensions is determined by measuring the intensity of the scattered light. This gives the translational diffusion coefficient which is in connection with the Stokes-Einstein equation over the hydrodynamic radius (Rh) of the molecule. If the macromolecules aggregate this can be seen in an increase in the Rh.

The analysis can be done with a Frequency vs Rh diagram, it can show monomodal or bimodal distributions. Monomodal distribution of a protein means that it is highly probable that the protein produces some crystals whereas a non-specific aggregation gives probably no crystals or crystals of poor quality. Mixtures of monomers and dimers are not allowed in crystal formation processes because they hinder the crystallization. This means, for crystallization it is very important to have a homogeneous protein where molecules are able to position in well-ordered aggregates (Bergfors, 1999).

1.1.1.3 Mass Spectroscopy

In mass spectroscopy the sample gets ionized which can cause that parts of the sample get fragmented. The ions are accelerated through an electric and magnetic field, due to this magnetic field the direction of the ions is altered. The magnitude of the deflection depends on the mass-to-charge ratio of the ions and means that lighter ions are more deflected than heavier ions. The mass analyser sorts the ions according to the different masses of the fractions – the ions are separated according to a mass-charge-ratio (Finehout & Lee, 2004).

1.1.2 Principles before Crystallization

For a protein crystallographic project some protein crystals are needed, but before starting the crystallization there are some important facts that have to be taken into account. The most important principles are listed below.

Homogeneity – For crystallization one of the most important properties is high purity and homogeneity of the protein. Crystallization process will be taken only in a presence of identical units in the sample. In this case a uniform population of the unit components is requite because this is the only way to allow the incorporation of the units in a crystal lattice.

Solubility – The protein has to be soluble to be able to crystallize it. This means that a monodisperse solution without non-specific oligomers, aggregates and molecular clusters is needed.

Stability – The protein has to be kept as stable as possible, it is not allowed to observe any denaturation, significant conformational changes or formation of oligomers. It has to be stable in the presence of ligands and in different buffers (McPherson, 2004).

Supersaturation – Crystals grow in a system that is not in an equilibrium, which is why the state of supersaturation has to be created by for example changing the pH, temperature or concentration of precipitant.

Association – It is important to avoid a precipitate, non-specific aggregation and phase separation to form a periodically repeating, three-dimensional array. The crystals form then by self-association.

Nucleation – Limited nucleation is important for the size, the number and the quality of the resulting crystal. This means the nucleation has to be adjusted by physical and chemical properties.

Variety – It is important to grow crystals under a variety of different conditions and from different polymorphs and not to just use the very first crystal formed due to the fact that macromolecules form some crystals under different parameters (e.g. biochemical, chemical, physical)

Control – The system has to be kept at an optimal state until the crystal is formed. It is not allowed to have some perturbations like fluctuations or shock to get some good quality crystals.

Impurities – It is important to avoid some impurities in the mother liquor and the incorporation of them in the lattice. Impurities can appear in the preparation of the macromolecule or also from some reagents.

Preservation – After the crystal growing is finished, the crystal should be protected from degrading or loss of quality by stabilizing them. Stabilization is done by temperature change or addition of more precipitating agent (Bergfors, 1999).

1.1.3 Storage of Proteins

In general proteins can be kept at the range of the temperatures from 4°C to -70°C and they should not be frozen and defrosted too often – because of this, proteins should be stored in aliquots. A good option to store proteins is to freeze them in liquid nitrogen and to store them at -70°C. Furthermore, it is recommended to store proteins in concentrated form rather than in diluted form due to the reduction of loss e.g. by absorption. It is important to check the activity and stability of the protein before freezing because different proteins tolerate different freezing temperatures and can be depredated during storage (Bergfors, 1999).

1.1.4 Crystallization Conditions – Precipitants, Temperature, Buffer and pH

Precipitants – There are four groups of precipitants: salts, organic solvents, polymers and non-volatile organic compounds, where salts and organic solvents are the most common once (McPherson, 1990, 1982).

Salts are used as precipitants due to their salting out effect which means that the salt dehydrates the protein. If the ion strength of the solution increases – the concentration of the ions gets higher – the protein solubility decreases exponentially which means that the protein has to neutralize the surface charges. The proteins interact with each other and due to this interaction an ordered arrangement in a crystal lattice is formed (Collins & Washabaugh, 1985).

The Hofmeister series describes the salting out effect of ions for negatively charged proteins.

$SO_4^{2-} > HPO_4^{2-} > CH_3COO^- >$ citrate$^{3-} >$ tartrate$^{2-} > HCO_3^- > CrCO_3^- > Cl^- > NO_3^- > ClO_3^-$

For positively charged proteins the series is reversed (Guilloteau *et al.*, 1992).

An organic solvent as precipitant behaves similar to salts in rivalry for water, but moreover the organic solvent decreases the dielectric screening capacity, resulting in the rise of the effective electrostatic force strength which lets the molecules attract to each other.

Crystallization process by the polymers as precipitants is similar to crystallization procedure by salts and organic solvents as precipitant. Organic polymers as PEGs dehydrate the protein sample and decrease the effect the dielectric screening capacity as well. Furthermore, organic polymers as precipitants disturb the solvents innate construction and forms a more complex network resulting in the macromolecular crystal formation (McPherson, 1990).

Temperature – Crystallization experiments are normally provided at 4°C or at room temperature but crystallization of the different proteins can also happen over a range of temperatures (less than 0°C to 60°C).

Protein solubility is in high contact with temperature. One can divide the solubility in normal and retro-grade solubility. With normal solubility an increasing solubility is observed with an increasing temperature. This occurs in low ionic strength conditions, e.g. PEG or MPD solutions. Retro-grade solubility means that the proteins at higher ionic strength are better soluble at 4°C than at 25°C.

Crystallization can be optimized with temperature gradients. The protein gets dissolved in a buffer at higher temperature and then slowly is cooled down causing the solubility of the protein to decrease and an increase of supersaturation (Bergfors, 1999).

Buffers and pH – Buffers and pH are probably the most important conditions for crystallization of a protein, this means the right choice of the buffer and the pH is essential for a good result.

The buffer concentration of the protein solution and the drops is different. For the protein solution, where the protein is dissolved in, HEPES (10mM, pH=7) or Tris (10mM, pH=8) is used. Sometimes it is needed to add some salt (NaCl) that the protein stays in solution. For the drops – the buffer in the reservoir – a concentration of 0.1M is used and it is important to calibrate the pH after the salt is added to the buffer solution.

To choose the right pH can be tricky, one can begin to screen in a pH range from 4-9 for example with a 1 or 1.5 pH units interval. After this one can fine tune the pH by using smaller intervals (0.05-0.1 pH units) in the pH range where some crystals were formed (Bergfors, 1999).

1.1.5 Formation of the Crystal

In general protein crystals form from aqueous solutions, the mother liquors (McPherson, 2004). To form a protein crystal, the concentration of the protein solution has to be increased up to the limiting concentration where the protein will no longer stay in solution which means the solution is no longer homogeneous. When this solubility limit is reached a new phase appears, the region of supersaturation which is a non-equilibrium condition where most of the protein molecules pass the solubility limit. In the region of supersaturation the protein starts to aggregate which means a solid state forms and the equilibrium is re-established (McPherson, 1999; Asherie, 2004).

The aggregation of the protein is a two-step process.

The first stage is the nucleation where the protein forms an ordered state, an amorphous precipitate or microcrystals –this is a stable complex. An amorphous precipitate is formed if

the protein concentration is above saturation and means that if the saturated state is reached too fast the precipitate prevails the system. Amorphous precipitate grows much faster than crystals itself (Rhoses, 2006).

After the saturation limit is passed the metastable phase is reached, where the formed nuclei will grow further without forming new nuclei and the supersaturation will decrease because of the decreasing amount of nutrient. This means the best way to get a few and large single crystals is to grow them very slowly and to pass the saturation line only slightly (Kutá Smatanová, 2002)

With a solubility diagram the crystal formation can be described (Fig. 1).

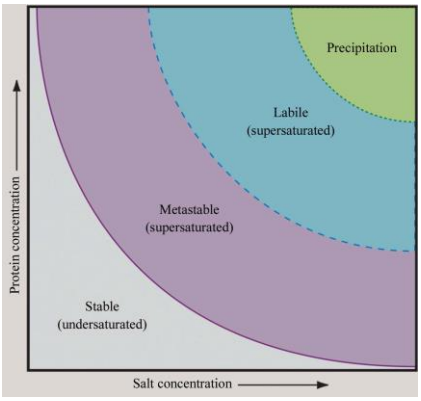

Figure 1. Phase diagram for the description of protein crystallization (McPherson, 1999)

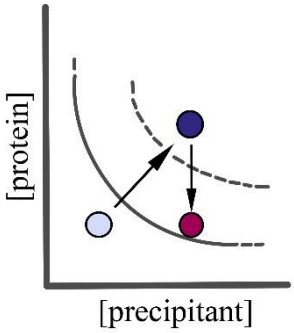

Figure 2. Ideal strategy to grow large crystals (Rhodes, 2006)

The diagram is divided into two regions – undersaturation and supersaturation where the supersaturation region is subdivided again into metastable zone, labile (nucleation) zone and precipitation zone. In the undersaturated zone there is no crystallization possible. When the salt and/or the protein concentration is increased the solubility line is reached where the precipitant and the solution is in equilibrium. Further increasing leads to the metastable phase where no nuclei form but crystals from seeds can grow. After this region the labile phase is reached where nuclei can form and also crystals grow. The last region is the precipitation zone which is very high supersaturated and is called like this due to the fact that a precipitate – an amorphous aggregate – is formed (Ducruix & Giege, 1992; McPherson, 2004). The best strategy to grow large crystals is to start crystallizing in the undersaturated region, increase the concentrations to get to the labile region and then go back to the metastable region as can be seen in Figure 2. (Rhodes, 2006).

1.1.5.1 Crystal Structure

For a macromolecule crystal it is almost impossible to reach a size as large as 1mm. Protein crystals of this size are often twinned which means that some crystals grow together or they cannot be used due to imperfection. That a protein crystal can be used for crystallography it had to have at least a size of 0.2mm (Rhodes, 2006). Nowadays already a size of 0.03mm is enough.

Protein crystal composition varies from 25 to 90% solvent which depends on the individual macromolecule, in average the composition is approximately 50%. The remaining volume is filled with protein or nucleic acid. This means that the protein crystal resembles an ordered gel with channels through which solvent and some small molecules can freely diffuse (McPherson, 2004).

The protein crystal is held together by weak interactions like salt bridges, hydrogen bonds or hydrophobic interactions, this means that the protein crystals stick together (Kutá Smatanová, 2002; McPherson, 2004). Due to this weak interactions protein crystals are very fragile and can be crushed easily for example with a needle (Rhodes, 2006).

1.1.5.2 Crystal Geometry and Symmetry

A crystal is a periodically arrangement of some atoms in the 3D space. The atoms are arranged in a crystal lattice which is constructed of identical unit cells. The unit cell is the smallest part of the lattice which is repeated in different directions over and over again (Rhodes, 2006).

Crystal structures are classified to their symmetry which is divided in three different types of symmetry: rotation, reflection and inversion.

Proteins can only form some crystals with rotational symmetry because they are single-chiral molecules. For reflection and inversion symmetries both chiral forms of the molecule are required (McPherson, 1999).

In crystallography only 2-fold (180°), 3-fold (120°), 4-fold (90°) and 6-fold (60°) rotation symmetries can be used because for example some 5-fold or 8-fold rotation symmetries cannot fill the space completely.

The space groups are defined by the symmetry elements in the unit cell, furthermore, the space groups belong to the seven crystal systems.

The seven crystal systems (cubic, trigonal, hexagonal, tetragonal, orthorhombic, monoclinic and triclinic) can be combined with the lattice centering operations (primitive = P or R, base-centered = A, B or C, body-centered = I, and face-centered = F) and results in the 14 distinct lattices, which is known as Bravais lattices. (Lockwood & Macmillan, 1978)

In crystallography there are 11 enantiomorphic pairs of space-group types, the allowed screw rotation systems which can be written as the Hermann-Mauguin symbols (2_1, 3_1, 4_1, 4_2, 4_3,6_1, 6_2, 6_3, 6_4, 6_5). If all crystallographic point groups and lattice types are combined, a total of 230 unique space group symmetries are formed but only 65 space groups can be used for chiral molecules like proteins (Hahn, 2002; McRee, 1999).

1.2 Crystallization of Membrane Proteins

Membrane proteins have some hydrophilic and hydrophobic parts and are enclosed in the lipid bilayer. The hydrophilic part is oriented to the solvent and the hydrophobic part is oriented to the interior of the lipid bilayer.

To use the membrane proteins, they first have to be solubilized from the lipid bilayer with a mild detergent, which imitates the lipid bilayer. The detergent coats the hydrophobic parts of the proteins and forms a protein-detergent micelle (Bergfors, 1999). This covering of the proteins large hydrophobic parts is important due to stabilization, otherwise it might be that they interact non-specifically which results in aggregation and precipitation of the protein. Solubilisation of the protein can be tricky because of the detergents volume and flexibility it decreases the crystallization success (Ostermair *et al.*, 1995; Ostermair & Michel, 1997).

There are three ways to form membrane protein crystals which are called 2D crystals, type I and type II crystals (Fig.3).

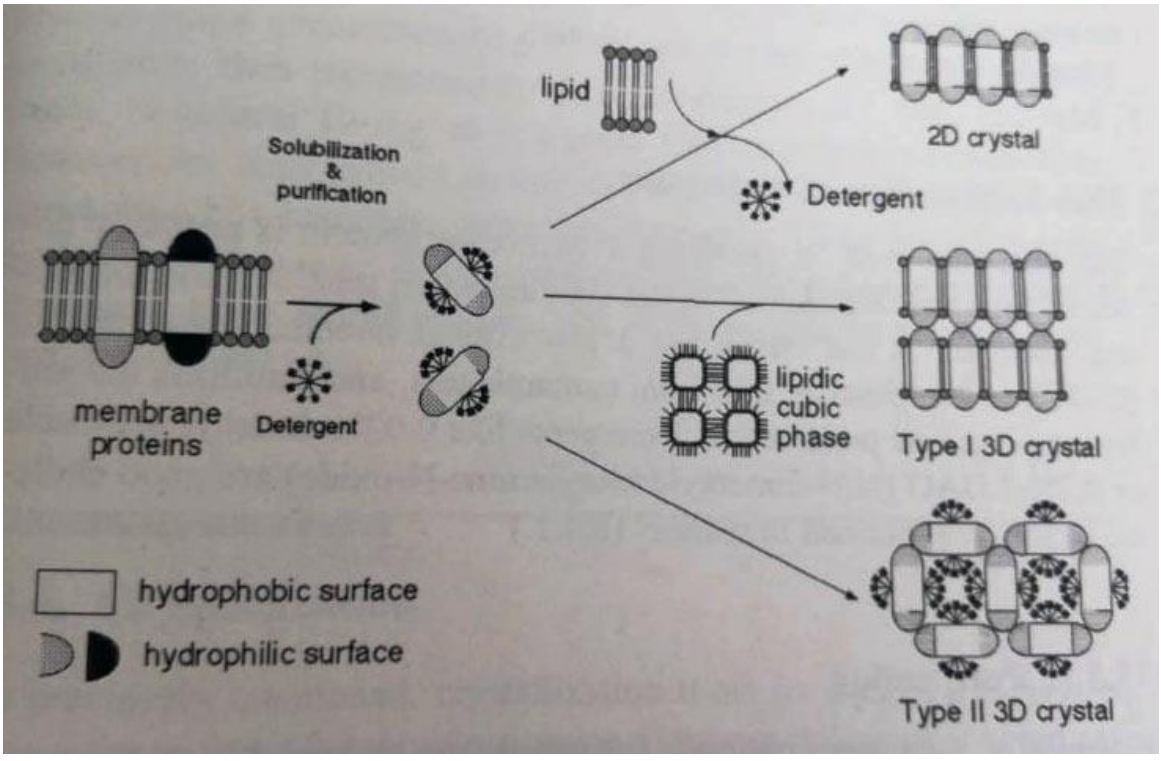

Figure 3. Representation of solubilisation, purification and the different types of membrane protein crystals (Bergfors, 1999)

Type I and type II crystals are the two important ones, while 2D crystals are some restructured bio-membranes.

Type I crystals are formed by stacking 2D crystals one over each other and stabilizing them through some hydrophobic and polar interactions between the protein and the lipid. The lipid, more precise, the lipidic cubic phase is a 3D persistent lipid phase acting as a membrane protein solvent (Bergfors, 1999).

To grow the more common type II crystals the presence of some detergents is needed. Stabilization of the crystal is reached by the polar hydrophilic surfaces of the membrane protein and due to the fact that the detergent micelles have to fit in the space between the proteins (Ostermeier & Michel, 1997)

1.3 Crystallization Techniques

In crystallizaiton several crystallization techniques can be used like sitting drop, hanging drop, microbatch under oil and Counter-diffusion (free interface diffusion) which have all their advantages and disadvantages and are presented below.

1.3.1 Sitting Drop

In the sitting drop method (Fig. 4 and 5), a small protein solution droplet is placed on a concave sitting drop post and some precipitant is added to the drop, this is then equilibrated against a reservoir solution. The protein solution-precipitant mixture can have a volume from 1-40µL.To isolate the system a transparent sealing tape is used. The precipitant concentration of the reservoir is higher than the starting concentration of the precipitant in the droplet, but after some time an equilibrium between the reservoir and the droplet will form, which results in a raise of relative supersaturation of the protein (Hampton research Corporation, 2006).

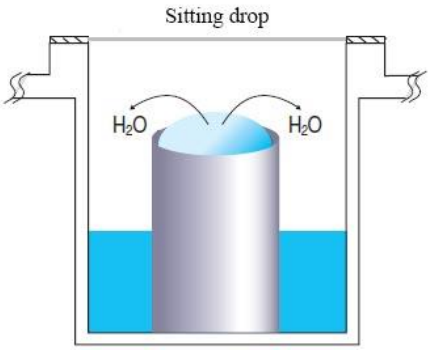

Figure 4. Schematic representation of the sitting drop techniue (vapour diffusion) (modified from Hampton research Corporation, 2006)

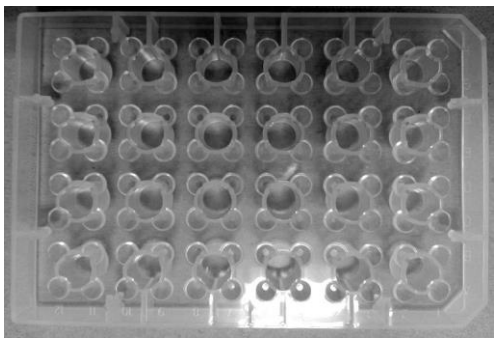

Figure 5. Sitting drop plate.
(Picture created by the author)

1.3.2 Hanging Drop

In the hanging drop method (Fig. 6 and 7), the protein-precipitant drop is placed on a siliconized glass cover slide and some precipitant is filled in a reservoir. Then the glass cover slide is put upside down over the reservoir to seal the system. Since the drop can fall down if it is too big, the volume is limited to 8-10µL.

Like in the sitting drop method there will be some equilibrium between the reservoir and the droplet and the relative supersatiration of the protein drop is again increased (Hampton research Corporation, 2006).

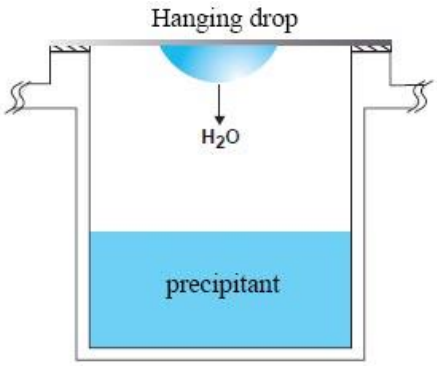

Figure 6. Schematic representation of the hanging drop technique (vapour diffusion) (modified from Hampton research Corporation, 2006)

Figure 7. Hanging drop plate. (Picture created by the author)

1.3.3 Microbatch under Oil

In microbatch under oil crystallization (Fig. 8 and 9) a small protein solution drop mixed with the precipitant is pipetted in a plate filled with paraffin oil and covered with a lid. The advantages of this method are the very small volumes needed, almost no evaporation of concentration of the drop and the ability to control the sample conditions (Hampton research Corporation, 2006).

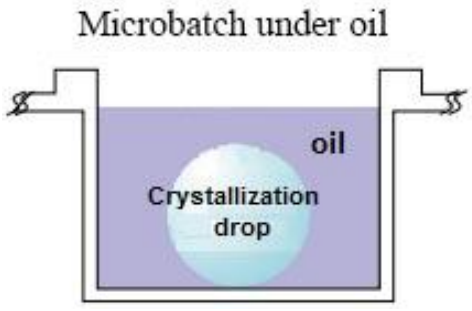

Figure 8. Schematic representation of the microbatch under oil technique (vapour diffusion) (modified from Hampton research Corporation, 2006)

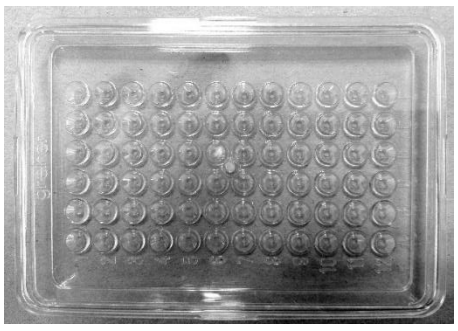

Figure 9. Microbatch under oil plate. (Picture created by the author)

1.3.4 Counter-diffusion (free interface diffusion)

In general, there are two solutions – the protein sample solution and the precipitant – placed in a capillary (Fig. 10 and 11) and then a concentration gradient is formed due to diffusion of the two solutions against each other. Because of the concentration gradient the supersaturation probability is increased where a crystal can form. To isolate the system, the capillary is sealed with some wax (Hampton research Corporation, 2006).

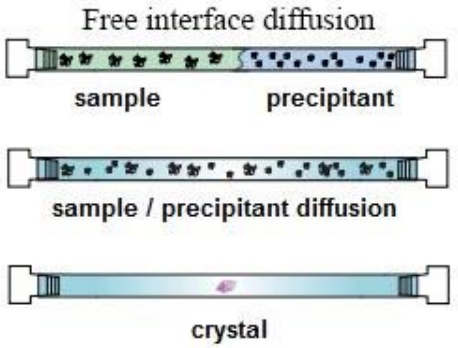

Figure 10. Schematic representation of the microbatch under oil technique (vapour diffusion) (modified from Hampton research Corporation, 2006)

Figure 11. Capillary for free interface diffusion. (Picture created by the author)

1.4 Testing of the Crystals for the Protein Nature

Ones the crystals grown we have to be sure that they are the crystals of our interest but not a component of the precipitant that we used in crystallization trials. There are several ways to clarify the crystals nature and most of them will destroy our crystals: x-ray diffraction, dye test, dehydration and crush test. The most confident technique is X-ray diffraction. The dye and the crush test are the commonly used in the laboratory to check if the crystal is of macromolecular nature, meaning a protein, or a salt crystal.

Dying test is performed by adding a small amount (0.1-1 µl) of Methylene Blue, also known as methylthioninium chloride, directly to the drop containing the crystals. Small molecules of dye will specifically bind to the protein to form the complex especially by the basic and aromatic amino acids. The dye only colours the protein crystal due to penetration of small dye molecule through the protein solvent channels. Salt crystals would stay colourless with this dye (Bergfors, 1999; Jena Bioscience GmbH, Germany).

Another straight forward way to check the crystals nature is a crush test. Protein crystals can be easily destroyed by tipping at it whit a needle due to its high solvent content and poor

mechanical properties. In contrast, salt crystals are really hard to destroy, they crush only with a high amount of applied force (Bergfors, 1999).

The most trustful proof of the crystals nature is the X-ray diffraction pattern. This can be performed only on the home source diffractometers or at the synchrotron. Beside the proof that the grown crystal is a protein crystal it yields information about the crystal symmetry and maximum resolution. For the X-ray diffraction experiment the crystal is mounted in an X-ray beam which is interacting with the crystal itself. The beam is diffracted by the crystal and the pattern is formed resulting in reflection spots in circular arrangements. Using the information from the X-ray diffraction experiment an electron density map can be produced (Drenth, 2007).

1.5 Protein Samples used for the Crystallization Experiment

For the crystallization experiments we use two freshly isolated and purified samples (lysozyme and human Oxidosqualene cyclase. Lysozyme is the model protein with known structure and known crystallization conditions for the crystal growth to learn all the crystallization techniques and trials. The second sample is the sample of our interest – hOSC that was expressed and purified at our partner's lab of Assoc. Prof Per-Olof Syrén from KTH Royal Institute of Technology (Stockholm, Sweden) in order to obtain the crystal structures of wild type of the enzyme and introduced point mutation variants.

1.5.1 Lysozyme

Lysozyme, also known as N-acetylmuramide glycanhydrolase, was first observed in tissues and secretions like tears and mucus by Fleming in 1922 (Afzal Mir, 1977). It is a low molecular weight protein with about 14.3kDA and well known sequence with about 129 amino acids (Howell, 1995).

Furthermore, Lysozyme can be found in large amounts in egg white, it is antibacterial, a good immuno-gene and enzymatically active (Young & Leung, 1970).

Lysozyme is produced by animals and is an enzyme that builds some part of the innate immune system. Moreover, it catalyses the hydrolysis of 1,4-beta-linkages in peptidoglycan (Manchenko, 1994). In addition, it is a well-known model protein that was already used in a lot of crystallization experiments. The reason is that it is very easy to crystallize to learn all the new techniques and methods lysozyme crystals will appear in a very short period of time.

1.5.2 Oxidosqualene Cyclase

Oxidosqualene cyclase (OSC), also known as lanosterol synthase, is a monotopic integral membrane protein in eukaryotic cells. Since the OSC is a monotopic membrane protein, it does not span the bilayer but it inserts into the membrane (Thoma *et al.*, 2004).

The main function of human oxidosqualene cyclase is the cyclization of 2,3-oxidosqualene to fused-ring compounds like the in plants found cycloartenol or the in mammals found lanosterol. Lanosterol is the most important one, it is a tetracyclic triterpenoid and almost all steroids are formed from it. In more detail it is needed in the cholesterol biosynthetic pathway because it is the initial four-ringed sterol intermediate. Therefore, OSC is an important target for the development of hypocholesterolemic drugs, which hinder the lanosterol synthesis (Thoma *et al.*, 2004; Lenhart *et al.*, 2002).

The conversion from 2,3-oxidosqualene cyclase to lanosterol involves several steps. The first step in the polycyclization is that 2,3-oxidosqualene forms the chair-boat-chair conformation by entering the active site cavity. After this the epoxide is protonated, because of this a cascade of ring-forming reactions is triggered which results in the protosterol cation. The backbone is rearranged by some 1,2-hydride and 1,2-methyl group shifts. Lanosterol is then formed after a last deprotonation (Fig. 12) (Thoma *et al.*, 2004).

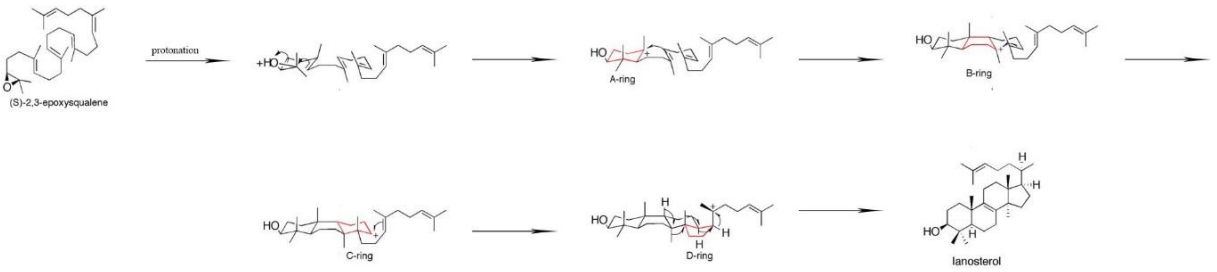

Figure 12. Catalytic mechanism for the polycyclization of 2,3-oxidosqualene cyclase to lanosterol (Thoma et al., 2004)

Moreover, oxidosqualene cyclase also catalyses the cyclization of 2,3:22,23-diepoxysqualene to 24(S),25-epoxylanosterol and then immediately 24(S),25-epoxycholesterol is formed, which is a ligand activator of the liver X receptor. Cholesterol synthesis is decreased by suppression of oxydosqualene cyclase and the 24(S),25-epoxycholesterol synthesis is raised selectively. Due to this dual mechanism, suppression of OSC reduces the plasma levels of low-density lipoprotein (LDL)-cholesterol (Huff & Telford, 2005).

The secondary structure of oxidosqualene cyclase consists of alpha helices, beta sheets and random coils. In detail human oxidosqualene cyclase (hOSC) consists of two α/ α barrel domains with a large central active-site cavity. Loops and three smaller β-structures connect the domains with each other. The positively charged (polar) amino-terminal region of OSC fills the space between the domains to stabilize the relative orientations (Thoma *et al.,* 2004). Human oxidosqualene cyclase has the highest sequence identity of 26% with one of the deposited structure of OSC, the Squalene-hopene cyclase (SHC) (RACB PDB ID 1GSZ).
SHC catalyses the cyclization of the linear triterpene squalene to hopanoids (Ruf et al. 2004). The molecular weight of human oxidosqualene cyclase is 83 kDA, it has a pI of 6.16 and it has about 732 amino acids with some natural mutations (Ruf *et al.,* 2004; Thoma *et al.,* 2004). An important inhibitor of human oxidosqualene cyclase is Ro48-8071 (fumarate), it has low-density lipoprotein cholesterol lowering activity and its pharmaceutical properties were tested as save in hamsters, squirrel monkeys and Göttingen minipigs. The amine of the Ro48-8071 inhibitor is meant to interact with the epoxide-opening region of the oxidosqualene cyclase, in more detail with its positive charge. The protosterol cation should be stabilized due to the interaction of the negative-point charge with the carbonyl of benzophenone (Lenhart *et al.*, 2002; Morand *et al.,* 1997).
In the partner's lab of Assoc. Prof Per-Olof Syrén from KTH Royal Institute of Technology (Stockholm, Sweden) was discovered that an activity of the hOSC protein is very sensitive to the thermodynamic parameters. In order to study this effect by X-ray crystallography three mutant forms were constructed (hOSC580W, hOSC443T and hOSC521W) with a few point mutation introduction. Variant 580W was introduced by substitution of 580S by W. The variant shows more "sever" effect and a complete reversed temperature dependence of an activity compared to the wild type of the hOSC protein with optimal temperature in 22°C instead of 37°C for the wild type protein. The activity of the variant hOSC443T were 443S was replaced by T is not so much dependent on the temperature any more. Both variants have the same activation energy as the wild type of the enzyme. Variant hOSC521W was constructed by substitution of 521F by W. The variant shows the opposite effect, so the activity is more depended on temperature than the activity of the wild type of hOSC enzyme. An activation energy of hOSC443T variant is higher than in wild type protein.

2. AIMS

- To learn basic and advanced crystallization techniques by the use of model protein.
- To characterise studied proteins and identify the crystallization conditions for the proteins crystal growth.
- To optimise initial crystallization conditions and prepare the crystals of hOSC and its mutant variant in diffraction quality.
- To model the 3D hOSC protein structure with secondary structure elements visualization.

3. MATERIALS AND METHODS

3.1 Chemicals

The chemicals were used as pure as possible (99,99% pure) due to the fact that already > 0.01% impurity can influence the crystal formation and the crystal quality.

Table 1. SDS-PAGE buffer composition

SDS-Gel		
Running Gel / Stacking Gel	**Sample buffer**	**Running buffer**
30% acrylamide mix	10% (w/v) SDS	25mM Tris-HCl
1.5M Tris (pH 8.8)	10mM Dithiothreitol, or beta-mercapto-ethanol	20% (v/v) Glycine
10% SDS	20%(v/v Glycerol	0.1% (w/v) SDS
10% ammonium persulfate (APS)	0.2M Tris-HCl (pH 6.8)	
TEMED	0.05% (w/v) Bromophenolblue	
Staining Buffer	**De-staining Buffer**	
Coomassie R250	Methanol	
Glacial acetic acid	Glacial acetic acid	
methanol	ddH_2O	
ddH_2O		
Marker		
Unstained molecular weight marker		

Table 2. Buffer composition of the hOSC protein purification procedure

hOSC sample preparation	
Extraction	**Purification**
Resuspension buffer – 50mM Tris-HCl, pH 7.5, 5% glycerol	Ni-NTA (washed in resuspension buffer)
0.2% Triton X-100 (stock 1% in resuspension buffer)	Washing mixture 1 – 50mM Tris-HCl, 5% glycerol, 50mM imidazole pH 7.5
	Washing mixture 2 – 50mM Tris-HCl, 5% glycerol, 0.8% ß-OG

Sample buffer
50mM potassium phosphate pH 7.5 with 0.2% Triton X-100

Table 3. Precipitant composition of the crystallized protein

Precipitant for hOSC and Lysozyme	
hOSC	**Lysozyme**
0.1M Tris-HCl	10% NaCl / 20% NaCl in Sodium acetate buffer pH 4.7
NH4-acetate	
5% Ethylene Glycerol	
30% Polyethylene Glycol (PEG-4000)	

Table 4. Other chemical used in the experiments

Other chemicals	
Crystal staining	JBS True Blue
Microbatch oil	Paraffin oil

3.2 SDS-PAGE

To check the molecular weight and the purity of the proteins a SDS-PAGE was done.

The SDS-PAGE gel is formed from a running gel 12% (10mL) and a stacking gel (2mL).

The running gel (Table 1) was prepared by mixing 3.3mL deionized water, 4.0mL 30% acrylamide mix, 2.5mL Tris (1.5M, pH 8.8) and 0.1mL 10% SDS. In the end 0.1mL 10% ammonium persulfate (APS) and 0.004mL TEMED are added to the solution to start polymerization. The mixture was then filled quickly in the gel casting form and the gel was covered with water to prevent drying out. After 30 minutes the gel was polymerized completely.

For the stacking gel (2mL) (Table 1) 1.4mL deionized water, 0.33mL 30% acrylamide mix, 0.25mL Tris (1.5M, pH 8.8) and 0.02mL 10% SDS, 0.02mL 10% APS and 0.002mL TEMED are combined and immediately filled on the top of the running gel. Then the comb was put in the stacking gel and gel was allowed to polymerize for another 30-60 minutes.

For the sample (hOSC-WT and hOSC-580W) preparation, 4µL protein, 2.5µL sample buffer (Table 1) and 3.5µL deionized water were mixed together to get a total sample volume of

10µL. For the Lysozyme sample 0.5µL Lysozyme, 2.5µL sample buffer and 7µL deionized water was mixed to get again a total volume of 10µL.

The gel was loaded with the marker (Unstained Protein Molecular Weight Marker) (Fig. 13) and the prepared samples (hOSC-WT 4.2mg/mL, hOSC-WT 1.3gm/mL, hOSC-580W 4.1mg/mL and hOSC-580W 3.4mg/mL), additionally a lysozyme sample was loaded once.

After the samples were loaded the gel run at 100V for approximately 60 minutes with the running buffer (Table 1). Then the gel was stained with Commassie Brilliant Blue (Table 1) to visualize the protein bands during 30 minutes and replaced to destaining solution (Table 1) for the period of 1-2 days until blue colour was removed from the gel.

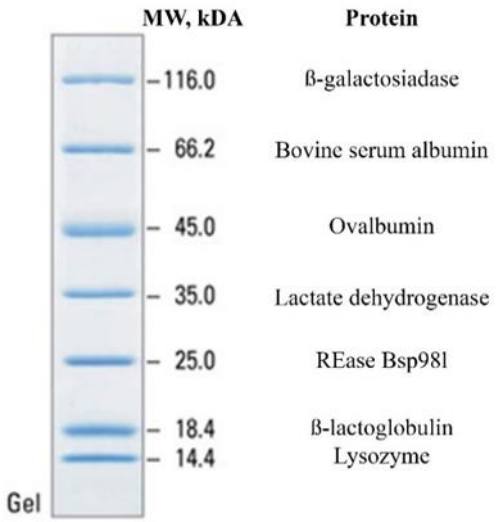

Figure 13. SDS-PAGE band ladder of the Unstained Protein Molecular Weight Marker. 8-16% Tris-glycine gel stained with coomassie blue gel stain.

3.3 Crystallization Techniques

3.3.1 Vapour-diffusion crystallization

Crystallization trials were performed by the sitting and hanging drop procedures of vapour-diffusion method (Bergfors 1999). Sitting drop technique was performed in 24 and 96 well plates (Fig. 5) (Hampton Research (HR) and Emerald BioSystems, USA). Hanging drop procedure was carried out in Limbro 24 well plates (HR, USA) (Fig. 7). Reservoirs contained 200 - 1000µL of crystallization reagents. The well droplets consisted of 4µL of total volume of different precipitant-protein composition. Crystallization trials plates were stored in 292K temperature.

3.3.2 Counter-diffusion method

Counter-diffusion crystallization was performed in single capillaries using two-layer configuration (Gavira *et al.,* 2006). Glass capillaries (Fig. 11) of 7mm lengths with different inner diameters of 0.5 and 0.8mm (Hirschmann, Germany) were placed into the Granada Crystallization Boxes (GCB®) (Triana Sci&Tech, Granada, Spain). Protein solution was filled to the capillaries with 30µL of different precipitant solutions. The filled capillaries were sealed by wax and stored at 292K temperature.

3.3.3 Microbatch crystallization

The Douglas Instruments, USA Vapor Batch 96 well plates (Fig. 9) were used to perform microbatch under oil method (Chayen *et al.,* 1999). Different precipitants and protein solutions were mixed directly in the well in a total volume of 4µL under 2 – 3mm of oil layer. Paraffin and silicon oils were used to test the protein sample crystallization capacity. Crystallization plates were stored in 292K temperature.

3.4 Crystallization of Lysozyme

To learn all the crystallization methods and techniques Lysozyme protein was used. To prepare the Lysozyme sample 100mg of Lysozyme was dissolved in 1mL of water to get a concentration of 100mg/mL as the initial concentration. This stock solution was diluted to prepare several protein concentrations of 10mg/mL, 20mg/mL, 30mg/mL and 40mg/mL.

As the percipient for the Lysozyme crystallization 10% NaCl in sodium acetate buffer pH 4.7 precipitant solution. To prepare 1L of sodium acetate buffer pH 4.7, 410.15mg anhydrous sodium acetate and 300.25mg (286µL) acetic acid were dissolved in 800mL distilled water and adjusted to pH 4.7 with HCl and NaOH. After pH calibration, the buffer solution was filled up to 1L.

The different crystallization methods: hanging drop, sitting drop vapour-diffusion, micro batch under oil and free interface diffusion were set up.

In the hanging drop method, the 20mg/mL protein solution was mixed with 10% NaCl in sodium acetate buffer pH 4.7 precipitant solution in different protein – precipitant ratios (1:1, 1:2, 1:3, 2:3, 3:1 and 3:2, in µL) and with different reservoir volumes (200µL, 250µL, 275µL and 300µL).

In the sitting drop method, the same protein and precipitant concentrations like in hanging drop method were used in different protein – precipitant ratios (1:1, 1:1.5, 1:2, 1:3, 1.5:2, 2:1, 2:1.5, 2:3, 3:1, 3:2, 3:4 and 4:3, in µL) and with a reservoir volume of 200µL.

In the microbatch under oil technique the 40mg/mL Lysozyme solution and the 10% NaCl in sodium acetate buffer pH 4.7 precipitant solution was used, again with different protein – precipitant ratios (1:2, 1.5:1, 2:1, 2:1.5, 2:2, 2:3.5, 2:5, 3:1, 3:2, 4:1, 4:3 and 5:2 in µL).
In the free interface diffusion method, the capillaries were filled with 30µL of the 40mg/mL Lysozyme solution and the 10% NaCl in sodium acetate buffer pH 4.7 precipitant solution.
To check the protein nature of the grown crystals and not salt crystals, the formed crystals were dyed with JBS True Blue. 0.5µL of JBS True Blue was added to the drop with the crystal and during the period of 30-60 minutes the protein crystals were coloured by blue.

3.5 Crystallization of hOSC-WT and hOSC-580W

3.5.1 Protein Preparation

The hOSC-WT and hOSC-580W, were purified in a cooperation laboratory. The proteins were extracted by suspending 3mL/g pellet resuspension buffer (Table 2) and the cells were disrupted by sonication (4x – 1s on, 1s off; 1min; 80% amplitude), 0.2% Triton X-100 was added. The mixture was incubated for 1h with end-over-end shaking on ice and cell debris was removed by centrifugation at 4°C (18000rpm, 30min). The supernatant was added to 0.75mL/g cell of Ni-NTA. The mixture was again incubated for 1h with end-over-end shaking on ice.
The proteins were purified by Immobilized Metal Ion Affinity Chromatography (IMAC) by washing with 3.75mL of washing mixture 1, followed by washing with 7.5mL of washing mixture 2 (Table 2). The proteins were eluted with 9 x 1mL of Tris-HCl, glycerol, ß-OG and imidazole (pH = 7.5) (Table 2).
Finally, the fractions were washed with washing mixture 2 again and the concentrated samples were analysed with Bradford which gave the following protein concentrations (Table 5). The proteins were stored in a sample buffer (Table 2) for further use.

Table 5. Original concentrations of the hOSC-WT and the hOSC-580W

Protein	Concentration (mg/mL)	mL
hOSC-WT	4.2	0.5
hOSC-WT	1.3	0.5
hOSC-580W	4.1	1.0
hOSC-580W	3.4	0.75

3.5.2 Protein Precipitant

The needed precipitant for crystallization of hOSC and was prepared by mixing 3.152g Tris-HCl (0.1M), 4.62g ammonium acetate, 10g ethylene glycole (5% w/v) and 60g polyethylene glycerol (PEG 4000, 30%). The pH was adjusted to 8.5 with HCl and NaOH.

3.5.3 Crystallization Methods

To crystallize human oxidosqualene cyclase (hOSC) and hOSC mutation 580W the initial crystallization trial was performed using a sitting-drop vapour-diffusion technique with the original concentrations (Table 5) at room temperature. The precipitant was a mixture of Tris-HCl (pH 8.5), NH_4-acetate, ethylene glycol (5%) and polyethylene glycol (30%).

The second step was to optimize the crystallization conditions, by changing the protein concentrations. The original protein concentrations and the optimized concentrations are presented in Table 6.

Table 6. Original concentrations and optimized concentrations of the hOSC-WT and the hOSC-580W

	original concentration [mg/mL]	optimized concentrations [mg/mL]		
hOSC-WT	4.2	3.8	3.8	3.0
hOSC-WT	1.3	1.1	0.8	0.5
hOSC-580W	4.1	3.8	3.5	3.0
hOSC-580W	3.4	3.1	2.8	2.5

To get the best possible results, different crystallization methods were set up for hOSC crystallization. For the experiments the methods vapour diffusion (sitting drop and hanging drop), batch crystallization (microbatch under oil) and counter-diffusion (free interface diffusion) were performed.

In the initial crystallization trial with original hOSC concentrations (Table 5), following protein – precipitant ratios 1:1, 1:2, 1:4, 2:1, 2:2, 2:3, 3:1, 3:2, 4:1 and 4:2 (in µL) were pipetted to the crystallization plate. 200µL precipitant was filled in the reservoir and the plate was stored at room temperature for one week. In the first observations light to heavy precipitation and clear drops were observed and after another check one month later needles formed in some drops. The ratios with the best results were used for the optimization of the crystal conditions. Based on these ratios the original protein concentrations were diluted like presented in Table 6.

The optimized concentrations (Table 6) were used in protein - precipitant ratios – 3:1, 4:1 and 5:2 (in µL). The drops were pipetted to the crystallization plate and stored at room temperature for several weeks.

In the hanging drop method, the optimized concentrations (Table 6) were used in protein - precipitant ratios – 3:1, 4:1 and 5:2 (in µL). Protein and precipitate were pipetted on the siliconized glass cover slide and 400µL precipitant were filled in the reservoir. The glass cover slide with the drop on it was flipped and sealed the reservoir for several weeks at room temperature.

Like in the sitting drop and the hanging drop method the optimized protein concentrations (Table 6) with protein – precipitant ratios – 3:1, 4:1 and 5:2 (in µL) were used. In addition, trials with the original protein concentrations in a protein – precipitant ration of 5:2 (in µL) were pipetted to the oil filled crystallization plate and stored at room temperature.

The original protein concentrations (Table 2) were used to perform the counter-diffusion method. The capillaries of inner diameter of Ø0.8mm were filled with the solutions to form some concentration gradient and sealed with some wax to isolate the system.

3.5.4 Summary of Crystallization Methods

For a better outline all used crystallization techniques are summarized with the corresponding proteins, concentration and ratios in the following Table 7.

Table 7. Summary of crystallization techniques, concentrations and ratios of hOSC-WT and hOSC-580W

	Protein	**Concentration**	**Ratio**
Sitting Drop	hOSC-WT hOSC-580W	new concentration	3:1 4:1 5:2
Hanging Drop	hOSC-WT hOSC-580W	new concentration	3:1 4:1 5:2
Microbatch under Oil	hOSC-WT hOSC-580W	new concentration / original concentration	3:1 4:1 5:2 / 5:2
Counter-diffusion (free interface diffusion)	hOSC-WT hOSC-580W	original concentration	

3.5.5 Testing of Crystals

To check the protein nature of the grown crystals and not salt crystals, the crystals were dyed or destroyed. To dye the crystal, 0.5µL of JBS True Blue were added to the drop containing the crystal and after 30-60 minutes the drop were examined under the microscope to observe the coloured crystals. To destroy the crystal, it was gently tipped against it with a needle.

3.6 Molecular Modelling

Molecular modelling is a collection of computer based techniques for prediction, representing and manipulating the structures and reactions of molecules that are dependent on the three dimensional structures. The methods are used in the fields of computational chemistry, drug design, computational biology and materials science to study molecular systems ranging from small chemical systems to large biological molecules and material assemblies. The common feature of molecular modelling methods is the atomic level description of the molecular systems.

3.6.1 Homology modelling in Phyre2 server

Phyre2 is a 3D protein structure prediction and analyzation web portal. It can build the 3D structure on the base of amino acid sequences, predict ligand binding sites and analyses amino acids in a protein sequence.

In general structure modelling of a protein sequence occurs in four steps, gathering homologous sequences, fold library scanning, loop modelling and side chain placement.

In the first step – gathering homologous sequences – the protein sequence is scanned against a sequence data base (no sequence with >20% identity) and then forms a sequence profile. This sequence profile, meaning the multiple-sequence alignment is used for secondary structure prediction of the sequence.

In the second step – fold library scanning – the sequence is again scanned against a database with proteins of known structure and the resulting alignments are used to form a crude backbone-model.

In step three – loop modelling – loops are corrected by fragmentation and clustering of the structure database.

In step four – side chain placement – the final model is formed by addition of some amino side chains (Kelley et al. 2015).

The hOSC-WT and hOSC-580W sequences were applied to perform the structure prediction by homology modelling in Phyre2.The final structures were downloaded and visualized in the in PyMOL program (Schrödinger LLC).

3.6.2 Visualization of the Structures in PyMOL

PyMOL is a software package for molecular visualization, 3D structures animating and rendering, created by Warren Lyford DeLano.

For visualization of the 3D protein structure the coordinates containing file was opened with PyMOL. The coordinates of every atom at the structure inserted into the program can be shown as cartoon, sticks, surface and much more. If the protein structure can by shown as cartoon helixes, strands and loops can be seen. In the surface option, for example, holes in the structure can be determine. If the protein structure is shown as sticks, all the amino acids structures can be seen, more over the whole sequence can be shown for example in carton and a chosen amino acid can be shown as stick. The program allows to show hydrogens, all the individual atoms and structures, colours for every part of the sequence can be changed and label chains, residues, segments and atoms can be labelled by insertion of the atom/residue names or element symbols.

The predicted structures of hOSC-WT and hOSC-580W from Phyre2 server were analysed and superimposed with the structure of SHC (PDB ID: 1GSZ) (Lenhart *et al.,* 2002). The visualization of the 3D structures and elements and the images were provided by PyMOL.

3.6.3 Theroretical pI and Molecular Calculation

Theoretical pI and molecular weight of the hOSC-WT on the base of the amino acids sequence were calculated at the ExPASy web server. ExPASy means Expert Protein Analysis System and is the SIB Bioinformatics Resource Portal that provides access to scientific databases and software tools in different areas of life sciences including proteomics, genomics, phylogeny, systems biology, population genetics, transcriptomic and so on. (Artimo *et al*. 2012). Compute pI/Mw is a tool which allows the computation of the theoretical pI (isoelectric point) and Mw (molecular weight) for a list of UniProt Knowledgebase (Swiss-Prot or TrEMBL) entries or for user entered sequences (Bjellqvist *et al*., 1993, 1994; Gasteiger *et al*., 2005)

4. RESULTS AND DISCUSSION

4.1 Crystallization of Lysozyme

To check the purity of the Lysozyme a SDS-PAGE (Fig. 19) was done and it was approved that the sample was pure enough for further experiments.

Lysozyme crystallization performed with all discussed crystallization methods and 10% NaCl in sodium acetate buffer pH 4.7 as precipitating agent yielded different results for the different methods.

In the hanging drop vapour diffusion method first 1D needle clusters (Fig. 14a, c) were observed in the period of one week in the following conditions with protein-precipitant drop ratio 1:2, 2:3 and 3:2 (in µL) with the reservoir volumes 275µL, 200µL, 300µL, respectively. 2D needle cluster (Fig. 14a) were grown in a drop with the protein-precipitant drop ratio 1:1 (in µL) and the reservoir volume 275µL and in a drop with protein-precipitant drop ration of 2:3 (in µL) and a reservoir volume of 200µL. Big 3D crystals (Fig. 14 b, c and d) were grown in a drop with a protein-precipitant drop ratio of 1:2, 1:3, 2:3, 3:1, 3:2 (in µL) at all reservoir volumes.

In sitting drop vapour diffusion method 3D crystals (Fig. 15a, b) of different size and shape were observed at all the conditions.

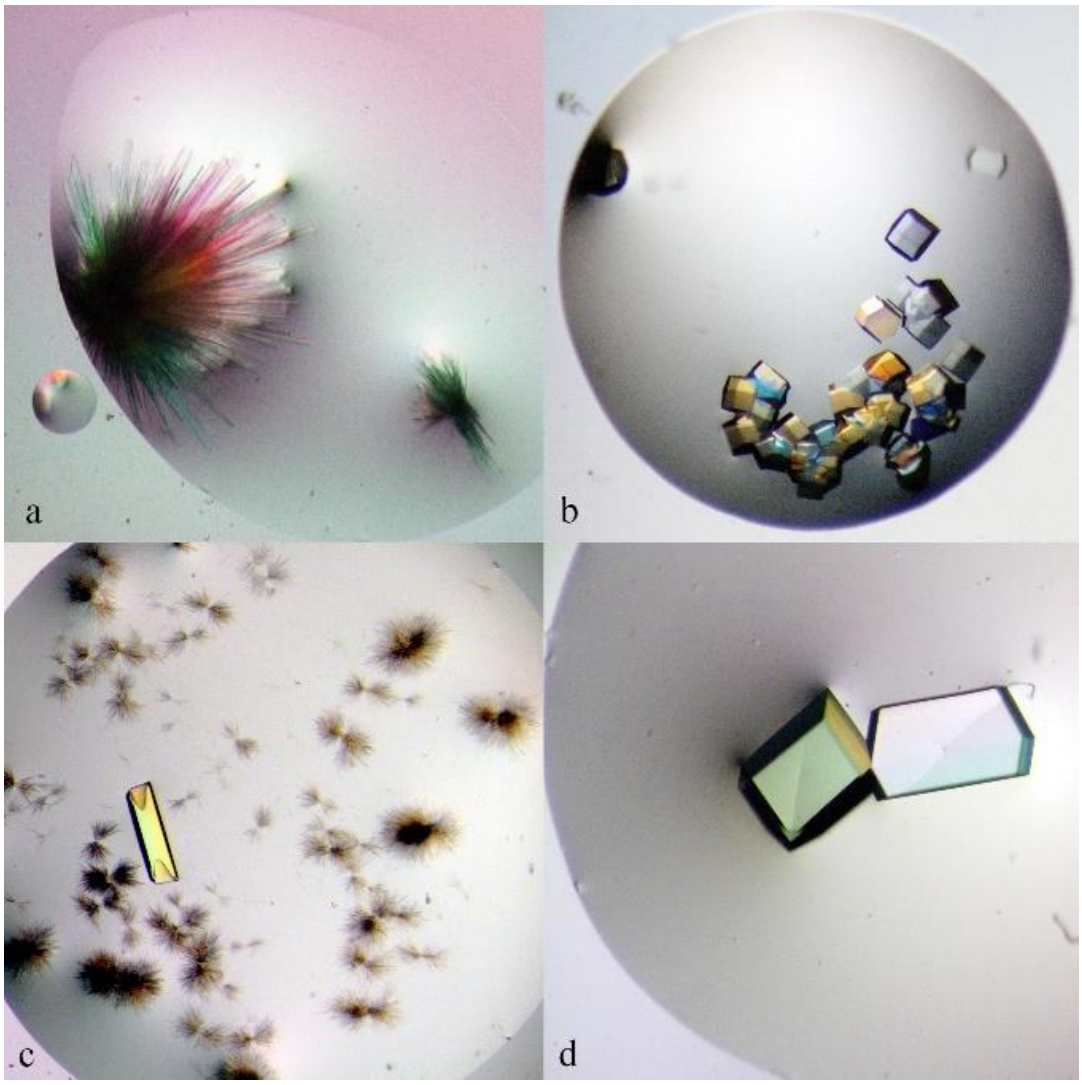

Figure 14. Lysozyme crystals in hanging drop vapour diffusion method: (a) 1D and 2D needle clusters, (b) various 3D crystals of different size, (c) crystal and 1D needle clusters, (d) two well shaped 3D crystals (Pictures created by the author)

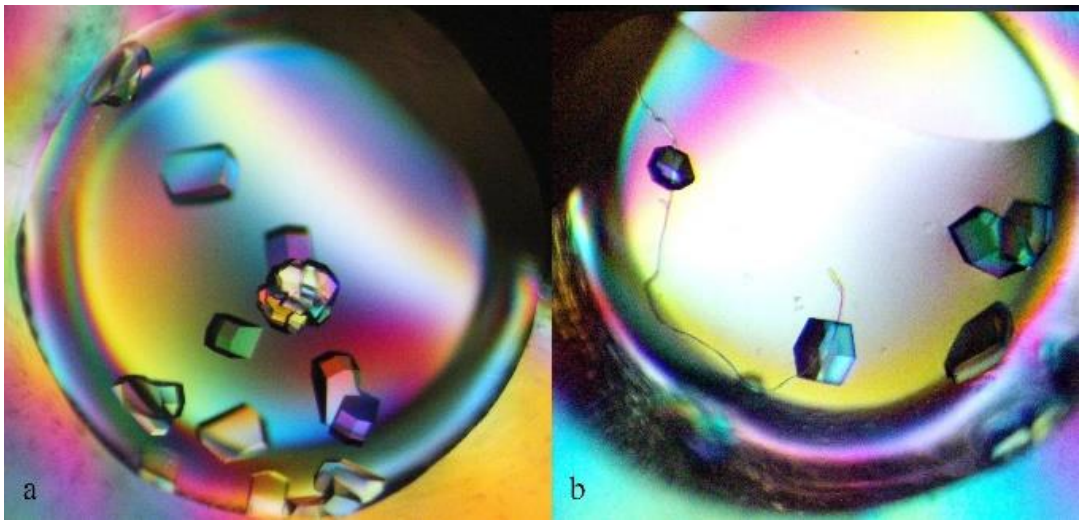

Figure 15. Lysozyme crystals in sitting drop: (a, b) various crystals of different size and shape (Pictures created by the author)

In microbatch under oil technique, 1D needle clusters (Fig. 16a) and big 3D crystals (Fig. 16b) grew within several days at room temperature. 1D needle clusters grew at lower concentration of protein and drop ratios of 2:3.5, 2:4 and 2:5 (in µL). 3D crystals were observed at the rest of the Lysozyme concentrations and the drop ratios. In the free interface diffusion technique big 3D needle clusters and 3D crystals were observed (Fig, 17).

The crystals were tested for the protein nature by dyeing them with JBS True Blue and by the crush test. The crystals absorbed the dye and became blue (Fig. 18a, c, d) and were destroyed (Fig. 18b, c, d) easily, what proved that the crystals were real protein crystals.

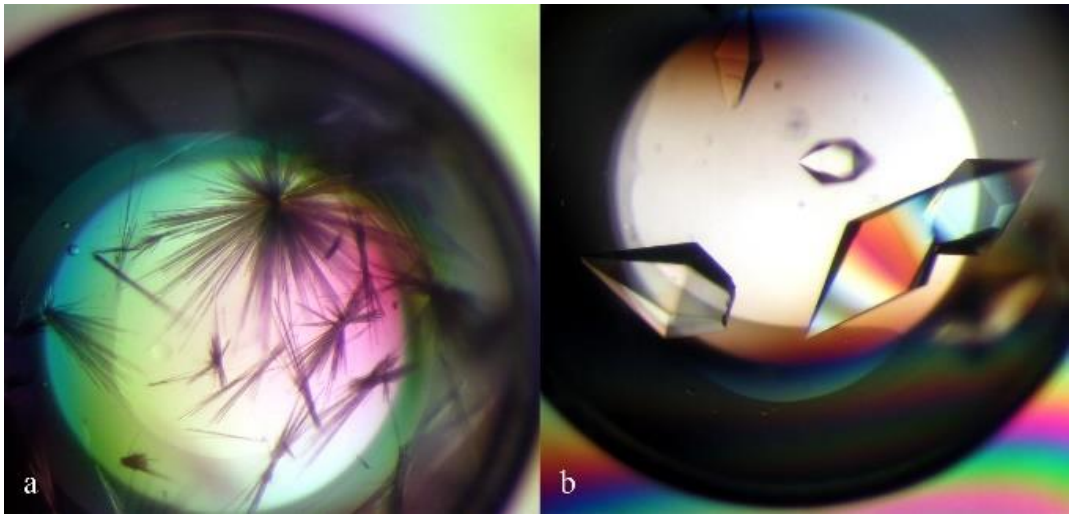

Figure 16. Lysozyme crystals in microbatsch under oil: (a) 1D needles, (b) crystals (Pictures created by the author)

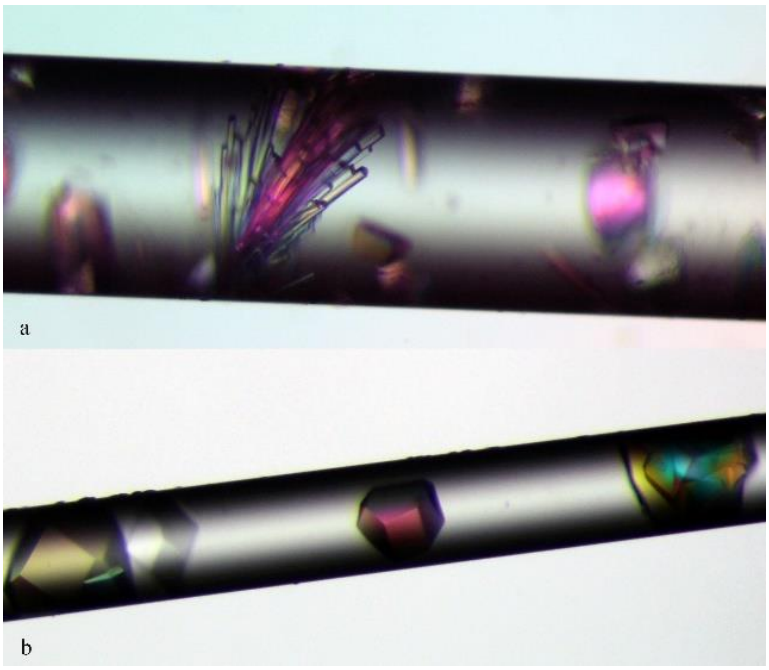

Figure 17. Lysozyme crystals in capillaries: (a) fibers, (b) crystals of different size and shape (Pictures created by the author)

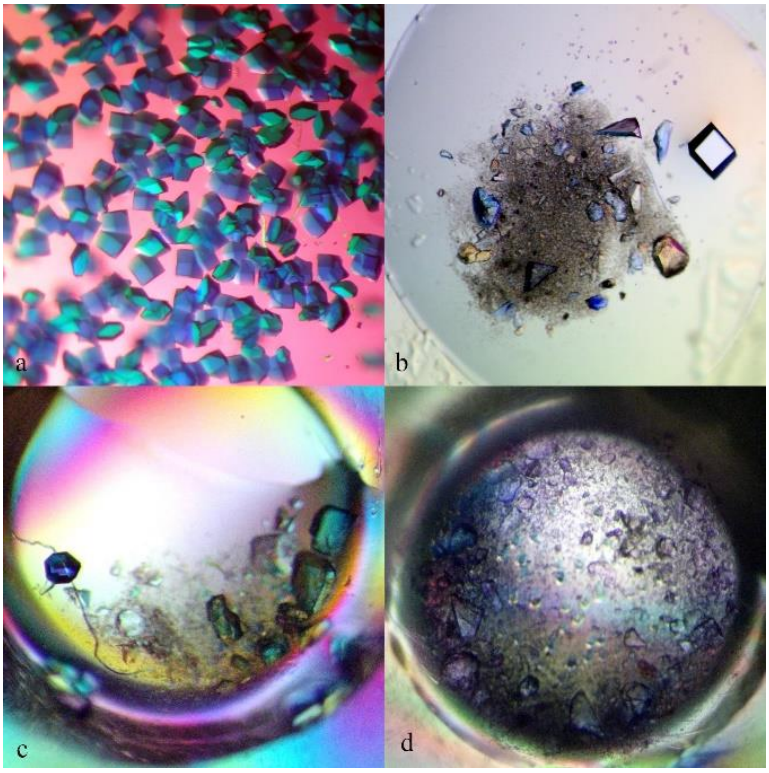

Figure 18. Dyed and destroyed Lysozyme crystals: (a) dyed crystals with JBS True Blue, (b) destroyed crystal in hanging drop, (c, d) destroyed and dyed crystals in sitting drop (Pictures created by the author)

4.2 Crystallization of hOSC-WT and hOSC-580W

In the SDS-PAGE of hOSC-WT and hOSC-580W (Fig. 19) a smear over the whole gel was observed, which indicates that the protein was not completely pure, containing some impurities of other proteins. The sample was purified and prepared in a cooperation laboratory. The impurities could be appeared during storage and transporting procedure from Sweden. Anyway, just these samples were available in very small amount and therefore they were used in the experiments. The protein can be purified only in the cooperation laboratory but we are working on the improvement on the protein purification protocol to produce a better quality sample, which is still in the progress.

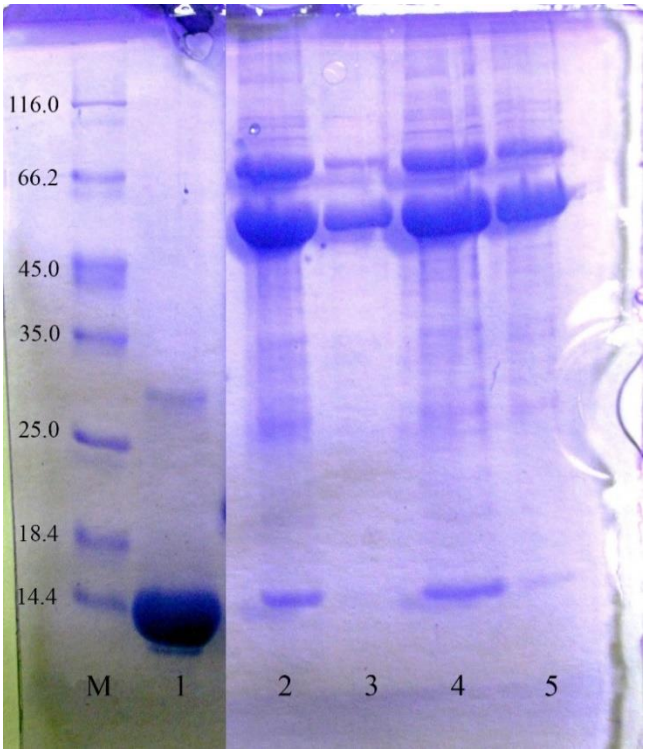

Figure 19. SDS-PAGE of (1) Lysozyme, (2) hOSC-WT 4.2mg/mL, (3) hOSC-WT 1.3mg/mL, (4) hOSC-580W 4.1mg/mL and hOSC-580W 3.4mg/mL. (M) The marker is an Unstained Protein Molecular Weight Marker. (Picture created by the author)

The initial crystallization experiments for human oxidosqualene cyclase and hOSC 580W mutant variant were started with sitting drop vapour diffusion method with the following protein concentrations 1.3, 4.2mg/mL for hOSC-WT and 3.4 and 4.1mg/mL for hOSC-580W. 0.3M NH_4-acetate, ethylene glycol (5%) polyethylene glycol 4000 (30%), Tris-HCl (pH 8.5) as precipitating agent was applied at room temperature. Due to the limitations of the amount of the samples we used the precipitant composition that resulted in a successful crystallization of hOSC in 2004 by A. Ruf and his colleagues (Ruf *et. al.*, 2004).

First observations yielded light to heavy precipitate and clear drops in a couple of days. In the period of three weeks needles (Fig. 20) of different size appeared. Bigger needles formed from the hOSC-580W with a concentration of 3.4mg/mL (Fig. 21) and 4.1mg/mL (Fig. 22) with the protein-precipitant ratio 2:3 (in µL). The crystals were dyed with JBS True Blue to prove the protein nature of them. After the dyeing the needles were intensively blue (Fig. 21, 23). Moreover, the needle was easily destroyed and so this is a further prove.

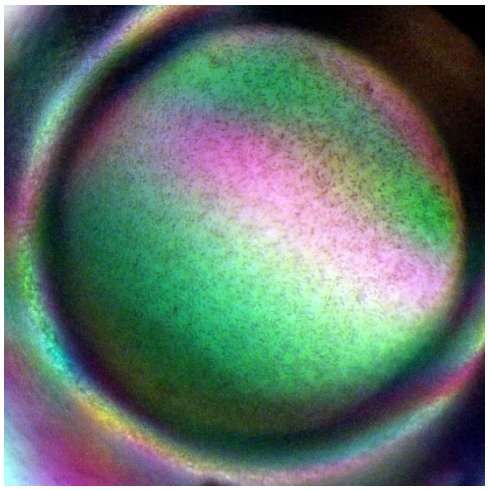

Figure 20. Observation after the initial crystalization trial with the original protein concentrations and sitting drop method. Tiny needles of hOSC-WT 4.2mg/mL 3:1 protein – precipitant ratio. (Picture created by the author)

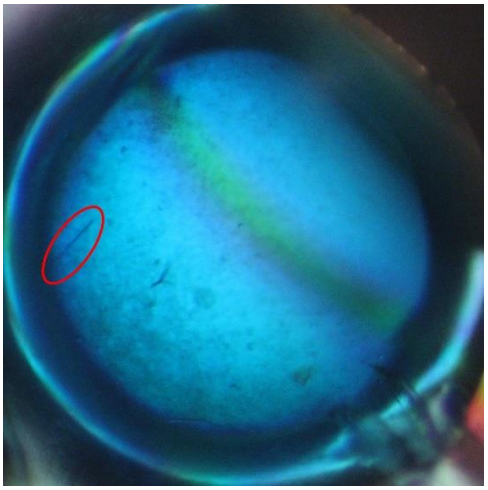

Figure 21. Observation after the initial crystalization trial with the original protein concentrations and sitting drop method. Dyed needle of hOSC-580W 3.4mg/mL 2:3 protein – precipitant ratio. (Picture created

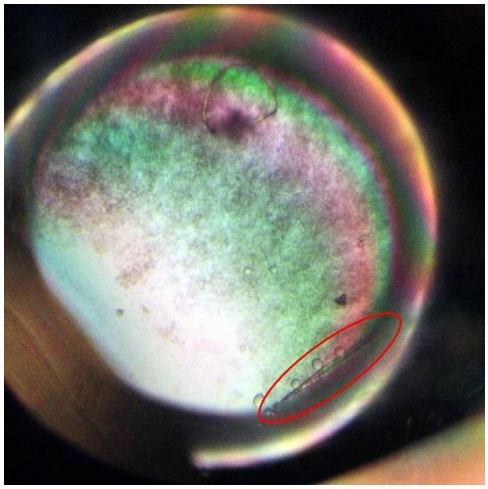

Figure 22. Observation after the initial crystalization trial with the original protein concentrations and sitting drop method. Big needle of hOSC-580W 4.1mg/mL 2:3 protein – precipitant ratio. (Picture created by the author)

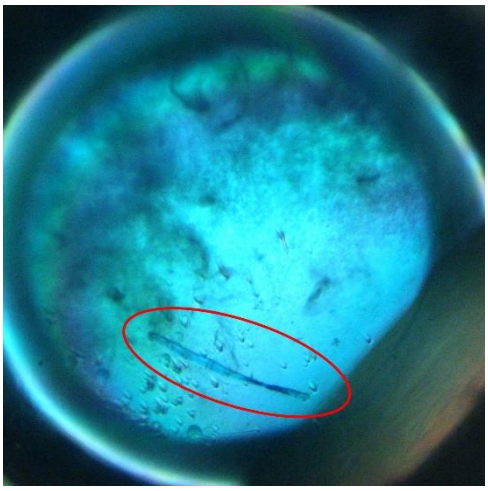

Figure 23. Observation after the initial crystalization trial with the original protein concentrations and sitting drop method. Dyed big needle of hOSC-580W 4.1mg/mL 2:3 protein – precipitant ratio. (Picture created by the author)

The second step was to optimize the crystal size and shape by changing the protein concentrations and application of different crystallization techniques and methods. The following optimized concentrations were used, 0.5, 0.8, 1.1, 3.0, 3.8mg/mL for hOSC-WT and 2.5, 2.8, 3.0, 3.1, 3.4, 3.5, 3.8 and 4.1mg/mL for hOSC-580W. The ratios of the initial crystallization trial with the best results were used for the optimization of the crystal conditions. The best results yielded drops with the protein – precipitant ratio of

3:1 and 4:1 (in µL). These two ratios and an additional ratio of 5:2 were used with the new protein concentrations for sitting drop, hanging drop and microbatch under oil crystallization trials. In the period of two weeks the optimisation process yielded a lot of drops containing tiny needles (Fig. 24 and Fig. 25). Approximately two weeks later a small 2D crystal (Fig. 26 and 27 in detail) grew in one drop with hOSC-WT (1.1mg/mL) protein – precipitate ratio of 5:2 (in µL) by hanging drop method.

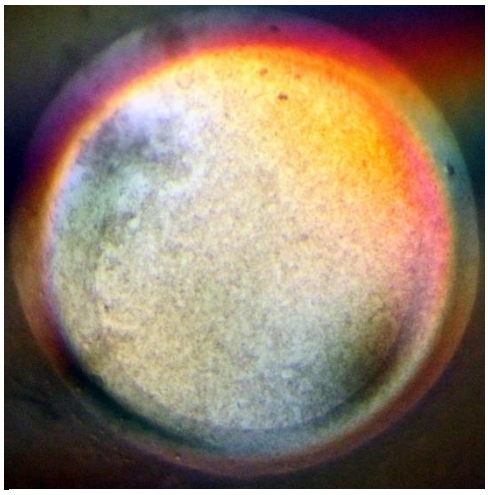

Figure 24. Observation after the optimization of the crystallization conditions. Needles in a drop of microbatch under oil method hOSC-580W 4.1mg/mL (original concentration) 5:2 protein – precipitant ratio. (Picture created by the author)

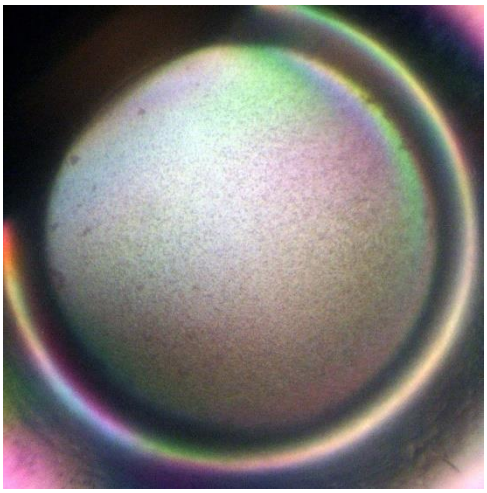

Figure 25. Observation after the optimization of the crystallization conditions. Needles in drop of sitting drop method hOSC-WT 3mg/mL (new concentration) 4:1 protein – precipitant ratio. (Picture created by the author)

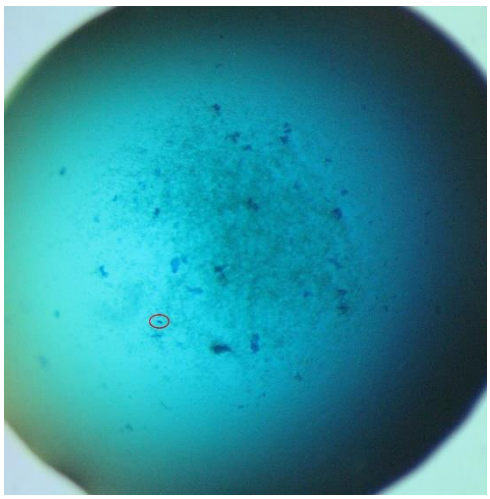

Figure 26. Observation after the optimization of the crystallization conditions. Crystal like structure in drop of hanging drop method hOSC-WT 1.1mg/mL (new concentration) 5:2 protein – precipitant ratio. (Picture created by the author)

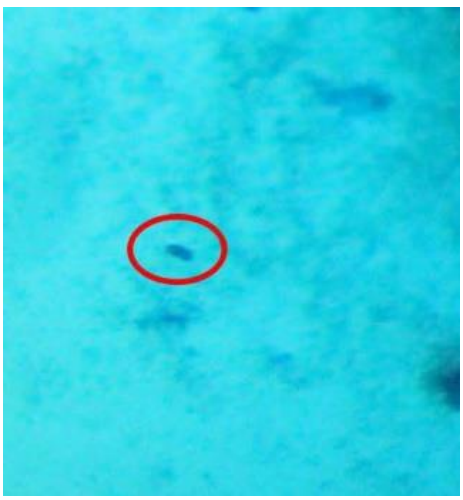

Figure 27. Observation after the optimization of the crystallization conditions. Detail of crystal like structure in drop of hanging drop method hOSC-WT 1.1mg/mL (new concentration) 5:2 protein – precipitant ratio. (Picture created by the author)

4.3 Modelling of 3D Structures of hOSC-WT and hOSC-580W

ExPASy server was used to compute the pI and the molecular weight of hOSC-WT, resulting in a pI of 6.16 and a molecular weight of 83.29kDa. To model the 3D structure of hOSC the multiple sequence alignment was provided in RCSB protein data base to find the closest sequence protein with known structure. The highest sequence identity was just 26%, structure of squalene hopene cyclase (PDB ID 1GSZ) (Lenhart *et al.*, 2002).

Squalene hopene cyclase (Fig. 29) was used as a template for this experiment, it consists of 631 amino acids and the sequence starts with Met at position 1 and ends with Arg at position 631, at position 580 it has the amino acid Tyr. Moreover, the crystal structure contains three chains – chains A, B and C (Fig. 28) and two domains per chain.

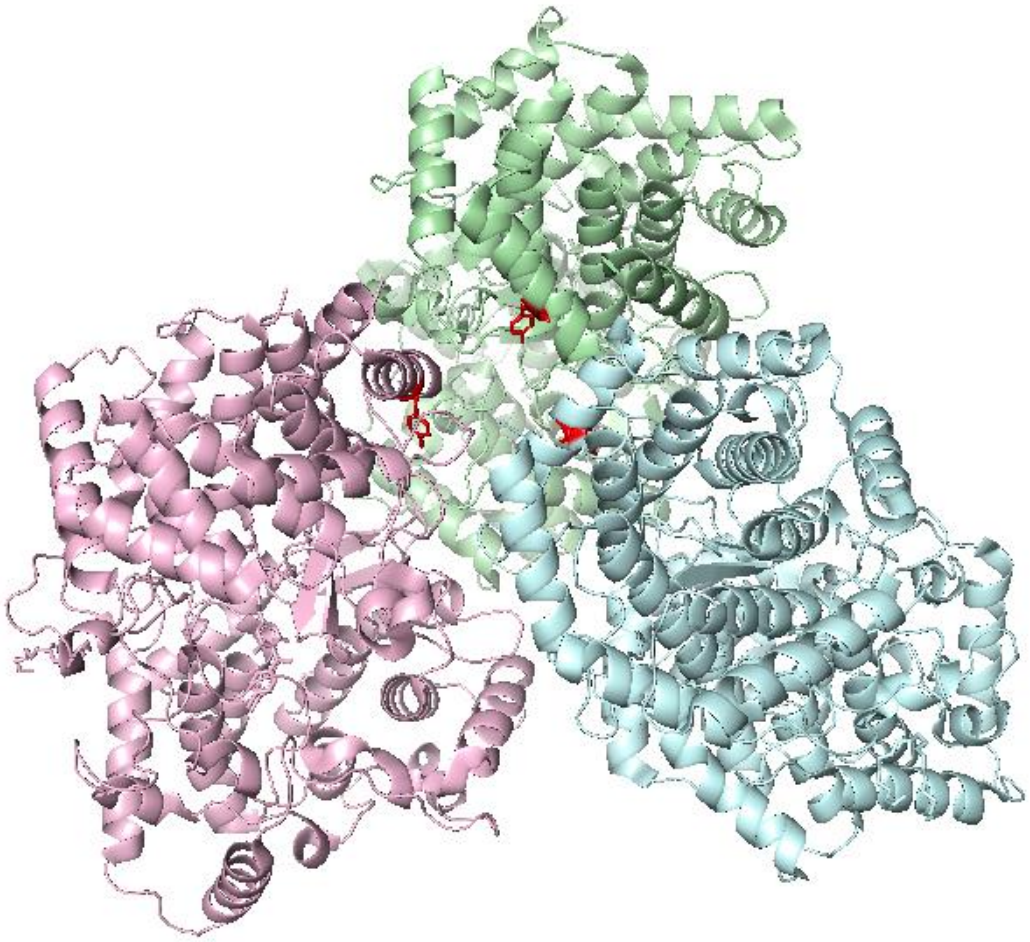

Figure 28. The ribbon representation of the asymmetric unit of the SHC with chain A in green, chain B in blue and chain C in rose. Tyr 580 is shown as a red stick.

The 3D structure of SHC contains 31 α-helices, 35 loops and 4 ß-sheets that are organized in two α-helical compact domains, so called α/α-barrels, connected by 4 β-sheets and loops (Fig. 28) (Lenhart *et al.*, 2002).

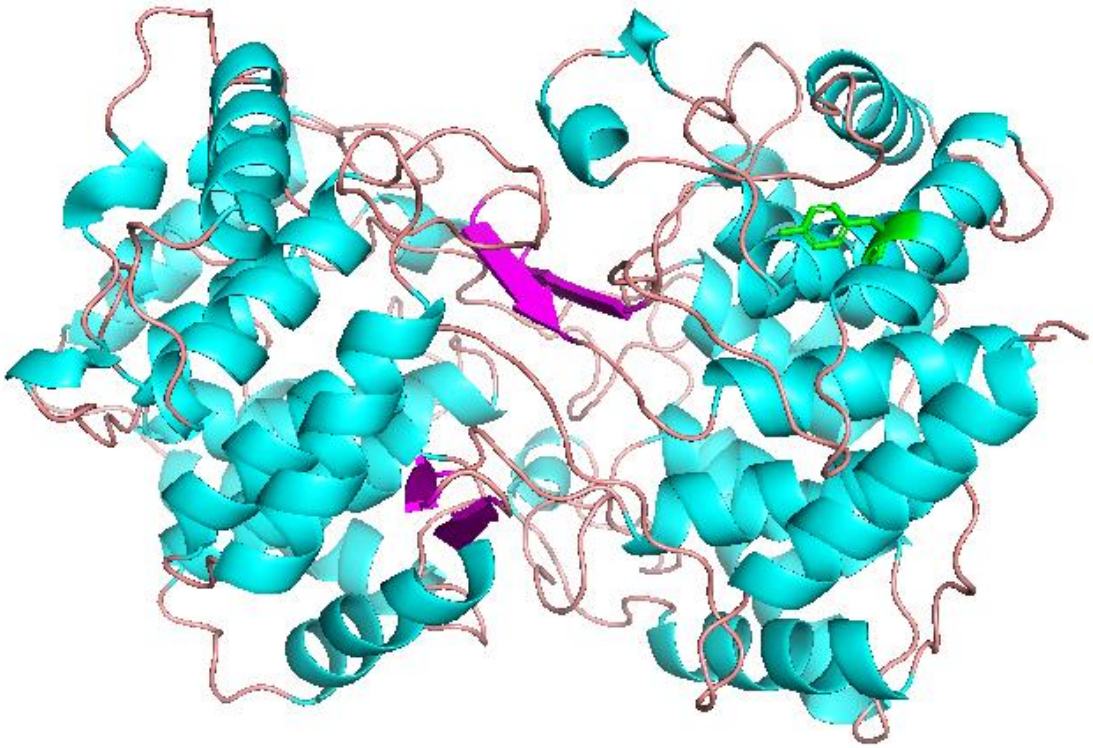

Figure 29. The Cα ribbon trace shows elements of the secondary structure of SHC with the α-Helices in cyan, the loops in rose and the ß-sheets in purple. Tyr 580 is shown as a green stick.

Phyre2 was used to predict the hOSC-WT protein structure by the homology modelling. The sequence of the wild type was used as a template for this and can be found in the Appendix 7.1. The 732 amino acids long hOSC starts with Met at position 1 and ends with Pro at position 732. The hOSC-WT has the amino acid Ser at position 580 and the mutated hOSC-580W has the amino acid Try at this position.

Phyre2 predicted with higher confidence (Appendix 7.2) that the hOSC-WT secondary structure has 28 α-helices, 33 loops and 4 ß-sheets. The secondary structure elements are organized in two α-helical domains which are separated by 4 ß-sheets and loops (Fig. 29).

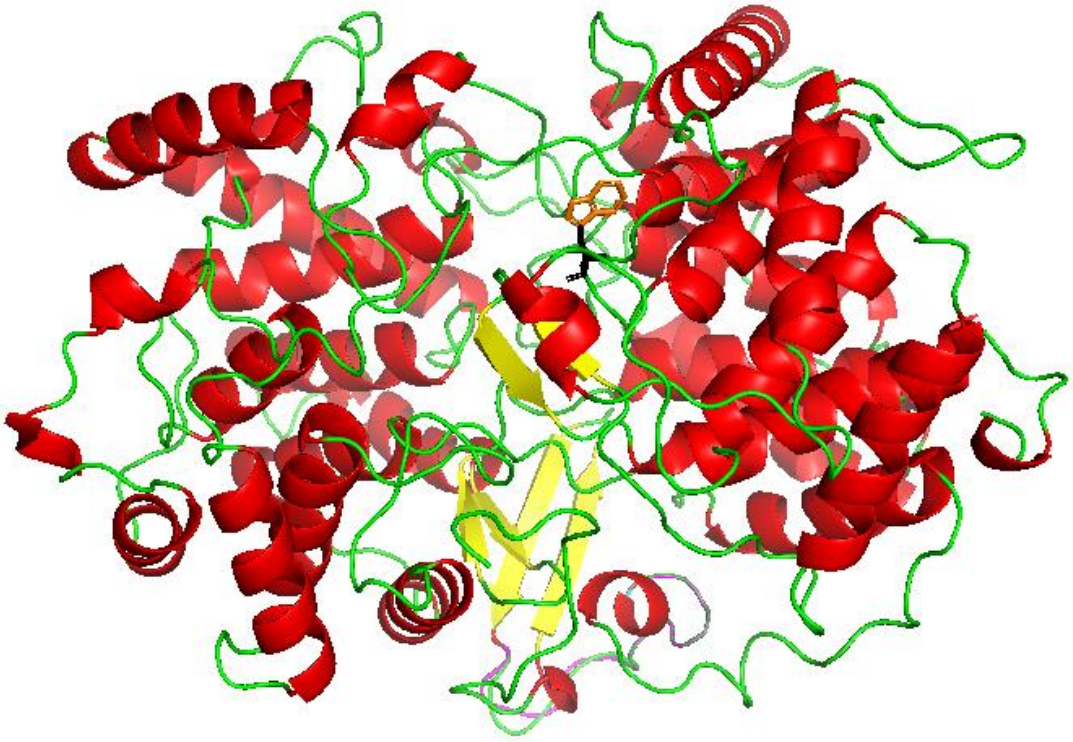

Figure 30. The Cα ribbon trace shows elements of the secondary structure of hOSC with the α-Helices in red, the loops in green and the ß-sheets in yellow. Try 580 is shown as an orange stick.

The structure of hOSC and SHC were superimposed to each other and compared (Fig. 31). Even if the structures are sequentially very low identity the secondary structure elements are very similar. The position of β-sheets, α-helices and domains is very close in both of the structures. Non-structured elements (loops) differ a lot.

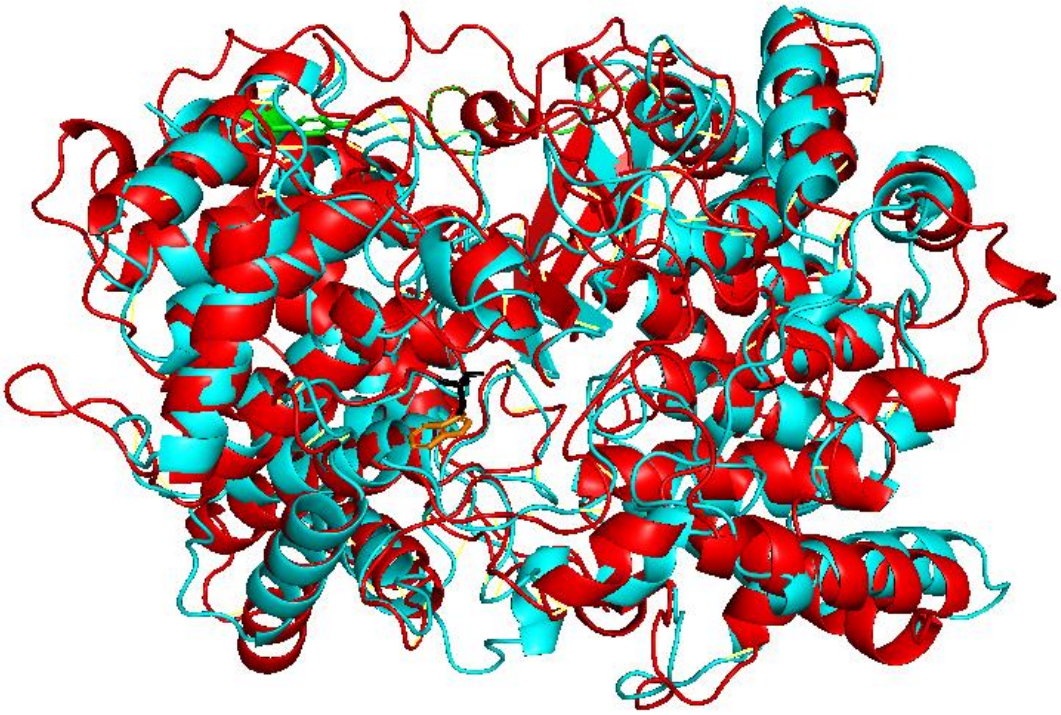

Figure 31. The Cα ribbon trace shows elements of the superimposed secondary structures of SHC in cyan and hOSC in red. Tyr 580, Ser 580 and Try 580 are shown as stick in green, black and orange respectively.

hOSC as well as SHC, has an active-site cavity (Fig. 32 a, b), which is between two domains in the centre of the molecule. Some inhibitors can bind to this active-site cavity like for example Ro 48-8071 (Lenhart et al. 2002, Morand et al. 1997). The active-site cavity of hOSC enzyme is more wide and opened which means that it is able to catalyse the conversion of bigger substrates.

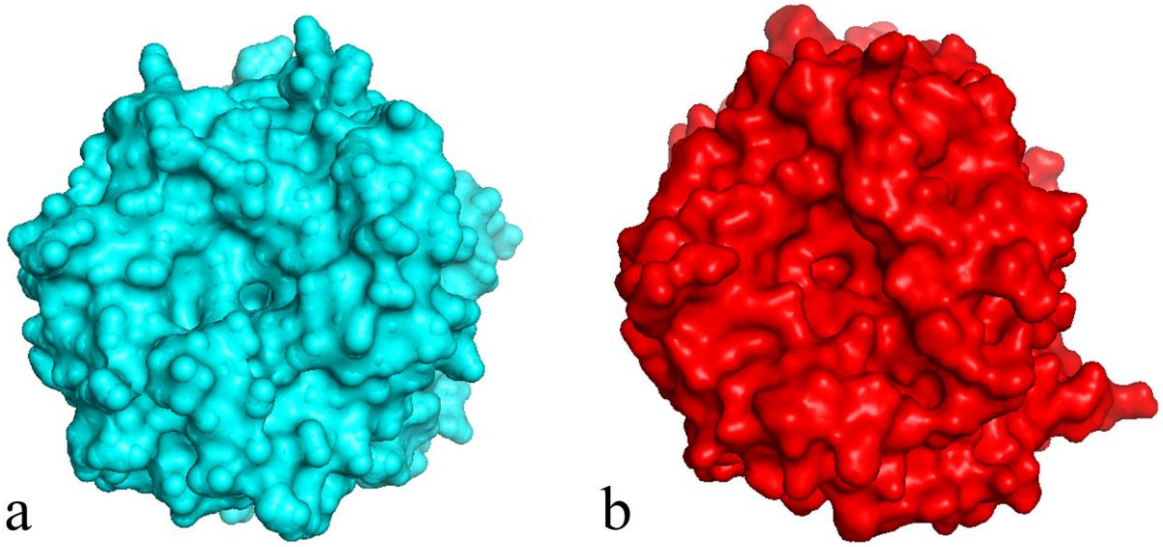

Figure 32. Visualization of solvent content
(a) with the active-site cavity of SHC in the centre of the molecule
(b) and the active-site cavity of hOSC in the centre of the molecule

The sequence of the mutant form (Appendix 7.1) was used as a template for the prediction of the structure in the Phyre2 (Appendix 7.3). The final structure of the mutant form was superimposed with the wild type structure to see the similarities and differences. The mutant form created a point mutation in position 580 by the substitution of the amino acid Ser (Fig. 33a) to Try (Fig. 33b). Figure 33c shows the superimposed secondary structure of hOSC-WT and hOSC-580W with its amino acids in detail.

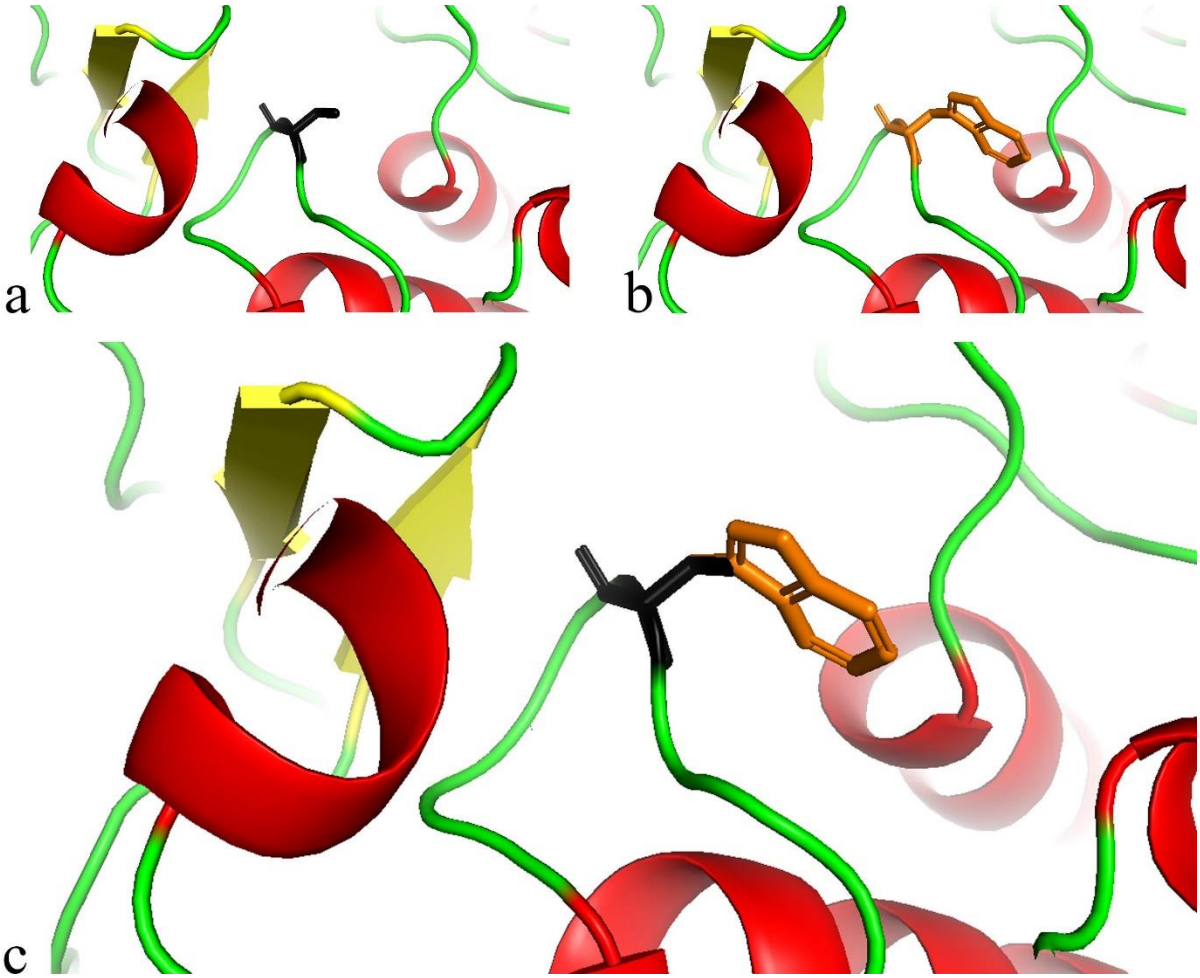

Figure 33. The Cα ribbon trace shows elements of the secondary structures of hOSC-WT and hOSC-580W with the α-Helices in red, the loops in green and the ß-sheets in yellow.
(a) Ser 580 of hOSC-WT shown as black stick in detail
(b) Try 580 of hOSC.580W shown as orange stick in detail
(c) Both, Ser and Tyr 580 shown as black and orange stick respectively

5. CONCLUSION

The goal of this thesis was to learn basic and advanced crystallization techniques and to identify the crystallization conditions for the crystal growth of the model protein Lysozyme. The second and global goal was to crystalize the monotopic integral membrane protein human oxidosqualene cyclase and its mutant variant and to optimize the initial crystallization conditions. Moreover, the 3D hOSC protein structure with the secondary structure elements was modelled and compared with the already known structure of oxidosqualene cyclase.

In order to fulfil these goals, several experiments were set up, starting with the crystallization of Lysozyme. Crystal structures of different quality, like 1D needle clusters, 2D needle clusters, 3D needle clusters and big 3D crystals were obtained by hanging drop technique, sitting drop technique, microbatch under oil technique and free interface diffusion technique. The crystal structures of Lysozyme, meaning size and shape, can be influenced by the reservoir volume, the different crystallization techniques, temperature and the protein-precipitant ratios. To crystalize human oxidosqualene cyclase and its mutant variant the procedure was started with some initial crystallization experiments by sitting drop vapour diffusion method and the original protein concentrations. This initial crystallization experiments resulted in light to heavy precipitate and clear drops within a few days and needles of different size within three weeks. The needles were proven to be of protein nature by dyeing test and crush test. Due to this observation the crystallization conditions were optimized by changing the protein concentrations und using different crystallization techniques. With the optimized conditions within two weeks a lot of tiny needles formed in a drop and again within two weeks a small 2D crystal formed in one drop of hOSC-WT with protein-precipitant ratio of 5:2 (in µL) by hanging drop method.

To model the 3D structure of hOSC, the already known squalene hopene cyclase was used as a template in this experiment. Although it has just 26% sequence identity, the secondary structure elements are very similar. Moreover, Phyre2 was used for the hOSC-WT and hOSC-580W protein structure prediction. This showed, with higher confidence, that the hOSC secondary structure has 28 α-helices, 33 loops and 4 ß-sheets. Additionally, the hOSC-WT has Ser at position 580 whereas the mutated hOSC-580W has Try at this position. Both, hOSC and SHC have an active-site cavity, which is found between two domains in the centre of the molecule and function as catalysts.

For further steps the colleagues from the cooperating laboratory have to optimize the protein purification protocol to produce a better quality sample and then the gained information from this work can be used for further experiments to grow quality 3D crystals in the future.

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7. APPENDIX

7.1 Protein Sequences

Sequence of 1GSZ

```
1   MAEQLVEAPA YARTLDRAVE YLLSCQKDEG YWWGPLLSNV TMEAEYVLLC HILDRVDRDR
61  MEKIRRYLLH EQREDGTWAL YPGGPPDLDT TIEAYVALKY IGMSRDEEPM QKALRFIQSQ
121 GGIESSRVFT RMWLALVGEY PWEKVPMVPP EIMFLGKRMP LNIYEFGSWA RATVVALSIV
181 MSRQPVFPLP ERARVPELYE TDVPPRRRGA KGGGGWIFDA LDRALHGYQK LSVHPFRRAA
241 EIRALDWLLE RQAGDGSWGG IQPPWFYALI ALKILDMTQH PAFIKGWEGL ELYGVELDYG
301 GWMFQASISP VWDTGLAVLA LRAAGLPADH DRLVKAGEWL LDRQITVPGD WAVKRPNLKP
361 GGFAFQFDNV YYPDVDDTAV VVWALNTLRL PDERRRRDAM TKGFRWIVGM QSSNGGWGAY
421 DVDNTSDLPN HIPFCDFGEV TDPPSEDVTA HVLECFGSFG YDDAWKVIRR AVEYLKREQK
481 PDGSWFGRWG VNYLYGRGAV VSALKAVGID TREPYIQKAL DWVEQHQNPD GGWGEDCRSY
541 EDPAYAGKGA STPSQTAWAL MALIAGGRAE SEAARRGVQY LVETQRPDGG WDEPYYTGTG
601 FPGDFYLGYT MYRHVFPTLA LGRYKQAIER R
```

Sequence of the wild type (WT):

```
  1 MTEGTCLRRR GGPYKTEPAT DLGRWRLNCE RGRQTWTYLQ DERAGREQTG LEAYALGLDT
 61 KNYFKDLPKA HTAFEGALNG MTFYVGLQAE DGHWTGDYGG PLFLLPGLLI TCHVARIPLP
121 AGYREEIVRY LRSVQLPDGG WGLHIEDKST VFGTALNYVS LRILGVGPDD PDLVRARNIL
181 HKKGGAVAIP SWGKFWLAVL NVYSWEGLNT LFPEMWLFPD WAPAHPSTLW CHCRQVYLPM
241 SYCYAVRLSA AEDPLVQSLR QELYVEDFAS IDWLAQRNNV APDELYTPHS WLLRVVYALL
301 NLYEHHHSAH LRQRAVQKLY EHIVADDRFT KSISIGPISK TINMLVRWYV DGPASTAFQE
361 HVSRIPDYLW MGLDGMKMQG TNGSQIWDTA FAIQALLEAG GHHRPEFSSC LQKAHEFLRL
421 SQVPDNPPDY QKYYRQMRKG GFSFSTLDCG WIVSDCTAEA LKAVLLLQEK CPHVTEHIPR
481 ERLCDAVAVL LNMRNPDGGF ATYETKRGGH LLELLNPSEV FGDIMIDYTY VECTSAVMQA
541 LKYFHKRFPE HRAAEIRETL TQGLEFCRRQ QRADGSWEGS WGVCFTYGTW FGLEAFACMG
601 QTYRDGTACA EVSRACDFLL SRQMADGGWG EDFESCEERR YVQSAQSQIH NTCWAMMGLM
661 AVRHPDIEAQ ERGVRCLLEK QLPNGDWPQE NIAGVFNKSC AISYTSYRNI FPIWALGRFS
721 QLYPERALAG HP
```

Sequence of the mutation (580W):

```
  1 MTEGTCLRRR GGPYKTEPAT DLGRWRLNCE RGRQTWTYLQ DERAGREQTG LEAYALGLDT
 61 KNYFKDLPKA HTAFEGALNG MTFYVGLQAE DGHWTGDYGG PLFLLPGLLI TCHVARIPLP
121 AGYREEIVRY LRSVQLPDGG WGLHIEDKST VFGTALNYVS LRILGVGPDD PDLVRARNIL
181 HKKGGAVAIP SWGKFWLAVL NVYSWEGLNT LFPEMWLFPD WAPAHPSTLW CHCRQVYLPM
241 SYCYAVRLSA AEDPLVQSLR QELYVEDFAS IDWLAQRNNV APDELYTPHS WLLRVVYALL
301 NLYEHHHSAH LRQRAVQKLY EHIVADDRFT KSISIGPISK TINMLVRWYV DGPASTAFQE
361 HVSRIPDYLW MGLDGMKMQG TNGSQIWDTA FAIQALLEAG GHHRPEFSSC LQKAHEFLRL
421 SQVPDNPPDY QKYYRQMRKG GFSFSTLDCG WIVSDCTAEA LKAVLLLQEK CPHVTEHIPR
481 ERLCDAVAVL LNMRNPDGGF ATYETKRGGH LLELLNPSEV FGDIMIDYTY VECTSAVMQA
541 LKYFHKRFPE HRAAEIRETL TQGLEFCRRQ QRADGSWEGW WGVCFTYGTW FGLEAFACMG
601 QTYRDGTACA EVSRACDFLL SRQMADGGWG EDFESCEERR YVQSAQSQIH NTCWAMMGLM
661 AVRHPDIEAQ ERGVRCLLEK QLPNGDWPQE NIAGVFNKSC AISYTSYRNI FPIWALGRFS
721 QLYPERALAG HP
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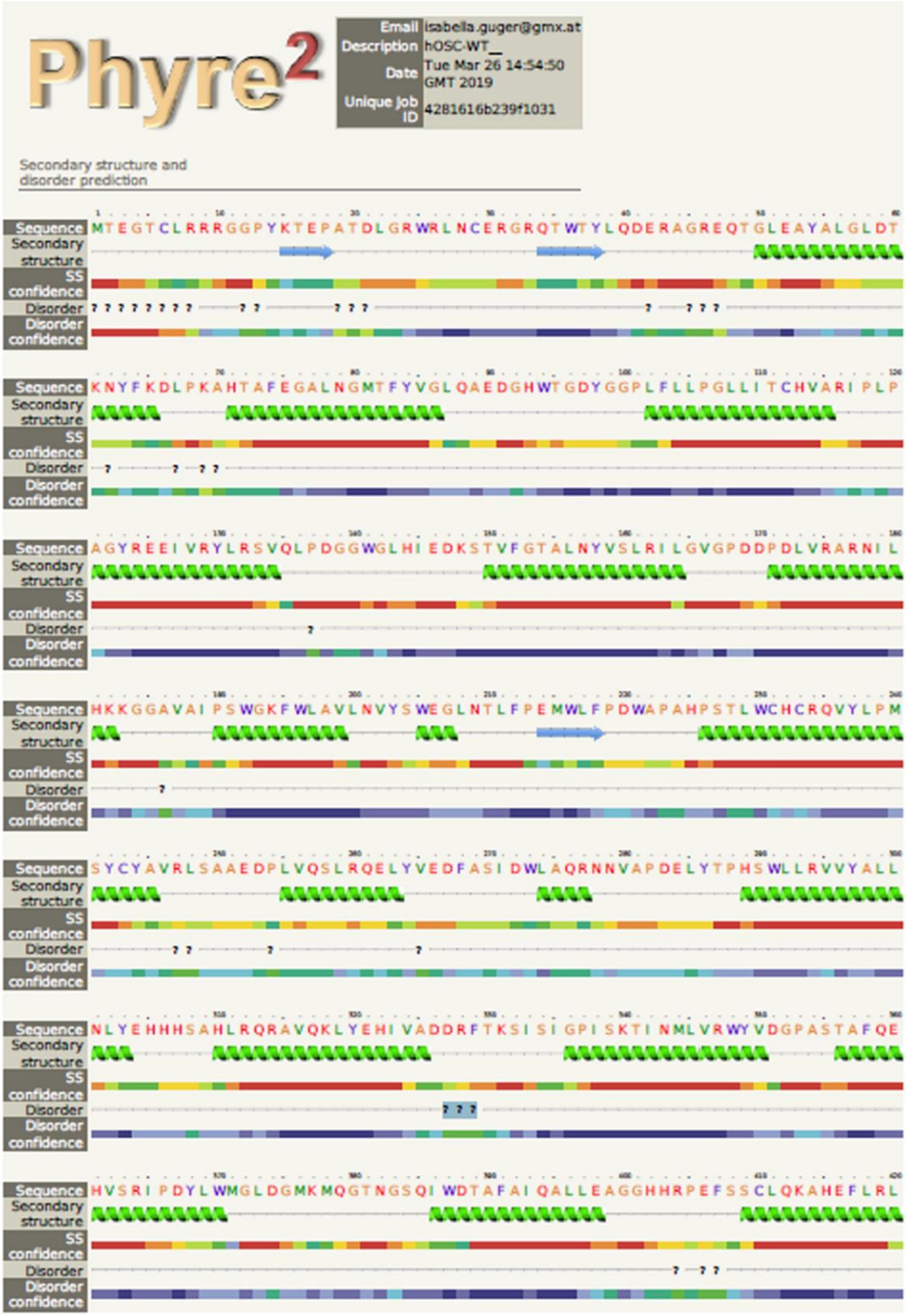
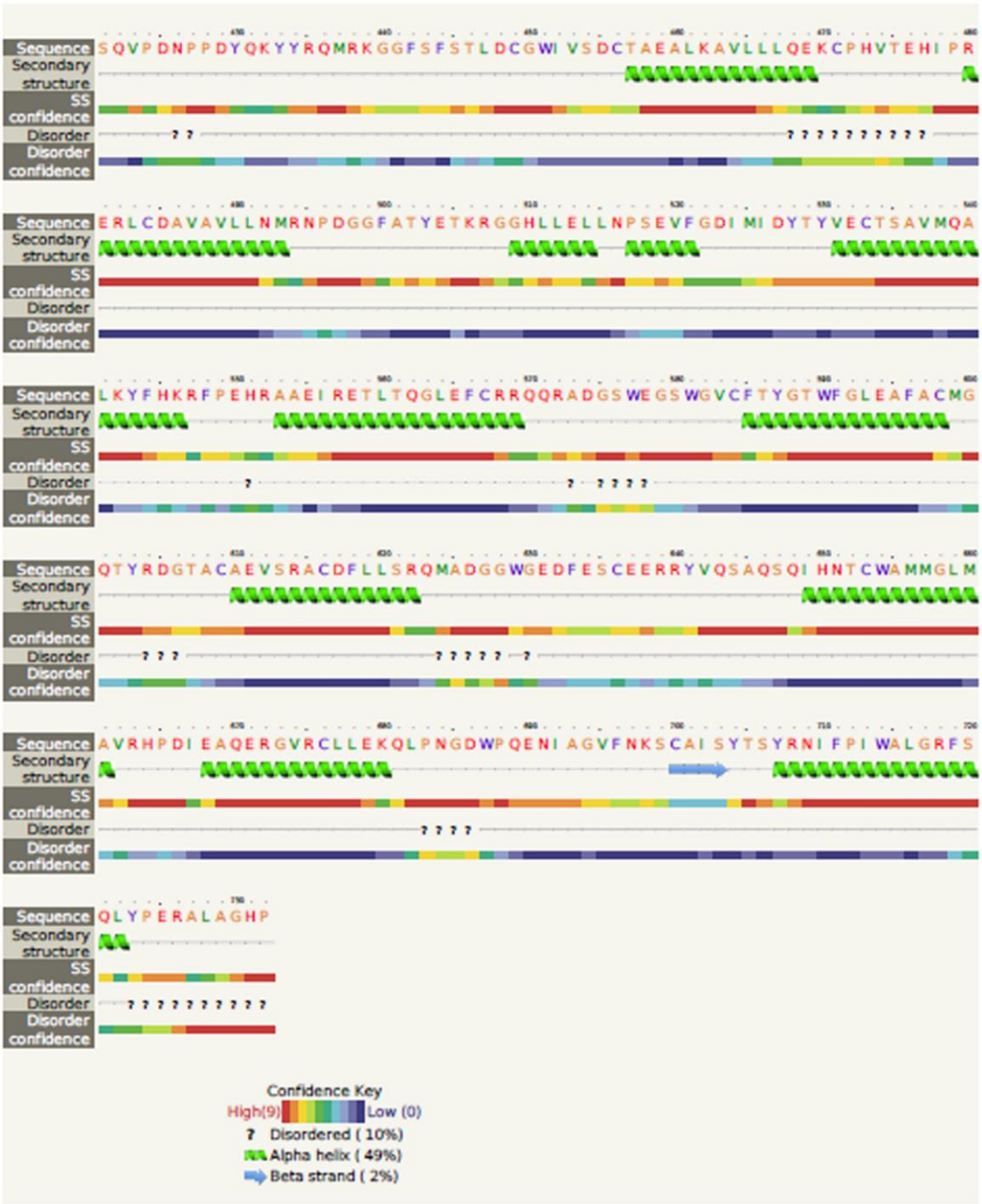
7.2 Data of Phyre2 server of hOSC-WT




Sequence
Secondary structure
SS confidence
Disorder
Disorder confidence
Confidence Key
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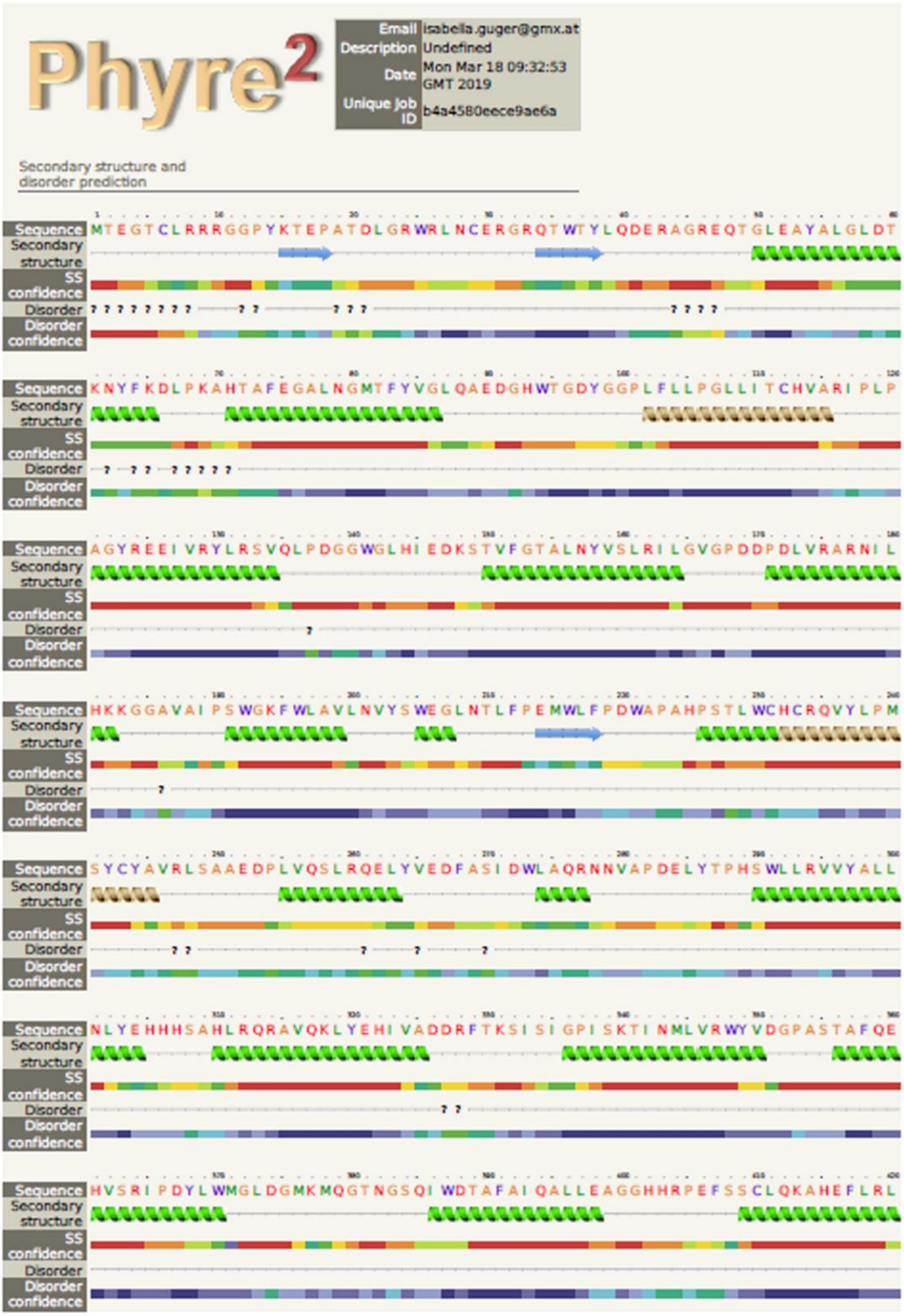
Low (0)
Disordered (10%)
Alpha helix (49%)
Beta strand (2%)

7.3 Data of Phyre2 server of hOSC-580W




Sequence
Secondary structure
SS confidence
Disorder
Disorder confidence
Confidence Key
High(9) Low (0)
? Disordered (9%)
Alpha helix (49%)
Beta strand (2%)
TM helix (9%)

7.4 Poster

Guger I., Kurtenc C., Syrenc P.-O., Kuta-Smatanova I., Prudnikova T.: Crystallization and structure-functional prediction of human oxidosqualene cyclase. Proteins in action – biophysical techniques for protein research, Materials structure in Chemistry, Biology, Physics and Technology 24, 2 (2017), p.106, Proteins in action – biophysical techniques for protein research, 26 -28 June 2017, Ceske Budejovice,Czech Republic.

Crystallisation and structure-functional prediction of human oxidosqualene cyclase

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Introduction

Oxidosqualene cyclase (OSC) is a monotopic integral membrane protein in eukaryotic cells. It catalyses the formation of the first sterol scaffold, the lanosterol, which is very important step for the development of hypocholestrolemic drugs [1], [2]. Human oxidosqualene cyclase (hOSC) 83kDa has the highest sequence identity 26% with one of the deposited structure of OSC (RSCB PDB ID 1GSZ). The protein hOSC was crystallised and the structure was predicted by homology modelling [3] with 100% confidence for all the residues. A superimposition of the structures reviled a higher similarity in the secondary structure elements. The hOSC protein consisted of two α/ α barrel domains with a large central cavity enclosed by loops and a short five stranded sheet linking the barrel domains. An activity of the protein is very sensitive to the temperature. In order to avoid this several mutant forms were constructed. One of the variant hOSCT580W shows more "sever" effect with optimal temperature in 22°C instead of 37°C for the wild type protein. The hOSC structural information will provide a basis for further studies of the human oxidosqualene cyclases and the development of the application in hypocholestrolemic drug design.

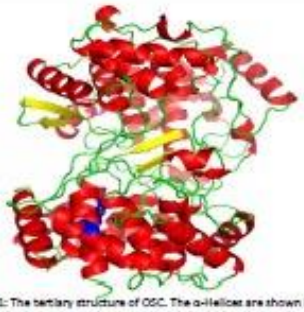


Fig.1: The tertiary structure of OSC. The α-Helices are shown in red, the loops are shown in green and the β-sheets are yellow. The Tyrosine is shown in blue.

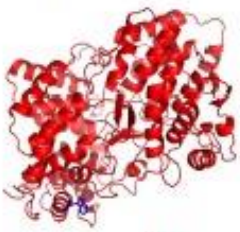


Fig.2:

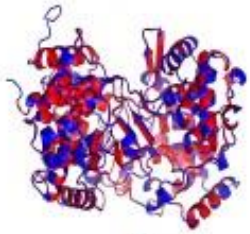


Fig.3:

Fig.4:

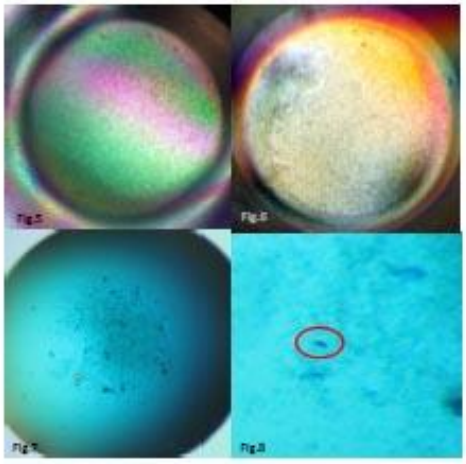
Fig.2: The tertiary structure of the mutation 850W with the amino acid Tryptophan in blue and the α-Helices, β-sheets and loops in red.

Fig.3: The tertiary structure of OSC (in red) superimposed with the mutation 580W (in blue).

Fig.4: The amino acids of the OSC and the mutation 850W in detail. The Tyrosine of the OSC is shown in red and the Tryptophan of the mutation 580W in blue.

Crystallization

We tried to crystallise the human oxidosqualene cyclase (hOSC) and the hOSC mutation 580W. In the first crystallisation experiment we started with the original concentrations (1.3mg/ml and 4.2mg/ml for the wild type and 3.4mg/ml and 4.1mg/ml for the mutation). The precipitant was a mixture of Tris-HCl (pH=8.5), NH4-acetate, ethylene glycol (5%) and polyethylene glycol (30%). In the fist observations we were able to observe light to heavy precipitation and some clear drops. After another check we found some needles in some drops (Fig.5).
The second step was to optimize the crystallisation conditions, because of this we changed the concentrations of the proteins (1.3mg/ml was diluted to 1.1mg/ml, 0.8mg/ml and 0.5mg/ml and 4.2mg/ml was diluted to 3.8mg/ml, 3.5mg/ml and 3mg/ml for the wild type; 3,4mg/ml was diluted to 3.1mg/ml, 2.8mg/ml and 2.5mg/ml and 4.1mg/ml was diluted to 3.8mg/ml, 3.5mg/ml and 3mg/ml) and used different crystallisation methods like hanging drop, sitting drop, micro batch and capillary. In the first observation after the optimisation process we found a lot of drops with needles in there (Fig.6) and approximately four weeks later we observed a small crystal like structure in one drop (Fig.7 and Fig.8 in detail).
This first crystallization conditions can now be used for further work and optimizations to get nice crystals and results in the future.



References

[1] A. Ruf, F. Müller, B. D'Arcy, M. Odhie, E. Kusznir, C. Handschin, O. H. Morand, R. Thoma (2004). The monotopic membrane protein human oxidosqualene cyclase is active as monomer. Biochem Biophys Res Commun. 315(2):247-54.
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[3] L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass, M. J. Sternberg (2015). The Phyre2 web portal for protein modelling, prediction and analysis Nat Protoc. 10, 845-858.