UNIVERSITY OF TARTU
Faculty of Science and Technology
Institute of Technology

Vladimir Gruzdev

DNA unwinding and annealing activity OpIrc3 of the thermotolerant yeast *Ogataea polymorpha*

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Supervisor(s):

Professor Juhan Sedman, MSc Vlad Julian Piljukov

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Abstract:

Helicases of Superfamily 2 are one of the most diverse groups of helicases that play crucial role in RNA and DNA metabolism. The Superfamily 2 DNA helicase Irc3 is essential for mitochondrial DNA maintenance in *Saccharomyces cerevisiae*. Recently proposed assumption that Irc3 is a functional homolog to RecG helicase, provides a new insight on Irc3 activity. Research conducted demonstrated the ability of Irc3 to anneal and unwind DNA substrates.

Keywords: DNA helicase, yeast, annealing, unwinding

CERCS: P310 Proteins, enzymes

Termotolerantse Ogataea polymorpha proteiini OpIrc3 DNA-d lahti ja kokku keeramise aktiivsus

Lühikokkuvõte:

Superperekond 2 helikaasid on üks kõige mitmekesisemaid helikaaside rühmi, millel on RNA ja DNA metabolismis ülioluline roll. Superperekond 2 DNA helikaas Irc3 on oluline mitokondriaalse DNA stabiilsuse säilitamiseks Saccharomyces cerevisiae's. Hiljuti väljapakutud hüpotees, et Irc3 on RecG helikaasi funktsionaalne homoloog, loob uue suuna Irc3 aktiivsuse uurimiseks. Antud töös läbiviidud uuringud näitasid Irc3 võimet DNA substraate kokku ja lahti keerata.

Võtmesõnad: DNA helikaas, DNA lahti keeramine, DNA kokku keeramine

CERCS: P310 Proteiinid, ensüümid

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TERMS, ABBREVIATIONS AND NOTATIONS

SF – superfamily

SF2 – superfamily 2

ssDNA – single stranded DNA

dsDNA – double stranded DNA

HD – helicase domain

mtSSB – mitochondrial single strand binding protein

POLγ – polymerase γ

oriH - heavy strand mtDNA replication origin

oriL - light strand mtDNA replication origin

POLRMT - mitochondrial RNA polymerase

INTRODUCTION

Helicases are generally a group of ATP-dependent proteins that play major role in the metabolism of RNA and DNA participating in several processes like replication, repair, recombination, transcription, chromosome segregation, and telomere maintenance (Wu, 2012). Typically, helicases unwind DNA, RNA or DNA-RNA helix by applying the energy derived from nucleoside triphosphate hydrolysis, however there are several helicases which also have annealing activity together or separately from the unwinding or show only ATP-based translocating activity (Wu, 2012). Disruption of their activities is often associated with different diseases such as cancers, developmental defects, and neurodegenerative diseases (Fairman-Williams et al., 2010). For our research we chose *S. Cerevisiae* as a model organism as it provides a great opportunity to systematically analyze the effects of various enzymes on the stability of mtDNA as 'budding yeast' can grow without a functional respiratory chain. Although it suits well for our research, it still possesses some drawbacks, in *vitro* studies of *S. Cerevisiae* are complicated due to protein instability. One of the major drawbacks in assays used previously was the rapid inactivation of Irc3 at temperatures higher than 30℃, therefore limited thermal instability altered the biochemical and structural studies of protein, on the contrary homolog encoded by the genome of *Ogataea polymorpha* stays active at the temperatures exceeding 40℃ (V.-J. Piljukov et al., 2022). Taking this into account we decided to use the $Irc3_{op}$ in our research and to develop a new assay optimized for this protein.

1 STRUCTURE OF SF2 DNA HELICASES

Based on conserved features of their sequences helicases are divided into 6 main groups – superfamilies (SF) and corresponding subgroups in accordance with their motifs – a set of highly conserved amino acid sequences. SF1 and SF2 typically form monomeric or dimeric structures and SF3-SF6 form higher count multimers (Fairman-Williams et al., 2010). The motif sequences are commonly known to reside in a core region of 200-700 amino acids, separated by stretches of low sequence but high length conservation (Raney et al., 2013). So far there have been described 9 primary helicase motifs which regulate the structural and biochemical functions of the SF2 (Q, I, Ia, Ib, II, III, IV, IVa, V, Va, and VI) spread among two main domains of the conserved helicase core which consist of RecA-like folds – helicase domains HD1 (motifs: Q, I, Ia, Ib, II, and III) and HD2 (motifs: IV, IVa, V, Va and VI) (Beyer et al., 2013). Motifs I and II (DE(H/A)H and SAT, respectively) – SF2 helicase motifs, places Irc3 proteins to SF2 RNA/DNA helicases (V.-J. Piljukov et al., 2022). The highest level of sequence conservation over the SF2 is in residues that arrange binding and hydrolysis of triphosphate (motifs I, II, VI). SF2 motif Q arranges the adenine base (Ding et al., 2015). Motifs Ia, Ib, IV, IVa, V are creating large-scale contacts with the phosphodiester backbone of the DNA lattice and motif III is responsible for ATP hydrolysis energy transmission into the motor function. Motifs I, II, and VI supposedly act as a helicase motor by using the energy obtained by consuming NTPs for translocation along ssDNA/RNA and DNA/RNA duplex unwind (Hall & Matson, 1999). Motifs (I, Ia, II, III, IV, V, and VI) manage the energy obtained from ATP hydrolysis into unwinding and remodeling (Ding et al., 2015). There is no clear evidence what motifs are of the most importance for the annealing, therefore it is considered that all motifs are used to some extent.

2 SF2 HELICASE IN DNA REPLICATION AND RECOMBINATION

In the process of DNA replication, helicases translocate along the dsDNA unwinding it into ssDNA, creating template for complimentary DNA strand formation (Bleichert et al., 2017). Human SF2 replicative helicase TWINKLE is essential for *in vivo* mitochondrial DNA (mtDNA) synthesis. TWINKLE in combination with mtDNA polymerase γ (POLγ) and mitochondrial ssDNA-binding protein (mtSSB) forms a replisome which synthesises ssDNA molecules. Instead of common lagging and leading strands, mtDNA is unwound to rich in guanine and rich in cytosine heavy and light strands, respectively. The initiation of heavy strand mtDNA occurs at the heavy strand mtDNA replication origin (oriH), located in the mtDNA main non-coding region. After completing around 67% of heavy strand mtDNA synthesis, the replisome passes the light strand mtDNA replication origin (oriL) producing the parental H strand as a single strand. Mediated by its ssDNA conformation, oriL forms a stem-loop structure and the mitochondrial RNA polymerase (POLRMT) initiates primer synthesis from a poly-dT stretch in the single-stranded loop region. POLRMT random RNA synthesis on the displaced strand is prevented by mtSSB bound to ssDNA (Falkenberg, 2018). After the primer synthesis is over POLRMT is replaced by POLγ initiating the synthesis of light stranded DNA (Jemt et al., 2011).

Homologous recombination is crucial throughout meiosis for DNA repair, replication, and exchange of genetic material between parental chromosomes. The phases of recombination involve complex reorganization of DNA structures and the successful completion of these steps depends on the activities of several helicase enzymes. The helicases of different superfamilies coordinate the processing of broken DNA ends and the subsequent formation and disassembly of recombination intermediates which are essential for template-based DNA repair. Importance of recombination is increased when replication forks as well as transcription machinery collide or encounter lesions in the DNA template. The successful recombination is likewise regulated by helicases in these cases, allowing normal cell growth and maintaining genomic integrity (Huselid & Bunting, 2020). Mitochondrial DNA integrity is crucial for cellular energy metabolism and the genetic recombination is a key feature of this metabolism (Gaidutšik et al., 2016). Irc3 provides a great material for the description of recombinant and recombinant like processes in mtDNA, its similarity to RecG helicase brings a new insight on recombination in yeast mtDNA (Sedman et al., 2017). SF2 DNA helicase Irc3 of *Saccharomyces cerevisiae* is suggested to be a mitochondrial branch migration enzyme (Gaidutšik et al., 2016). It is assumed that Irc3 is a

functional homolog to RecG of *Escherichia coli*, and as RecG, also participates in recombination-dependent repair by facilitating branch migration or resolving Holliday junctions. *In vitro* experiments suggest, that Irc3 has branched DNA-specific helicase and branch migration activity and therefore is involved in recombination processes by maintaining the stability of mtDNA through replication fork regression stimulation and branch migration, as well as inhibition of irregular recombination (Sedman et al., 2017).

3 SF2 HELICASE ANNEALING AND UNWINDING ACTIVITY

Common helicase activity is to unwind dsDNA in ATP-dependent manner. The mechanism of the unwinding differs between different SF and their subdivisions, the unwinding can be based on translocation on nucleic acid (NA) (Lohman & Fazio, 2018), however there is also translocation without unwinding and unwinding without translocation (Fairman-Williams et al., 2010). Also, researchers suggest that some helicases, in the presence or absence of nucleoside triphosphate can anneal complementary strands of NA (Byrd, 2012). SF2 RecG of *Escherichia coli* – specific branch migration helicase that unwinds forked DNA to form four-stranded Holliday junctions (McGlynn & Lloyd, 2002). Irc3 is considered to be a yeast functional homolog to RecG (Gaidutšik et al., 2016). Double stranded DNA translocase formed by RecG helicase domains pulls parental strands across a wedge domain that is only capable of accommodating two single strands of DNA, one for each of channels flanking the wedge. As a result of such DNA translocation leading and lagging strands are unwound from their respective parental template strands, thus allowing the template strands to rewind. Moreover, to facilitate annealing of leading and lagging strands the displaced strands are extruded in proximity. Therefore, as the translocase continues pulling the template duplex, a Holliday junction is formed at the wedge domain (McGlynn & Lloyd, 2002).

Human ATP-dependent SF2 HepA-related protein (HARP) is an annealing helicase that rewinds DNA (Yusufzai & Kadonaga, 2010). Mutations of HARP genes cause rare autosomal recessive pleiotropic disease, the Schimke immuno-osseous dysplasia (SIOD), which commonly leads to death in early age (Ghosal et al., 2011). HARP commonly takes part in the damaged replication fork repair and restart. Preferentially HARP binds to fork DNA structures, ssDNA or dsDNA, binding to fork DNA stimulates its ATPase activity (Yusufzai & Kadonaga, 2010). Also *in vitro* HARP has ATP-dependent helicase activity and can anneal ssDNA complimentary strands bound by replication protein A (RPA). It is suggested that HARP annealing activity is essential due to its function *in vivo*, it was evident that some of mutations in SIOD patients were caused by HARP mutant proteins with malfunctioned ATPase and helicase rewinding activity (Ghosal et al., 2011).

4 Irc3

Irc3 is a monomeric, double-stranded DNA-dependent helicase of the SF2 family. Its main function is to maintain the integrity of the mitochondrial genome in *Saccharomyces cerevisiae* and it is responsible for DNA branch migration (Gaidutšik et al., 2016) Although Irc3 is not yet well studied, it has been suggested to unwind the nascent lagging strand in a 3'-5' direction at the replication fork (V. Piljukov et al., 2020). It has been proven that the activity of Irc3 is dependent on the length of the ATPase stimulating dsDNA molecule - longer molecules stimulate better (Sedman et al., 2017). Moreover, the Y-shaped DNA forks or Holliday junctions also stimulate ATPase activity. Irc3 is proven to share several biochemical functions with RecG helicase and therefore believed to share the unwinding and remodeling activity (Gaidutšik et al., 2016).

5 THE AIMS OF THE THESIS

- Purify recombinant OpIrc3 protein from His-10-SUMO-OpIrc3 pet24d (+) construct
- Measure and quantify annealing activity of OpIrc3
- Measure and quantify unwinding activity of OpIrc3

6 EXPERIMENTAL PART

In this chapter the methods and materials used in the research are described. All procedures were conducted in the University of Tartu, Institute of Molecular and Cell Biology. All experiments were conducted under supervision of Prof. Juhan Sedman and MSc Vlad Julian Piljukov.

6.1 MATERIALS AND METHODS

6.1.1 Materials

pET-24d-His-10-SUMO (Institute of Molecular and Cell Biology, UT)

BL21(DE3)RIL (Institute of Molecular and Cell Biology, UT)

RFSO1 (TAG Copenhagen A/S)

RFSO2 (TAG Copenhagen A/S)

RFSO3 (TAG Copenhagen A/S)

6.1.2 Plasmid transformation

The 1µl of pET-24d-His10-SUMO-Irc3$_{op}$ plasmid (with kanamycin (KAN) and chloramphenicol (CAM) resistant marker) was transformed into 50µl BL21(DE3)RIL competent cells, cooled down on ice for 30 mins and then heat shocked at 37℃ for 2 mins. After that, cells were added to 0.5ml of LB/2xYT medium (containing 10mM glucose and 20mM MgSO4) and kept at 37℃ for 30 mins. After 30 mins period culture medium was inoculated on 2 Petri dishes (each containing 50µg/ml KAN and 25µg/ml CAM) and left incubating for 16 hrs. at the 37℃.

6.1.3 Cell cultures growth and overexpression

After incubating for 16 hrs., we have picked 3 colonies and divided them between 3 tubes containing LB medium with KAN and CAM inside. After that, cells were incubated for 3 hrs. in a rotating shaker at 37℃ and 181 rpm. During the incubation step we prepared 2l of LB medium in in 4 flasks (each carrying 0.5l of medium with KAN and CAM). After the

incubation we transferred 4ml of cell culture medium from one tube into all 4 flasks and left for further incubation at 37℃ and 181 rpm for 60 mins. After 60 mins passed, we have measured the OD and proceeded with calculations, we expected that the cell medium OD will be in range of 0.4-0.6 after 4 hours of incubation stating that we have the required number of cells for further procedures.

6.1.4 Inducing protein expression

After the OD of cell medium in all four flasks was in the expected range, we induced it with 0.5mM isopropylthio-β-galactoside (IPTG) per each flask and left in rotating shaker for 16 hrs at 20℃ and 181 rpm.

6.1.5 Handling the cell cultures

After inducing cells for 16 hrs., we pelleted them in centrifuge (4500 rpm) at 4℃ for 20 mins. Then the pellet was resuspended in 30ml of ST buffer, pelleted in centrifuge (4500 rpm) at 4℃ for another 15 mins. After final centrifugating the supernatant was discarded and pellet snap frozen in liquid nitrogen and stored at -80℃.

6.1.6 Protein purification

Before proceeding with protein purification, pellet obtained earlier was melted on ice. After melting the pellet, it was resuspended in LB buffer (50mM Tris-HCl pH 8.0, 500mM NaCl, 0.1% Tween, 2mM β-mercaptoethanol, 10% glycerol). Next 1g of lysosome was added and samples were incubated for 60 mins on end-over-end shaker at 4℃. After incubation, cells were sonicated 50% power 3x20s (BANDELIN SONOPLUS HD 2070) to lyse the cells. The lysate was subjected to centrifugation at 15000 rpm for 20 mins at 4℃. Next, supernatant was incubated with 2ml of Ni-NTA agarose beads [50% matrix to liquid] (Qiagen) on end-over-end shaker for 2 hrs. at 4℃ and then washed with 5 bed volumes of LB buffer containing 50mM imidazole. Proteins bound to the column were eluted using LB buffer containing 400mM imidazole (approximately 500µg of protein from each liter of culture). 10xHisSUMO-OpIrc3 fusion protein was subjected to 50µg of Ulp1 protease cleavage at 4℃ for 16 hrs., to separate the N-terminal SUMO-tag from the Irc3 pro-

tein. Sample was diluted with 0mM NaCl LB buffer to up final 300mM monovalent salt concentration (400mM imidazole was considered to fully dissociate) and filtered through a 5ml HiTrap Heparin HP column (GE Healthcare). Protein was washed with 5 bed volumes of FB buffer (50mM Tris-HCl pH 8.0, 300mM K-Glu pH 8.0, 2mM DTT. 10% glycerol) containing 300mM K-Glu and protein eluted with FB buffer containing 1000mM K-Glu. Fractions containing pure Irc3 were pooled, snap-frozen in liquid nitrogen and stored in small aliquots at -80°C. Protein fraction concentrations were determined by NanoDrop Microvolume Spectrophotometer (E=50770 and M=68.6 kDa).

6.1.7 Preparation of Native 10% PAGE gel

For Irc3 activity studies we have prepared the 10% Native PAGE gels (2.5ml 30% Acrylamide/Bis solution, 1.5ml 10xTBE, 75µl APS, 5µl TEMED,) with 0.75mm gel comb. Before using the PAGE gels, they first were pre-electrophoresed at 100V for 90 mins. To obtain a higher resolution of small fragments we used TBE buffer (10.8% Tris, 5.5% Boric Acid, 4% 0.5M EDTA pH 8.0, and MiliQ up to 1l of total solution).

6.1.8 Preparation of DNA substrates

We prepared DNA substrates using RFSO1, RFSO2, and RFSO3F oligonucleotides (RFSO3F carrying a fluorescein marker). Following substrates were annealed: 123F [10µM RFSO1, 10µM RFSO2, 9µM RFSO3, MiliQ, and 5xAnnealing buffer (250mM Tris-HCl pH 7.5, 50mM MgCl2, 250 mM NaCl)], 23F (10µM RFSO2, 9µM RFSO3F, MiliQ, and 5xAnnealing buffer), and 3F (10µM RFSO3F, MiliQ, and 5xAnnealing buffer). The sequences of the oligonucleotides are shown in Table 1.

Oligonucleotide name	Length (nt)	Sequence (5'-3')
RFSO1	50	GTCGGATCCTCTAGACAGCTCCATGATCAC-TGGCACTGGTAGAATTCGGC
RFSO2	50	CAACGTCATAGACGATTACATTGC-TACATGGAGCTGTCTAGAGGATCCGA
RFSO3	25	TAGCAATGTAATCGTCTATGACGTT

Table 1. List of nucleotides used to construct DNA substrates (Zegeye et al., 2012).

6.1.9 DNA unwinding

To study the unwinding activity of Irc3 we have developed an assay based on tracking the activity of helicase during different time points. By loading the samples on the PAGE gel, we could see how the unwinding of 123F is happening, knowing that the lower molecular weight of the DNA is the lower the bands will be. We expected to see three bands forming on different levels signalling that the 123F has been unwound firstly to 23F and then to F3. Due to the fluorescein marker, it was easy to detect those cofactors. For this procedure we prepared an SBM buffer (0.3% SDS, 10mM EDTA, 20% Glycerol, 1xTBE, and 4.5µl of Bromphenolblue) that would stop branch migration and making it easier to load the samples onto the gel. Samples containing 50nM OpIrc3 and 10nM DNA substrate (123F) were studied during 6 timepoints – 0-5 mins with 1 min interval. Reaction started by protein addition of 50nM OpIrc3 to a Master Mix (MM) [1xReactionMix(100mM K-Glu, 50mM Tris-HCl pH 8, and 1mM DTT), 10nM 123F, MiliQ, 5mM ATP + 5mM MgCl2)] starting the reaction, then the sample was taken from the MM every minute, mixed with SBM buffer, and stored on ice. Additionally, we added two control samples – one with protein and substrate being boiled for 5 mins at 95℃ and another with no protein added to the substrate kept at 30℃ for 5 mins straight. After the procedure was done, samples were loaded onto PAGE gel and left for electrophoresis for 180 mins at 100V. After the electrophoresis was done, the gel was analysed by using Amersham™ Typhoon™ Biomolecular Imager (**Figure 1)**. Resulted images were subjected to signal quantification by ImageJ software and analysed in Microsoft Excel.

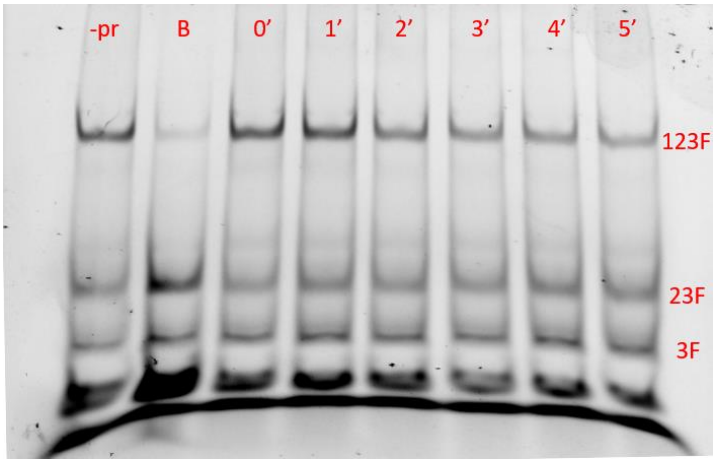


Figure 1. DNA unwinding: 0' – zero-minute time point, 1'-5' time points with 1 minute interval, -pr – no protein added, B – boiled at 95℃. 123F substrate, 23F substrate, 3F substrate.

6.1.10 DNA annealing

For studying annealing activity of Irc3 we decided to conduct two different experiments – one with the ATP present and one without ATP. In this case we used an equimolar mix of not previously annealed 10nM 23F and RFSO1 substrates. Time points were 0 minutes (0'), 2.5', 5', 10', 15', and 20'. This time after the PAGE gel electrophoresis we expected to see substrates annealing – forming 123F. For this procedure we used SBM buffer, 50nM OpIrc3, and equimolar 10nM 23F and RFSO1. Reaction started by protein addition of 50nM OpIrc3 to Master Mix [1xReactionMix(100mM K-Glu, 50mM Tris-HCl pH 8, and 1mM DTT), 10nM 123F, MiliQ, 50mM ATP + 50mM MgCl2)] and the second sample introduced to the Master Mix without ATP. After conducting the experiment, samples were loaded onto PAGE gel and left for electrophoresis for 180 mins at 100V. After the electrophoresis was done, the gel was analyzed by using Amersham™ Typhoon™ Bio-molecular Imager (**Figure 2)**. Resulted images were then subjected to signal quantification by ImageJ software and analyzed in Microsoft Excel.

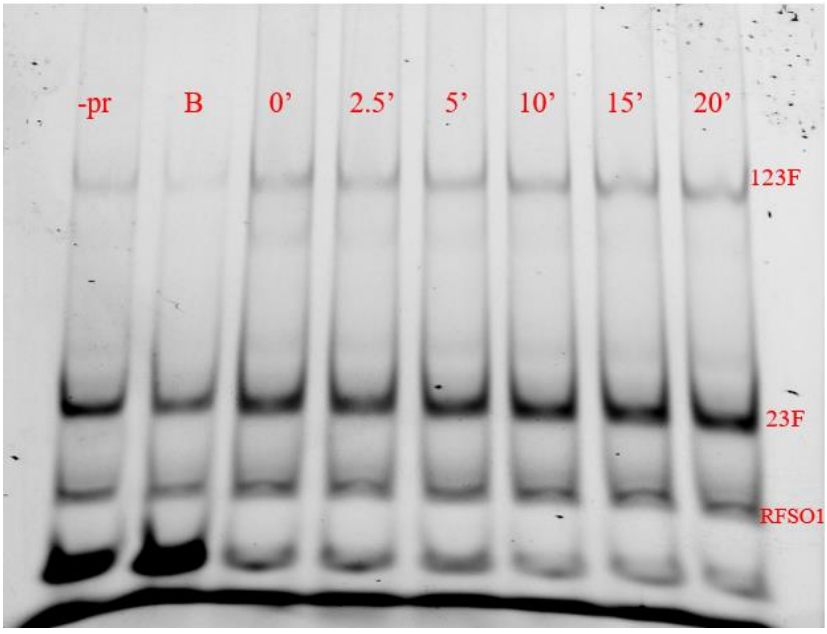


Figure 2. DNA annealing (no ATP); 0' – zero-minute time point, 2.5'-20' time points, -pr – no protein added, B – boiled at 95°C, 123F substrate, 23F substrate, RFSO1 oligonucleotide.

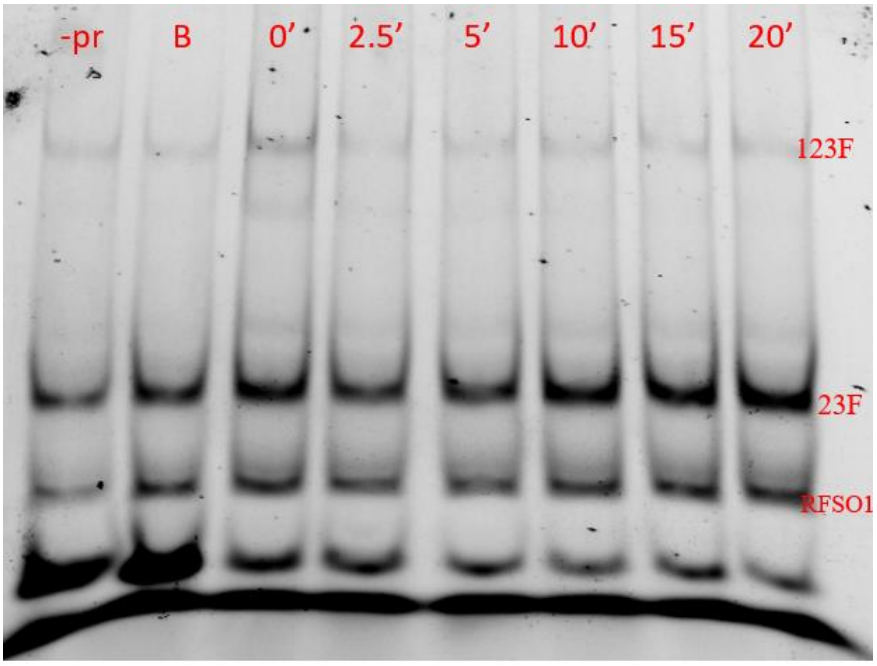
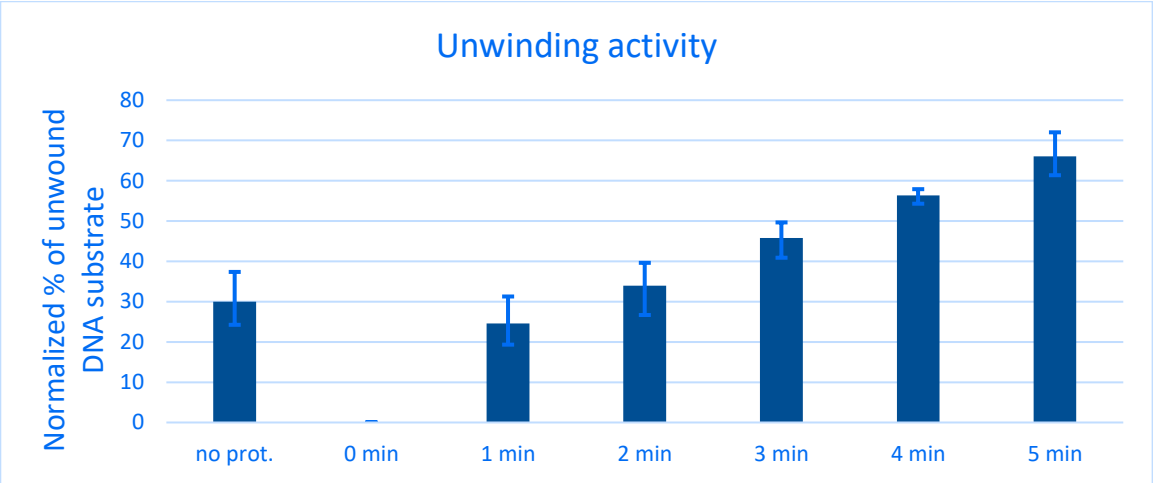


Figure 3. DNA annealing (with ATP); 0' – zero-minute time point, 2.5'-20' time points, -pr – no protein added, B – boiled at 95℃, 123F substrate, 23F substrate, RFSO1 oligonucleotide.

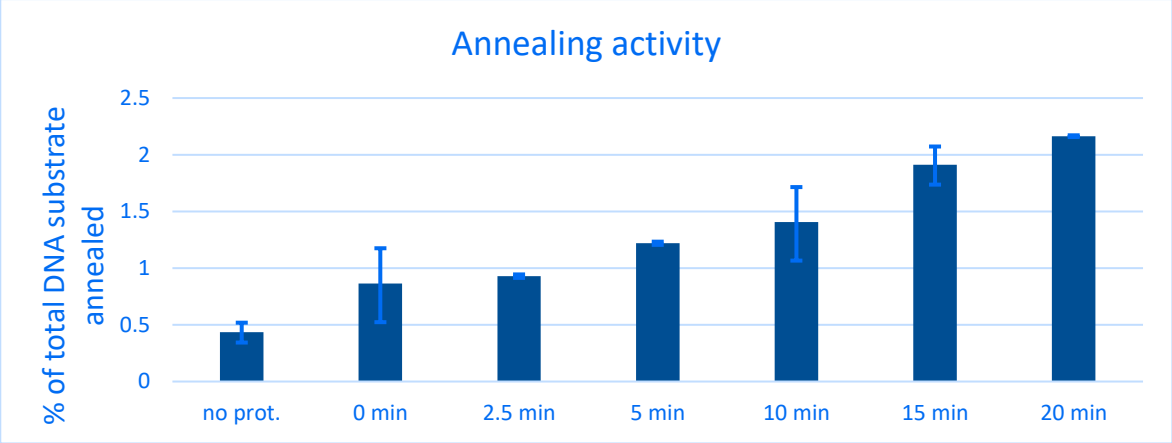
6.2 Results and Discussion

The assay developed by our research group differs from commonly used approaches. The most common way to study the activity of helicases is by incubating a helicase with ATP and a radiolabelled DNA duplex. Terminating the reaction and analysing the products using the PAGE gel (Belon & Frick, 2008). Our approach is based on fluorescence; the fluorescein marker tagged to 5' of RFSO3 and later when analysing the PAGE, we could distinguish the positions 123F, 23F and RFSO3.

The data gathered by conducting two sets of experiments under the same conditions for unwinding and annealing activity let us quantitively analyse the behaviour of Irc3 helicase over time. As for unwinding, the analysis (**Graph 1**) shows us that percentage of the unwound product grows over time significantly, reaching up to 66% of DNA substrate unwound during first 5 minutes of reaction. This data can be compared with other known SF2 helicases. For example, comparing the data obtained from studying RecQ DNA helicases, in this experiment the percent of DNA unwound is determined by normalizing reactions between negative control – no RecQ, and positive control boiled for 5 mins, the experiment lasted for 5 mins and conditions highly correlate to ours (Sharma et al., 2005). The comparison between these two studies reveals the advantage of RecQ in terms of unwinding activity. At the same time points of 5 minutes, results differ drastically with Irc3 unwinding approximately 34% of DNA substrate and RecQ unwinding approximately 82% of the DNA substrate (Sharma et al., 2005). Nevertheless, the results of our research show that the unwinding activity of Irc3 is present and with the assay optimizations the faster and more efficient unwinding activity could be achieved. Analysis of annealing activity (**Graph 2**) showed poor results with only 2% of total DNA substrate annealed in 20 mins period and only in case of ATP being absent. The set of experiments with the ATP present resulted in no annealing activity (**Figure 3)**. Compared to our research, the quantitative analysis of RecQ helicase enzymes WRN and BLM (Machwe et al., 2006) has shown the percent of substrate reaching up to 40% in the presence of ATP and 80% without ATP for BLM and up to 40% in the presence of ATP and approximately 60% without ATP for WRN-E84A (Machwe et al., 2006). Interestingly, in both cases the presence of ATP inhibited the annealing activity of helicases and this correlates to our research. Even with such a significant difference in the results, the annealing activity of Irc3 is still evident and with further optimizations of the assay better results might be achieved.



Graph 1. Normalized percentage of unwound DNA substrate over time; normalized by 0 min point (0 min point counted at 100%). Results were gained by quantitative analysis. At no protein sample (no prot.) average percent equals to ≈ 30.00% and at protein sample at point 5 mins (5') the average percent equals to 66.03%.



Graph 2. Percentage of total DNA substrate annealed over time; results taken by average measurement of the two similar sets of experiments. Results were gained by gel quantitative analysis. At no protein sample (no prot.) average percent equals to 0.43% and at protein sample at point 20 mins (20') the average percent equals to 2.16%.

SUMMARY

This study aims to examine the unwinding and annealing activity of the thermotolerant yeast *Ogataea polymorpha* helicase Irc3 ($Irc3_{op}$).

For this study, the pET-24d-His10-SUMO-$Irc3_{op}$ plasmid was transformed into BL21(DE3)RIL competent cells, cell cultures were grown and induced for protein overexpression. The recombinant protein was purified, obtained aliquots of pure $Irc3_{op}$ were stored and used for further studies.

Unwinding activity of $Irc3_{op}$ was studied through conducting a set of duplicate experiments held under the same conditions. Samples containing 50nM OpIrc3 and 10nM DNA substrate (123F) were prepared during 6 timepoints – 0-5 mins with 1 min interval, additionally two control samples (one boiled at 95℃ for 5 mins and one without protein present) were added. After preparation, the samples were loaded onto 10% PAGE gel and left for 180 mins for electrophoresis under 100V. The gel was analysed by Amersham™ Typhoon™ Biomolecular Imager. Later the data was quantified by ImageJ software and analysed in Microsoft Excel.

Over time annealing activity of $Irc3_{op}$ was studied through conducting a set of duplicate experiments (one set in the present of ATP and one set without ATP) under the same conditions. Samples containing equimolar mix of not previously annealed 10nM 23F and RFSO1 substrates. Time points were 0 minutes (0'), 2.5', 5', 10', 15', and 20',with sample being prepared at each time point, additionally two control samples (one boiled at 95℃ for 5 mins and one without protein added) were added. After preparation, the samples were loaded on the 10% PAGE gel and left for 180 mins for electrophoresis under 100V. The gel was analysed by Amersham™ Typhoon™ Biomolecular Imager. Later the data was quantified by ImageJ software and analysed in Microsoft Excel.

Results gained from the quantitative analysis of the experimental data and other research comparison, suggests that unwinding activity of $Irc3_{op}$ is evident and easily detectable, while also being comparable to other SF2 helicases.

Results gained from the quantitative analysis of the experimental data and other research comparison, suggests that unwinding activity of $Irc3_{op}$ is evident and detectable, however when compared to other SF2 helicases, it tends to show lower annealing activity.

REFERENCES

Belon, C. A., & Frick, D. N. (2008). Monitoring helicase activity with molecular beacons. *BioTechniques*, *45*(4), 433–442. https://doi.org/10.2144/000112834

Beyer, D. C., Ghoneim, M. K., & Spies, M. (2013). Structure and Mechanisms of SF2 DNA Helicases. In M. Spies (Ed.), *DNA Helicases and DNA Motor Proteins* (Vol. 767, pp. 47–73). Springer New York. https://doi.org/10.1007/978-1-4614-5037-5_3

Bleichert, F., Botchan, M. R., & Berger, J. M. (2017). Mechanisms for initiating cellular DNA replication. *Science*, *355*(6327), eaah6317. https://doi.org/10.1126/science.aah6317

Byrd, A., K. (2012). Superfamily 2 helicases. *Frontiers in Bioscience*, *17*(7), 2070. https://doi.org/10.2741/4038

Ding, H., Guo, M., Vidhyasagar, V., Talwar, T., & Wu, Y. (2015). The Q Motif Is Involved in DNA Binding but Not ATP Binding in ChlR1 Helicase. *PLOS ONE*, *10*(10), e0140755. https://doi.org/10.1371/journal.pone.0140755

Fairman-Williams, M. E., Guenther, U.-P., & Jankowsky, E. (2010). SF1 and SF2 helicases: Family matters. *Current Opinion in Structural Biology*, *20*(3), 313–324. https://doi.org/10.1016/j.sbi.2010.03.011

Falkenberg, M. (2018). Mitochondrial DNA replication in mammalian cells: Overview of the pathway. *Essays in Biochemistry*, *62*(3), 287–296. https://doi.org/10.1042/EBC20170100

Gaidutšik, I., Sedman, T., Sillamaa, S., & Sedman, J. (2016). Irc3 is a mitochondrial DNA branch migration enzyme. *Scientific Reports*, *6*(1), 26414. https://doi.org/10.1038/srep26414

Ghosal, G., Yuan, J., & Chen, J. (2011). The HARP domain dictates the annealing helicase activity of HARP/SMARCAL1. *EMBO Reports*, *12*(6), 574–580. https://doi.org/10.1038/embor.2011.74

Hall, M. C., & Matson, S. W. (1999). Helicase motifs: The engine that powers DNA unwinding. *Molecular Microbiology*, *34*(5), 867–877. https://doi.org/10.1046/j.1365-2958.1999.01659.x

Huselid, E., & Bunting, S. F. (2020). The Regulation of Homologous Recombination by Helicases. *Genes*, *11*(5), 498. https://doi.org/10.3390/genes11050498

Jemt, E., Farge, G., Bäckström, S., Holmlund, T., Gustafsson, C. M., & Falkenberg, M. (2011). The mitochondrial DNA helicase TWINKLE can assemble on a closed circular template and support initiation of DNA synthesis. *Nucleic Acids Research*, *39*(21), 9238–9249. https://doi.org/10.1093/nar/gkr653

Lohman, T. M., & Fazio, N. T. (2018). How Does a Helicase Unwind DNA? Insights from RecBCD Helicase. *BioEssays*, *40*(6), 1800009. https://doi.org/10.1002/bies.201800009

Machwe, A., Lozada, E. M., Xiao, L., & Orren, D. K. (2006). Competition between the DNA unwinding and strand pairing activities of the Werner and Bloom syndrome proteins. *BMC Molecular Biology*, *7*(1), 1. https://doi.org/10.1186/1471-2199-7-1

McGlynn, P., & Lloyd, R. G. (2002). Genome stability and the processing of damaged replication forks by RecG. *Trends in Genetics*, *18*(8), 413–419. https://doi.org/10.1016/S0168-9525(02)02720-8

Piljukov, V., Garber, N., Sedman, T., & Sedman, J. (2020). Irc3 is a monomeric DNA branch point-binding helicase in mitochondria of the yeast *Saccharomyces cerevisiae*. *FEBS Letters*, *594*(19), 3142–3155. https://doi.org/10.1002/1873-3468.13893

Piljukov, V.-J., Sillamaa, S., Sedman, T., Garber, N., Rätsep, M., Freiberg, A., & Sedman, J. (2022). *Irc3 of the thermotolerant yeast* Ogataea polymorpha *is a branched-DNA-specific mitochondrial helicase* [Preprint]. Biochemistry. https://doi.org/10.1101/2022.03.21.485104

Raney, K. D., Byrd, A. K., & Aarattuthodiyil, S. (2013). Structure and Mechanisms of SF1 DNA Helicases. In M. Spies (Ed.), *DNA Helicases and DNA Motor Proteins* (Vol. 767, pp. 17–46). Springer New York. https://doi.org/10.1007/978-1-4614-5037-5_2

Sedman, T., Garber, N., Gaidutšik, I., Sillamaa, S., Paats, J., Piljukov, V. J., & Sedman, J. (2017). Mitochondrial helicase Irc3 translocates along double-stranded DNA. *FEBS Letters*, *591*(23), 3831–3841. https://doi.org/10.1002/1873-3468.12903

Sharma, S., Sommers, J. A., Choudhary, S., Faulkner, J. K., Cui, S., Andreoli, L., Muzzo-lini, L., Vindigni, A., & Brosh, R. M. (2005). Biochemical Analysis of the DNA Unwinding and Strand Annealing Activities Catalyzed by Human RECQ1*[boxs]. *Journal of Biological Chemistry*, *280*(30), 28072–28084. https://doi.org/10.1074/jbc.M500264200

Wu, Y. (2012). Unwinding and Rewinding: Double Faces of Helicase? *Journal of Nucleic Acids*, *2012*, 1–14. https://doi.org/10.1155/2012/140601

Yusufzai, T., & Kadonaga, J. T. (2010). Annealing helicase 2 (AH2), a DNA-rewinding motor with an HNH motif. *Proceedings of the National Academy of Sciences*, *107*(49), 20970–20973. https://doi.org/10.1073/pnas.1011196107

Zegeye, E. D., Balasingham, S. V., Laerdahl, J. K., Homberset, H., & Tønjum, T. (2012). Mycobacterium tuberculosis RecG binds and unwinds model DNA substrates with a preference for Holliday junctions. *Microbiology*, *158*(8), 1982–1993. https://doi.org/10.1099/mic.0.058693-0

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