Optimization of affinity-based tubulin purification and polymerization kinetics from goat brain and mung seedlings

A thesis submitted towards the partial fullfilment of
BS-MS dual degree programme
from May 2022 to April 2023

by

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under the guidance of

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Certificate

This is to certify that this dissertation entitled "*Optimization of affinity-based tubulin purification and polymerization kinetics from goat brain and mung seedlings*" submitted towards the partial fulfilment of the BS-MS degree at the Indian Institute of Science Education and Research, Pune represents original research carried out by **K. T. Abdul Rishad**, under the supervision of **Dr. Chaitanya A. Athale** during the academic year May 2022 to April 2023.

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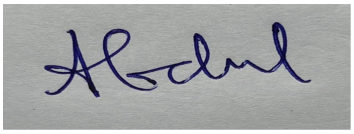
Date:
30/04/2023

Declaration

I, **K. T. Abdul Rishad**, hereby declare that the matter embodied in the report titled "*Optimization of affinity-based tubulin purification and polymerization kinetics from goat brain and mung seedlings*" is the results of the investigations carried out by me at the "Indian Institute of Science Education and Research Pune" from the period May-2022 to April-2023 under the supervision of **Dr. Chaitanya A. Athale** and the same has not been submitted elsewhere for any other degree.



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K T Abdul Rishad	Writing
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K T Abdul Rishad	Visualization
K T Abdul Rishad	Validation

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Abstract

Microtubules (MT) form cytoskeletal polymers consisting of $\alpha-$ and $\beta-$tubulin dimers. Understanding MT polymerization kinetics can shed light on intrinsic physiological properties and diversification (Jain *et al.*, 2021). In previous work, GST-TOG-1/2 column-based affinity purification was devised by Widlund *et al.* (2012), as an alternative to activity-based purification.

Here, I report the optimization of expression and purification conditions for GST-TOG-1/2 protein, make a column and test binding efficiency of an existing one and measure the effect of crowdants on goat brain and mung tubulin polymerization kinetics. The TOG-1/2 protein was successfully overexpressed in *Escherichia coli* BL21-DE3 *Rosetta* cells from a plasmid pGEX-6P-1. Complexation of 5.7 mg of purified TOG-1/2 with 1 ml of NHS-activated sepharose resin resulted in very poor binding, which when tested with BSA was found to be due to lack of activity of the NHS-sepharose material. As an alternative, I purified tubulin using a pre-existing column and found 23 to 48% elution of goat brain tubulin depending on column volume and salt used- either ammonium sulphate ($(NH_4)_2SO_4$) or potassium chloride (KCl). Polymerization kinetics of goat brain and mung seedling tubulin, were performed to optimize conditions for activity testing. Glycerol as a crowdant was tested on goat brain and mung tubulin, and the effect of 10% and 15% glycerol increased polymerization for goat tubulin and slightly for mung tubulin.

Thus, I have optimized isolation of GST-TOG-1/2, attempted to make an affinity chromatography column, established the elution conditions of previously prepared affinity columns and optimized conditions for polymerization kinetics of goat brain and mung tubulin in presence of crowdants.

Contents

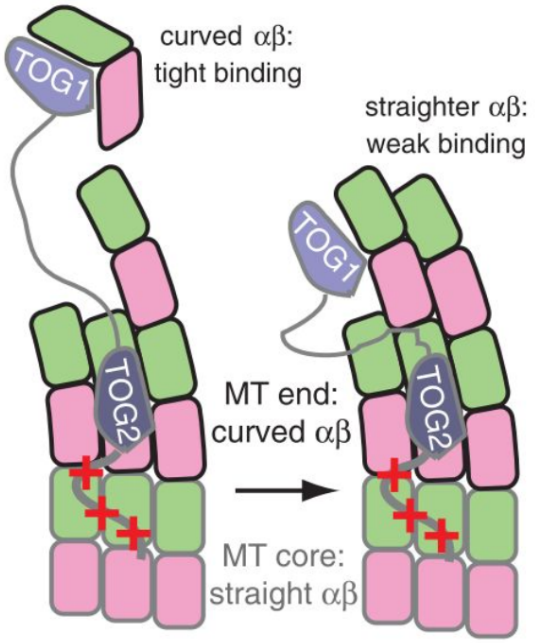
Chapter 1

Introduction

Microtubule is a eukaryotic cytoskeleton polymeric protein made up of heterodimeric monomers, α and β tubulin, in which the monomers are arranged in a head-to-tail formation giving rise to polarity in the polymer. Due to the existence of polarity, there are proteins called molecular motors, which walk in an anterograde (from cell centre to periphery) or retrograde (from periphery to cell centre) to transport intra-cellular cargo. Hence, it is involved in crucial cell physiology eg. cell division and organelle positioning (Alberts *et al.*, 2002). Tubulin protein is known to be a highly conserved protein across eukaryotic species, but *in-vitro* studies of tubulin extracted from plant and animal sources show differences in biophysical activity e.g. bulk and single filament polymerization kinetics. Hence, this makes tubulin protein, a perplexing and a crucial protein to conduct *in-vitro* and *in-vivo* studies on it from a variety of sources, to understand their different biophysical characteristics and pinpoint the reason for their distinct properties.

Purification strategies adopted for tubulin isolation:

Usually, natural sources of tubulin are preferred over a few successful recombinant systems to express tubulin for *in-vitro* experimentation/assays of tubulin as there are certain complications involved in expressing plant and animal tubulin in lower eukaryotic recombinant systems (Widlund *et al.*, 2012). In natural sources, mammalian brain tissues eg. Bovine and porcine brains are considered the standard sources of tubulin for *in-vitro* work as they have an enriched amount of tubulin. Currently, to extract tubulin from a given tissue source, the most efficient method is activity-based isolation of tubulin (Castoldi and Popov, 2003) based on the working principle that the amount of active tubulin will get filtered from the cell lysate after two rounds of temperature-dependent polymerization-depolymerization cycle, from the



(a)

Figure 1.1: (a) shows the conformation based affinity of TOG towards tubulin. TOG1 domain shows great affinity towards the unpolymerized form of tubulin, TOG2 domain may recognise microtubule plus end (MT end) and "+++" is a region which provides affinity towards microtubule lattice (Taken from Ayaz *et al.* (2012)), reproduced with permission (attached as a supplement).

inactive tubulin fraction, which is unable to polymerize maybe due to distorted conformation of tubulin. Two rounds of polymerization and depolymerization cycle (pol-depol) occur by switching temperature from 37 ^{o}C to 4 ^{o}C, to extract tubulin from a given cell lysate. It is well-known experimentally, that microtubule depolymerizes at a lower temperature (i.e 4 ^{o}C) and polymerizes at an optimum 37 ^{o}C, but by using two rounds of pol-depol cycle, it helps in increasing the fraction of polymerization-competent tubulin apart from the inactive or polymerization incompetent tubulin fraction from a given source. The method is straightforward and less laborious. The limitation of activity-based purification is that if the plant or animal tissue doesn't have tubulin in enriched amounts, the recovery of active or polymerization-competent tubulin fraction from the cell lysate will be significantly lower than compared to the recovery obtained from a standard source of tubulin for example, mammalian brain tissue. To purify tubulin from non-standard sources, Affinity based purification is preferred. As it will be more effective for tubulin purification from a given source, even if the source has a low concentration of tubulin. In affinity-based purification, TOG (Tumour overexpressed gene) domains are the ligands (which are complexed with NHS-ester-activated sepharose beads) and show great affinity towards unpolymerized tubulin fraction or curved conformation of tubulin.

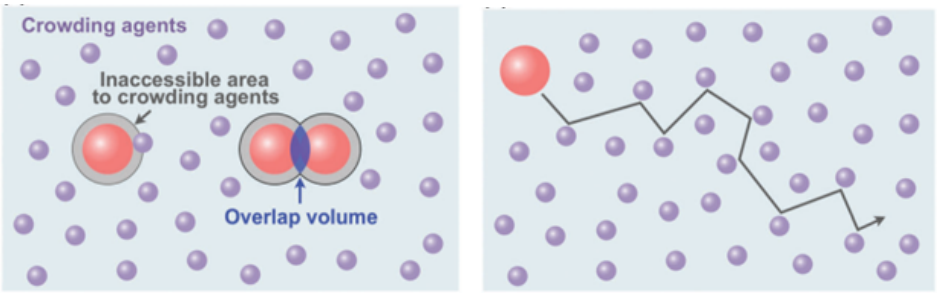
Role of TOG1/2 in MT polymerization and use for affinity purification:

TOG domains are part of the XMAP215/Dis1 protein family which is known to promote microtubule polymerization. Stu2p, natively expressed in *Saccharomyces cerevisiae* is a member of the XMAP215/Dis1 protein family, has two pairs of TOG domains (TOG1 and TOG2), and according to previous work on stu2p, (Al-Bassam *et al.*, 2006) has shown that TOG domains show affinity towards unpolymerized tubulin fraction, which exists in a curved conformation than compared to tubulin in the polymerized state which is present in straight conformation as shown in Fig. 1.1(a), and hence Widlund *et al.* (2012) considered to use stu2p TOG domains (TOG1-TOG2) and recombined it with Glutathione S-transferase (GST tag) sequence and incorporated in an appropriate expression system (*E.coli* BL21 DE3 Rosetta cells). The proteins were purified using a glutathione affinity column and complexed with N-hydroxysuccinimide-ester-activated (NHS) sepharose beads, to prepare GST-TOG-1/2 immobilized affinity column and was ready to be used to purify tubulin from any standard and non-standard source. After loading the cell lysate sample, certain wash buffers (ATP and GTP wash buffer) are used to remove proteins which are forming non-specific interactions with affinity

column or chaperone proteins interacting and forming complexes with tubulin. Elution of tubulin is performed using ionic strength using a gradient of ionic salt buffers. Widlund *et al.* (2012) and Hotta and Hashimoto (2020) have demonstrated the purification of tubulin using affinity purification from many lower and higher eukaryotic species with their own optimization of steps involved in the preparation, hence it seemed it was important to optimize each and every step involved in the process of TOG-based affinity purification as the technique is well known and needs to have standardized conditions as part of validation and reproduction of the results using experimental data.

Effects of crowdants on bulk polymerization of tubulin *in-vitro*:

Crowding is a phenomenon in which a crowdant molecule occupies a significant fraction of volume, imparting a negative effect on diffusion as shown in Fig. 1.2(b) or shifting the biochemical equilibria of a system. In cells, it is known that macromolecules (eg. lipids and proteins) occupy around 20-30% of the volume of the cell, hence they are considered a crowded environment. They affect the system mainly by volume exclusion as shown in Fig. 1.2(a), which results in increasing the thermodynamic activity of the system (Ellis, 2001; Zimmerman and Trach, 1991).



(a) (b)

Figure 1.2: (a) and (b) illustrates effects of crowding via volume exclusion and negative effect on diffusion, respectively (Taken from Hata *et al.* (2018)), reproduced with permission (attached as a supplement).

Due to the important physiological role of microtubules in cells (eg. Cell division), it is important to study and understand the polymerization kinetics of tubulin, under the effect of crowdant (glycerol) from standard and non-standard sources of tubulin.
There are two manners of studying tubulin polymerization dynamics: (I) Single filament polymerization kinetics and (II) Bulk polymerization kinet-

ics. Single filament polymerization kinetics study employs the use of high-resolution microscopy eg. Interference reflection microscopy (IRM) and TIRF (Total internal reflection microscopy), focusing on the single microtubule filament's parameters eg. Rate of catastrophe and rescue of a single filament. Bulk polymerization kinetics employs the use of spectrophotometry and the parameters we estimate from the experimentation data are the averaged outcome of the bulk tubulin reaction mix. The advantage of bulk tubulin polymerization kinetics as it helps in understanding the effects of physical parameters such as diffusion, or drugs (eg. microtubule polymerization promoter and inhibitor), using a simple, easy-to-use method of experimentation compared to the high-resolution microscopy method. In theory, Bulk tubulin polymerization has three distinct phases: the lag phase (nucleation phase), the log phase (elongation phase) and the plateau phase (equilibrium phase). It is a spontaneous polymerization as the nucleus/seeds are formed spontaneously and the entire reaction probably follows a mechanism known as nucleation-dependent polymerization (Sabareesan and Udgaonkar, 2014). But in single filament polymerization kinetics, pre-formed nuclei are used to observe microtubule polymerization dynamics and hence specifically, deal primarily with the elongation phase of microtubules.
In a recent study by Molines *et al.* (2022), it has been shown in a single filament polymerization kinetics study, that there is a decreasing trend of microtubule polymerization rate with an increasing concentration of glycerol (crowdant). Also, there are still unresolved questions in relation to the effect of various crowdants (varying sizes, charged or inert) on the nucleation and elongation stages of microtubule polymerization. Hence, I wanted to test the effect of glycerol (crowdant) concentration on bulk tubulin polymerization kinetics on both mung and goat brain tubulin to test whether there is a common characteristic change observed in their bulk polymerization kinetics.

Chapter 2

Materials and Methods

2.1 Expression of GST-TOG-I/II protein from *E.coli* BL21 DE3 transformant cells:

Plasmid pGEX-6P-1 encoding stu2 (1-590) was obtained from Dr Minhaj Sirajuddin, iNSTEM, Bangalore, based on a previously reported work by Widlund *et al.* (2012). The plasmid pGEX-6P-1 Stu 2 (1-590 aa) contains recombinant sequence for GST-TOG-1/2, which were transformed into *E.coli* BL21 (DE3) Rosetta, expressed and purified as described with modifications. The primary culture was made by reviving cells from stored glycerol stock of *E.coli* BL21 DE3 transformant cells (Roy *et al.*, 2021), using 5 ml Terrific Broth medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH2PO4, and 72 mM K2HPO4; HiMedia) along with 100 ug/ml ampicillin and 15 ug/ml chloramphenicol. The primary culture was incubated for 17 hours at 37 ^{o}C and 180 rpm using a shaker incubator (SHEL LAB S16, Sheldon Manufacturing, Cornelius, OR, USA). A 0.2% primary inoculum was taken for the secondary culture of 1 L TB medium along with 100 ug/ml ampicillin and 15 ug/ml chloramphenicol. The secondary culture was incubated at 37 ^{o}C and 180 rpm (SHEL LAB SSI10 Large Capacity Shaking Incubator, Sheldon Manufacturing, Cornelius, OR, USA). At 1-hour intervals, 1 ml of culture was taken periodically to measure the OD at 600 nm. After OD reached 0.1, the time interval was then changed to 30 minutes. When OD ranges between 0.6 and 0.8, 0.2 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to the secondary culture and later incubated for 18 hours at 18 ^{o}C at 180 rpm. The culture was centrifuged at 6000 rpm for 15 mins at 4 ^{o}C (F12-6x500 LEX, Sorvall RC+ Centrifuge, Thermo scientific). The supernatant was discarded and the cell pellet was resuspended using wash buffer I (1X PBS, 1 mM $MgCl_2$, 1 mM ATP, 1 mM

DTT and 0.2 mM PMSF; 10X PBS stock: 27 mM KCl, 15 mM KH_2PO_4, 81 mM Na_2HPO_4, 1.37 M NaCl, pH=7.4). Resuspended cells were centrifuged at 6000 rpm for 10 mins at 4 ^oC (Centrifuge 5810 R, Eppendorf) and later, the supernatant was decanted. The cell pellets were flash-frozen using liquid nitrogen and were stored at -80 ^oC for future downstream protein purification steps.

2.2 Purification of GST-TOG-I/II from *E.coli* BL21 DE3 transformant cells:

The induced *E.coli* BL21 DE3 culture cell pellets were resuspended using the wash buffer I and the cells were lysed using sonication (Pulse: 1 sec; Pause: 3 sec; time: 10-15 mins; Amplitude: 60%). The lysed cells were later centrifuged at 8000-12000 rpm for 1 hour at 4 ^oC (Centrifuge 5810 R, Eppendorf). Glutathione sepharose 4B resin (GE Healthcare) column was prepared in PD-10 column (5 ml (CV) per 1 L culture pellet). The column was later equilibrated using 4 CV 1X PBS and 1 CV wash buffer I (1X PBS, 1 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.2 mM PMSF) sequentially. The supernatant was loaded onto the column and later, the flow through was recycled two times. The column was washed with wash buffer I (1X PBS, 1 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.2 mM PMSF) and wash buffer II (1X PBS, 1 mM $MgCl_2$, 0.2 mM PMSF) sequentially (5 CV each). GST-TOG-1/2 protein was eluted using a gradient of glutathione elution buffer (10, 20 and 40 mM glutathione, 1X PBS; Stock: 100 mM reduced glutathione in Milli-Q, pH = 8.0). All the GST-TOG-1/2 protein elutions were pooled together and dialyzed in dialysis buffer (0.1 M $NaHCO_3$, 0.1 M NaCl, pH=8.2) for three rounds using Snakeskin dialysis tubing (10 KDa cutoff; thermo scientific) and later concentrated via centrifuge filter Amicon ultra-15 (30 KDa cutoff; Merck Millipore). The concentrated protein sample was flash-frozen using liquid nitrogen and stored at -80 ^oC. Samples from each step were run on an SDS-PAGE gel using a coomassie blue stain. Protein quantification was done by using Bradford and BCA assay using BSA as a control (n CV means n times the column volume).

The column was regenerated using column regeneration I (0.1 M tris pH 8.5, 0.5 M NaCl, 3.5 mM SDS), distilled water, column regeneration II (0.1 M sodium acetate pH 4.5, 0.5 M NaCl, 3.5 mM SDS) and distilled water sequentially 2CV each. Later, the column was stored at 4 ^oC with 20% ethanol (Widlund *et al.*, 2012; Hotta and Hashimoto, 2020; Roy *et al.*, 2021). Most reagents unless otherwise mentioned were obtained from Sigma-Aldrich,

Mumbai.

2.3 Quantitative analysis of protein concentration

Protein estimation via BCA assay (Smith *et al.*, 1985): BCA Reagent A and Reagent B are mixed in a 50:1 ratio (BCA working reagent). The working reagent was mixed with an unknown protein sample, a set of serial diluted BSA (bovine serum albumin) samples and a blank solution in an 8:1 ratio respectively. Incubated for 30 mins at 37 ^{o}C. Later, cool for 2-3 minutes. The samples were loaded into a 96-well UV-transmissible half-area plate (Corning, USA) and measured the absorbance at 562 nm using a spectrophotometer (Varioskan Flash2, ThermoScientific, USA). A fit line is obtained in an absorbance vs. protein concentration plot using the serial diluted BSA solution.

Protein estimation via Bradford assay (Bradford, 1976): Bradford reagent and protein sample are mixed in a 20:1 ratio. Using a set of serial diluted BSA (bovine serum albumin) samples and a blank solution, to obtain a fit line in an absorbance vs protein concentration plot to determine the concentration of the given unknown protein sample. The reaction mix is later, incubated for 10 mins at room temperature. The samples were loaded into a 96-well UV-transmissible half-area plate (Corning, USA) and measured the absorbance at 595 nm using a spectrophotometer (Varioskan Flash2, ThermoScientific, USA).

2.4 Preparation of GST-TOG-1/2 affinity column:

The dialyzed GST-TOG-1/2 pooled elution (20 mg is required per ml NHS-activated sepharose resin) is mixed gently with $MgCl_2.6H_2O$ powder (final concentration: 80 mM). 1 ml (CV: Column volume) of NHS-activated sepharose 4 fast flow (Binding capacity: 30 mg/ml) was loaded into an empty PD-10 column, and later the column was rinsed with 3.75 CV of 1 mM HCl. The protein sample was loaded into the column and the flow through was recycled n-times (for 1-2 hours). Bradford assay was used to determine the concentration of final flow through to evaluate coupling efficiency. 3 CV of Quenching solution (0.5 M ethanolamine, 0.5 M NaCl, pH=8.3) was loaded onto the column three times and 15 minutes of incubation was given each time. 10 CV of 6X PBS and 5 CV of 1X PBS were used as wash buffers,

sequentially. Lastly, 10 CV of storage buffer was loaded into the column and the stopper was added until the storage buffer volume reached 1-2 CV. The column was stored in -20 ^{o}C.

2.5 GST-TOG- 1/2 column-based affinity purification of tubulin

The column setup was maintained under 4 ^{0}C. GST-TOG-1/2 column was equilibrated with 1X BRB-80, later loaded with purified goat brain tubulin and Incubated the column for 15 minutes on ice. The column was washed with GTP wash (5 CV, column volumes; GTP wash: 100 μM GTP mixed in 1X BRB-80 and Milli-Q) and again incubated for 15 minutes on ice. The column was washed again with GTP wash (3 CV; GTP wash: 100 μM GTP mixed in 1X BRB-80 and Milli-Q). KCl/$(NH_4)_2SO_4$ elutions were used to elute out the protein (KCl/$(NH_4)_2SO_4$ elutions: 50, 100, 250 and 500 mM KCl/$(NH_4)_2SO_4$ mixed in 100 μM GTP, 1X BRB-80 and Milli-Q each), using 1 TV (tubulin volume) for each elution. The column was regenerated using regeneration buffers: 10 CV 1X PBS, 10 CV 10X PBS and 5 CV 1X PBS. The GST-TOG-1/2 column was stored under -20 ^{o}C for future use, after adding 3xCV of storage buffer (1X PBS in 50% glycerol) (n CV means n times the column volume and similarly n TV means n times the tubulin volume).

2.6 Qualitative analysis of GST-TOG-1/2 elution sample SDS PAGE gel analysis

SDS-PAGE gels were quantitatively analyzed by intensity analysis of bands using the ImageJ intensity function (Schindelin *et al.*, 2012). Normalized band intensity (N.B.I) is:

$$N.B.I = \frac{\sum X_i}{\sum X_{tub}} \tag{2.1}$$

Where, $\sum X_i$ represents the area under the intensity-distance curve of the i_{th} elution band and $\sum X_{tub}$ represents the area under the intensity-distance curve of the tubulin band*, which was loaded into the column.
(*If a dilution of purified tubulin is taken into the gel, we need to multiply with the factor of dilution to make it corresponding to the intensity of the tubulin loaded into the GST-TOG-1/2 column.)

2.7 In-vitro bulk tubulin polymerization assay

The reaction mix was 1X BRB80, 10% glycerol, 1 mM GTP, 1 mM $MgCl_2$ (for mung bean tubulin) and 5 mM $MgCl_2$ (for goat brain tubulin) and added either mung bean or goat brain tubulin, to study their respective kinetics. The reaction mix was prepared on ice and the start point of the polymerization reaction was when the polymerization mixture was loaded into a 96-well UV-transmissible half-area plate (Corning, USA) in the spectrophotometer (Varioskan Flash2, ThermoScientific, USA and EnSight multimode plate reader, PerkinElmer, USA) compartment and incubated at 37 ^{o}C. The polymerization of tubulin was assessed spectrophotometrically at 340 nm.

The absorbance output was blank-subtracted and path length corrected. A time-dependent absorbance (A_t) kinetics equation was fitted to the absorbance vs. time plot (Jain *et al.*, 2021).

$$A_t = A_0 + \frac{A_{max} - A_0}{1 - e^{-r*(t-t_{1/2})}} \tag{2.2}$$

Where, A_t = Absorbance at time t; A_0 = Initial absorbance; A_{max} = maximum absorbance; r = polymerization rate; $t_{1/2}$=half-maximal time of polymerization kinetics.

Pathlength correction is used to normalize it to the sample absorbance value in a standard cuvette of fixed pathlength (1 cm) by loading solvent buffer of a given sample volume into a microplate well, Incubating the microplate at 37 ^{o}C and measurng absorbance at 975 and 900 nm.

$$\text{Pathlength correction} = \frac{A_{975nm}^{microplate\smile} A_{900nm}^{microplate}}{A_{975nm}^{cuvette\smile} A_{900nm}^{cuvette}} * 10 \text{ mm} \tag{2.3}$$

where $A_{975nm}^{microplate}$ and $A_{900nm}^{microplate}$ represents absorbance reading of sample taken from a microplate at 975 and 900 nm respectively. Similarly, $A_{975nm}^{cuvette}$ and $A_{900nm}^{cuvette}$ represents absorbance reading of sample taken from a standard cuvette at 975 and 900 nm respectively.

$$A_{corrected} = A_{raw} * \frac{10 \text{ mm}}{\text{Pathlength correction}} \tag{2.4}$$

Where, $A_{corrected}$ represents the corrected absorbance reading, A_{raw} represents the raw absorbance reading taken from the microplate reader.

2.8 Data analysis

All graphs were generated using Python 3.8 with MATPLOTLIB libraries running on a Windows10 computer. Optimizing functions to data was performed using the Scientific Python Optimize libraries.

Chapter 3

Results

3.1 Optimization of transformant *E.coli* BL21 DE3 cells induction condition for optimum expression of GST-TOG-1/2 protein

Optimization of GST-TOG-1/2 induction conditions for transformant *E.coli* BL21 DE3 cell culture was done over 0.2 mM (literature value) and 0.1 mM of IPTG concentration. The reason being in previous literature (Hotta and Hashimoto, 2020; Widlund *et al.*, 2012), 0.2 mM concentration of IPTG was taken to induce the transformant *E.coli* BL21 DE3 cells, hence a lower concentration of IPTG (0.1 mM) was examined if it will result in more protein recovered in the supernatant or less protein aggregation compared to the standard 0.2 mM IPTG induction condition. According to Fig. 3.1, all the bands are equally similar in their band intensity qualitatively. Still, quantitatively, further analysing the image using the intensity function of ImageJ, the 0.2 mM IPTG supernatant was a bit more intense than the 0.1 mM IPTG supernatant band, suggesting more protein of size 85-100 KDa (Expected size of GST-TOG-1/2: 94 KDa) is greater in production in the 0.2 mM IPTG condition than in 0.1 mM IPTG condition. Further, the 0.2 mM pellet band is quantitatively lighter than the 0.1 mM pellet band (intensity function of ImageJ), suggesting that the protein aggregation was more significant in the 0.1 mM IPTG condition than compared 0.2 mM IPTG condition. This suggests that IPTG concentration lower than 0.2 mM doesn't increase the recovery of protein in the supernatant.

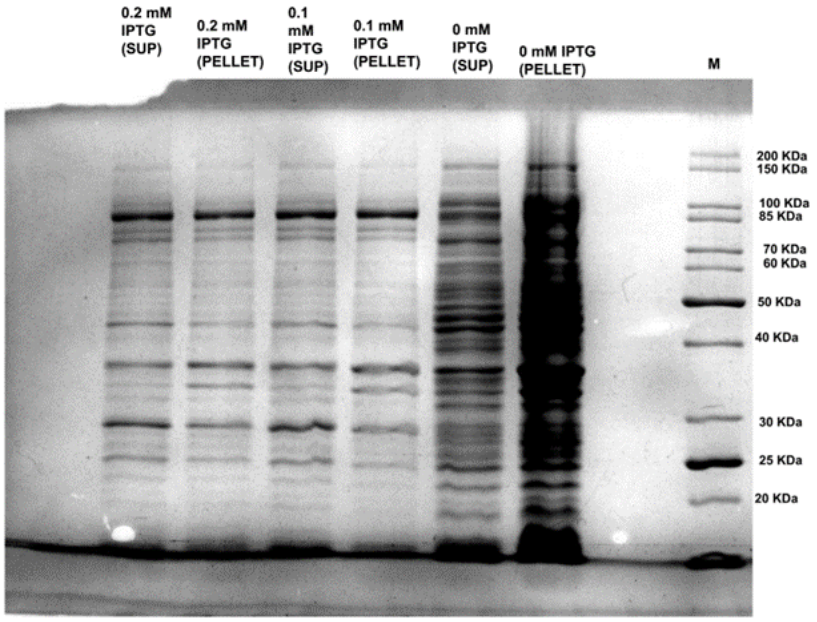
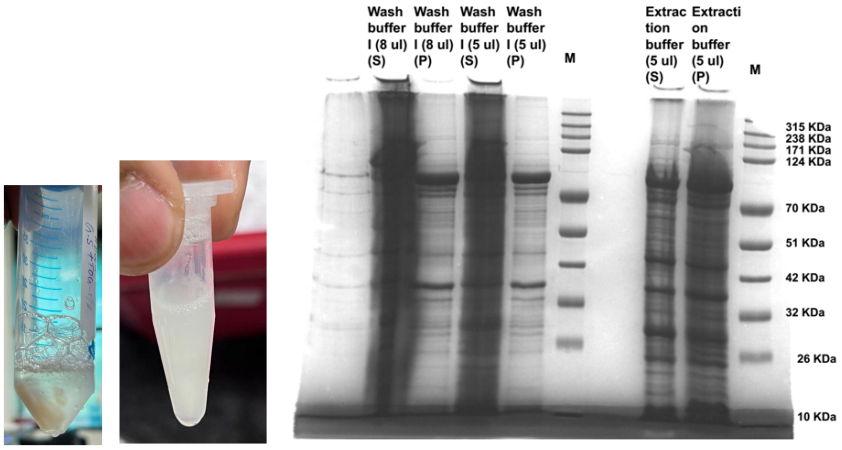


Figure 3.1: **Optimization of GST-TOG-1/2 induction condition using IPTG as an inducer in the secondary culture of *E.coli* BL21 DE3 transformant cells**. 0 mM IPTG represents uninduced cell culture and 0.1 and 0.2 mM IPTG represents induced cell culture with varying concentrations of IPTG inducer. For each condition, the culture was sonicated and centrifuged, to extract the pellet and supernatant for SDS-PAGE analysis.

3.2 Optimization of GST-TOG-1/2 purification condition from transformant *E.coli* BL21 DE3 culture cell pellet:

Resuspension condition for transformant *E.coli* BL21 DE3 culture cell pellet: The resuspension of Transformant *E.coli* BL21 DE3 cell pellets were necessary by using a suitable buffer condition for further cell lysis using sonication. In previous work over GST-TOG-1/2 affinity column, non-ionic detergents were used as a component in the resuspension buffer (eg. 0.01% triton X-100 or 0.1% tween-20 along with 1X PBS, 1 mM $MgCl_2$, 1mM DTT, 1mM ATP and 0.2 mM PMSF). It was suggested to ensure cell lysis. But, During the experimentation to resuspend the transformant *E.coli* BL21 DE3 culture cell pellet, the cells were not properly resuspended using the buffer containing triton X-100. It appeared the cells were all lysed due to detergent present in the buffer and appeared to be forming a clot-like structure. So, the same buffer condition except for any non-ionic surfactants as a component was considered for experimentation, which resulted in proper cell pellet resuspension, according to Fig. 3.2(a) and (b). According to Fig. 3.2(c), the extraction buffer (without sonication), the cell pellet was observed to be lysed a bit due to the presence of non-ionic detergent, but the wash buffer resuspension condition (after sonication), seems to look like the there is an issue with the supernatant, as it appears smeared over its gel lane, most probably due to improper DTT or PMSF concentration as it seems that the proteins are degraded by protease action. Hence, All stock solutions were re-prepared for the preparation of wash buffer I and it was decided that, Wash buffer I was apt to be used as a resuspension buffer as it has given a proper resuspend cell pellet for sonication.

Optimization of glutathione sepharose 4B Column condition: Glutathione sepharose 4B affinity column works on the principle that Glutathione S-transferase (present on the recombinant protein GST-TOG-1/2 at the N-terminal) will show affinity towards its substrate glutathione and later, the recombinant protein can be eluted using a gradient of reduced glutathione elution buffers. According to Fig. 3.3(a) and (b), It shows that 1 ml of glutathione sepharose 4B resin (Binding capacity: 5 mg/ml) volume was insufficient to bind and elute out the GST-TOG-1/2 protein from 670 ml *E.coli* BL21 DE3 culture cell pellet (Expected GST-TOG-1/2 weight: 3.35 mg). By referring Hotta and Hashimoto (2020), which suggested 5 ml resin volume for protein recovered per litre culture cell pellet and hence, only by increasing the resin volume to 3 ml, a proper amount of elution qualitatively occurred as seen in Fig. 3.3(c) and (d). But, there appeared



(a) (b) (c)

Figure 3.2: **Optimization of resuspension buffer condition**. (a) represents *E.coli* BL21 DE3 culture cell pellet resuspended in buffer containing triton X-100 and (b) represents *E.coli* BL21 DE3 culture cell pellet resuspended in buffer without triton X-100. (c) represents SDS-PAGE (10% resolving) was run with supernatant and pellet from conditions shown in fig. 3.2(a) and (b). The volume of sample load into the gel well is mentioned atop along each lane. ul: microlitre

significant non-specific proteins in the elutions. To reduce the amount of non-specific proteins in the elutions, wash buffer II was modified by the addition of 0.1 mM of reduced glutathione, based on the principle that weakly binding proteins elute early using a lower concentration of elution condition. But, according to Fig. 3.3(e), it seems the wash buffer II step didn't elute out the non-specific proteins from the column, which could be due to a very low concentration of reduced glutathione present in the modified wash buffer II.

Activity of purified GST-TOG-1/2 protein using *in-vitro* bulk tubulin polymerization kinetics: It has been explained Widlund *et al.* (2011), that combined TOG1-TOG2 (TOG domains) will show an increase in microtubule growth, as their presence will minimally (compared to entire stu2p protein) work to help in the polymerization of microtubule filaments. Hence, I tested the bulk polymerization kinetics of goat brain tubulin in the presence and absence of purified GST-TOG-1/2, expecting an enhancement of the microtubule polymerization kinetics curve in the presence of GST-TOG-1/2 compared to tubulin polymerization in absence of GST-TOG-1/2. According to fig. 3.4, it clearly indicates that the GST-TOG-1/2 is active

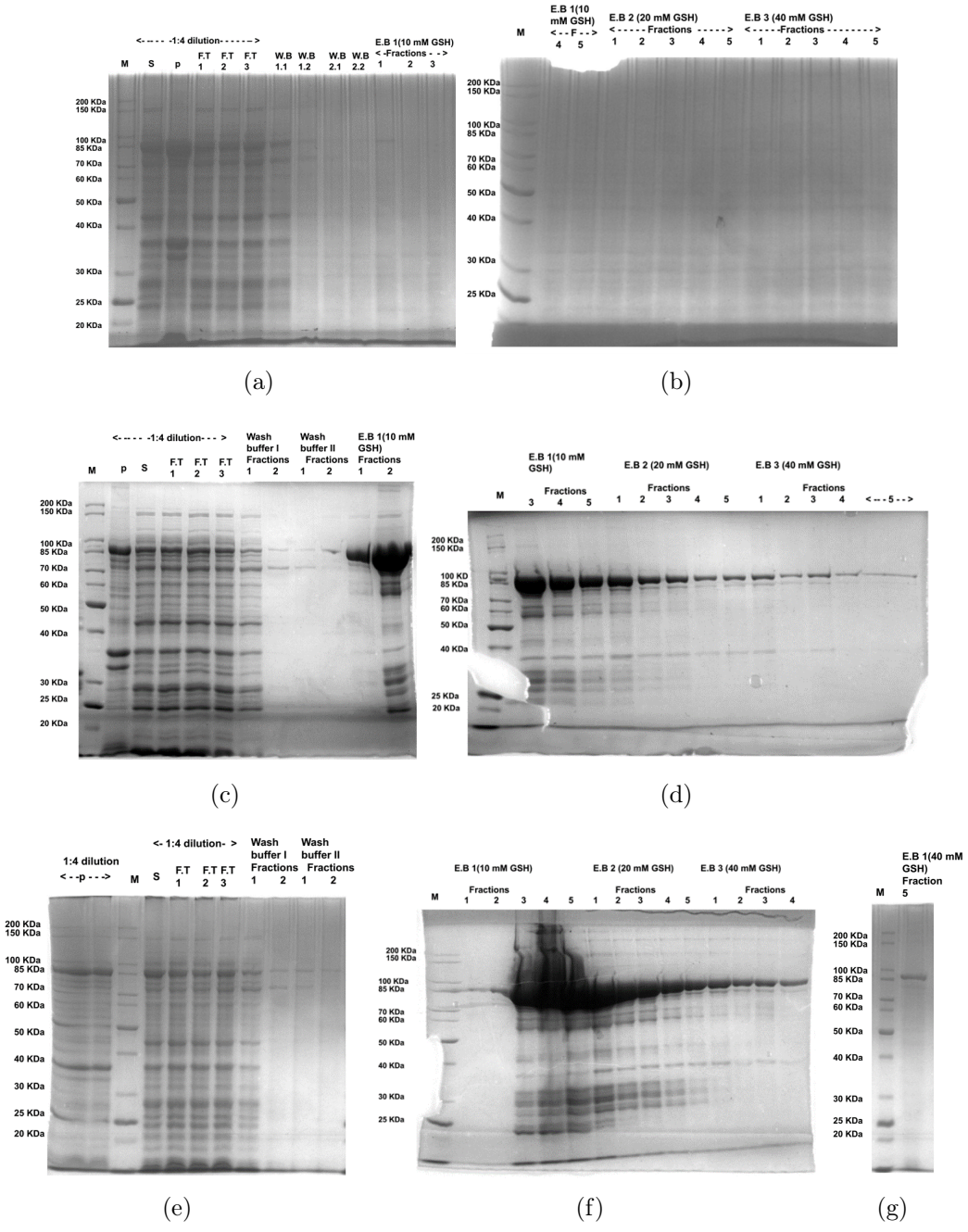


Figure 3.3: **Optimiztion of purification of GST-TOG-1/2 using glutathione sepharose 4B affinity column**. (a) and (b) represents purification of GST-TOG-1/2 from 670 ml *E.coli* BL21 DE3 culture cell pellet, using 1 ml glutathione sepharose 4B resin. (c) and (d) represents purification of GST-TOG-1/2 from 670 ml *E.coli* BL21 DE3 culture cell pellet, using 3 ml glutathione sepharose 4B resin. (e), (f) and (g) represents purification of GST-TOG-1/2 from 1340 ml *E.coli* BL21 DE3 culture cell pellet, using 6 ml glutathione sepharose 4B resin. 19

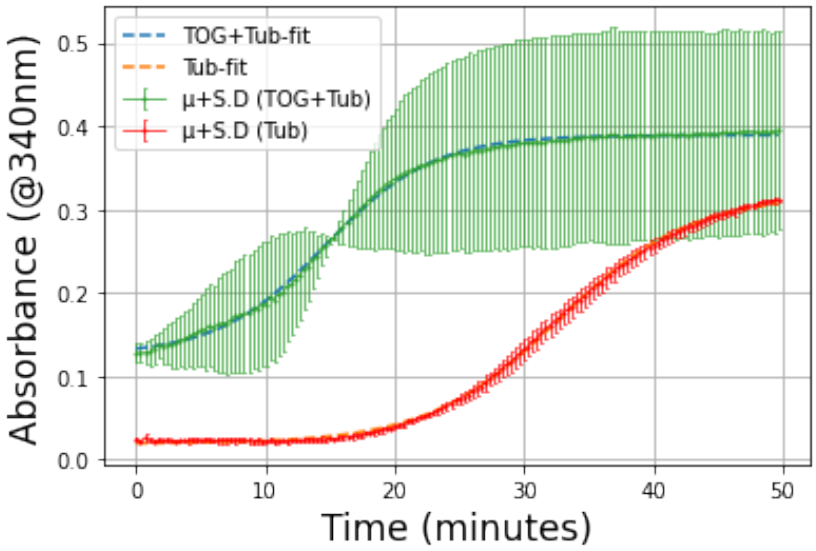
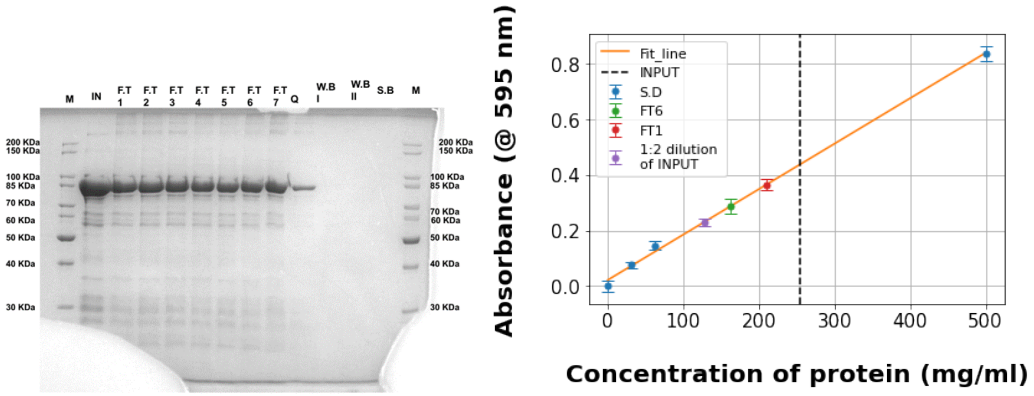


Figure 3.4: **Activity of purified GST-TOG-1/2 via bulk tubulin polymerization kinetics assay using goat brain tubulin**. The figure illustrates the bulk kinetics of two scenarios, (i) Tub: represents the standard condition for goat brain tubulin polymerization in the absence of GST-TOG-1/2 (n=3) and (ii) TOG+Tub: represents the standard condition for goat brain tubulin polymerization in the presence of GST-TOG-1/2 (n=2) (Error bar: 1*std dev; [Tub]: 15 μM, [GST-TOG-1/2]: 1.3 μM).

as it is able to enhance the bulk tubulin growth kinetics curve of goat brain tubulin.

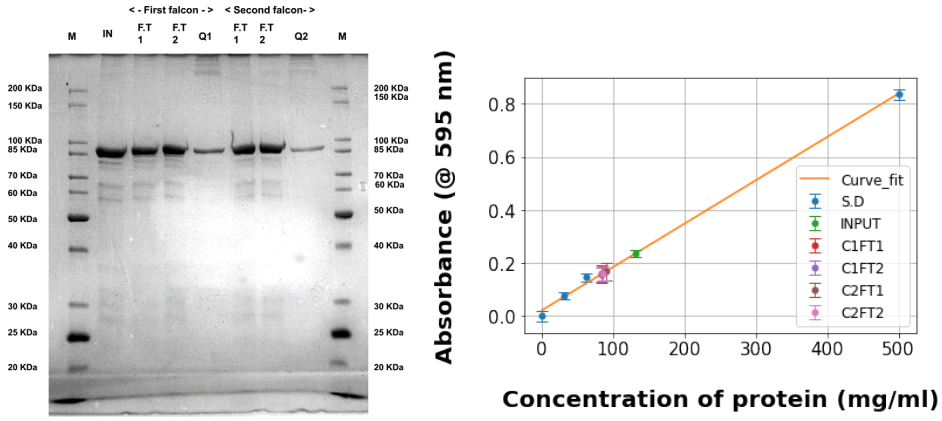
Optimization of GST-TOG-1/2 complexation with NHS-activated sepharose beads conditions: According to Fig. 3.5(a), It appears after examining flow through 1-7 (F.T 1 - F.T 7), qualitatively that there is less than 50% fraction of GST-TOG-1/2 that has coupled to 1 ml NHS-activated sepharose resin column. In this column run, only around 5.732 mg of dialyzed GST-TOG-1/2 (Stock: 0.26 mg/ml of 60 ml) was loaded into the column to test the coupling efficiency of the NHS-activated sepharose resin. Theoretically, all of the protein should have coupled as the coupling capacity of NHS-activated sepharose 4 fast-flow is 30 mg of coupled protein per ml of NHS-activated sepharose resin but according to Fig. 3.5(b) and later calculations, quantitatively only around 2.319 mg of protein-coupled to 1 ml NHS-activated sepharose resin column, indicating that the coupling efficiency was less than or around 40%. This suggests that NHS-activated sepharose 4 fast-flow resin may have a reduced binding capacity.

To test the binding capacity of NHS-activated sepharose, the complexation was tested using a lower volume of NHS-activated sepharose resin (400 μl) with using the 10 ml x2 of Flow through 7 (FT7 from Fig. 3.5(a)) as input for 400 μl x2 NHS-activated sepharose resin containing falcon. After each incubation period, the resin and flow through were separated using centrifugation at 7000g for 10 mins at 4 ^{o}C (Centrifuge 5810 R, Eppendorf). According to Fig. 3.6, qualitatively and quantitatively, there was less than 50% complexation of GST-TOG-1/2. As only a low amount of protein (around 1.2 mg) was loaded into each falcon compared to the binding capacity of NHS-activated sepharose resin for 400 μl (12 mg) and still the coupling efficiency is less than 50%, it suggests that NHS-activated sepharose 4 fast-flow resin may have a reduced binding capacity.



(a) (b)

Figure 3.5: **Complexation of 5.732 mg GST-TOG-1/2 with 1ml NHS-activated sepharose 4 fast flow resin**.(a) Complexation of GST-TOG-1/2 with 1 ml NHS-activated sepharose resin. The figure depicts SDS-PAGE for all the steps followed in the complexation process. IN: The Purified and dialyzed GST-TOG-1/2 protein (5.732 mg) as input, F.T1-F.T7: sequential 7 flow-through (Total incubation period: 2 hours), Q: Quenching step, W.B I and II: Wash buffer I and II and S.B: Storage buffer. (b) Bradford assay curve containing standard BSA data points (S.D) with a fit line (R^2:0.99), INPUT (dashed line): Input GST-TOG-1/2 (determined by doubling the concentration of 1:2 dilution of input sample, FT1: flow-through 1, FT6: flow-through 6. The error bar represents 1*standard deviation



(a) (b)

Figure 3.6: **Complexation of 10 ml x2 of used GST-TOG-1/2 (from flow through 7, fig. 3.5(a)) with 400 μl x2 NHS-activated sepharose resin**. The figure depicts SDS-PAGE for all the steps followed in the complexation process. IN: F.T7 from Fig. 3.5 as input, F.T 1 and F.T 2: 2 sequential flow-through (Total incubation period: 4 hours), Q1 and Q2: Quenching step. Two falcons were used, each containing 400 μl NHS-activated sepharose 4 fast-flow resin. (b) Bradford assay curve containing standard BSA data points (S.D) with a fit line (R^2:0.99), IN: Input GST-TOG-1/2 (F.T7 from Fig. 3.5), C1FT1: Column no. 1 flow-through 1, C1FT2: Column no. 1 flow-through 2, C2FT1: Column no. 2 flow-through 1, C2FT2: Column no. 2 flow-through 2. The error bar represents 1*standard deviation

To test the binding capacity of NHS-activated sepharose using BSA (Bovine serum albumin) as a control protein sample, the complexation was tested using a lower volume of NHS-activated sepharose resin (400 μl). Using 1.6 mg/ml (10 ml) of BSA and 0.8 mg/ml (10 ml) of BSA in dialysis buffer (0.1 $NaHCO_3$, 0.1 NaCl, pH = 8.2) as total input for two separate 400 μl NHS-activated sepharose resin (Total binding capacity: 12 mg) containing falcons, respectively. After an incubation period of 2 hours at 4 ^{o}C, the resin and flow through were separated using centrifugation at 7000g for 10 mins at 4 ^{o}C (Centrifuge 5810 R, Eppendorf). According to Fig. 3.7, qualitatively there was less than 50% complexation in overloading (10 ml of 1.6 mg/ml sample) and underloading (10 ml of 0.8 mg/ml sample) of BSA with respect to the bonding capacity of 12 mg for 400 μl NHS-activated sepharose resin, it confirms that NHS-activated sepharose 4 fast-flow resin has a reduced binding capacity.

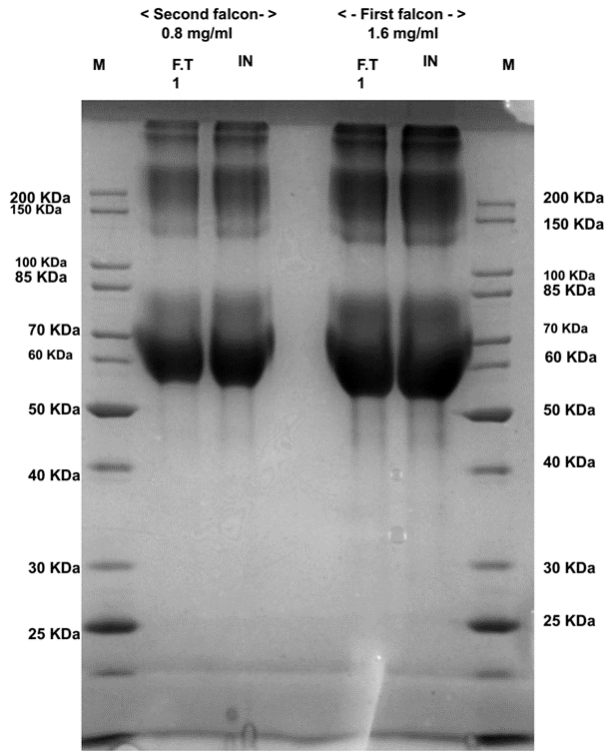


Figure 3.7: **Complexation of 1.6 mg/ml (10 ml) and 0.8 mg/ml (10 ml) of bovine serum albumin in dialysis buffer with two separate 400 μl NHS-activated sepharose resin**. The figure depicts SDS-PAGE for all the steps followed in the complexation process. IN: Input protein fraction, F.T 1: First flow-through (Total incubation period: 2 hours at 4 ^{o}C), Expected size of BSA: 66 KDa. Two falcons were used, each containing 400 μl NHS-activated sepharose 4 fast-flow resin.

3.3 Testing binding and elution efficiency of pre-made GST-TOG-1/2 columns using pre-purified goat brain tubulin

In previous work by Roy *et al.* (2021), they had prepared two GST-TOG-1/2 affinity columns of 1 and 2 ml, respectively and were stored at -20 ^{o}C. The binding and elution conditions for these columns have to be tested and optimized via a gradient of 10-500 mM ionic salt elution buffer (preferably using KCl or $(NH_4)_2SO_4$ as suggested by Widlund *et al.* (2012) and Hotta and Hashimoto (2020) by using pre-purified goat brain tubulin (purified using pol-depol method (Jain *et al.*, 2021)). According to Fig. 3.8(c) and Fig.

3.9(d), it shows that the total tubulin elution fraction of $(NH_4)_2SO_4$ elution, shows almost double the fraction of tubulin eluted compared to KCl elution for 1 ml TOG-1/2 column, suggesting that $(NH_4)_2SO_4$ salt improves the elution efficiency of 1 ml TOG-1/2 column. According to Fig. 3.8(d), it shows that 2 ml TOG-1/2 column KCl elution shows around 48.1% elution of loaded tubulin, which with the previous result suggests that the elution fraction of the loaded tubulin will be more than 50-60% of tubulin eluted from the column if $(NH_4)_2SO_4$ salt is used as elution, theoretically speaking but this needs to be tested experimentally.

3.4 Characteristics of bulk tubulin polymerization kinetics of goat and mung bean tubulin

Fig. 3.10 shows the differences in the activity of bulk tubulin from two distinct sources. The goat brain tubulin kinetics curve compared to mung bean tubulin (purified using pol-depol method (Jain *et al.*, 2021)) is smooth and easily distinguishable phases of polymerization. The heavy fluctuations as quantified in Fig. 3.10(e) in the kinetics curve of mung bean tubulin could be due to the low concentration (0.57 μM) of mung bean tubulin (Stock mung bean concentration = 0.67 μM, determined by BCA assay (Smith *et al.*, 1985)). In Fig. 3.11 and 3.12, we can observe the effect of glycerol (crowdant) on goat brain and mung bean bulk tubulin polymerization kinetics. Qualitatively, there is a significant difference in the kinetic curves of goat brain tubulin compared to the mung bean tubulin kinetics curve. After fitting the logistic growth equation 2.2 (Jain *et al.*, 2021) to all the mean polymerization kinetics curves, we observe that there is a slight increase in Amax (mean maximum absorbance (at asymptote)), polymerization rate (r) and a significant decrease in the t1/2 (half maximal time of polymerization reaction) with an increasing concentration of glycerol (crowdant) between the mean parameter value of 10% and 15% glycerol goat brain tubulin reaction mix.

There isn't a significant dissimilarity between the mean parameter value of 10% and 15% glycerol mung bean tubulin reaction kinetics curve. It could be due to low precision between each replicate of a sample due to handling error or due to low concentration of mung bean tubulin. Hence, the test needs to be repeated using a greater concentration of mung bean tubulin to observe a significant result.

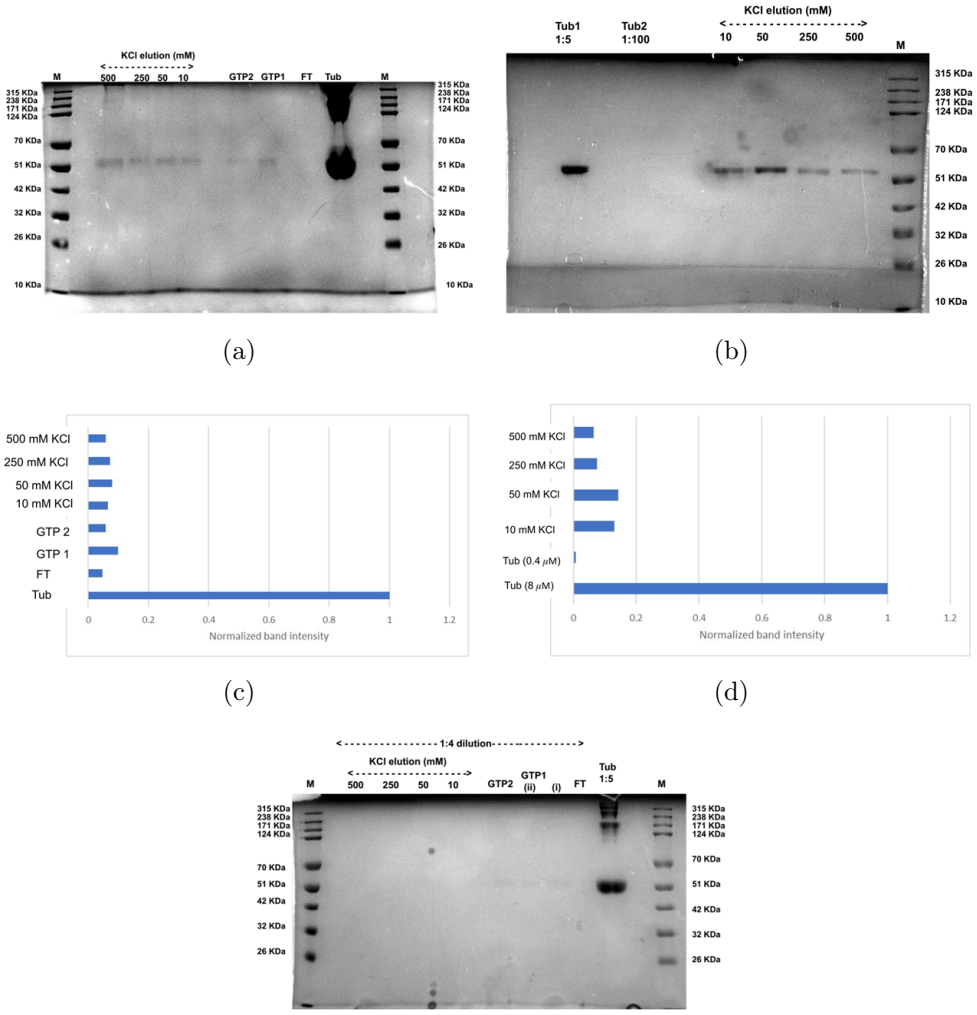


Figure 3.8: (a) The SDS-PAGE (10% resolving) was run with the loaded and eluted fractions from 1 ml TOG-1/2 column SDS-PAGE gel analysis([Tub]=4 μM, FT: Flow-through, 10,50,250 and 500 are respective 10 mM, 50mM, 250mM, and 500 mM KCl elution samples).(b) The second column of 2 ml TOG1/2 column SDS-PAGE gel analysis ([Tub1]=8 μM, [Tub2]=0.4 μM, 10,50,250 and 500 are respective 10 mM, 50mM, 250mM, and 500 mM KCl elution samples).(c) Normalized band intensity Vs. elutions of 1 ml TOG column (using SDS-PAGE gel image in fig. 3.8(a)).(d) Normalized band intensity Vs. elutions of 2 ml TOG column (using SDS-PAGE gel image in fig. 3.8(b); [Tub1] band was multiplied by a factor 5 to match the intensity of tubulin band which was loaded into the column: 40 μM). (e) SDS-PAGE gel analysis of 2 ml GST-TOG-1/2 column elutions using KCl as elution buffer (All the elutions except tub are 1:4 diluted, [Tub(1:5)]= 8 μM).

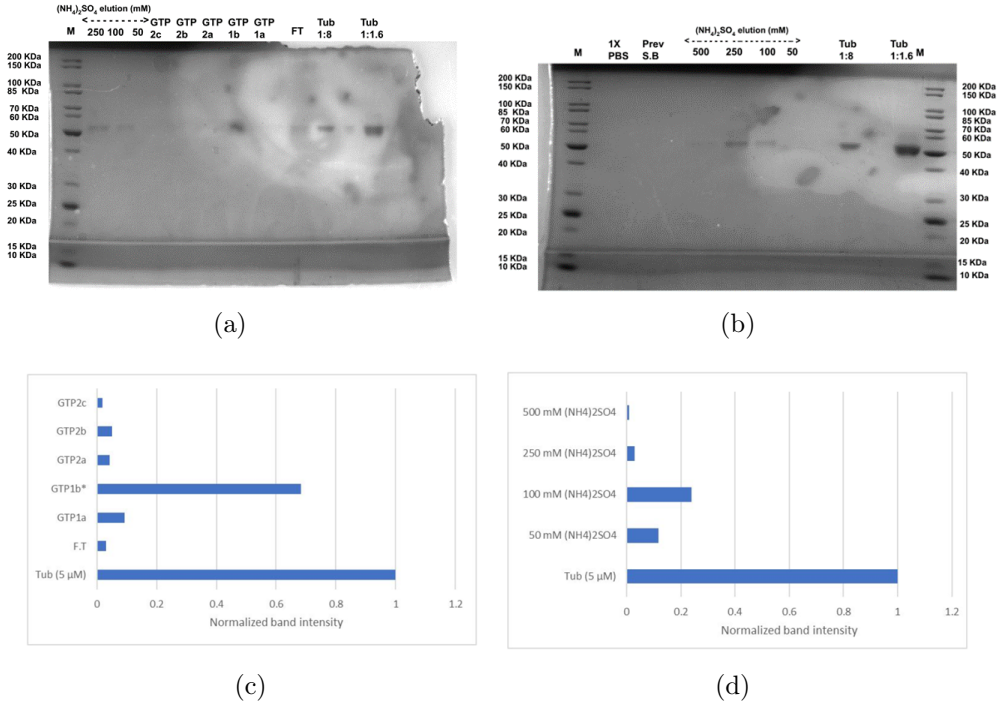
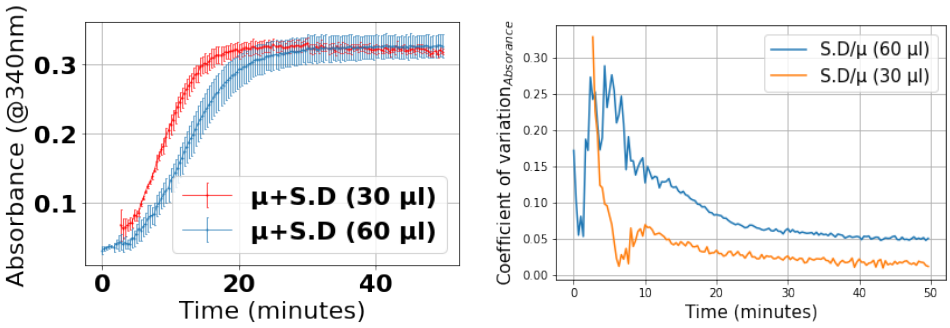
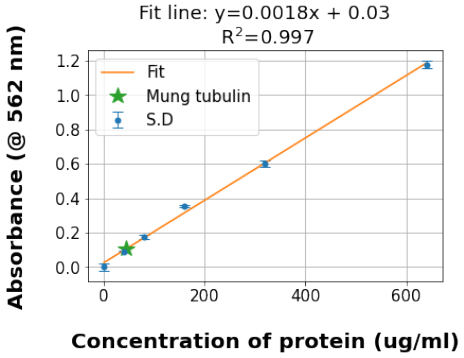


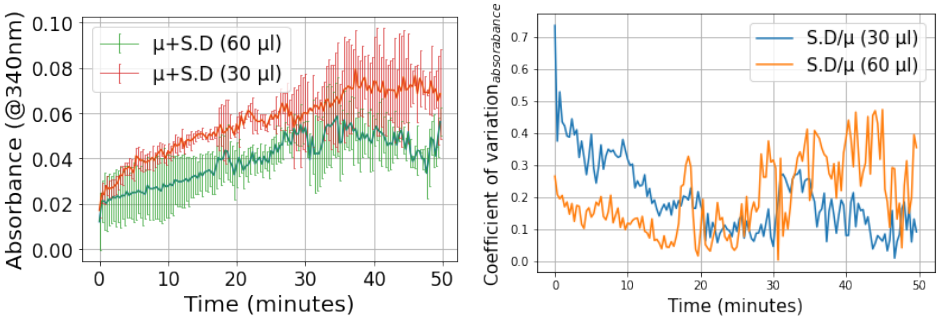
Figure 3.9: (a) The SDS-PAGE (10% resolving) was run with the loaded and eluted fractions from 1 ml TOG-1/2 column SDS-PAGE gel analysis([Tub(1:1.6)]=3.125 μM;[Tub(1:8)]=0.625; FT: Flow-through; first GTP wash: GTP 1a,1b; second GTP wash: GTP 2a,2b,2c; 50mM,100mM,250mM, and 500 mM $(NH_4)_2SO_4$ elution samples).(b) The second SDS-PAGE gel is the continuation of eluted fractions from 1 ml TOG-1/2 column SDS-PAGE gel analysis ([Tub(1:1.6)]=3.125 μM;[Tub(1:8)]=0.625; 50mM,100mM,250mM and 500mM are $(NH_4)_2SO_4$ elution samples; S.B prev: previous run storage buffer; 1X PBS: elution from regeneration step).(c) Normalized band intensity Vs. elutions of 1 ml TOG column (using SDS-PAGE gel image in fig. 3.9(a)).(d) Normalized band intensity Vs. elutions of 1 ml TOG column (using SDS-PAGE gel image in fig. 3.9(b)).



(a) (b)



(c)



(d) (e)

Figure 3.10: (a) Goat brain tubulin bulk polymerization kinetics under 10% glycerol concentration for 30 and 60 μl ([Tub]: 30μM) and (b) represents the plot of coefficient of variation of the absorbance reading vs. time for fig. 3.10(a). (c) Absorbance measured at 562 nm Vs. Concentration of BSA (μg/ml) plot, represents the linear plot derived from the BCA assay.(d) Mung bean tubulin bulk polymerization kinetics under 10% glycerol concentration for 30 and 60 μl ([Tub]: 0.57μM)(μ and S.D represents mean and 1*standard deviation respectively).(e) represents the plot of coefficient of variation of the absorbance reading vs. time for fig. 3.10(d)

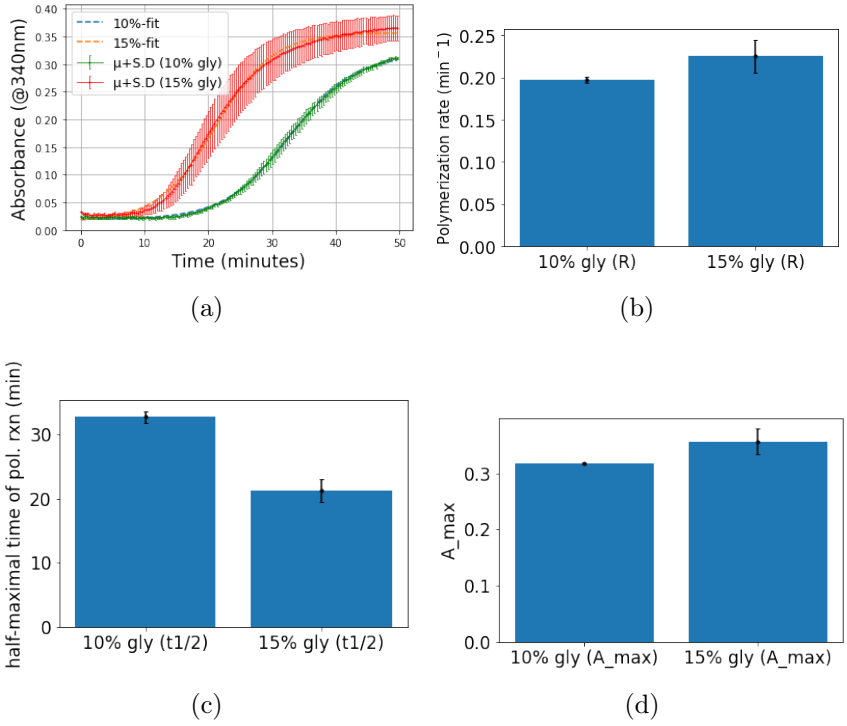


Figure 3.11: (a) Effect of glycerol (crowdant) on goat brain tubulin bulk polymerization kinetics under 10 and 15% (v/v) glycerol ([Tub]=15 μM); 10% - μ+S.D and 15% - μ+S.D means 10% and 15% (v/v) glycerol bulk tubulin polymerization kinetics condition, μ+S.D represents the mean and standard deviation for each sample (n=3); 10%-fit and 15%-fit are tubulin kinetics fit line for mean-10% line and mean-15% line (v/v) glycerol condition respectively, using equation 2.2. (b) Mean polymerization rate Vs. crowdant concentration in the reaction mix.(c) Mean half maximal time of polymerization reaction Vs. crowdant concentration (v/v) in the reaction mix.(d) Mean maximum absorbance (at asymptote) Vs. crowdant concentration (v/v) in the reaction mix. (Error bar: 1*std dev.; p-value<0.05 for half-maximal time of pol. rxn plot and p-value>0.05 for polymerization rate plot and mean maximum absorbance (at asymptote) plot: two sample t-test)

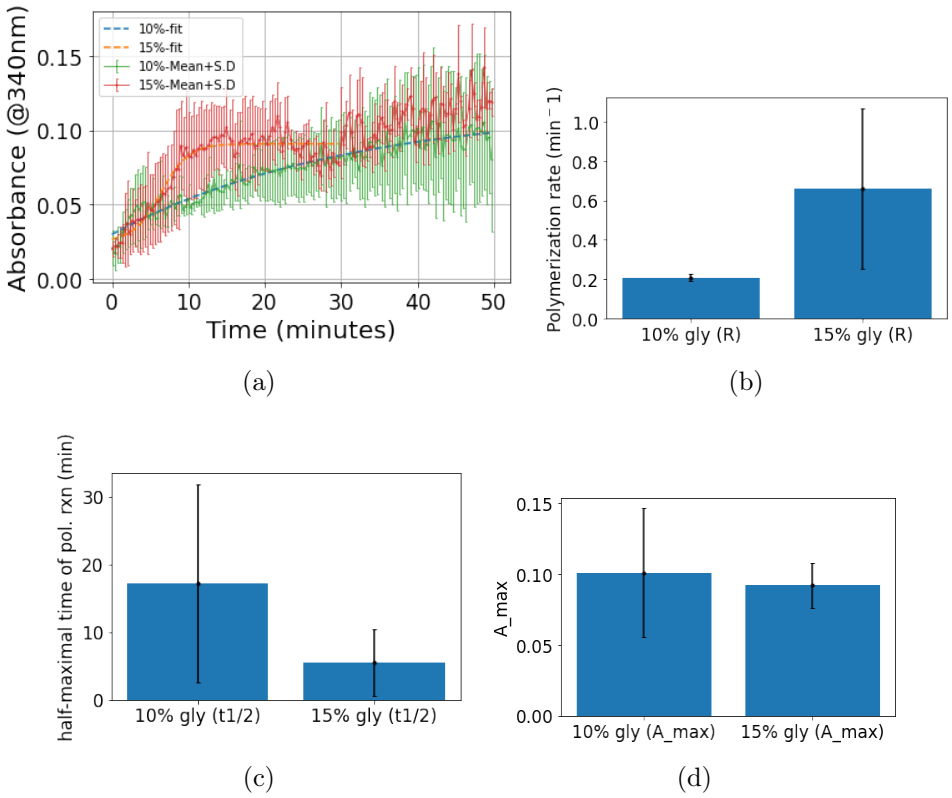


Figure 3.12: (a) Effect of glycerol (crowdant) on mung bean tubulin bulk polymerization kinetics under 10 and 15% (v/v) glycerol ([Tub]=0.53 μM); 10% - mean+S.D and 15% - mean+S.D means 10% and 15% (v/v) glycerol bulk tubulin polymerization kinetics condition, mean+S.D represents the mean and standard deviation for each sample (n=3); 10%-fit and 15%-fit are tubulin kinetics fit line for mean-10% line and mean-15% line (v/v) glycerol condition respectively, using equation 2.2. (b) Mean polymerization rate Vs. crowdant concentration in the reaction mix.(c) Mean half maximal time of polymerization reaction Vs. crowdant concentration (v/v) in the reaction mix.(d) Mean maximum absorbance (at asymptote) Vs. crowdant concentration (v/v) in the reaction mix. (Error bar: 1*std dev.; p-value>0.05 for all the plots, test: two sample t-test)

Chapter 4

Conclusions and Discussion

All the steps (Induction, extraction and purification condition) involved in the preparation of GST-TOG-1/2 were optimized, standardized and validated using experimental data with previous literature (Widlund *et al.*, 2012; Hotta and Hashimoto, 2020). The purified GST-TOG-1/2 protein is active as shown in fig. 3.4, but there was insufficient complexation of GST-TOG-1/2 protein due to the low binding capacity of NHS-activated sepharose beads. Optimization of elution conditions for pre-made TOG affinity column (Roy *et al.*, 2021) leads to suggest that, $(NH_4)_2SO_4$ as an elution buffer has an increased elution efficiency compared to KCl elution buffer, which may be due to higher ionic strength. By examining the effect of glycerol (crowdant) on bulk tubulin polymerization from standard and non-standard sources, there appears an increase in the rate of polymerization reaction with increasing glycerol concentration (from 10% to 15% v/v) for goat brain tubulin. But in the case of mung bean tubulin, it appears as a slight increase in the rate of polymerization reaction with increasing glycerol concentration (from 10% to 15% v/v). Hence, the assay needs to be repeated for mung bean tubulin with a greater concentration, to avoid loss of precision by increasing the signal-to-noise ratio, to suggest a common characteristic change in the bulk kinetics curve of tubulin from standard and non-standard sources.
In future, a fresh batch of the resin, combined with more tubulin purification can be used to examine kinetics of polymerization of multiple tubulins from non-standard sources and interestingly, according to my work on bulk polymerization and Molines *et al.* (2022) work on single filament polymerization, there appears a discrepancy observed in microtubule growth rate with respect to bulk and single filament dynamics data with an increasing concentration of glycerol (crowdant), which needs to be addressed.

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Appendix A

Additional data

A.1 Computational analysis of bulk tubulin polymerization data points using python:

1) Goat brain tubulin bulk tubulin polymerization kinetics curve analysis by fitting a logistic equation 2.2 mentioned in Jain *et al.* (2021) for gaining insights over the physical parameters.

```
import matplotlib.pyplot as plt
import numpy as np
import pandas as pd
from scipy.optimize import curve_fit

# file destination
PATH =('location of the file')
Data = pd.read_table(PATH)

# Fixes fonts for all elements of all plots.
font = {'family' : 'DejaVu Sans',
        'weight' : 'light',
        'size'   : 17}
plt.rc('font', **font)

# Rename all of the column names for ease
X=Data["Results of Photometric1"].to_frame().reset_index()

#list of all the time points from 0 to 2980,
edit it according to the new new time
```

```
points range
T=list(range(0, 3000, 20))

#Creating seperate dataframes for all
blanks and all replicates of
different glycerol concentrations.

#db10 represent blank containing
the reaction mix with 10% glycerol
without any tubulin.

d101,d102,d103 represents three replicates
for 10% glycerol concentration.

i=1
db=pd.DataFrame()
d101=pd.DataFrame()
d102=pd.DataFrame()
d103=pd.DataFrame()
while i<=600:
        if X["level_2"][i]==" G01":
                db=db.append(X[i:i+1])
        if X["level_2"][i]==" G02":
                d101=d101.append(X[i:i+1])
        if X["level_2"][i]==" G03":
                d102=d102.append(X[i:i+1])
        if X["level_2"][i]==" G04":
                d103=d103.append(X[i:i+1])
        i=i+1

i=1
D1=[]
D2=[]
D3=[]
D4=[]
while i<=150:
     n1=float(d101["level_7"][i])/0.180007-
     float(db["level_7"][i])/0.180007
     n2=float(d102["level_7"][i])/0.180007-
     float(db["level_7"][i])/0.180007
     n3=float(d103["level_7"][i])/0.180007-
```

```
    float(db["level_7"][i])/0.180007
    n4=(n1+n2+n3)/3
    D1.append(n1)
    D2.append(n2)
    D3.append(n3)
    D4.append(n4)
    i=i+1

#the time unit is changed from seconds to minutes
H=[]
for i in range(len(T)):
    x=T[i]/60
    H.append(x)

# Absorbance curve fitting function
a=A0 (initial absorbance)
b=Amax (Maximum absorbance (at equilibrium phase))
c=r (Polymerization rate)
d=t1/2 (half-maximal time of polymerization kinetics)

def f(x, a ,b ,c ,d):
    return a +((b-a)/ (1 + np.exp(-c*(x-d))))

#parameters and covariance values for
each replicate curve with their fit
params, pcov = curve_fit(f,H,D1)
params1, pcov1= curve_fit(f,H[8:],D2[8:])
params2, pcov2= curve_fit(f,H,D3)

print("R1fit: A0, Amax, r, t1/2:", params.round(3))
print("R2fit: A0, Amax, r, t1/2:", params1.round(3))
print("R3fit: A0, Amax, r, t1/2:", params2.round(3))

#eg. params is a set of numbers in which
at [0] position gives A0, at [1] gives Amax,
at [2] gives r and at [3] gives t1/2.
a,b,c,d=params
A,B,C,D=params1
aa,bb,cc,dd=params2
```

```
#fit line set.
y_line = f(H, a, b, c, d)
y_line1= f(H,A,B,C,D)
y_line2=f(H,aa,bb,cc,dd)

#Standard deviation of the polymerization
rate parameter

ravg1=(params[2]+params1[2]+params2[2])/3
Qerr=[]
x=2*(np.sqrt(((ravg1-params[2])**2+
(ravg1-params1[2])**2+
(ravg1-params2[2])**2)/3))
Qerr.append(x)
print(ravg1)
print(Qerr)

#Standard deviation of t1/2 parameter

tavg1=(params[3]+params1[3]+params2[3])/3
N=[params[3],params1[3],params2[3]]
Nerr=[]
n=2*(np.sqrt(((tavg1-params[3])**2+
(tavg1-params1[3])**2+
(tavg1-params2[3])**2)/3))
Nerr.append(n)
print(Nerr)
print(tavg1)

yerr=[]
i=0
while i<150:
    x=(np.sqrt(((D1[i]-D4[i])**2+
    (D2[i]-D4[i])**2+
    (D3[i]-D4[i])**2)/3))
    yerr.append(x)
    i+=1

#plot for tubulin in 10% v/v glycerol (three replicate):
plt.plot(H,D1, '*',markersize=5,label="R1")
plt.plot(H,y_line, label="R1fit")
```

```
plt.plot(H,D2, 'o', markersize=5,label="R2")
plt.plot(H,y_line1, label="R2fit")
plt.plot(H,D3, '+', markersize=5,label="R3")
plt.plot(H,y_line2, label="R3fit")
plt.plot(H,D4, label="Mean")
plt.errorbar(H,D4,yerr=yerr,
fmt='-o', markersize=1,label="error bar"
,capsize=0.5, linewidth=0.7)
plt.title("Absorbance Vs. Time
(for 10% v/v glycerol)\n")
plt.ylabel("Absorbance (@340nm)", size=20)
plt.xlabel("Time (minutes)", size=20)
plt.grid()
plt.legend(bbox_to_anchor=(1.01,1.15), loc="upper left")
plt.show()
```

A.2 Computational analysis of standard BCA assay data using python:

1) By fitting a linear equation through a standard line for differential diluted BSA solution by measuring absorbance at 562 nm as mentioned in Smith *et al.* (1985) for gaining insights over the unknown concentration of the given sample.

```
import matplotlib.pyplot as plt
import numpy as np
import pandas as pd
from scipy.optimize import curve_fit
from sklearn.linear_model import LinearRegression

PATH =('location of the file')
Data = pd.read_table(PATH)

# After assorting (collection into
proper sets, pathlength correction,
blank subtraction) the data
for each concentration of BSA,
given an unknown protein absorbance value.

#Below function and module
```

```
can be used to fit a line
through the standard points of BSA.

def f(x, a ,b):
    return a*x+b

params, pcov = curve_fit(f,Conc,Abs)
a,b=params
y_line = f(np.array(Conc), a, b)

model = LinearRegression()
model.fit((np.array(Conc).reshape((-1, 1))), Abs)
r_sq = model.score((np.array(Conc).reshape((-1, 1))), Abs)
print(f"coefficient of determination: {r_sq}")
print(f"intercept: {model.intercept_}")
print(f"slope: {model.coef_}")
y_pred = model.predict((np.array(Conc).reshape((-1, 1))))
print(f"predicted response:\n{y_pred}")

intercept={round(model.intercept_,2)}
```

SUPPLEMENT

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14) **Additional Terms for Specific Products and Services.** If a User is making one of the uses described in this Section 14, the additional terms and conditions apply:

a) ***Print Uses of Academic Course Content and Materials (photocopies for academic coursepacks or classroom handouts).*** For photocopies for academic coursepacks or classroom handouts the following additional terms apply:

i) The copies and anthologies created under this License may be made and assembled by faculty members individually or at their request by on-campus bookstores or copy centers, or by off-campus copy shops and other similar entities.

ii) No License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied) (ii) permit "publishing ventures" where any particular anthology would be systematically marketed at multiple institutions.

iii) Subject to any Publisher Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the academic pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to no more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular anthology, whether photocopied or electronic, at more than one institution of learning;

E) in the case of a photocopy permission, no materials may be entered into electronic memory by User except in order to produce an identical copy of a Work before or during the academic term (or analogous period) as to which any particular permission is granted. In the event that User shall choose to retain materials that are the subject of a photocopy permission in electronic memory for purposes of producing identical copies more than one day after such retention (but still within the scope of any permission granted), User must notify CCC of such fact in the applicable permission request and such retention shall constitute one copy actually sold for purposes of calculating permission fees due; and

F) any permission granted shall expire at the end of the class. No permission granted shall in any way include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied).

iv) Books and Records; Right to Audit. As to each permission granted under the academic pay-per-use Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any photocopies sold or by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this License for any reason.

b) ***Digital Pay-Per-Uses of Academic Course Content and Materials (e-coursepacks, electronic reserves, learning management systems, academic institution intranets).*** For uses in e-coursepacks, posts in electronic reserves, posts in learning management systems, or posts on academic institution intranets, the following additional terms apply:

i) The pay-per-uses subject to this Section 14(b) include:

A) **Posting e-reserves, course management systems, e-coursepacks for text-based content,** which grants authorizations to import requested material in electronic format, and allows electronic access to this material to members of a designated college or university class, under the direction of an instructor designated by the college or university, accessible only under appropriate electronic controls (e.g., password);

B) **Posting e-reserves, course management systems, e-coursepacks for material consisting of photographs or other still images not embedded in text,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorization: to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above, including any necessary resizing, reformatting or modification of the resolution of such requested material (provided that such modification does not alter the underlying editorial content or meaning of the requested material, and provided that the resulting modified content is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms), but not including any other form of manipulation, alteration or editing of the requested material;

C) **Posting e-reserves, course management systems, e-coursepacks or other academic distribution for audiovisual content,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorizations: (i) to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above; (ii) to display and perform the requested material to such members of such class in the physical classroom or remotely by means of streaming media or other video formats; and (iii) to "clip" or reformat the requested material for purposes of time or content management or ease of delivery, provided that such “clipping” or reformatting does not alter the underlying editorial content or meaning of the requested material and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms. Unless expressly set forth in the relevant Order Conformation, the License does not authorize any other form of manipulation, alteration or editing of the requested material.

ii) Unless expressly set forth in the relevant Order Confirmation, no License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied or, in the case of Works subject to Sections 14(b)(1)(B) or (C) above, as described in such Sections) (ii) permit "publishing ventures" where any particular course materials would be systematically marketed at multiple institutions.

iii) Subject to any further limitations determined in the Rightsholder Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the electronic course content pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular materials, whether photocopied or electronic, at more than one institution of learning;

E) electronic access to material which is the subject of an electronic-use permission must be limited by means of electronic password, student identification or other control permitting access solely to students and instructors in the class;

F) User must ensure (through use of an electronic cover page or other appropriate means) that any person, upon gaining electronic access to the material, which is the subject of a permission, shall see:

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- a statement to the effect that such copy was made pursuant to permission,
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G) any permission granted shall expire at the end of the class and, absent some other form of authorization, User is thereupon required to delete the applicable material from any electronic storage or to block electronic access to the applicable material.

iv) Uses of separate portions of a Work, even if they are to be included in the same course material or the same university or college class, require separate permissions under the electronic course content pay-per-use Service. Unless otherwise provided in the Order Confirmation, any grant of rights to User is limited to use completed no later than the end of the academic term (or analogous period) as to which any particular permission is granted.

v) Books and Records; Right to Audit. As to each permission granted under the electronic course content Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any electronic copies used by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this license for any reason.

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15) **Miscellaneous.**

a) User acknowledges that CCC may, from time to time, make changes or additions to the Service or to the Terms, and that Rightsholder may make changes or additions to the Rightsholder Terms. Such updated Terms will replace the prior terms and conditions in the order workflow and shall be effective as to any subsequent Licenses but shall not apply to Licenses already granted and paid for under a prior set of terms.

b) Use of User-related information collected through the Service is governed by CCC's privacy policy, available online at www.copyright.com/about/privacy-policy/.

c) The License is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the License or any rights granted thereunder; provided, however, that, where applicable, User may assign such License in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in any new material which includes the Work(s) licensed under this Service.

d) No amendment or waiver of any Terms is binding unless set forth in writing and signed by the appropriate parties, including, where applicable, the Rightsholder. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by or on behalf of the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the License described in the Order Confirmation, which terms are in any way inconsistent with any Terms set forth in the Order Confirmation, and/or in CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

e) The License described in the Order Confirmation shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such License shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.

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