Development of a novel biochemical assay for the identification of promiscuous compounds

by

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Supervised by Dr. Raymond Hewer

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Science, University of KwaZulu-Natal, Pietermaritzburg, from January 2017 to December 2018, under the supervision of Dr. Raymond Hewer. The studies represent original work by the author and none of this work has been submitted for the award of any degree or examination at any university. All authors of data and any other information has been acknowledged accordingly by reference.

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DECLARATION 1: PLAGIARISM

I, **Nomfundo Ntombela**, declare that:

1. The research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work.

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This thesis is dedicated to my mother, Miss Khanyisile Ntombela, Mahlobo, Dlukula, Mpangazitha, thank you so much for your prayers and support.

ABSTRACT

Promiscuous compounds, specifically aggregate-based inhibitors that result in false positives in biochemical activity assays present a serious and increasing interference to early-stage drug discovery processes. Although under-used, a number of purpose-specific assays have been developed to enable the identification of promiscuous inhibitors. To advance on these efforts, this study aimed to develop and optimise a novel thermal shift assay to concurrently identify both true and promiscuous inhibitors. In particular, stem bromelain selected as the model protein for this study was successfully isolated from the bromelain mixture through molecular exclusion chromatography and shown to be enzymatically active in the titrimetric assay (gelatin digestive unit/gm enzyme of 2024.36-2085.5). The radial diffusion assay predicted that the promiscuous compound Epigallocatechin gallate and the known aggregate based-promiscuous compound Congo Red activated the digestion of gelatin by stem bromelain. In the thermal shift assay, bromelain yielded a melting temperature of ~75-76 °C which then shifted by 9 °C in the presence of a true inhibitor E-64. A similar shift was surprisingly observed in the presence of Epigallocatechin gallate and both these compounds were similarly not affected by the presence of a detergent (0.004% sodium dodecyl sulfate). The protein was aggregated in the presence of Congo Red, however, the addition of the detergent effectively restored the protein to its original melting temperature. Both Epigallocatechin gallate and Congo Red demonstrated cytotoxicity. As a proof of concept, this study showed that in addition to identifying true inhibitors, the detergent-based thermal shift assay can be successfully employed to identify promiscuous inhibitors and to determine different mechanisms.

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ABBREVIATIONS

°C	Degrees Celsius
3D	Three dimensional
β	Beta
µg	Microgram
µg/ µL	Microgram/ microlitre
µL	Microlitre
µM	Micromolar
CC_{50}	Cytotoxic concentration 50
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1- propanesulfonate
CMC	Critical micellar concentrations
CO_2	Carbon dioxide
CR	Congo Red
CETSA	Cell-based thermal shift assay
dH_2O	Distilled water
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DSC	Differential scanning calorimetry
DSF	Differential scanning fluorimetry
DSLS	Differential static light scattering
DTT	Dithiothreitol

E-64	*N*-[*N*-(L-3-*trans*-carboxyirane-2-carbonyl)-L-leucyl] agmatine
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
g	Gram
GDUs	Gelatin digestive units
H_2O_2	Hydrogen peroxide
HEK 293T cells	Human embryonic kidney 293 cells with the SV40 T-antigen
HRM	High resolution melting
HTS	High throughput screening
IC_{50}	Inhibitory concentration 50
kDa	Kilo Dalton
MEC	Molecular exclusion chromatography
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar
MTT	3-4,5 dimethylthioazol-2,5 diphenyl tetrazolium bromide
nm	Nanometer
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
QSAR	Quantitative structure-activity relationship
RCCs	Redox recycling compounds
SB	Stem bromelain
SH	Thiol
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SPR	Surface Plasmon Resonance
TPCK	Nα-Tosyl-L-lysine chloromethyl ketone
TSA	Thermal shift assays
T_m	Melting temperature
UK	United Kingdom
USA	United States of America
x g	Relative centrifugal force

Chapter 1: Introduction

1.1. Drug discovery and development

The process of drug discovery and development is complex, it involves several steps and as such, it takes years before a drug reaches the market (Figure 1.1). Additionally, the process of producing a successful drug can range from $1.3 billion to $12 billion of costs as stated in Herper, 2012. Early-stage drug discovery is the initial phase of the discovery and development pipeline (Figure 1.1). This phase is initiated in search of suitable medical products to treat clinical conditions and diseases whose clinical needs are not met, as reviewed in Hughes *et al.*, 2011.

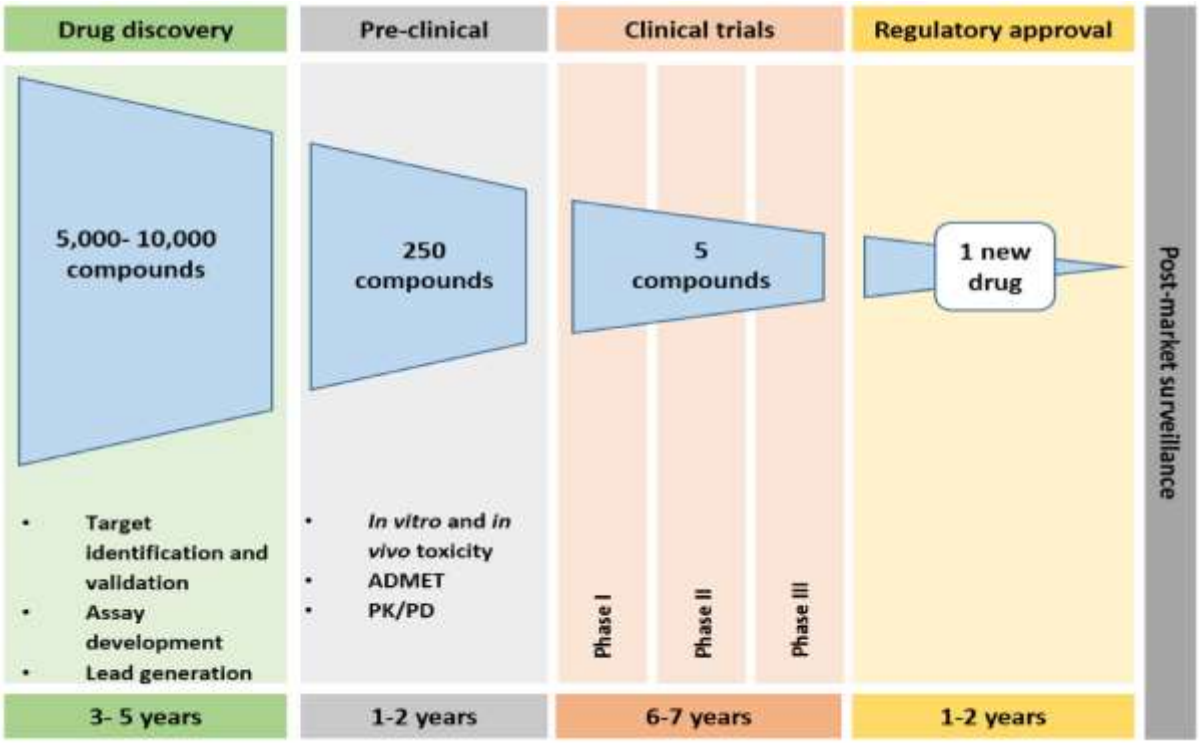


Figure 1.1: The drug discovery development and pipeline. The process of drug development highlighting different stages of the pipeline and the estimated duration of each stage. Drug discovery is the initial stage of this process with several assays used to generate leads, followed by the pre-clinical where lead compounds undergo further validation both *in vitro* and *in vivo*. Once validated, the next stage is the clinical trial which is divided into three phases. The success of these stages promote the approval stage that comes before the drug is available in the market. Figure obtained from Matthews *et al*., 2016.

Like any other scientific process, drug discovery starts with intensive research about a potential drug candidate before developing a theory that promoting, blocking or modifying the interactions of a pathway, or protein or a target may lead to a therapeutic benefit for a clinical condition or disease (Hughes *et al.*, 2011). Driven by this theory, the proceeding steps usually involve the selection of a potential target that is further validated before progressing into the lead discovery phase. Once at this phase, approximately 5 000 - 10 000 compounds undergo thorough tests in the quest for one potential marketed drug. Out of the compounds tested, approximately 250 compounds proceed into preclinical development as leads for further validation (Figure 1.1) (Hughes *et al.*, 2011). If a lead proves to be effective, it progresses to clinical development and eventually into a marketed drug (Alvarez and Shoichet, 2005).

1.2. Different approaches to lead identification

Drug discovery uses several approaches to acquire novel drug compounds, these include non-random screening and clinical observation. The random and rational drug design approaches, both extensively used when seeking new compounds are discussed below.

1.2.1. Rational drug design

Unlike the traditional try-and-error methods used in drug discovery, the rational approach uses a collection of computational methods whose success is not subject to chance alone, but mostly dependent on the initial knowledge of the effect that a compound will have on a specific target (Parrill and Reddy, 1999; Baldi, 2010; Kalyani *et al*., 2013). Briefly, this approach involves designing small molecules called HITS with desired or even predefined properties for a target whose function may be known or unknown (Mandal *et al*., 2009). These HITS are compounds that bring about desired effect on a specific target, they are identified by screening libraries of compounds against specific proteins for further development into potential leads (Wunberg *et al*., 2006). Ligand-based and structure-based drug design sum up the facets of rational drug design as shown in Figure 1.2. The key difference is that structure-based drug design exploits information of a known or predicted target structure (Bohacek *et al*., 1996). Whereas in ligand-based drug design information about the compound (ligand) that binds to the target is exploited (Marrone *et al*., 1997; Kurogi and Guner, 2001; Aparoy *et al*., 2012). The ligand-based design is often used in the absence of a three-dimensional (3D) structure of the target. Using several tools such as the 3D quantitative structure-activity relationship (QSAR), ligand-based drug design has led to the identification of pharmaceutical products that have contributed to drug discovery. Examples include the acetylcholinesterase inhibitor donepezil hydrochloride used in patients with Alzheimer's disease under the market name Aricept (Kawakami *et al*., 1996).

1.2.2. Random drug discovery

This approach which is highly dependent on chance revolves around the random screening of thousands of molecules in hope of finding compounds that have activating or inhibiting properties towards a target. The screened compounds which are often sourced from synthetic chemicals, marine or microbial origin and natural plant products undergo a series of tests before a compound can be optimized as a lead (Boehm *et al*., 2000; Kalyani *et al*., 2013). Even though it went through a period of being overlooked in the past, random drug design has improved over the years through the development of new technologies (Figure 1.2), some of which are discussed below.

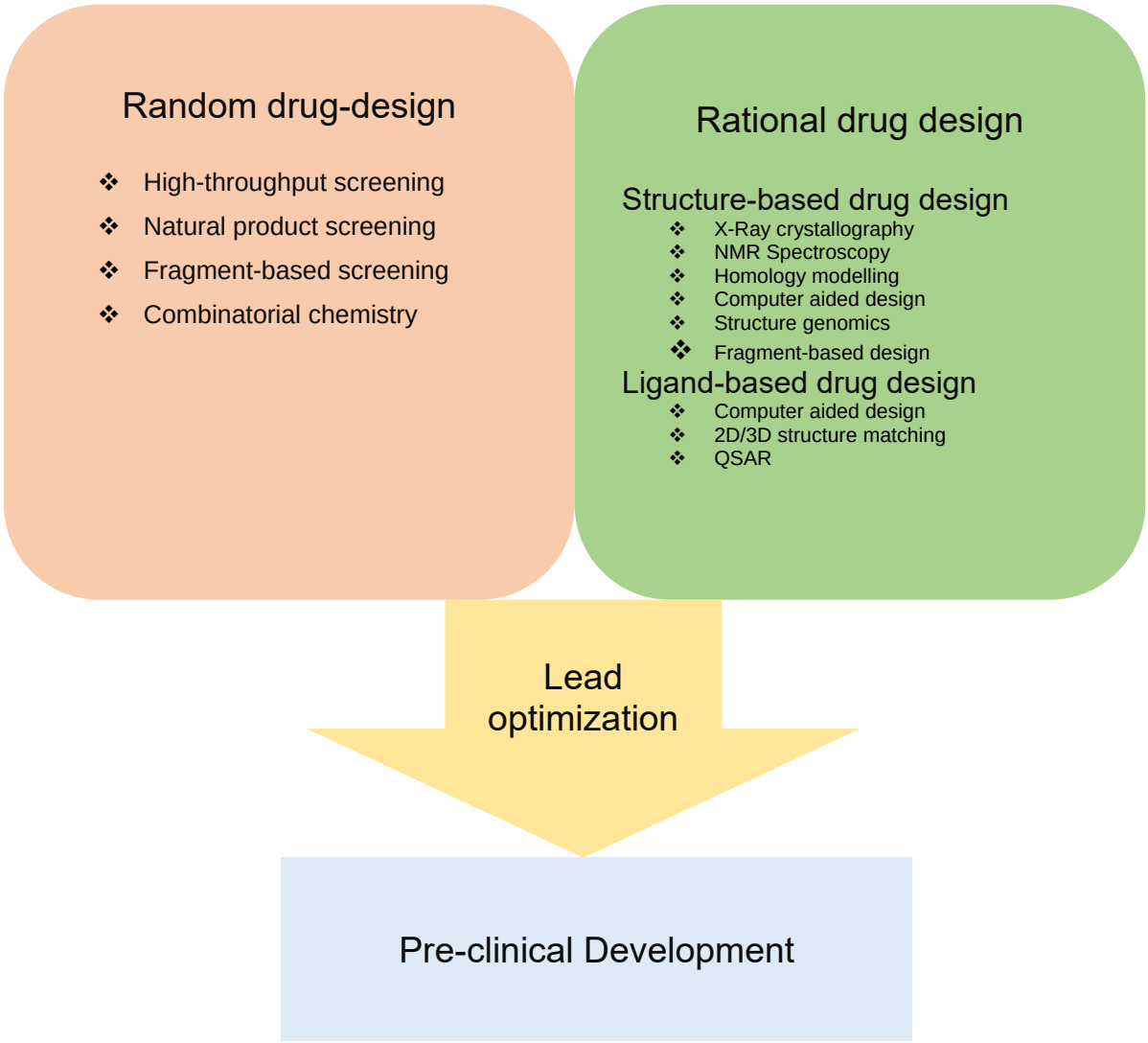


Figure 1.2: An overview of random and rational approach in drug discovery. The lead identification approaches; random and rational drug design showing different techniques applied for each approach. These all promote lead optimization and pre-clinical development. The modified image was adapted from Hewer *et al*., 2012.

1.2.2.1. Combinatorial chemistry

Combinatorial chemistry encompasses the chemical synthesis that enables the generation of a large array of chemically diverse compounds commonly referred to as a library (Darvas *et al*., 2001). This is achieved through a systematic linkage of chemical building blocks to explore all possible combinations (Figure 1.3). The solid phase is employed during compound synthesis where chemicals are put on resin beads which can be easily washed off or removed once the linkage or reaction is complete (Ohlmeyer *et al*., 1993).

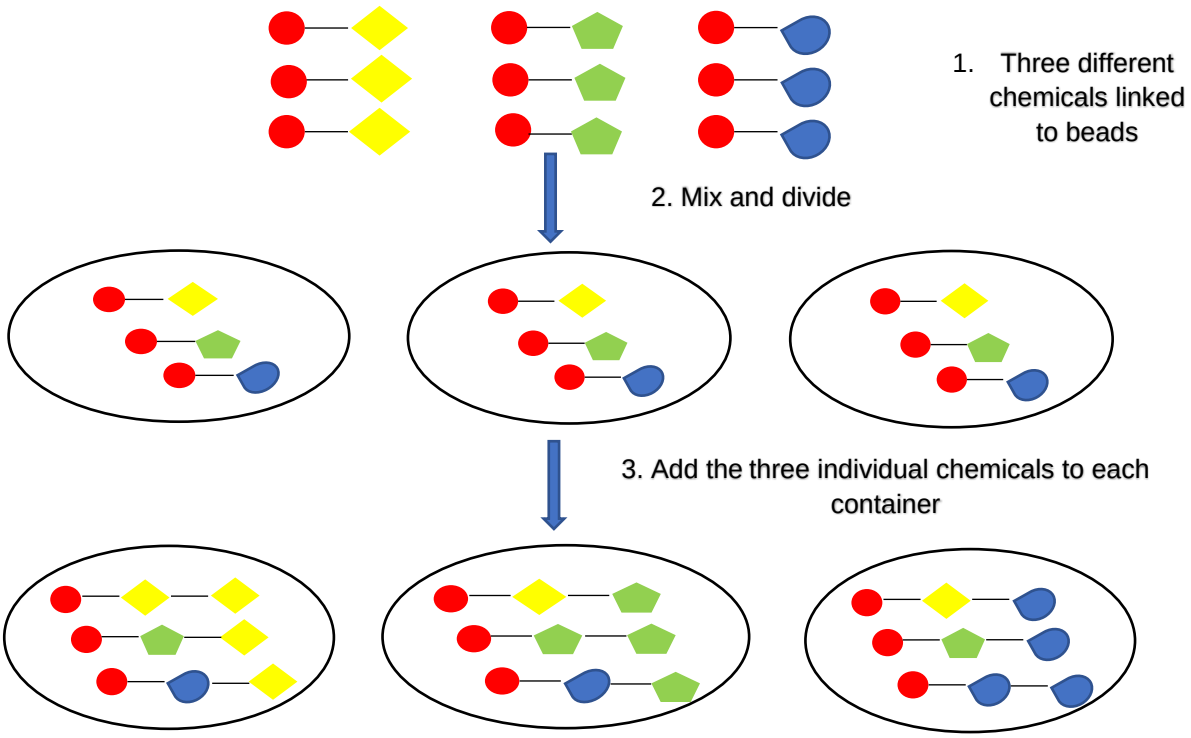


Figure 1.3: A typical combinatorial chemistry process. An illustration of how compounds are synthesized using combinatory chemistry, the red circles represent the beads that link each chemical. The chemicals are then mixed and divided into three containers (1), where all the containers have identical sets (2). The chemicals shown in 1 are then added to the three containers to obtain all possible combinations (3).

This highly automated technique used for lead identification has elevated random drug discovery as it produces large libraries of compounds in a shortened time (Miertus *et al*., 2000). Following the testing of these lead compounds against potential drug targets is a lead optimization technique with similar properties as combinatorial chemistry. This technique is called parallel synthesis, and it uses more focused methods to further produce a great number of variants of the potential leads. Another dominantly used technology in drug discovery is

called the high-throughput screening (HTS) (Jadhav *et al.,* 2009; Chan *et al.*, 2009). Combinatorial chemistry runs parallel with HTS; where combinatorial chemistry made possible the generation of huge numbers of new compounds to feed into the screening machines, and HTS could run through the new combinatorial compound libraries at a fast rate (Miertus *et al*., 2000; Darvas *et al*., 2001; Jadhav *et al.,* 2009).

1.2.2.2. High throughput screening

HTS is a process whereby hundreds of thousands of samples are assayed and screened against specific targets under given conditions (Szymański *et al*., 2011). This process is guided by four elements, the first one being suitably arranged compound libraries which are normally obtained from combinatorial synthesis or products extracted from natural plants (Broach and Thorner, 1996). The samples or libraries used can be of different biochemical nature such as live cells, protein, chemical compounds or peptides (del Álamo *et al*., 2016). The second element involves the development of a screening assay, where assay conditions are validated to screen the arrayed compound library (Broach and Thorner, 1996). Thirdly, it requires a robotic workstation for which assays are usually automated in the form of 96-well plates. The final element is the analysis which requires a computerized system to handle and analyze data (Broach and Thorner, 1996). Currently, with the help of the robotic equipment that systematizes this process, more than 100,000 samples can be screened and analyzed a day. It is through this advantage that HTS is popular in science since it enables the generation of a reliable database of lead compounds in a short amount of time (Janzen, 2014). However, the actual preparation steps involved in HTS; sample preparation, sample handling, and data analysis can take days (Dragiev *et al*., 2011).

Over the years, HTS has become a cohesive part of drug discovery processes. This is because the method enables the rapid identification of HITS or small molecules that can further be generated into leads that specifically inhibit enzyme function (Feng *et al*., 2005; Wunberg *et al.*, 2006). The main goal of HTS is to identify HITS that are active at low a concentration. The lower the concentration at which the compound is found to be effective, the more likely that it will exhibit specificity, and as a result, the less likely that it will have undesired side effects (Broach and Thorner, 1996).

1.3. Promiscuous compounds as a challenge facing drug discovery

Promiscuous compounds are defined as non-specific small molecules that can interact (inhibit or activate) with more than one target (Ingólfsson *et al*., 2014). These targets can vary in structure, function and even originate from different families. In their pursuit to understand these compounds, Hu and Bajorath described compound promiscuity under assay conditions as the presence of specific interactions amongst micro molecules and a variety of biological targets (Hu and Bajorath, 2013). According to this definition, promiscuous compounds not only interact with targets, but they can also interact with each other, hence they are called promiscuous due to their unselective nature. The unintentional identification of promiscuous compounds wastes time and resources which can be dedicated to pursuing compounds that can be further developed into potential drugs (Roche *et al*., 2002). A number of compounds (toxoflavin, rhodanines etc.) have been exposed to have promiscuous activity, however, regardless of this knowledge, such compounds are still being used as potential leads (Bruns and Watson, 2012).

1.3.1. Typical behaviors of promiscuous compounds

Promiscuous compounds are usually identified by their puzzling behaviors in biochemical assays. These include a steep-dose response curve, concentration-dependent inhibition, flattened structure-activity relationship, and inhibition of unrelated targets. In 2012, Bruns and Watson developed a set of 275 rules that guide the identification of compounds that interfere with biological assays so that they can be eliminated in screening sets (Bruns and Watson, 2012). Mostly, these rules are based on the structure (for example, ring, halogen, aldehyde etc) and function (protecting groups, chelators etc.) of the compounds (Bruns and Watson, 2012).

The dose-response curve explains the relationship between the activity of a compound (the effect) and its concentration (the given amount), (Walters and Namchuk, 2003). One common indication of promiscuity among HITS is a high Hill slope shown in a steep dose-response curve. Hence, compounds with a slope of much greater than one should not be regarded as HITS in biochemical assays. For promiscuous compounds, inhibition increases with an increase in concentration which is expected. A comparison of the acceptable Hill slope and the rejected one is shown in Figure 1.4, ‘compound a’ has a Hill slope of 1.3 which makes the compound acceptable whereas ‘compound b’ has a higher Hill slope of 8.6 which makes it unacceptable.

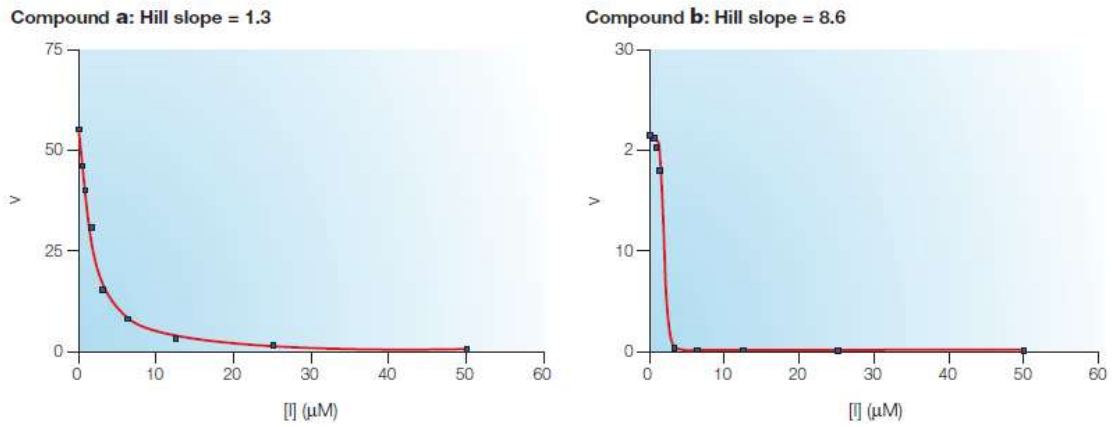


Figure 1.4: Dose-response curves comparing two different compounds with the same IC_{50}. Image adopted from Walters and Namchuk, 2003.

1.3.2. Mechanism of action

Although promiscuous compounds have received a lot of research attention over the years, still not much is certain about their mechanism of inhibition or stoichiometry towards the target. One of the common mechanisms amongst promiscuous compounds are the aggregate inducing compounds which results in false positives. Table 1.1 shows examples of other promiscuous compounds known as Pan Assay Interference Compounds (PAINS) which have been identified in high throughput screens. These compounds were identified by their ability to act as promising, but misleading HITS against different and unrelated targets (Dahlin *et a*l., 2015; Dahlin and Walters, 2016). As shown in Table 1.1, these compounds can interact with the target using three mechanisms of action, namely; redox recycling, metal complexation, and covalent modification.

Table 1.1: Different mechanisms for pan assay interference compounds toward promiscuous inhibition.

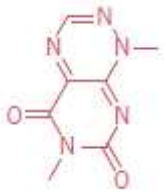
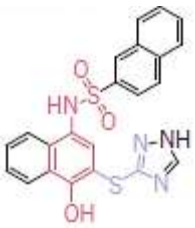
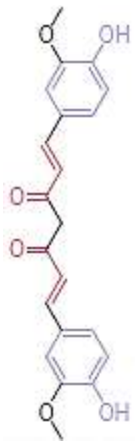
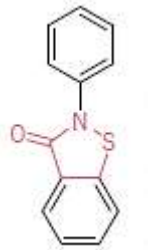
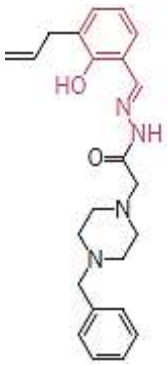
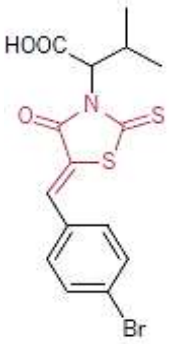
Mechanism	Examples		Description
Redox recyclers	Toxoflavin 	Phenol-sulphonamides 	Compounds produce hydrogen peroxide which activates and inactivate different proteins and breaks down into molecules that give false signals.
Covalent modification	Curcumin 	Isothiazolones 	These compounds react chemically with proteins in a non-specific, non-drug like way. They also disturb the response of membrane receptors.
Metal complexation	Hydroxyphenyl 	Ene-rhodanine 	Compounds sequester metal ions that inactivates proteins.

Table information obtained from Baell and Walters, 2014.

1.3.2.1. Redox recycling compounds

Redox recycling compounds (RCCs), also known as redox recyclers, produce hydrogen peroxide in the presence of strong reducing agents such as dithiothreitol (DTT) (Figure 1.5) (Baell and Walters, 2014). In HTS assays, such reducing agents are common components found in assay or enzyme storage buffers used to maintain catalytic activity and/or folding of target proteins (Lor *et al*., 2007; Johnston *et al*., 2008; Soares *et al*., 2010). The hydrogen peroxide produced by RCCs oxidizes cysteine residues that are vital to keeping target proteins in their active form (Tierno *et al*., 2007; Baell and Walters, 2014). In search of compounds that inhibit caspase-8, Smith and co-workers conducted an HTS of more than 700,000 compounds which generated about 1% of HITS (Smith *et al*., 2002). However, 85% of the primary group of compounds tested were found to be RCCs as they inhibited the enzyme upon the production of hydrogen peroxide in the presence of the reducing agent DTT (Smith *et al*., 2002).

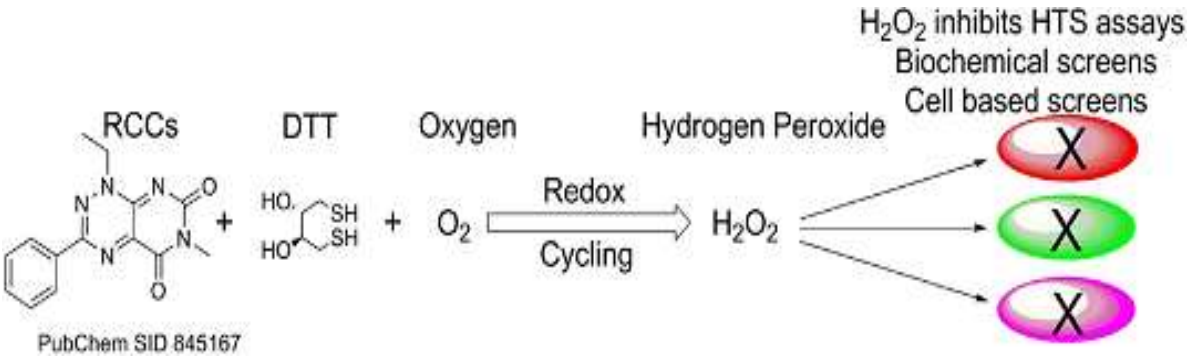


Figure 1.5: The generation of hydrogen peroxide by redox recycling compounds in the presence of a reducing agent. Figure obtained from Johnston, 2011.

1.3.2.2. Covalent modification

Covalent modification occurs as result of the removal or attachment of a molecule to a protein or enzyme (Figure 1.6). Often, the contributing molecule provides a functional moiety that alters the function, catalytic activity and structure of the enzyme (Berg *et al*., 2002). Consequently, a new substrate might then be required for the modified enzyme. Covalent modification is an enzyme regulatory strategy that can be both irreversible and reversible (Hinman *et al*., 2006; Tizón *et al*., 2015). Examples of covalent modification include but not limited to phosphorylation and acetylation (Berg *et al*., 2002). Promiscuous compounds Epigallatocatchin-3-gallate (EGCG) and curcumin are amongst the compounds reported to

interact with proteins through both covalent modification and redox recycling (Ishii *et al*., 2008; Baell and Walters, 2014; Farhan *et al*., 2016).

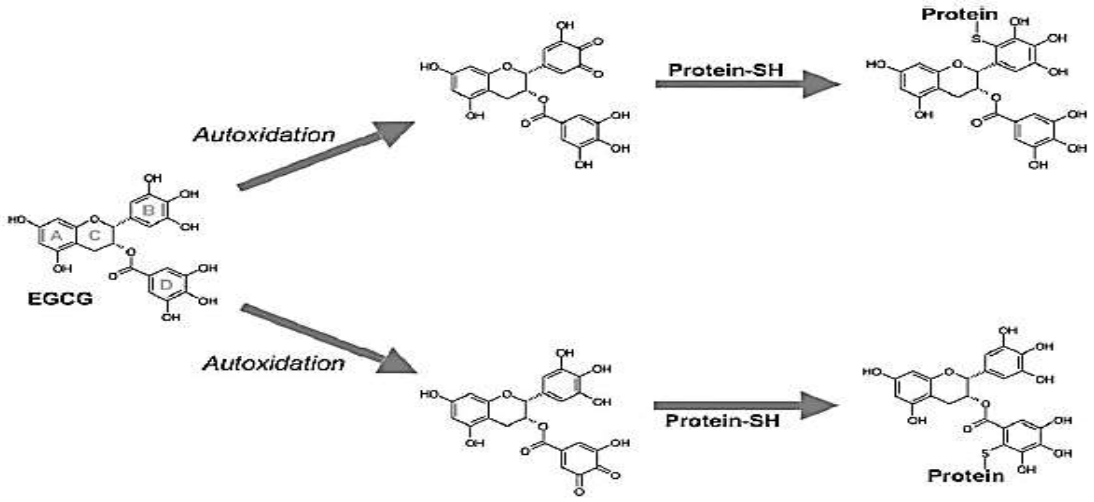


Figure 1.6: An example of covalent modification of a protein by EGCG through autoxidation. Figure obtained from Ishii *et al*., 2008.

1.4. Aggregate-based promiscuous compounds

Of the known types of promiscuous compounds, aggregate-based compounds remain dominant. Despite their dominance, not much is known about aggregate structure, concentration, stoichiometry and even their affinity to targets remains inconclusive. However, it is common knowledge that these small molecules form colloids of 30-400 nm in diameter and their existence results in false positives in biochemical assays (Doak *et al*., 2010). As per their name, these microscopic molecules are formed by spontaneous self-association which leads to aggregation at micromolar concentrations in aqueous solutions (Doak *et al*., 2010). These molecules are problematic and are challenging to identify because they do not affect spectral assays, do not appear to be chemically reactive and they pass internal filters previously used to identify promiscuous compounds (Shoichet, 2004). The internal filters include interference with assay measurements such as absorbance and fluorescence; instability; reactivity and compounds that do not possess both oxygen and nitrogen which are considered to lack drug-ability (Bruns and Watson, 2012). Figure 1.7 shows a typical example of non-specific inhibition by aggregate forming compounds.

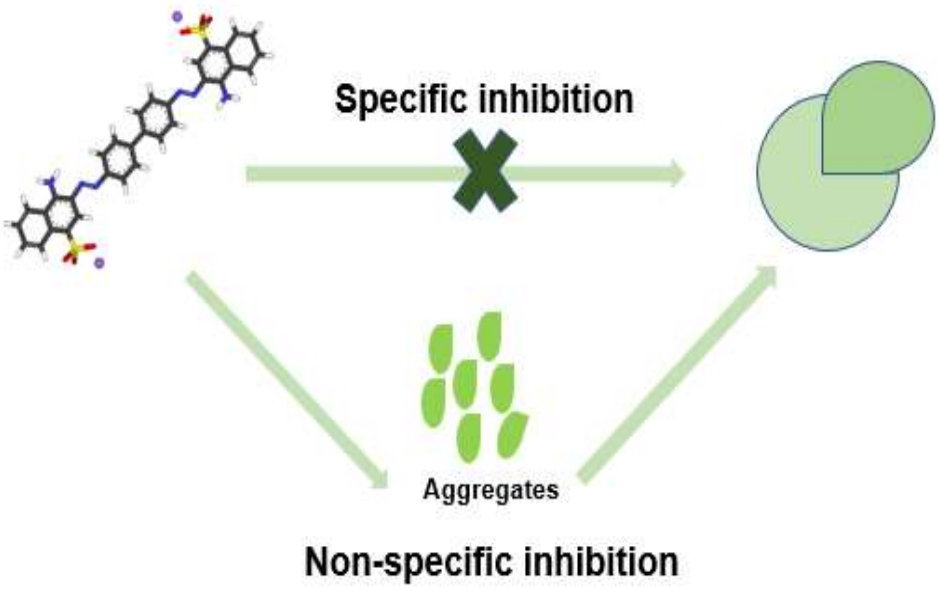


Figure 1.7: Enzyme inhibition through aggregation. The process that results in the formation of the aggregates through self-association of single compounds which non-specifically inhibit the enzyme.

1.4.1. Signatures of aggregate-forming compounds

McGovern and colleagues were the first to identify aggregation as a mechanism for non-specific inhibition (McGovern *et al.*, 2002). Even though their initial intention was to identify inhibitors for β-lactamase, the compounds tested showed puzzling behaviours. Initially, these compounds were thought to interact through covalent inhibition, however, this speculation was soon rooted out since inhibition of these compounds showed to be reversible through dilution which is not a trait of covalent inhibitors (McGovern *et al.*, 2002). Inhibition of three unrelated proteins, being non-competitive and steep dose-response curves were amongst the other odd behaviours identified in the tested compounds. In 2003, McGovern and co-workers further investigated 100 small compounds (including clinical drugs, leads and screening HITS) to further understand this mechanism for non-specific inhibition (McGovern *et al.*, 2003). Of the compounds screened, approximately 50 were reported to show inhibition of various dissimilar enzymes in a time-dependent fashion (McGovern *et al.*, 2003). These results led to the properties that help identify and distinguish the aggregate-forming inhibitors from well-behaved inhibitors. The promiscuous inhibitors were found to be sensitive to detergents, ionic strength and enzyme concentration which was seen to be different for the well-behaved, or true inhibitors (McGovern *et al*., 2003). Table 1.2 shows various properties of aggregate-based promiscuous inhibitors and two of these properties (sensitivity to increased enzyme concentration and effect of incubation) are discussed briefly below.

1.4.2. Sensitivity to increased protein concentration

One of the well-known signatures of aggregate-based promiscuous compounds is their sensitivity towards changes in enzyme concentrations. In the work done by McGovern and co-workers in 2002, they found that increasing the concentration of the enzyme or target resulted to a drastic decrease in inhibition effect of the aggregate based compound at micromolar concentrations (McGovern *et al.*, 2002). Conversely, this was not the case with true or well-behaved inhibitors, since increasing the protein concentration did not interfere with inhibition. Experimentally, testing for this feature can be done by performing a screening assay with an increased enzyme concentration (10-fold) (McGovern *et al.*, 2002). Normally, this test is done with the inhibitor present at an inhibitory concentration 50% as determined by the same enzyme system before the increase in enzyme concentration.

1.4.3. Effect of incubation time

Inhibition by aggregate forming inhibitors increases when these compounds are pre-incubated with the enzyme 5 minutes before adding the substrate, which is different when compared to the same aggregate forming inhibitors without pre-incubation (McGovern *et al.*, 2002). Incubation is reported to not be specific for the formation of aggregators since there are inhibitory mechanisms that operate in a time-dependent manner such as covalent binding (Alvarez and Shoichet, 2005). This effect can be tested by adding an incubation period to the protocol. If the inhibitor shows time dependence behavior, apparent inhibition should decrease in the absence of the incubation period. Normally, this assay is performed with the inhibitor at the IC_{50} concentration obtained in the typical assay of incubation (Alvarez and Shoichet, 2005). For aggregate inhibitors, the inhibition should decrease by 50% to about 40% without incubation. As a control well behaved, non-time dependent inhibitors of the target should not show an appropriate difference in inhibition with or without inhibition period.

Table 1.2: Comparison of the typical behaviours of aggregate-forming inhibitors and well-behaved inhibitors.

Experimental features	Aggregate-based inhibitors	Well-behaved inhibitors	References
Decreased inhibition in the presence of a detergent	✓	X	Feng and Shoichet, 2006
Inhibition of unrelated enzymes	✓	X	Seidler *et al*., 2003
Decreased inhibition in the presence of albumin	✓	Occasionally	Alvarez and Shoichet, 2005
Inhibition in orthogonal assays	X	✓	Thorne *et al.*, 2010
Decreased inhibition in the presence of increased enzyme concentration	✓	X	McGovern *et al.*, 2002
Formation of particles detectable by light scattering	✓	X	Seidler *et al*., 2003
Inhibition of wild-type and mutant proteins	✓	X	Ravikumar *et al*., 2004
Decreased inhibition in the presence of increased ionic strength	✓	Occasionally	McGovern *et al.*, 2002
Increased inhibition with incubation time	✓	Occasionally	McGovern *et al*., 2002

1.4.4. Mechanism of interaction between aggregators and protein

Although not much is known about how these aggregate inhibitors achieve their function, a number of possible mechanisms of action have been discussed (Coan *et al*., 2009). These include chemical reactivity, denaturing, sequestering, endpoint interference and restrained dynamics. This study will focus on three of these mechanisms and they are discussed below.

1.4.4.1. Sequestering

Enzyme or protein sequestering is one of the common mechanisms that result in promiscuous inhibition (McGovern *et al*., 2003). Sequestering occurs at certain conditions and specific micro-molar concentrations, where small molecules self-associate to form microscopic colloidal aggregate particles that are 30-400nm in diameter in aqueous solutions (Thorne *et al.*, 2010). The aggregate forms colloids which cover or aggregate the enzyme thereby sequestering it from the reaction mixture (Figure 1.8C). It then appears as though the enzyme has been inhibited since its activity cannot be detected in the solution.

1.4.4.2. Enzyme unfolding and restrained dynamics

When the aggregate-based inhibitors interact with the protein or the enzyme, a disruption of the protein's structure occurs. This disruption interferes with the function of proteins hence resulting in non-specific inhibition. In 2009, Coan and co-workers reported that the association of colloidal aggregate with an enzyme or protein might result to partial denaturing of the enzyme and this might be a possible inhibitory mechanism (Figure 1.8A) (Coan *et al*., 2009). Once the enzyme's structure has been denatured or partially unfolded in the presence of the aggregate, it becomes less stable, leading to none or less activity. Hence, it appears as if the enzyme has been inhibited (Coan *et al*., 2009). Kinetic experiments have shown that solvent denaturants do not decrease potency or affect the aggregators (Coan *et al*., 2009). Additionally, other studies have shown that the decrease in thermodynamic stability of an enzyme does not increase the potency of aggregate-forming compounds (Coan *et al*., 2009). This mechanism is general since it does not require any specific feature of the aggregate structure or the enzyme. What this mechanism requires are non-polar residues (present in all enzymes) which associate with the non-polar surface of the aggregate inhibitors which is also always present. This explains the unselective and non-specific nature of aggregate inhibitors with soluble proteins. Another possible mechanism which is referred to as the restrained dynamic interaction is shown in Figure 1.8B. Instead of unfolding, the aggregate inhibitor might solidify the enzyme which restricts any possible movement. Therefore, the enzyme cannot interact or bind the inhibitor or substrate. Again, it will appear as though the enzyme is inhibited whereas its' structure and hence activity are restrained.

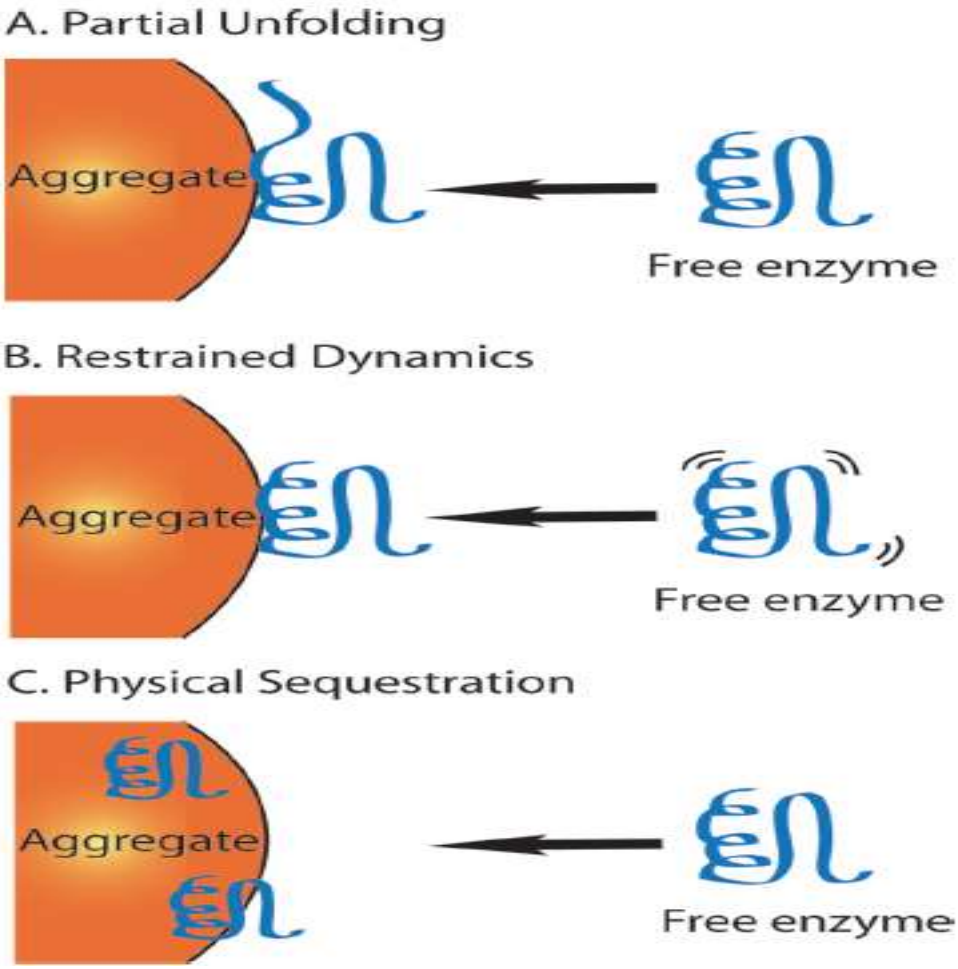


Figure 1.8: Three models of inhibitory mechanisms of aggregate-based promiscuous compounds. The promiscuous aggregate inhibitor (orange), interacts with the enzyme (blue ribbons) and the black arrows show how they proceed with the interaction. A. shows interaction with the aggregate can denature the protein leading to partial unfolding. B. shows the interaction with the enzyme restrains its dynamics and restricts its' catalytic function. C. aggregates form colloids that sequester the enzyme. Figure obtained from Coan *et al*., 2009.

1.4.5. Known compounds previously identified as aggregators

Generally, aggregators are highly conjugated and hydrophobic molecules (Coan and Shoichet, 2008). However, predicting which compounds aggregate with a glance has proven to be difficult since aggregation depends on several factors such as pH, temperature, concentration and others listed in Table 1.2.

1.4.5.1. Congo Red

Congo Red is a benzidine-based stain that belongs to the family of Azo dyes (Figure 1.9). It is one of the first direct dyes which can have direct contact or can directly dye cotton without needing a fixation substance (Frid *et al*., 2007; Yaneva and Georgieva, 2012). This synthetic dye was first discovered in 1884 by Paul Böttiger, a young German scientist who first used it as a pH indicator (Steensma, 2001; Frid *et al*., 2007; Yaneva and Georgieva 2012). It was later that Congo Red was discovered to detect amyloid beta in cells since it binds and aggregates the beta conformation of the protein, this remained the major function of Congo Red in biochemical assays (Wood *et al*., 1983). However, recent studies showed that Congo Red binds to other proteins of different configuration and these proteins were unrelated (McGovern *et al*., 2002). This led to the conclusion that this dye resulted in non-specific binding, hence, it was identified as one of the promiscuous aggregate compounds. McGovern and colleagues demonstrated that the presence of Congo Red in biochemical assays aggregated proteins of unrelated natures such as chymotrypsin, β-lactamase and dihydrofolate reductase (McGovern *et al*., 2002).

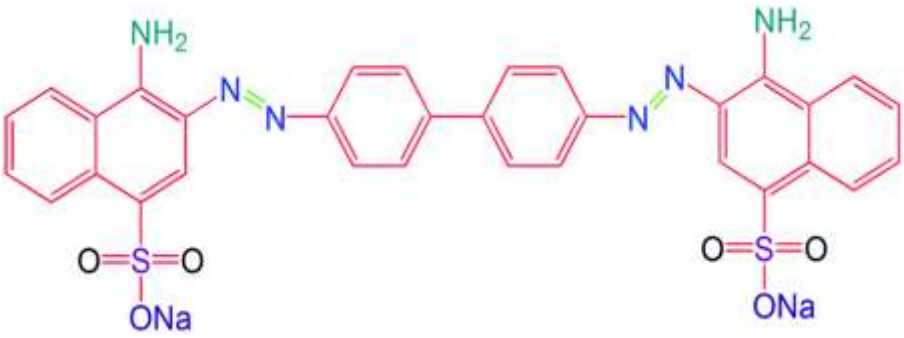


Figure 1.9: The chemical structure of Congo Red. The benzidine-based structure of Congo Red consisting of a hydrophobic center with two phenyl rings connected by diazo bonds. Figure was taken from Chowdhury and Bhattacharyya, 2015.

1.4.6. Methods used to identify aggregate-based promiscuous compounds

Due to their dominance and the identification properties mentioned above, aggregate forming inhibitors can be detected using a number of techniques and assays. Gurgen Tumanian, Allison Doak, Teague Sterling, John Irwin, and Brian Shoichet created Aggregate advisor which is a tool used to advise which compounds are likely to aggregate. Using conical SMILES of potential aggregators, aggregate advisor compares the structure of the compound with that of some known and previously identified aggregators (advisor.bkslab.org)**.** Most identified aggregates are reported to be dye conjugated, examples of such are Congo Red and

methylene blue (Bruns and Watson, 2012). Other techniques include; detergent based, dynamic light scattering and the Surface Plasmon Resonance assays.

1.4.6.1. Detergent-based assays

Promiscuous inhibitors are sensitive to detergents like Triton X100 (non-ionic), CHAPS (zwitterionic) and Tween 20 (non-ionic). This is due to the detergents' ability to adjust the aggregation process of small molecules by decreasing their surface tension, this is an important factor used in the detection of these inhibitors (McGovern *et al*., 2002). Although this method is less direct than other methods that measure the physical formation of particles, it is more stable, requires less time and more susceptible to miniaturization (Feng and Shoichet, 2006). Feng and Shoichet reported that molecules whose inhibition is higher in the absence of detergents are likely to be inhibiting via the formation of promiscuous aggregate-based inhibitors. They justified this by proving that in the presence of detergents the inhibition was decreased which means that the aggregate inhibitors were detected (Feng and Shoichet, 2006). The level at which promiscuous inhibition is decreased depends on the type of compound, nature and the concentration of the detergent (Ryan *et al.*, 2003). This is evident in an experiment conducted where two aggregate inhibitors (Congo Red and Rottlerin) were exposed to the presence of 0.02 CMC CHAPS, the Congo Red lost its inhibitory activity whereas the Rottlerin maintained some inhibitory effect (Ryan *et al*., 2003). Furthermore, when the same inhibitors were exposed to 0.02 CMC-Tween 20, Congo Red managed to retain its inhibitory whereas Rottlerin was no longer inhibitory (Ryan *et al*., 2003).

1.4.6.2. Dynamic light scattering

Dynamic light scattering (DSL) method is another alternative for the detergent-based assay and it is used to detect and quantify the size of micro-aggregates. The DLS technique involves determining the variation of scattering intensity of particles in solution that illuminate under a coherent light source over time (Chan *et al*., 2009). The DLS Plate Reader is the typical instrument used when examining the light scattering properties of a dilution series of known aggregating inhibitors. It is commonly seen that there is a direct proportionality between the compound concentration and the DLS instrument light scattering, however, when aggregating compounds are detected there is a dramatic decrease compared to the non-aggregating compounds. This method is reported to be more direct than the detergent-based assay (Feng and Shoichet, 2006). Conversely, it is a low throughput and detection takes a longer period of time. It is also not easy to interpret the measurements produced by DLS during the evaluation of insoluble compounds which leads to the formation of fit errors (Lorber *et al.*, 2012). These errors are generated from the DLS particle-sizing model that is based on

Mie-theory calculation which takes on scattering from spherical particles of regular size. However, aggregates form inconsistent, irregular shapes of fibrous tendrils and uneven clumps. Chan and associates reported that even though DLS quantify the size of scattered particles in solution, it failed to characterize aggregation of the compounds in their experiment, which was seen by a noticeable difference in readings for each small molecule (Chan *et al.*, 2009).

1.4.6.3. Surface plasmon resonance

Surface Plasmon Resonance (SPR) assays are a common tool used to detect and characterize inhibitors involved in promiscuous binding and to determine the degree of interaction between aggregates and the protein (Karlsson, 2004). Therefore, aggregating compounds can be directly detected in SPR assays through an optical biosensor surface by monitoring the rate at which proteins interact with the small molecules in kinetic reactions (Chan *et al*., 2009). Furthermore, the use of this assay enables the differentiation between the compound aggregation and compound affinity. Additionally, the results obtained from the SPR provide other useful information about reversibility, stoichiometry, and alterations in the behaviour of compound over a series of concentrations (Giannetti *et al*., 2011).

1.5. Thermal shift assays

Thermal shift assays (TSA) is a collective term given to thermal denaturing methods in biochemical assays mostly used to study ligand binding to purified proteins. These biophysical techniques are inexpensive and present quick methods for measuring the thermal stability and transitions of proteins under different conditions (Niesen *et al.*, 2007). The unfolding transitions of proteins are explored in a temperature gradient shown in a sigmoidal melting curve that deduces the melting temperature (T_m) of the protein. The 'shift' is then caused by the binding of a ligand to a protein which increases the T_m to a higher temperature, hence, resulting in a thermal shift. In drug discovery, the thermal shift assays are used for the identification of possible HITS and for low molecular molecule studies (Zubrienė *et al.*, 2009).

The thermal shift assays cover several approaches and applications of both high throughput and low throughput nature (Table 1.3) which are widely used in biochemical assays, structural biology and have contributed to drug discovery (Patel *et al*., 2008; Thompson *et al*., 2008). Traditionally, these assays employ temperature-regulated instruments such as real-time PCR machines, thermal cyclers or heating plate readers to monitor the changes in fluorescence, absorbance and circular dichromic (Chavan *et al*., 1994; Morton *et al*., 1995). The cell-based

thermal shift assay (CETSA) and the differential scanning fluorimetry (DSF) are the most used thermal shift methods and they are briefly discussed below.

1.5.1. Cell-based thermal shift assays

The CETSA method, first described in 2013 by Molina and co-workers is used for target engagement in drug discovery and it explores the principle that ligand binding relies on the stabilization of the bound protein (Molina *et al*., 2013). This principle is comparable to that of the traditional TSAs, however, CETSA is broader as it is applicable to living cells, cell lysates and tissues (Ishii *et al*., 2017). Following the discovery that several proteins unfold and precipitate in a similar manner as purified proteins when heated in cells, the CETSA approach quantifies the amount of soluble protein remaining in cells that have been heated at gradual temperatures in the presence or absence of a ligand (Molina *et al*., 2013; Molina and Nordlund, 2016). The CETSA is a useful tool in a number of stages in the drug discovery pathway due to its versatility, it can also be used in HTS since it can be performed in a physiologically relevant context.

1.5.2. Differential scanning fluorometry

DSF, initially known as the Thermofluor is a high throughput method which monitors protein thermal denaturation by fluorescence, and it is conducted in real-time quantitative polymerase chain reaction (qPCR) machine (Senisterra *et al*., 2012; Huynh and Partch, 2015). As shown in Figure 1.10a, DSF experiments generally require a few reagents; protein; ligand; assay buffer and the dye, these reagents are all placed in a PCR tube at a total volume of 25 µL. During these assays, the protein solution is heated at a temperature gradient ranging from 25 °C to 95 °C in the presence of environmentally sensitive dyes such as SYPRO Orange, 1-anilinonaphenthalen-8-sulfonic acid (1, 8-ANS), 2, 6-ANS to name a few (Pantoliano *et al*., 2001).

The baseline temperature of 25 °C does not denature the protein, however, an increase in heating causes a change in the conformation of the protein. A further increase in temperature unfolds the protein thereby introducing hydrophobic regions in the solution. The dye which is attracted to the hydrophobic regions binds to the unfolded protein resulting in an increase in fluorescence (Vollrath *et al*., 2014). As shown in figure 1.10b, when the fluorescence increases, it allows the determination of the melting temperature (T_m) of the protein which is indicated as the midpoint of the peak (Lo *et al*., 2004). Once the melting temperature is reached, the dye is released into solution and protein is aggregated which causes a decrease in fluorescence as seen in Figure 1.10b.

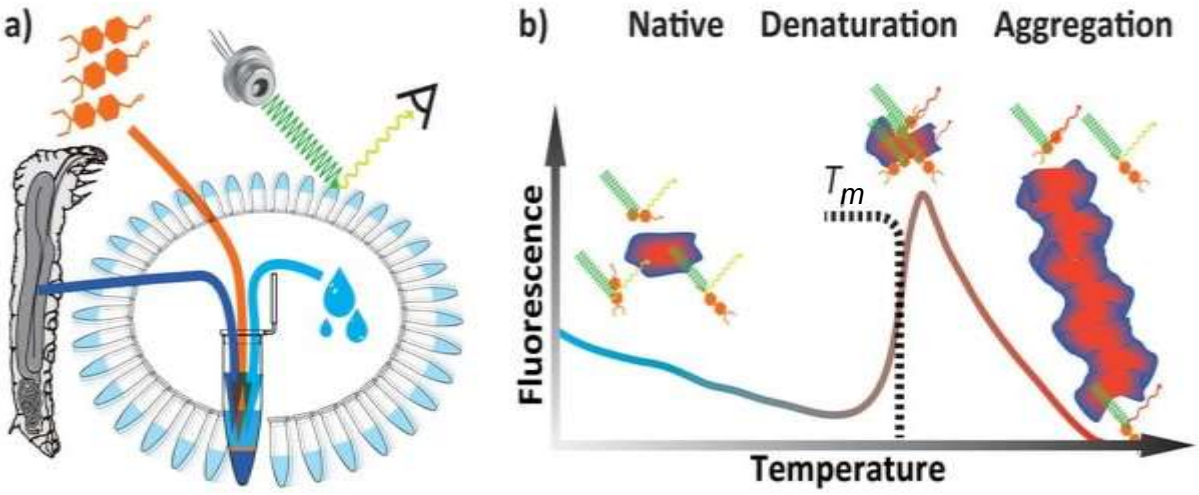


Figure 1.10: An illustration of the thermal shift assay. a), a typical example of sample preparation for the thermal shift assay. b) the thermal transitions of a protein showing three stages that take place during the temperature gradient; native, denaturation and aggregation. Figure obtained from Vollrath *et al*., 2014.

Table 1.3: Different thermal shift methods and their applications

Application	Approach	Throughput	References
Drug lead optimization	DSC DSF	Low Highest	Matulis *et al*. ,2005; Srinivasan *et al*., 2015
Enzyme mechanism	DSF	Highest	Lea and Simeonov, 2012
Parallel thermal shift assays	CETSA	Lowest	Molina *et al*., 2013
Protein stabilization	DSF	Highest	Alexandrov et al., 2008
Inhibitor screening	DSF	Highest	Grüneberg *et al*., 2001; Grøftehauge *et al*., 2015
Assigning function of unknown proteins	DSF	Highest	Carver *et al*., 2005
Protein-protein interaction	DSF	Highest	Kopec and Schneider, 2011
Mutation effects and characterization of variant proteins	DSLS DSF	Intermediate Highest	Hong *et al*., 2007; Allali-Hassani *et al*., 2009; Lavinder *et al*., 2009
Antibody formulations	DSLS	Intermediate	He *et al*., 2010; He *et al*., 2011

DSF-Differential scanning fluorimetry; CETSA-Cell-based thermal shift assay; DSLS-Differential static light scattering; DSC-Differential scanning calometry.

1.6. Stem bromelain

Bromelain is a non-toxic mixture of proteolytic enzymes present in the tissues of the pineapple plant and additional species of the family of *Bromeliaceae* (Hatano *et al*., 1998; Hale *et al*., 2005). These enzymes were first identified in the fruit flesh in the early 1890s (Chittenden *et al*., 1891) and have since been known as fruit bromelain (EC 3.4.22.33). Following their identification in the 1950s, proteolytic enzymes found in the fruit stem have since been known as stem bromelain (EC 3.4.22.32) and determined as having higher bromelain activity than the fruit bromelain equivalent (Taussig and Batkin, 1988). This mixture contains thiol-endopeptidases and many other non-characterised components such as peroxidases, phosphatase, carbohydrates and glycoproteins that do not exhibit proteolytic activity (Pavan *et al*., 2012).

1.6.1. Properties of stem bromelain

Commercially obtained bromelain is a yellow powder prepared by centrifugation, ultrafiltration, and lyophilization of cooled pineapple. Bromelain is approximately 33 kDa in molecular size and it is made up of 212 amino acids (Heinicke and Gortner, 1957). The enzyme activity of bromelain is determined with the specific substrates such as chromogenic tripeptides, casein, and gelatin. Bromelain is inhibited by N α (alpha)-Tosyl-L-lysine chloromethyl ketone (TPCK), *trans*-Epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64) protease inhibitor, iodoacetate and other elements such as silver, mercury and copper (Gautam *et al.*, 2010). The non-toxicity and effectiveness of bromelain after being administered orally has allowed it to be used for a number of therapeutic functions.

1.7. Hypothesis aim and objectives

The hypothesis of this study was that the thermal shift assay could be used in a novel manner to identify aggregate-based promiscuous inhibitors. This study aimed to optimise a detergent-based thermal shift assay using stem bromelain as a model enzyme.

Objective 1: To isolate the enzyme bromelain in a mixture using molecular exclusion Chromatography.

Objective 2: To determine the activity of bromelain using the titrimetric assay.

Objective 3: To determine the temperature profile of bromelain and the effect of the inhibitor (E-64).

Objective 4: To identify the effect of promiscuous compounds on the temperature profile of bromelain in the presence and the absence of the detergents.

Chapter 2: Methods and materials

2.1. Theoretical characterization of stem bromelain

The amino acid sequence of stem bromelain was obtained from UniProt (www.unipot.org; accession number P14518. Molecular properties such as effective size, melting temperature, extinction coefficient were obtained from ExPASy (www.expasy.org). The hydrophobicity plots were also obtained from ExPASy (ProtParam) according to the Kyte and Doolittle scale (Kyte and Doolittle, 1982).

2.2. Isolation of stem bromelain

2.2.1. Separation of proteins through molecular exclusion chromatography

Stem bromelain (Sigma, USA) was isolated by molecular exclusion chromatography (MEC) using the ÄKTAprime Plus fast protein liquid chromatography (FPLC) (GE Health Care Biosciences, Sweden). A 600 mm long column was packed with HiPrep 16/60 Sephacryl S200 high resolution (HR) resin which has a fractionation range from 5 to 250 kDa. The resin was equilibrated as described in Eyssen, 2014, and the column was washed with 60 mL Milli-Q water followed by equilibration with MEC buffer (50 mM NaH_2PO_4, 300 mM NaCl pH 8). The flow rate was set at 0.5 mL / min with pressure at 0.30 MPa and the injection valve set to inject prior to the run. A total of 1 mL bromelain solution (200 mM in 100 mM sodium acetate buffer containing 260 mM sodium chloride at pH 4.5) was centrifuged using the bench top centrifuge for 20 minutes at 3000 x g and loaded in the column. The column was washed at a flow rate of 1 mL/ min with 0.2 M NaOH (60 mL) followed by MEC buffer (160 mL), Milli-Q water (60 mL) and finally with 20% ethanol (120 mL).

2.2.2. Sample concentrating

Fractions containing peaks were pooled and concentrated through centrifugation at 6000 x g for 10 minutes and 4 °C in centrifugal filter units (Merck-Sigma, USA) with a 10 kDa cut off using a C0650 rotor in the Allegra x-22R centrifuge (Beckman Coulter, USA). The protein concentration of the pooled sample was determined using the NanoDrop (Thermo Fischer Scientific, USA) through the protein A280 option. Briefly, an amount of 2 µL of the elution buffer was used as a blank before measuring the protein concentration at the same volume.

2.2.3. Separation by SDS-PAGE

For the characterization of stem bromelain based on its size, SDS-PAGE was adopted. Freshly made reagents used in preparation of the gels that were made as per the Laemmli method (Laemmli, 1970). Briefly, 12.5% running gels were poured in between plates and left to polymerize for approximately an hour before pouring 4% stacking gels. The samples were run under non-reducing conditions. Precision Plus Protein Standards (Bio-Rad Laboratories, USA) were used as molecular weight markers and the run was completed at a constant current of 20 mA/ gel and at maximum voltage. The gels were stained with Coomassie Blue R-250 (Sigma, USA) and de-stained with a methanol acetic acid solution.

2.3. Evaluation of stem bromelain activity

The activity of commercial bromelain was determined according to the titrimetric assay previously described by Gautam *et al*., 2010. Briefly, bromelain (Sigma, USA) at concentrations 2 and 4 units/ mg enzyme (2290 units = 1 g) was prepared in 100 mM sodium acetate buffer containing 260 mM sodium chloride at pH 4.5, and added to 5% gelatin (Sigma, USA) at a 1:25 ratio. This sample was incubated at 45 °C under non-shaking conditions for 20 minutes followed by the addition of 0.1 mL of a 3% hydrogen peroxide (Merck-Sigma, USA). The pH was adjusted to 6.9 with 0.05 N sodium hydroxide (Acechem, RSA) and then mixed under constant swirling with 10 mL of the 37% formaldehyde (Merck-Sigma, USA). Thereafter the pH of this solution was adjusted to 7.8 using the 0.05 N sodium hydroxide (Merck-Sigma, USA). The volume of sodium hydroxide required to adjust the pH to 7.8 was recorded and this sample was designated as the test sample. The blank sample was run simultaneously with the test sample. Here, an amount of 25 mL of the gelatin solution was incubated at 45 °C for 20 minutes. After incubation, 1 mL of the enzyme solution was added and mixed by swirling. To this, 0.1 mL hydrogen peroxide was added, and the pH was adjusted to 6.9 by titrating with 0.05 N sodium hydroxide. An amount of 10 mL of 37% formaldehyde solution was added with constant swirling and the pH was finally adjusted to 7.8 using the sodium hydroxide solution. The volume required to adjust the control sample to pH 7.8 was recorded. The content of stem bromelain in solution was then calculated by means of the following equation (Gautam *et al.*, 2010):

$$\text{Units/mg enzyme} = \frac{(\text{Volume for Test} - \text{Volume for Blank})\ (N)\ (14)\ (1000)}{\text{mg enzyme/RM}}$$

Where: N= normality of sodium hydroxide,

14 = mg nitrogen/ mmole nitrogen,

1000 = milligram conversion,

mg enzyme = amount o/gram of bromelain present in a 1 mL solution

RM= reaction mix (for this experiment RM =1)

Similarly, this assay was used to monitor the change in absorbance during the reaction using yellow-dyed gelatin. Absorbance readings were taken at each step of the assay on a Bio Wave DNA spectrophotometer (Biochrom, UK) over a wavelength range of 300 – 500 nm. The enzyme activity was calculated in Gelatin digestive units (GDUs).

2.4. Radial diffusion assays

The radial diffusion assays were carried out as previously described in Gallagher *et al*., 1986. The activators (cysteine, Ethylenediaminetetraacetic acid (EDTA) and soybean trypsin inhibitor (Sigma, USA)), inhibitors (E-64, iodoacetate (Sigma, USA), and mercury) and compounds (Congo Red and EGCG (Sigma, USA)) were dissolved in distilled water and the Bromelain solution in the assay buffer. The 1% (m/v) agarose containing 0.1% gelatin was prepared through dissolving the agarose in assay buffer (100 mM sodium acetate buffer containing 260 mM sodium chloride at pH 4.5) then heating it in a microwave for complete dissolution. The gelatin solution prepared in distilled water was added to the liquefied agarose and mixed in a flask through gentle shaking. The agarose- gelatin solution was poured into a petri dish and allowed to solidify, the wells were punched in using a syringe leaving 0.5 cm between them.

An amount of 25 µL of the protein solution was added to 15 tubes marked E_1 – E_{15}, a further 25 µL of the assay buffer was added to tube E_1 and E_6 and to the rest of the tubes 25 µL of assay buffer containing 40 mM cysteine. All the tubes were incubated at 37 °C for 5 minutes in a water bath, 50 µL of assay buffer, assay buffer with cysteine, EDTA (200 mM), iodoacetate (100 mM), soybean trypsin inhibitor (100 mM), mercury (1.5 mg/ mL), EGCG (0.008 mM, 0.009 mM, 0.01 mM and 0.015 mM), Congo Red (0.008 mM, 0.009 mM, 0.01 mM and 0.015 mM) and E-64 (0.030 mM) were added to tubes E_1 – E_{15} respectively. The tubes were further incubated for 15 minutes at 37 ºC in a water bath and the solutions in each tube was used to fill up respective wells on the agarose-gelatin in a petri dish. The petri dish was placed in a plastic container based with a damp paper towel and incubated at room temperature overnight. The solid gel was stained using 1% Amido black dissolved in the distaining solution for nor less than 5 hours before distaining with a methanol-acetic acid-distilled water solution for 24 hours changing the distain at least 3 times.

2.5. Thermal shift assays

All thermal shift assays were run using qPCR clear tubes on the real time PCR machine (Corbett 5 Plex high resolution melt (HRM), Corbett, Australia) at a temperature profile of 25 to 95 °C with temperature increment increases of 0.2 °C, holding time of 2 seconds and the gain held at 7 as instructed in Niesen *et al*., 2007. The SYPRO orange dye (Thermo Fisher Scientific, USA) at 10X concentration was prepared fresh each time before use in a phosphate buffered saline (PBS) at pH 7.2. Stem bromelain solutions were prepared in a 100 mM sodium acetate buffer containing 260 mM sodium chloride at pH 4.5 and the protein concentration was confirmed each time using the NanoDrop (Thermo Fischer Scientific, USA). The inhibitors and compounds were prepared in dH_2O at 125 µM stock solutions.

2.5.1. Optimisation of dye concentration

For optimisation purposes, using the same assay buffer used in the titrimetric assay, different concentrations of SYPRO Orange dye were tested to find a suitable concentration for the protein and the assay. A stock of 5000X dye concentration was diluted using PBS pH 7.2 to a stock of 200X and concentrations of 10 to 80X with 10X increments were analysed in the assay. These concentrations were tested in the presence and the absence of 17 µg/ 25 µL stem bromelain. The reagents were added in the following manner to make a total volume of 25 µL; protein, assay buffer followed by the dye solution at a specific concentration.

2.5.2. Optimisation of protein concentration

Different concentrations of stem bromelain were subjected to the thermal shift assay for optimisation purposes as previously done in Vedadi *et al*., 2006. Once the concentration of the protein was confirmed using the NanoDrop, the solution was diluted into different concentrations of 5 to 29 µg/ 25 µL with 3 µg increments in triplicates at least five times. The samples were run in the presence of a final concentration of 10X SYPRO Orange and the reagents were added in a comparable manner as seen in section 2.5.1. The controls for these experiments were run either in the absence of dye or in the absence of the protein.

2.5.3. The effect of inhibitors on stem bromelain

Different concentrations of the protease inhibitors; E-64, iodoacetate and TPCK (Sigma, USA) were compared with stem bromelain at different concentrations in the thermal shift assay for optimisation purposes. The inhibitors were prepared in dH_2O and kept on ice. Concentrations of 29, 17 and 5 µg/ 25 µL stem bromelain were tested against 5-50 µM of the inhibitors. The reaction mixture containing the protein, assay buffer, the inhibitor was

incubated for 30 minutes on ice while shaking. After the incubation SYPRO orange dye was added at a final concentration of 10X dye and the samples were run under optimized conditions.

2.5.4. The effect of compounds on stem bromelain

Congo Red and EGCG were dissolved in dH_2O at a stock concentration of 125 µM and the solution was kept at room temperature and was made fresh each time for use. Concentrations of 5 to 20 µM with 1 µM increments Congo Red were tested in the presence of 5, 17 and 29 µg/ 25 µL stem bromelain in the manner previously describe in section 2.5.3, similarly different protein concentrations were also tested with just one concentration of Congo Red. EGCG was tested in a comparable manner.

2.5.5. Identification of promiscuous compounds

2.5.5.1. The protein-inhibitor and compound cocktail

Stem bromelain was tested with both the inhibitor and the compound present in the same tube, the experiments were run comparably with all the thermal shift assays with an exception that in this section the reagents were added as follows; stem bromelain, inhibitor, compound, buffer, thirty minutes incubation and thereafter the SYPRO orange dye prior run.

2.5.5.2. The dye aggregation principle

The protein was tested on its own, in the presence of the inhibitor, Congo Red and EGCG and the experiments were run as seen in sections 2.5.4 corresponding to each compound and inhibitor. After the run the samples were cooled on ice for five minutes before the addition of protein in all the tubes, these experiments were done in triplicates at least five times.

2.5.6. Optimisation of different detergents

The detergents Tween-20 (Sigma, USA), Triton-X100 (Sigma, USA) and SDS (Sigma, USA) were diluted to a stock of 1% using distilled water and stored at room temperature in a Para film sealed tube. Different concentrations 0.002%, 0.004%, 0.006%, 0.008%, 0.01 and 0.012% of each detergent were tested in the thermal shift assay and the reagents were added in the following pattern; detergent (in a specific concentration), assay buffer and the dye.

The protein was incubated with the detergent for approximately thirty minutes on ice before starting the reactions. The protein- detergent mixture was added first into the tube, followed by the inhibitor or the compound, the samples were then incubated for thirty minutes before

the addition of the dye. These experiments were tested in the presence and in the absence of the detergents.

2.6. Cytotoxicity assays

2.6.1. Cell culture

Human embryonic kidney 293 cells containing the SV40 T-antigen (HEK 293T cells) (Creative biogene, USA) were seeded in Dulbecco's Modified Eagle's Medium (DMEM) (Bio Whittaker, USA) containing 10% fetal bovine serum (FBS), 0.1% Penicillin streptomycin (x100) and 0.1% Gentamycin. The cells were maintained at 37 °C 5% carbon dioxide (CO_2) humidified incubator for 72 hours and grown in T75 culture flasks. Trypsinization to harvest cells after achieving confluence was routinely achieved by resuspending cells in trypsin (10x) followed by a 2 minutes incubation before adding an equal amount of DMEM medium. The cells were centrifuged at 200 x g for 5 min and pellet re-suspended in DMEM media by gently mixing to ensure solubility. Cell count was achieved by diluting cells with 0.4% Trypan Blue stain (Bio-Rad, USA) at a 1:1 ratio and counting which was completed on a T20 Automated Cell Counter (Bio-Rad, USA). The final cell concentration of 1 x 10^5 cells / mL was cultured in 15 mL DMEM media.

2.6.2. MTT assay

The Cytotoxicity effect of Congo Red and EGCG on HEK 293T cells was evaluated through the MTT (3-4,5 dimethylthioazol-2,5 diphenyl tetrazolium bromide) assay as described in Leila *et al*., 2016. Briefly, the cells were seeded at a concentration of 1 x 10^4 cells/ mL per well in a 96-well cell culture plate at a final cell volume of 100 µL per well and equilibrated at 37°C in an incubator of 5% CO_2 for 2 hours. Congo Red and EGCG were diluted in DMEM medium and used to treat the cells at different concentrations (0-200 µM). After the two-hour incubation, 100 µL of each dilution was pipetted into the wells while controls were not treated, and the plate was further incubated for 72 hours. After treatment 20 µL of MTT (Sigma, USA) (5 mg/ mL) solution was pipetted into each well and the plate was incubated for a further 2 hours in the 37°C and 5% CO_2 incubator. After incubation, the supernatant was discarded by overturning the plate. An amount of 200 µL of solvents SDS (10%); SDS (10%)/HCL (0.01 M) and SDS (10%)/ HCL (0.01M)/ Isopropanol (200 µL) was added to respective wells to dissolve formazan crystals. The absorbance was read at 590 nm using microplate reader. Data analysis was completed using GraphPad prism 7 where the halfway maximal cytotoxicity concentration CC_{50} was determined as the compound concentration that would reduce the cell viability by 50%. The cell viability was taken as the absorbance of the untreated cells at 100%.

2.7. Statistical analysis

All the Data collected was analyzed on GraphPad Prism 7 (GraphPad Prism, USA). Each experiment was presented in triplicates (n=3) and repeated at least five times. Statistical analyses were obtained from GraphPad Prism. The standard deviations are presented as error bars.

Chapter 3: Results

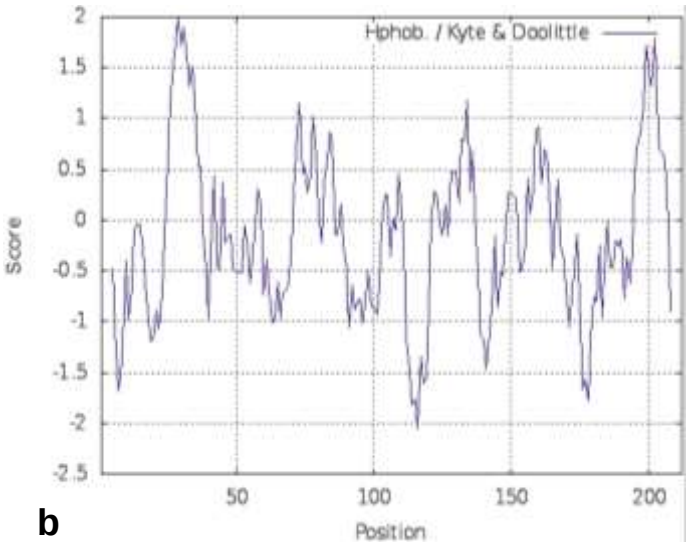
3.1. In *silico* theoretical analysis of stem bromelain

The theoretical physiological analysis of stem bromelain is shown in Figure 3.1. Stem bromelain is comprised of 212 amino acid residues and is 33 kDa in size as per previous reports (Margaretta *et al*., 2015; Ritonja *et al*., 1989). Furthermore, the theoretical melting temperature (T_m) of stem bromelain is reported to be subject to pH (Haq *et al*., 2005). Where at neutral pH the melting temperature is 71 °C and at acidic pH it is 77 °C (Figure 3.1a). The amino acid sequence of stem bromelain contains an equal amount of hydrophilic and hydrophobic amino acid residues. This observation corresponded with the hydrophobicity plot of stem bromelain shown in Figure 3.1b. As seen in this plot, an evident percentage of the hydrophobic residues (Indicated by positive scores) was observed on the surface of the protein. Briefly, positions 5-45 and 180-200 have the highest hydrophobicity score of 1.5 to 2 as previously analysed in Albee *et al*., 1997. An equal distribution of hydrophobic and hydrophilic amino acids was observed at the core of the protein (position 70-160).

Properties	Stem bromelain
EC number	3.4.22.32
Number of amino acids	212
Molecular weight	33 kDa
Melting temperature	71 °C [1] 77 °C [2]
Theoretical PI	8.60

[1] Basic pH [2] Acidic pH

a



b

Figure 3.1: Theoretical characterisation of stem bromelain including a) the molecular properties of stem bromelain and b) hydrophobicity plot of stem bromelain as obtained from www.expasy.org.

3.2. Biochemical characterization of stem bromelain

3.2.1. Isolation of stem bromelain

As stem bromelain is a crude mixture of proteins, molecular exclusion chromatography was conducted to separate proteins in the mixture. An elution profile of stem bromelain with

corresponding absorbances at 280 nm is shown in Figure 3.2. As seen in this figure, the first peak elutes at approximately 70 mL at an absorbance of 1250 mAu. A second peak, shouldering on the first peak is observed at approximately 120 mL with an absorbance of 600 mAu. Finally, a broad, shallow peak elutes at 200 mL with absorbance maxima of 300 mAu. Fractions comprising of each of the peaks were pooled.

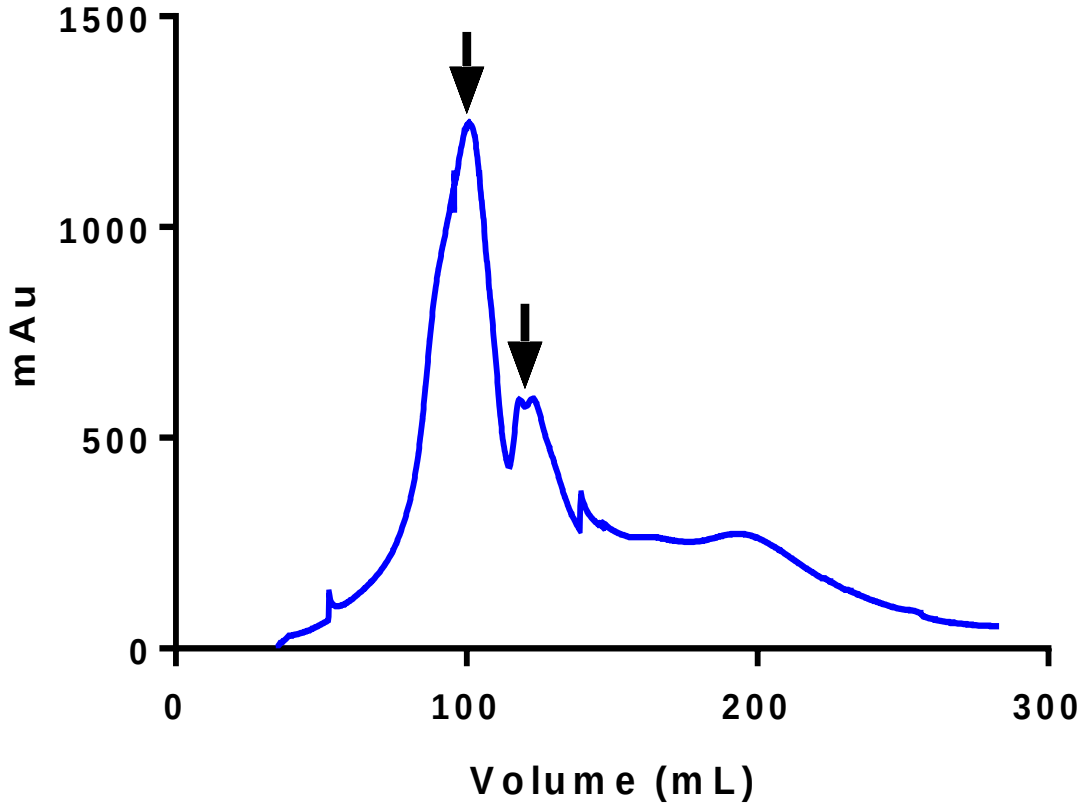


Figure 3.2: The molecular exclusion chromatographic (MEC) profile of bromelain mixture. Bromelain at a concentration of 200 mM was prepared in 100 mM sodium acetate buffer containing 260 mM sodium chloride at pH 4.5. The sample was run in a 600 mm column packed with HiPrep 16/60 Sephacryl S200 HR resin on an ÄKTA Plus purifier. The samples were eluted in the presence of MEC buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8) at a rate of 0.5 mL per minute. The two black arrows are an indication of the two peaks eluted at 70 mL and 120 mL.

3.2.2. Separation by SDS-PAGE

Following MEC, the pooled samples were concentrated using a 10 kDa centrifugation filter and separated by SDS-PAGE. Crude stem bromelain was observed to be a mixture, as separation resulted in five protein bands of different sizes and intensity (Figure 3.3). The first band was intense and observed at approximately 158 kDa, while less intense bands were observed at 33.11 kDa and 25 kDa. The fourth band observed at 15.6 kDa was narrow and less intense while the fifth band at 10.84 kDa was intense. A comparable SDS-PAGE analysis of extracted stem bromelain conducted by Gautam and colleagues yielded proteins of similar

sizes (Gautam *et al*., 2010). Pooled samples from peak 1 were collected in the filter unit (concentrate) and in the wash-through (filtrate) of the centrifugal filter and their separation is shown in lanes 3 and 4 of Figure 3.3 respectively.

The concentrate separated into three distinct protein bands at 33.11 kDa, 15.6 kDa and 10 kDa while no protein bands were detected in the filtrate (lane 4). Similarly, the concentrate for peak 2 separated into four distinct bands at 33.11 kDa, 26,25 kDa and 22 kDa (lane 5) while the filtrate contained no protein bands (lane 6). Proteins at 33.11 kDa, 15.6 kDa and 10.84 kDa present in peak 1 may have co-eluted in a complex or in an aggregate. It is likely that the > 200 kDa band observed in the crude mixture eluted in the void of the column.

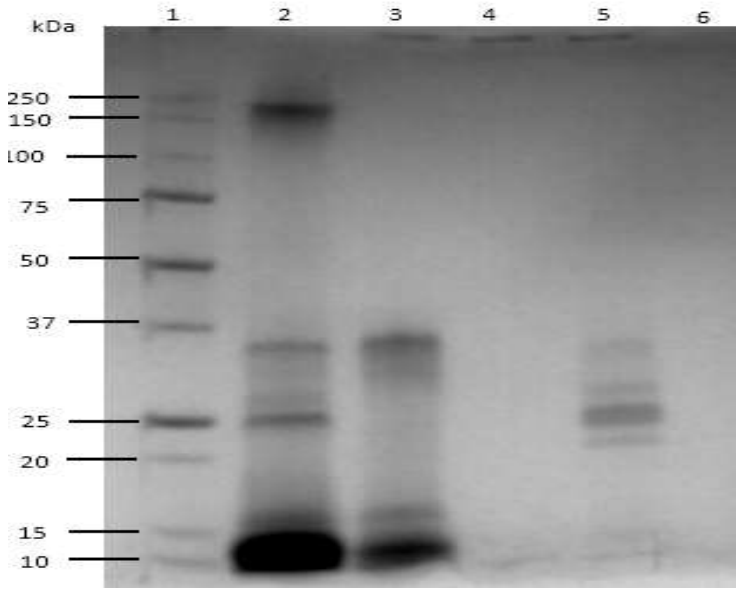


Figure 3.3: Analysis of bromelain samples separated on a 12.5% non-reducing SDS PAGE. All samples were loaded at 10 µL. Lane 1 is Precision Protein Plus Standards (250 – 10 kDa), lane 2 is a bromelain mixture (13 mM) prepared in phosphate buffered saline. Lane 3 and 4 are the concentrate and filtrate of the pooled fractions of peak of the MEC elution respectively, following centrifugal concentration. Similarly, lane 5 and 6 represent the concentrate and filtrate of the pooled fractions of peak 2 respectively following centrifugal concentration. The gel was run at 20 mA/ gel and stained with Coomassie Brilliant Blue.

3.3. Enzyme activity

3.3.1. Enzyme activity through gelatin digestive units

The enzymatic activity of stem bromelain was demonstrated through the titrimetric assay shown in Table 3.1. The assay monitors the degradation of the substrate gelatin as measured in gelatin digestive units (GDUs). The activity in GDUs was then calculated from the volume

of sodium hydroxide required to adjust the pH of the enzyme reaction mixture to pH 7.8. The 2-unit enzyme required over 0.6 mL more hydrogen peroxide to reach the required pH when compared with the 4-unit enzyme (Table 3.1). Since no purification tests were conducted for this experiment, the reaction mixture was set to be 1 rpm for both the 4 and 2-unit enzyme. Therefore at 1 rpm, the 2-units enzyme yielded 2085.5 GDU / mg enzyme whereas the 4-unit enzyme yielded 2024.36 GDU/ mg enzyme. This was found to be comparable with previous reports (Gautam *et al*., 2010).

Table 3.1: The titration volume of sodium hydroxide required to adjust the pH to 7.8 during the degradation of gelatin by 2-units and 4-units of bromelain enzyme.

	2-unit enzyme		**4-unit enzyme**	
	Blank (mL)	Test (mL)	Blank (mL)	Test (mL)
Volume of sodium hydroxide	13.600	14.700	12.900	14.000
	13.460	14.780	12.740	14.040
	13.640	15.000	13.000	14.060
	13.120	14.820	12.860	14.150
	13.600	14.600	12.660	14.200
Average	13.484	14.780	12.832	14.090
Standard deviation	0.192	0.133267	0.119733	0.073756
GDUs /mg enzyme	2085.5		2024.36	

3.3.2. The activity of bromelain through absorbance in the titrimetric assay

The activity of stem bromelain was further investigated through absorbance in the titrimetric assay as shown in Figure 3.4. Commercial gelatin produced no peaks on the spectrum and as a result yellow coloured gelatin was used for this experiment. Unlike commercial gelatin (see Table 3.1), less volume of sodium hydroxide was required to adjust the test and blank solutions to pH 7.8 in the presence of 4-unit enzyme and yellow coloured gelatin. Even with this divergence, the pattern shown in Table 3.1 was still maintained in the presence of coloured gelatin. The blank solution required less volume (2.5 mL) than the test solution (3.6 mL) to adjust the pH to 7.8. The activity of stem bromelain in GDUs was calculated to be 1770.11 units / mg enzyme. This notable decrease in activity was expected due to low volumes of sodium hydroxide titrated. The effect of each step of the assay is shown in Figure 3.4 where

notable shouldering peaks are observed at 420 and 435 nm. With reference to Figure 3.4, incubation at 45 °C and the addition of hydrogen peroxide had no effect on the assay. The same trend was observed in panel a, b and c. In these panels, the enzyme activity was higher for samples with stem bromelain (test) than the gelatin solution without enzyme (blank). However, the addition of sodium hydroxide decreased the activity of the test while increasing the activity of the blank with a notably enlarged difference in absorbance between them (Panel d). This trend was maintained in panel e and f, however further decrease in absorbance was observed upon the addition of formaldehyde and with the final titration with sodium hydroxide (panel e and f respectively).

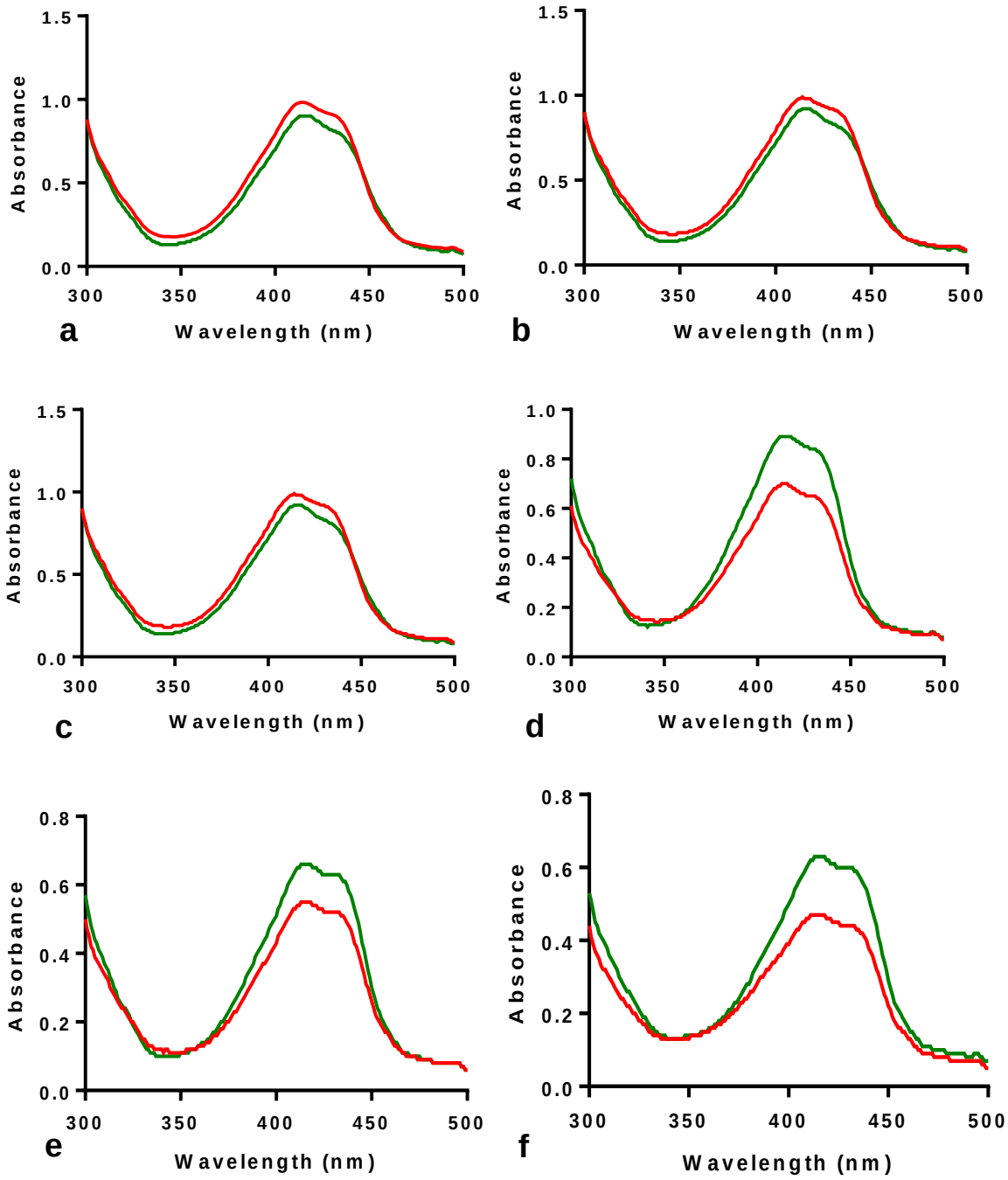


Figure 3.4: The absorbance profile of gelatin degradation in the bromelain titrimetric assay. The absorbance profile of yellow-dyed gelatin was tracked using the titrimetric assay in the presence of 4-unit bromelain enzyme (red) and absence of enzyme (green). Panel **a** is the absorbance profile before incubation at 45°C, panel **b** was taken after incubation at 45°C for 20 minutes, panel **c** was taken after the addition of 3% hydrogen peroxide, panel **d** represents the first titration to adjust solutions to pH 6.9, panel **e** shows the effect of formaldehyde within the solution and panel **f** shows the final titration to pH 7.8.

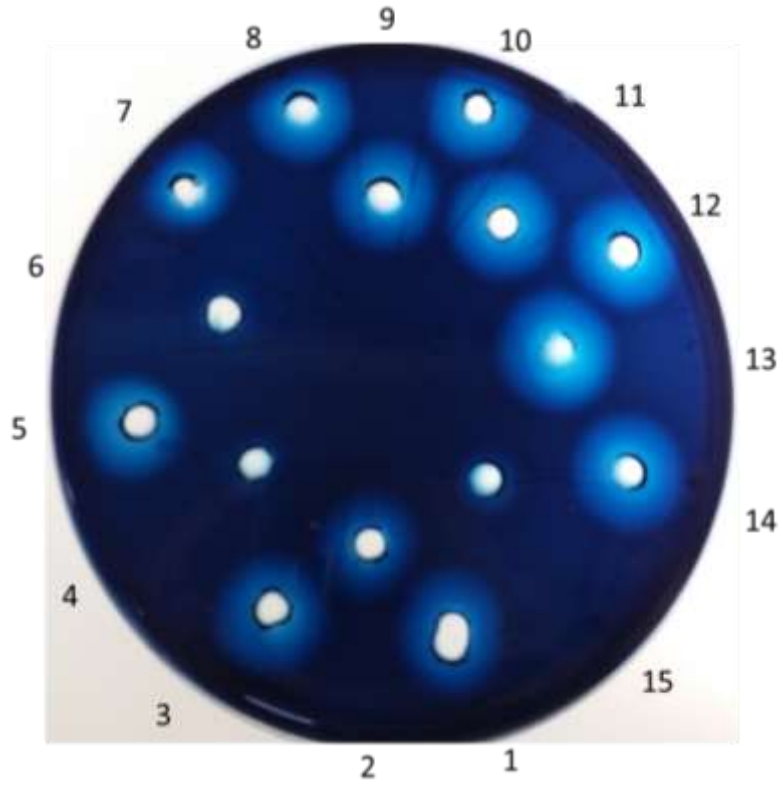
3.4. The effect of compounds on the activity of stem bromelain

The activation and inhibition of stem bromelain was studied on an agarose-gelatin diffusion assay. Table 3.2 lists the compounds that brought about inhibition or activation (no effect) of the enzyme. The list includes known promiscuous inhibitors Congo Red and EGCG which were tested at different low micro molar concentrations as previously advised (Heiser *et al*., 2000; Necula *et al*., 2007; Sehm *et al*., 2015). The inhibition and digestion are demonstrated by the zone of each well (Figure 3.5). With reference to Figure 3.5, the presence of a clear zone indicated digestion of gelatin (activation of enzyme) and the absence of it demonstrated the inhibition of the enzyme by the compound added (Gallagher *et al*., 1986). Therefore, the compounds that had no effect on stem bromelain (activators) in this experiment were EGCG, Congo Red, cysteine, EDTA and soybean trypsin inhibitor, as cleared zones were observed in these wells (Figure 3.5). Conversely, the enzyme was inhibited by iodoacetate, E-64 and mercury as stated in Table 3.2 and evident with the lack of cleared zones in Figure 3.5. Inhibition of stem bromelain by these inhibitors in different assays is reported in Chao and Liener., 1967; López-García *et al*., 2012; Masdor, 2013.

Table 3.2: The effect of inhibitors and activators in the digestion of an agarose-gelatin gel in the presence of stem bromelain.

Well number	Compound name	Digestion	Inhibition
1	Buffer	✓	x
2	Buffer with cysteine	✓	X
3	EDTA	✓	X
4	Iodoacetate	x	✓
5	Soybean trypsin inhibitor	✓	X
6	Mercury	x	✓
7-10	EGCG	✓	✓
11-14	Congo Red	✓	x
15	E-64	x	✓

Furthermore, the cleared zones observed differed in intensity, with Congo Red having the lightest wells at concentrations 0.009 mM, 0.010 mM and 0.015 mM, whereas cysteine has the darkest zoned well. Figure 3.6 quantifies the degree of bromelain inhibition by each compound through measuring the distance travelled by the cleared zone in mm. Congo Red travelled the longest distance (30 mm) whereas the shortest distance was travelled by mercury (0 mm). Granting that wells 7-10 contained EGCG, they were however observed to have travelled different distances (Figure 3.6). The inhibitors iodoacetate and E-64 both travelled 0.5 mm which shows that they did not completely supress the activity of the enzyme whereas mercury completely inhibited stem bromelain activity (distance 0 mm).



1- Buffer
2- Buffer with Cysteine
3- EDTA (20 mM)
4- Iodoacetate (100 mM)
5- Soybean trypsin inhibitor (100 mM)
6- Mercury (1.5 mg/ mL)
7- EGCG (0.008 mM)
8- EGCG (0.009 mM)
9- EGCG (0.010 mM)
10- EGCG (0.015 mM)
11- Congo Red (0.008 mM)
12- Congo Red (0.009 mM)
13- Congo Red (0.010 mM)
14- Congo Red (0.015 mM)
15- E-64 (0.030 mM)

Figure 3.5: The digestion of gelatin by bromelain in the presence of activators, inhibitors and compounds. An amount of 25 µL bromelain 0.3 mg/ mL bromelain stock was incubated with 50 µL of cysteine, EDTA (100 mM), iodoacetate (100 mM), soybean trypsin inhibitor (100 mM), Mercury (1.5 mg/ mL) Congo Red (8, 9, 10, and 15 µM), EGCG (8, 9, 10 and 15 µM) and E-64 (0.030 mM) in 1% agarose containing 0.1% gelatin gel and further incubated overnight. The gel was stained using a 0.15% Amido black solution and de-stained with a methanol-acetic acid-water solution.

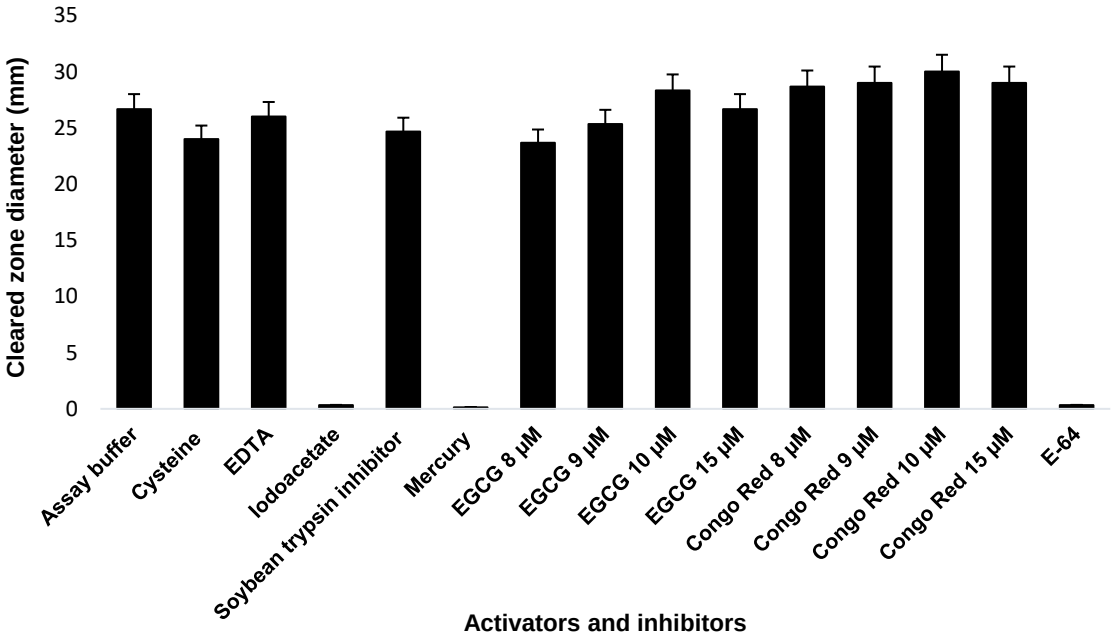


Figure 3.6: The activation and inhibition of stem bromelain in an agarose-gelatin gel. The distance (mm) travelled by the cleared zone measured across the diameter of the well after staining with Amido black.

3.5. The thermal stability of stem bromelain

3.5.1. Optimisation of dye concentration

Thermal shift studies were achieved through the DSF assay which uses fluorescent dyes such as SYPRO orange to monitor the transitions in the signal as the protein undergoes thermal unfolding. Hence, optimisation of a suitable dye concentration was considered as a crucial step in this study. The effect of eight different concentrations of SYPRO orange dye on the thermal unfolding of stem bromelain is shown in Figure 3.7. A general downward trend (shown by a black arrow) was observed at low temperatures of the thermal profile for both the fluorescence intensity curve (panel a) and the derivative curve (panel b). Following this trend, a plateau was observed which peaked at 75-76 °C (Figure 3.7). The different concentrations of dye follow this trend, however, differ in fluorescence intensity as in previous reports (Niedziela-Majka *et al*., 2015). Notably, the dye-protein relationship was observed as inversely proportional since the peaks increased with a decrease in dye concentration. With 80X dye having the lowest or no peak and 10X dye with the highest peak. In 2015, Niedziela-Majka and colleagues reported that 10X dye concentration also gave the highest peak in the presence of 0.005% protein. However, a decrease in this concentration showed a decrease in the fluorescence intensity which contradicted with the results obtained in this study. To further investigate this, different concentrations of dye were compared with different concentrations of stem bromelain (results not shown). An inversely proportional relationship

between the dye and the protein was still maintained and visible in fluorescent peaks. Therefore, from here on 10X dye concentration was regarded as an ideal concentration for this study.

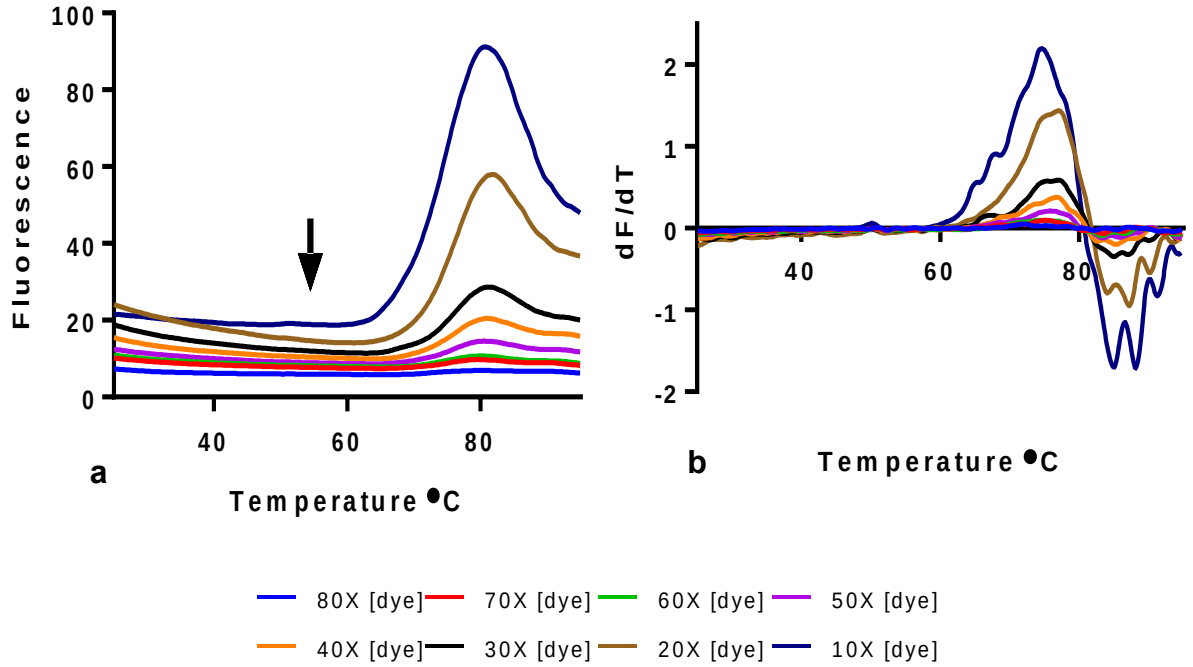


Figure 3.7: Optimization of SYPRO orange dye concentration in the presence of a fixed protein concentration. SYPRO orange dye was prepared in PBS pH 7.2 and was tested in the presence and the absence of a 17 µg / 25 µL of stem bromelain. Panels a and b represent the fluorescence and derivative curve respectively. The general downward trend is indicated by the black arrow. The temperature range was set at 25-95°C with temperature increment of 0.2 °C, holding time of 2 seconds and the gain held at 7.

3.5.2. Optimization of protein concentration

Different concentrations of stem bromelain were subjected to the DSF assay to assist in determining the melting temperature of stem bromelain. Generally, in thermal shift assays, the T_m is indicated as the halfway point of the apex in the fluorescence intensity curve (panel a) while in the first derivative plot (panel b) it is known as the highest point of the peak. The T_m of stem bromelain was observed to be at ~75-76 °C and was not affected by the change in protein concentration (Figure 3.8). However, the fluorescence intensity was observed to increase with an increase in protein concentration. As a result of increased fluorescence intensity, the peaks were observed to be sharper and more defined (see Figure 3.8). Comparably, in previous studies, the T_m of proteins remained the same for all protein concentrations tested (Pantoliano *et al*., 2001; Vedadi *et al*., 2006; Niesen *et al*., 2007; Lavinder *et al*., 2009).

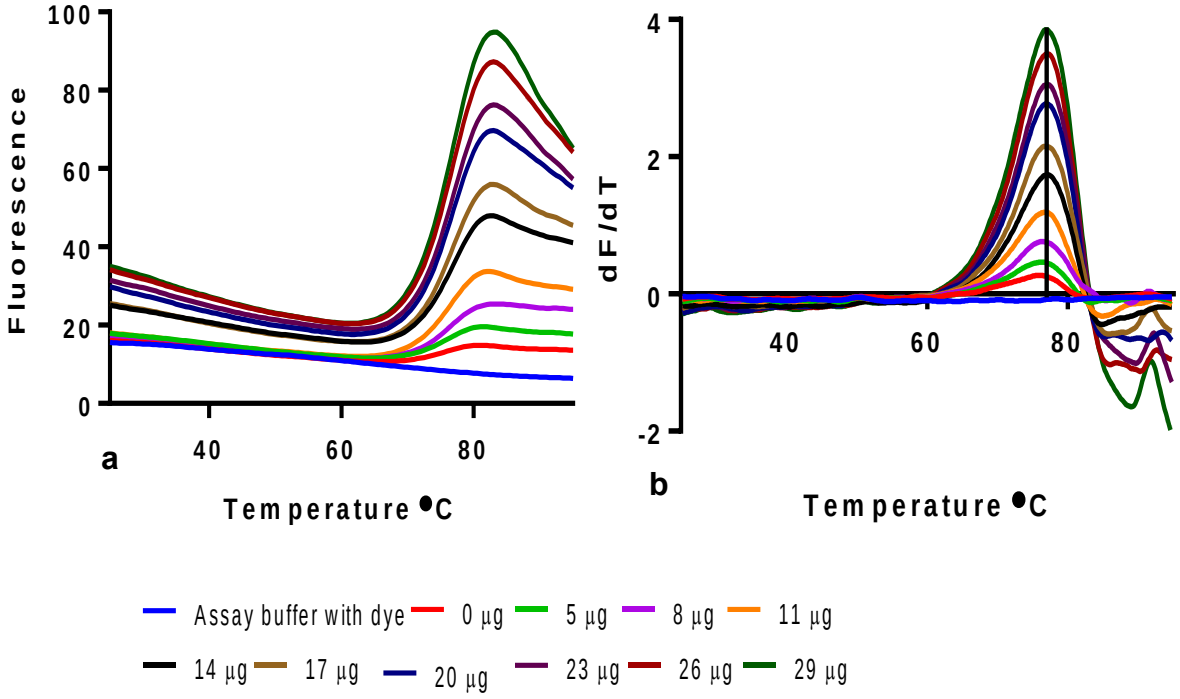


Figure 3.8: The unfolding transitions of stem bromelain at different concentrations in the presence of SYPRO orange dye. Stem bromelain was prepared in a 100 mM sodium acetate buffer containing 260 mM sodium chloride at pH 4.5. The SYPRO orange dye was present at a final concentration of 10X in the respective tubes. Each PCR tube contained a solution with 25 µL final concentration and was prepared in the following order; protein at a specific concentration, the assay buffer and lastly the SYPRO orange dye. Panel a and b represent the fluorescence curve and the first derivative curve respectively. The black vertical line represents the T_m also known as the melting temperature. The temperature range was set at 25-95 °C with temperature increments of 0.2 °C, holding time of 2 seconds and the gain held at 7.

3.5.3. The shift in the T_m of stem bromelain in the presence of E-64

DSF assays containing E-64, a true inhibitor of stem bromelain, were conducted in a dose-dependent manner. To cover the ranges, the inhibitor was tested in the presence of two different protein concentrations (17 and 29 µg / 25 µL). The T_m of stem bromelain was observed to be ~75-76 °C in the absence of the inhibitor but was increased to be ~84-86 °C in the presence of E-64, a ~9 °C shift to the right (Figure 3.9). Generally, the average shift upon ligand binding is known to be ~7 °C (Bia *et al*., 2019). Hence, the shift (~9 °C) obtained in this study was observed to be above this average. The p-value was between these two variants (protein and protein + inhibitor) was found to be 0.004963 which showed significance at $p < 0.05$. The effect of E-64 was observed to be moderately similar for each protein concentration with an exception for lower concentrations (5 and 10 µM) which showed no apparent effect (Figure 3.8).

Furthermore, sharper and more defined peaks were observed in the presence of higher concentrations of E-64. Besides the increase in fluorescence intensity, there was no prominent difference observed between the 17 and 29 µg / 25 µL thermal profiles (Figure 3.9).

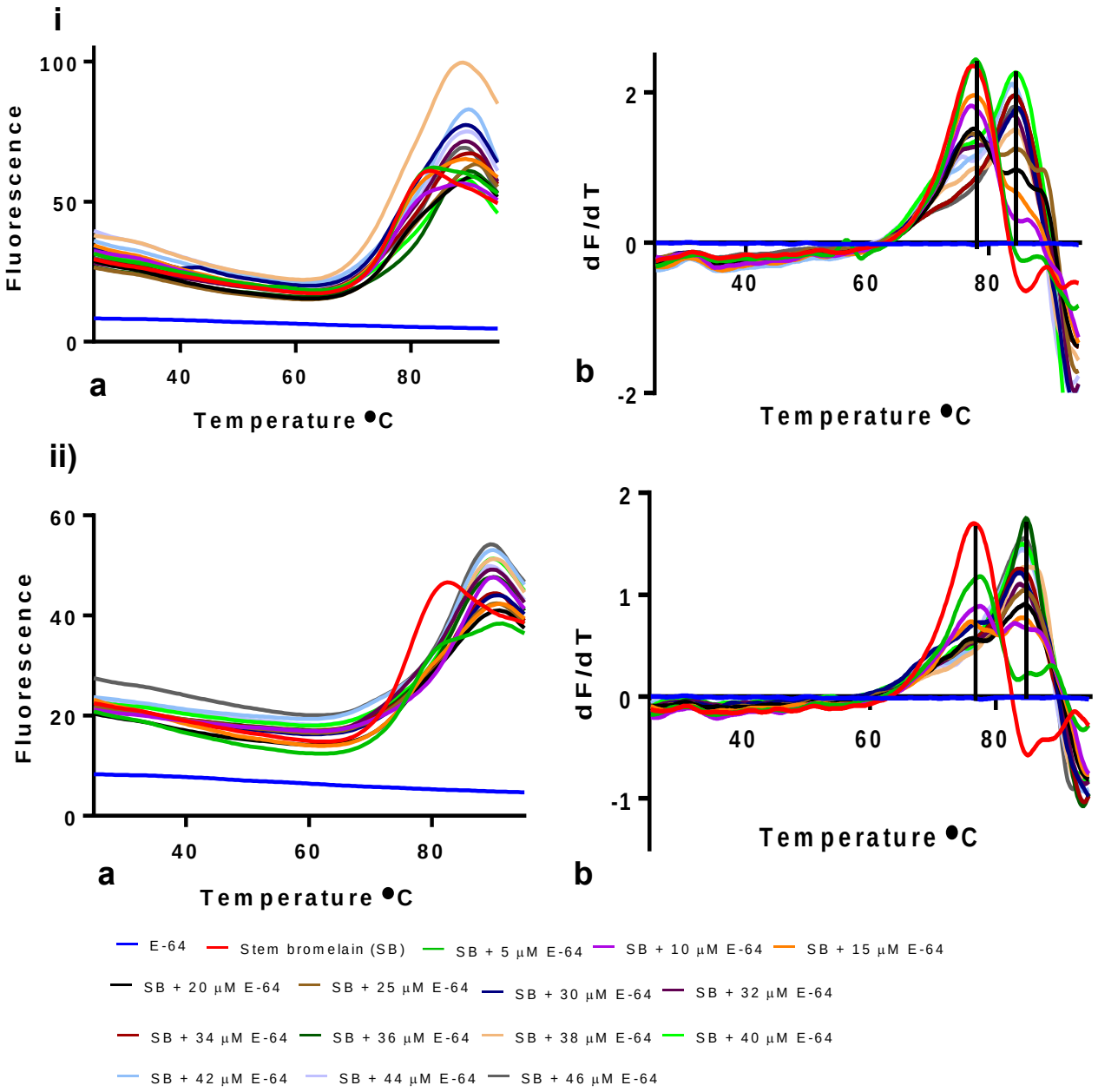


Figure 3.9: The effect of different concentrations of E-64 on different concentrations of stem bromelain. The inhibitor E-64 was dissolved in distilled water and tested at different concentrations in the presence of (i) 17 and (ii) 29 µg / 25 µL stem bromelain. Incubation of samples in ice was set for 30 minutes before adding SYPRO orange dye at a final concentration of 10X. Panel a and b represent the fluorescence curve and the first derivative curve respectively; the black vertical lines indicate the T_m. The temperature range was set at 25-95 °C with a temperature increment of 0.2 °C, holding time of 2 seconds and the gain held at 7.

3.5.4. The effect of Congo Red on the unfolding transitions of stem bromelain

The effect of the known promiscuous compound, Congo Red, on the melting profile of stem bromelain was evaluated in a similar manner as done for E-64 in the DSF thermal shift assay. The T_m of ~75-76 °C of stem bromelain was maintained in the presence of different concentrations of Congo Red. However, at protein concentrations 17 and 5 µg / 25 µL the effect of the compound was slightly different (Figure 3.10). A minor shift to the left was observed which appeared to be more prominent as the protein concentration decreased (see Figure 3.10 ii) and iii). Additionally, the presence of Congo Red was observed to decrease the fluorescence intensity in the thermal curve of stem bromelain. This was evident by low peaks which decreased with an increase in Congo Red concentration at all three tested protein concentration. Furthermore, this effect by Congo Red was observed to not only be dependent on the compound concentration but also highly dependent on the protein concentration. These results were expected as concentration dependent aggregation of proteins by promiscuous inhibitors have been reported (McGovern *et al.*, 2002).

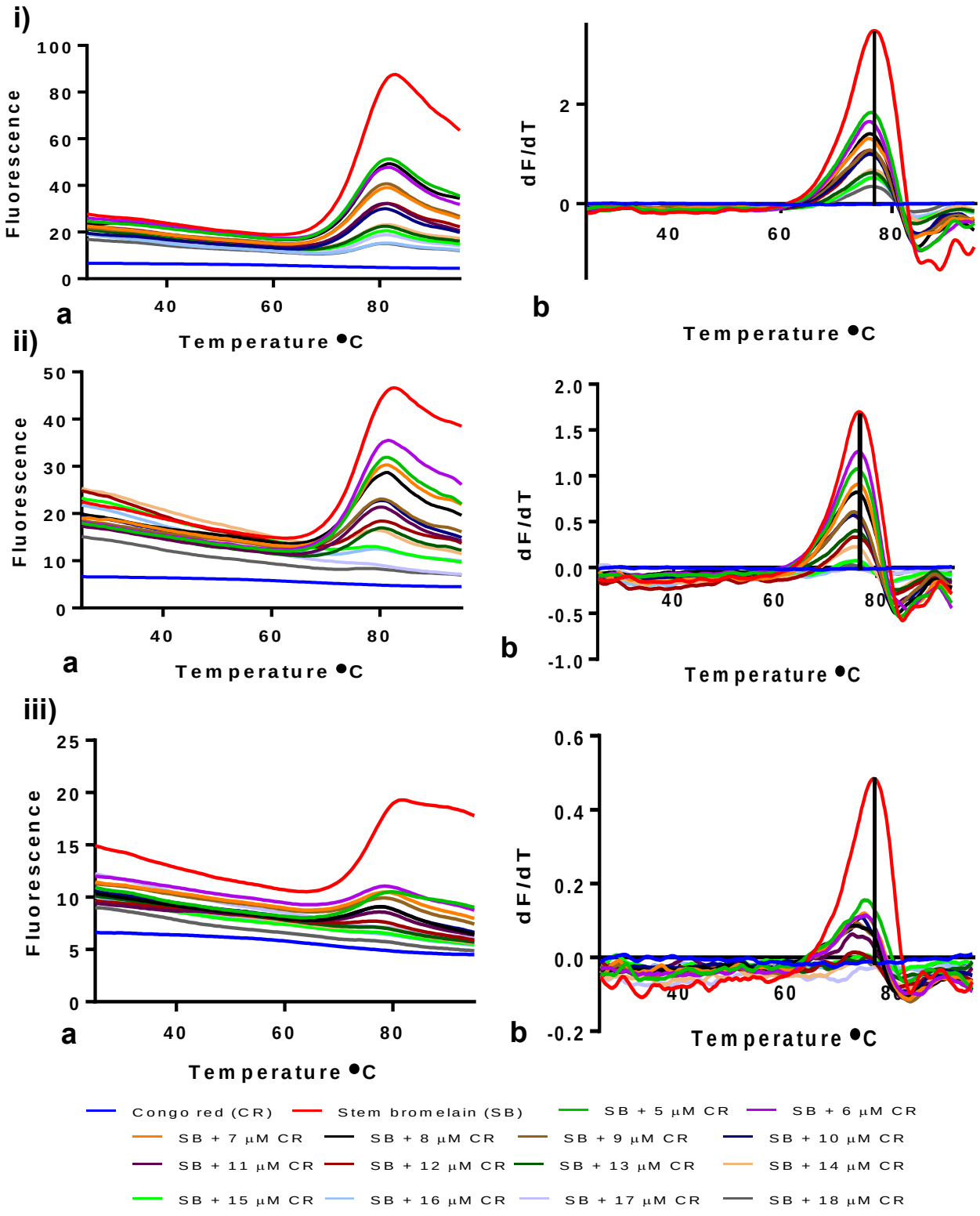


Figure 3.10: The unfolding transitions of stem bromelain in the presence of different concentrations of Congo Red. The aggregate promiscuous compound was dissolved in water and tested at low micromolar concentrations in a 25 µL solution consisting of the protein, buffer and the dye added after a 30 minutes incubation. Different concentrations of Congo Red were tested in the presence of i) 29, ii) 17 and iii) 5 µg / 25 µL stem bromelain respectively. Panel a and b represent the fluorescence curve and the first derivative curve respectively, while the T_m is indicated by the black vertical line. The temperature range was set at 25-95 °C with temperature increment of 0.2 °C, holding time of 2 seconds and the gain held at 7.

3.5.5. The effect of the compound EGCG

The compound EGCG was observed to have a different effect on stem bromelain when compared with Congo Red and E-64. Two shouldering peaks were observed in the presence of tested concentrations of EGCG which resulted in two different melting temperatures. As shown in Figure 3.11, the first peak was observed at ~75-76 °C and is identical to that of stem bromelain when tested on its own.

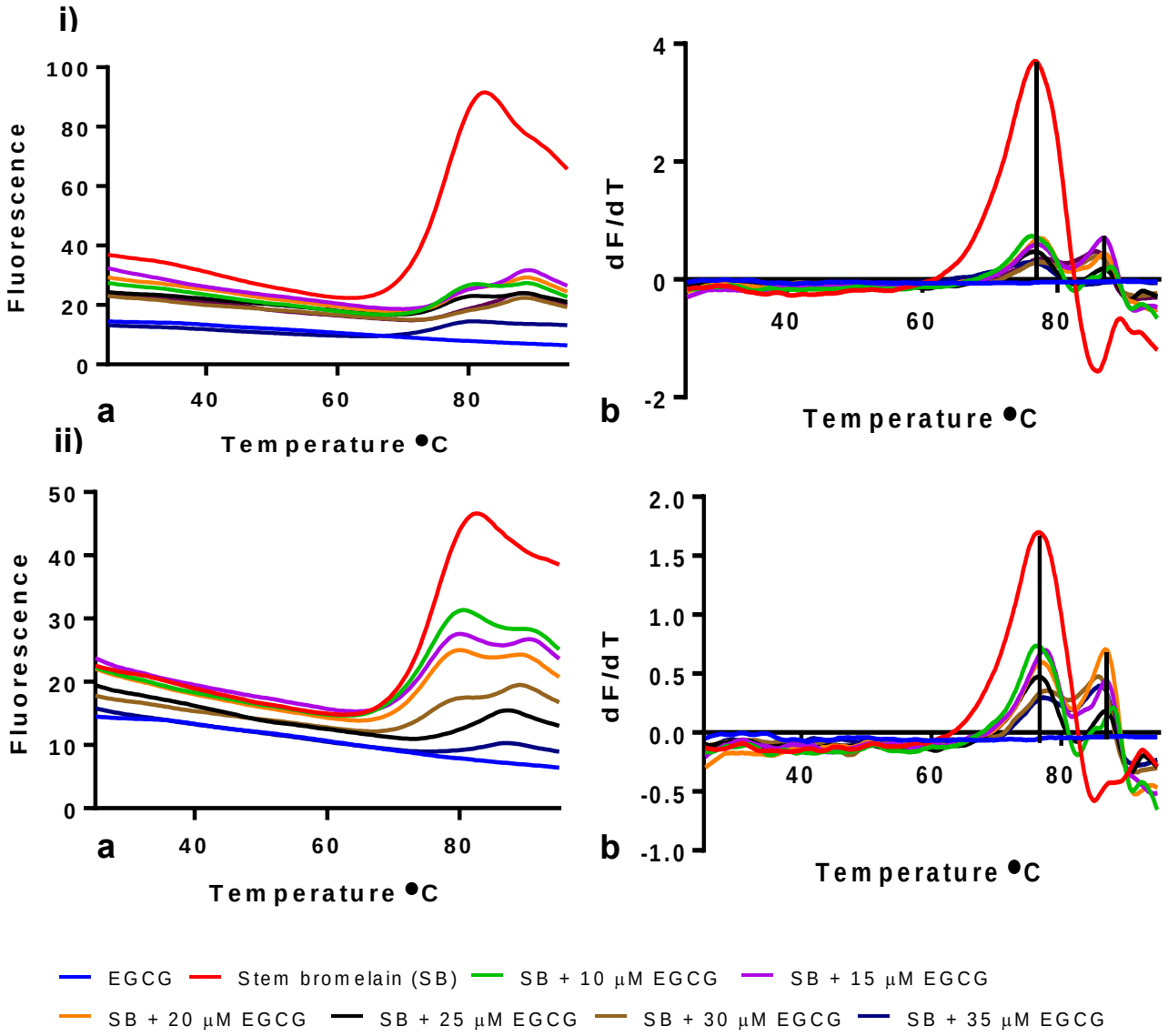


Figure 3.11: The unfolding transitions of stem bromelain in the presence of different concentrations of EGCG. EGCG was dissolved in water and tested at different concentrations in a 25 µL solution consisting of the protein, buffer and the dye added after a 30 minutes incubation. The symbols i) and ii) represent concentrations 29 and 17 µg/25 µL stem bromelain respectively. With panel a and b representing the fluorescence curve and the derivative curve respectively. The temperature range was set at 25-95 °C with temperature increment of 0.2 °C, holding time of 2 seconds and the gain held at 7.

The second peak was shifted to the right and was observed at ~87-88°C. However, at higher concentrations (35 µM) of EGCG the first peak was observed to fade while the second peak became dominant (Figure 3.11). Furthermore, the presence of EGCG resulted in low peaks when compared with the peak for stem bromelain in the absence of the compound. This decrease in fluorescence intensity was observed to be dependent on the concentration of EGCG, with 35 µM having the lowest peak while 10 µM had the highest peak at all tested protein concentrations (Figure 3.11).

3.5.6. Aggregation vs. unfolding

Up to this point, this study has explored the effect of compounds EGCG and Congo Red on the enzyme stem bromelain. However, the effect of these compounds on a stabilized protein-inhibitor complex had not been explored. Therefore, the effect of compounds on a stabilised complex (protein-E-64 complex) was studied to understand the mechanism of action of compounds EGCG and Congo Red. With reference to Figure 3.12, Congo Red maintained its mechanism of action since stem bromelain was aggregated in the presence and in the absence of E-64. Even with this observed aggregation, the melting temperature of stem bromelain in the absence (~75-76 °C) and in presence (~84-86 °C) of E-64 was also maintained. However, aggregation was not maintained in the presence of EGCG as an increase in fluorescence intensity was observed in the presence of the stabilised complex (Figure 3.12).

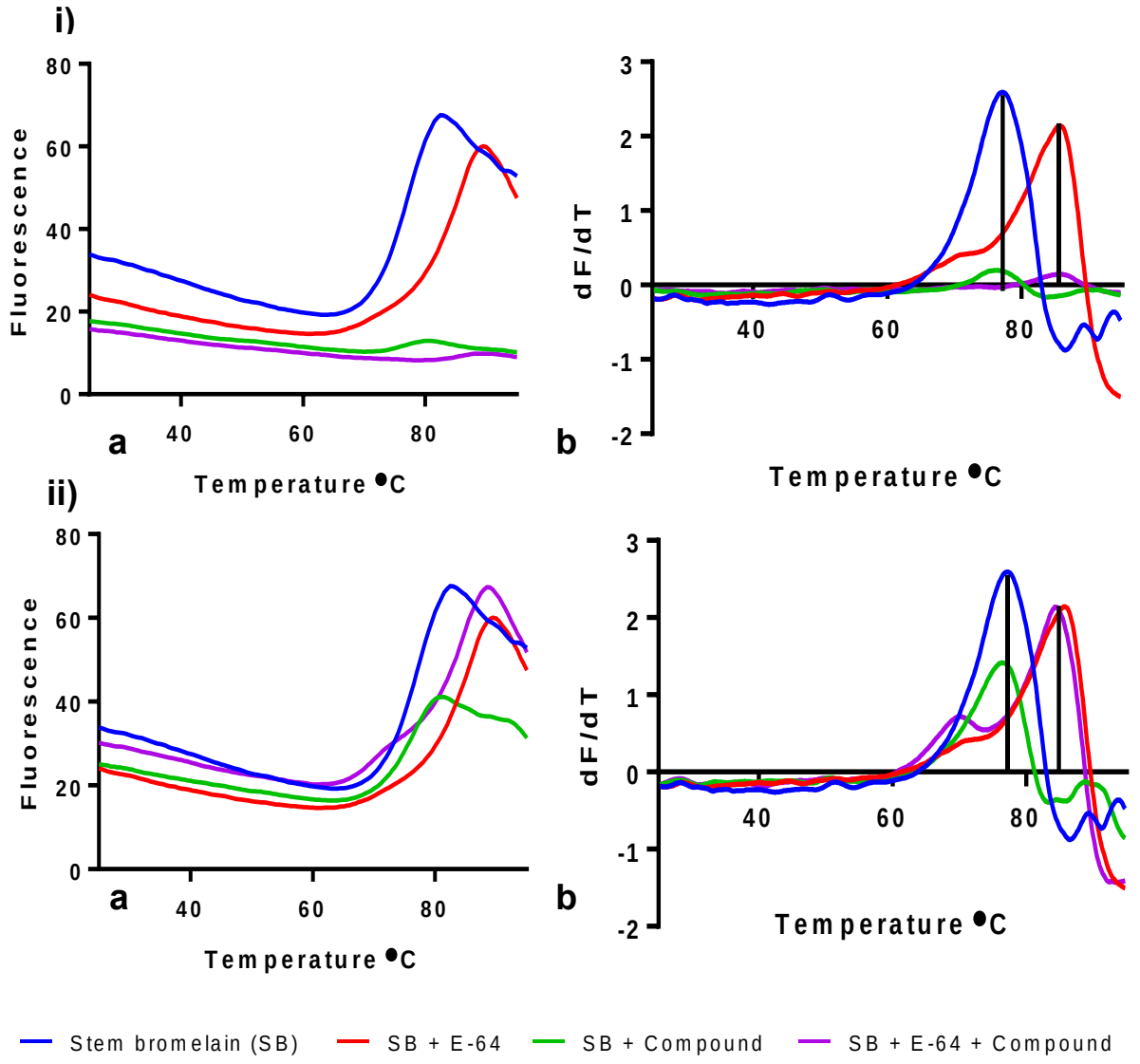


Figure 3.12: The effect of compounds on a stabilized complex. The compounds Congo Red and EGCG were added to a typical protein-inhibitor complex. An amount of 17 µg protein, 18 µM Congo Red, 35 µM EGCG and 10X SYPRO orange dye were present in respective tubes. i). protein-inhibitor complex with Congo Red ii). Protein inhibitor-complex with EGCG, with panels a and b representing the fluorescence and the derivative curves respectively, the two vertical lines represent the T_m. The temperature range was set at 25-95 °C with temperature increment of 0.2 °C, holding time of 2 seconds and the gain held at 7 for each run.

3.5.7. Peak recovery after the addition of more protein

The principle of the DSF TSA is that after binding, the dye is released in solution which results in the decrease in the peak. The hypothesis of this experiment was that during protein aggregation by Congo Red, the dye does not bind and thus is still available in the solution. To investigate this hypothesis, stem bromelain was tested in the presence of Congo Red and run on the thermal shift assay. After the run, the same sample was cooled and to it 10 µg/

25 µL of protein was added before undergoing a second run. This was done as the presence of 18 µM Congo Red aggregated the protein which completely removed the fluorescence peak (blue line Figure 3.13). The second run resulted in a recovered peak with a T_m of ~75-76 °C (red line Figure 3.13), which is identical to that of stem bromelain alone in the absence of Congo Red.

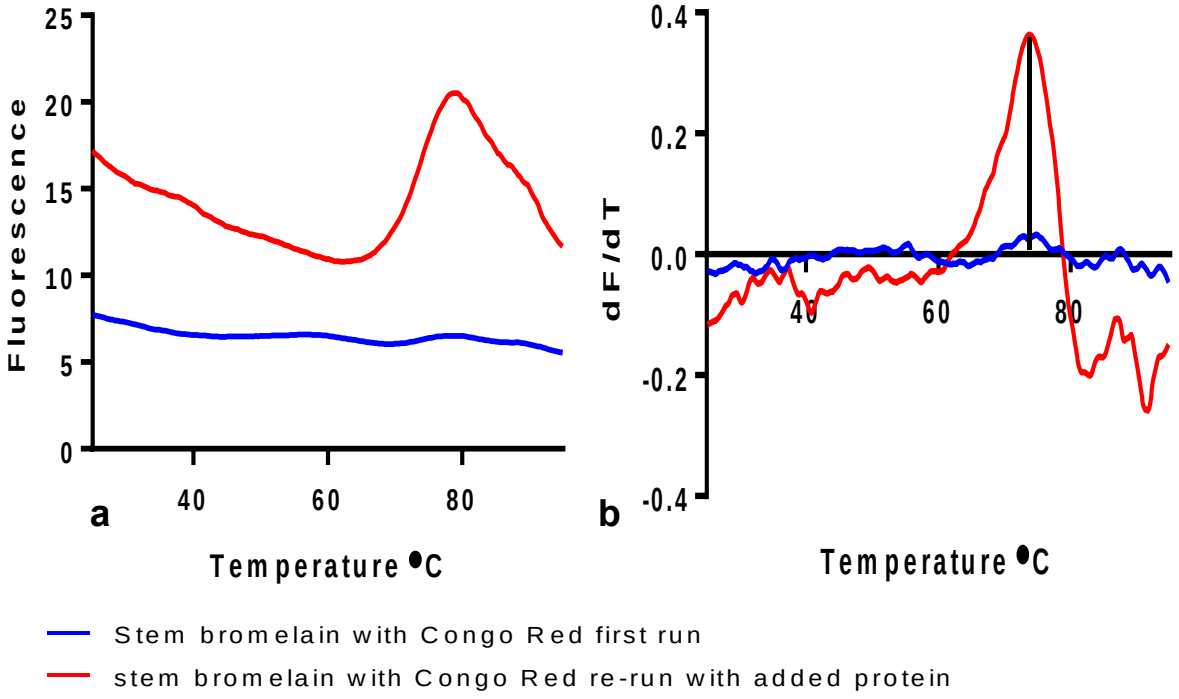


Figure 3.13: The recovery of a peak upon addition of protein. Stem bromelain was tested with Congo Red in the thermal shift assay, after the first run, 10 µg/ 25 µL of the protein was added and the assay was re-run in the absence of SYPRO orange dye. Results are presented in a fluorescence curve (Panel a) and derivative curve (panel b) and the black vertical line represents the T_m. The temperature range was set at 25-95 °C with temperature increment of 0.2 °C, holding time of 2 seconds and the gain held at 7 for both runs.

3.5.8. Effect of detergent

Detergent-based assays are extensively used to identify promiscuous compounds in drug discovery. As such, different concentrations of detergents were tested for their effect on the DSF assay to explore this knowledge. The presence of different concentrations of SDS increased the fluorescence intensity in the melt curve of stem bromelain (Figure 3.14). Additionally, a decrease in the T_m of stem bromelain was observed with increasing concentrations of SDS, this change was observed as a shift to the left (Figure 3.14). However, this shift was not observed in the presence of 0.004% SDS where the T_m of stem bromelain remained unaltered. A similar decrease in T_m was observed in previous reports (Yeh *et al.*,

2006; Alexandrov *et al*., 2008). In contrast, Tween-20 and Triton-X100 displayed high background noise, as a result the curve could not be detected and thus prevented the determination of the melting temperature of the protein (results not shown). The effect of SDS on the thermal profile of stem bromelain in the presence of E-64, Congo Red and EGCG was compared in Figure 3.15. The T_m of the protein with E-64 remained ~84-86 °C in the presence and in the absence of the detergent. Interestingly, the thermal profile of stem bromelain in the presence of Congo Red was observed to be affected by SDS (Figure 3.15 iii).

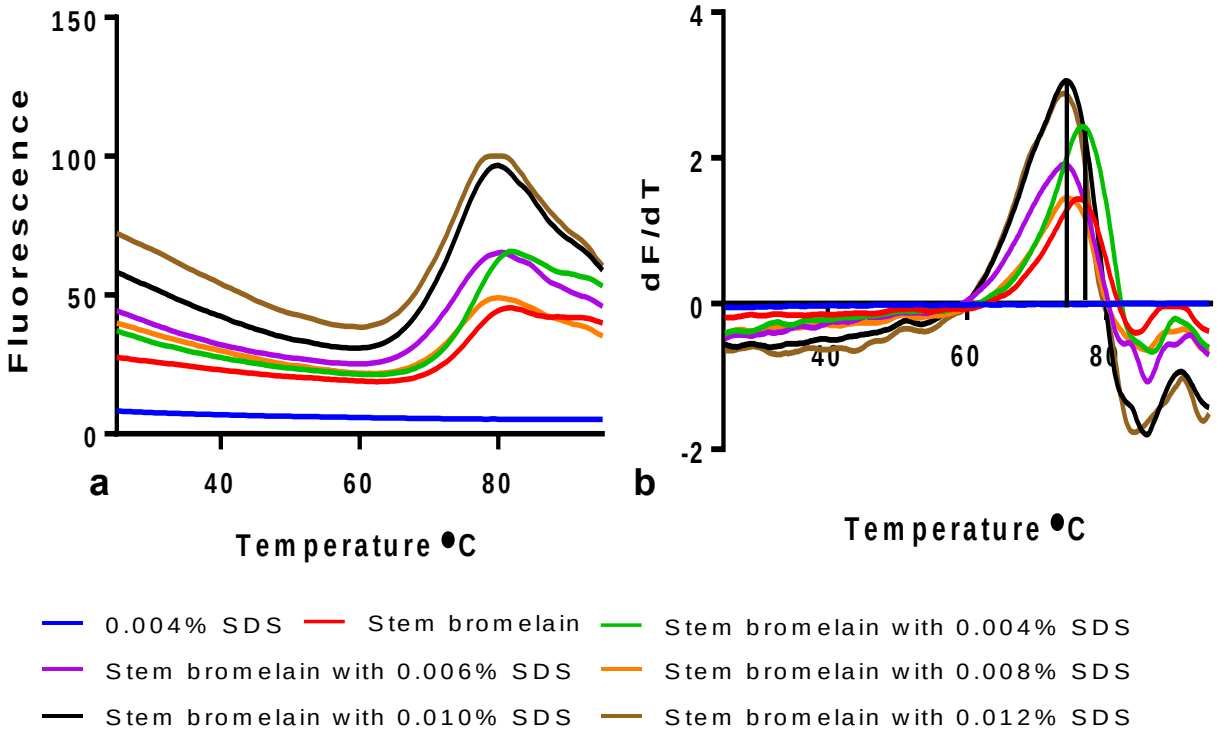


Figure 3.14: The effect of SDS on bromelain in the presence of SYPRO orange dye. Stem bromelain at 17µg/ 25 µL was incubated with different concentrations of SDS for 30 minutes prior run in the thermal shift assay. The final dye concentration present in each tube was 10X. The fluorescence and derivative curves are represented in panels a and b respectively, the T_m is indicated by the black vertical line. The temperature range was set at 25-95 °C with temperature increment of 0.2 °C, holding time of 2 seconds and the gain held at 7.

The peak previously aggregated by Congo Red in the absence of the detergent was recovered in the presence of the detergent. The recovered peak was observed at ~73-74 °C indicating a minimal shift to the left from the T_m of stem bromelain ~75-76 °C (Figure 3.15 iii). The thermal profile of stem bromelain in the presence of the compound EGCG was not altered by SDS (Figure 3.15 iv). Previous reports on detergent-based assays have indicated that aggregate-based promiscuous compounds are affected by detergents whereas detergents have no effect on true inhibitors (Feng and Shoichet, 2006; Feng *et al*., 2007).

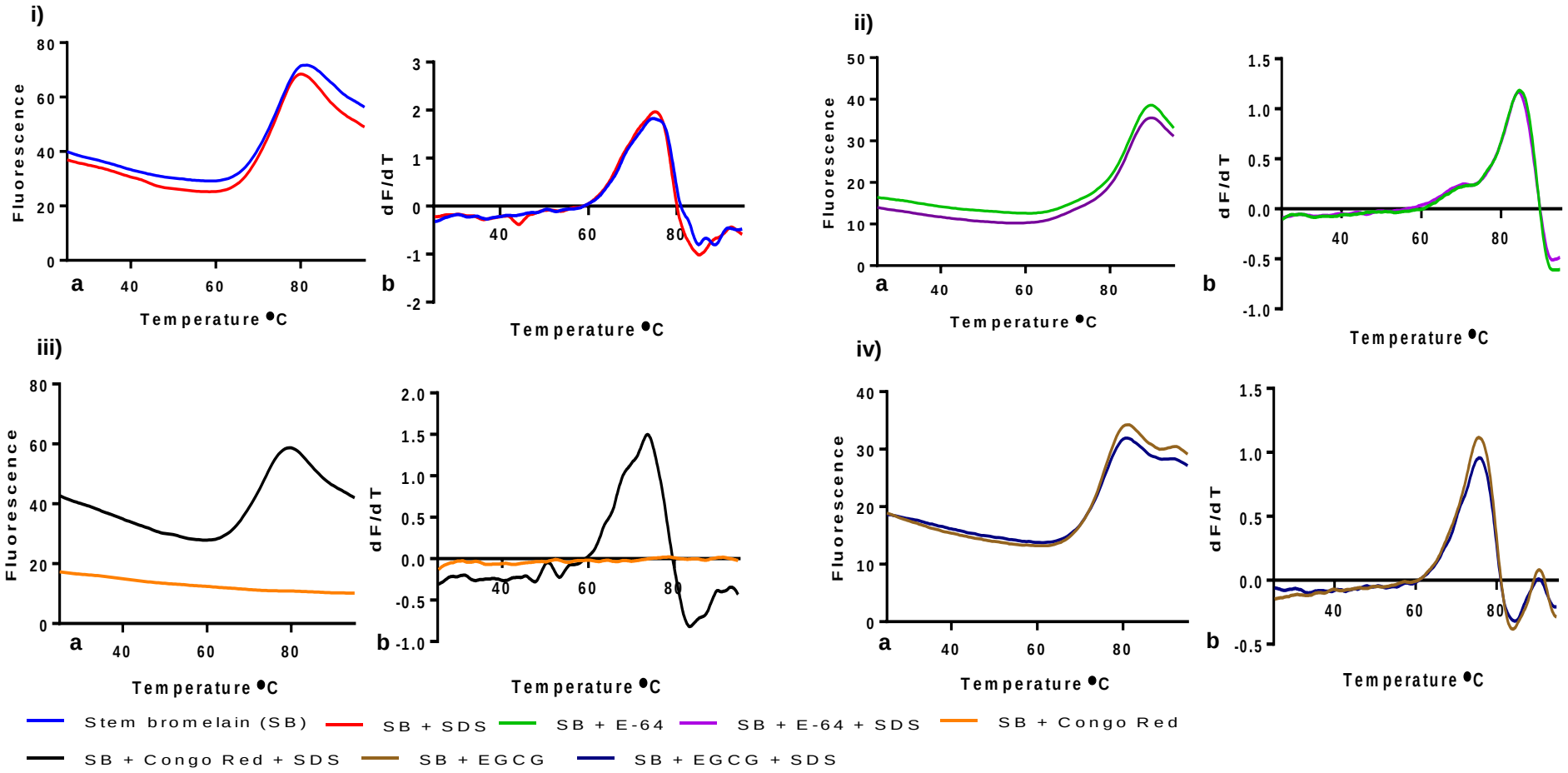


Figure 3.15: The effect of SDS on compounds and inhibitor in the presence of SYPRO orange dye. Stem bromelain (SB) at 17 µg/ 25µL was tested with the compounds in the presence of 0.004% SDS in the thermal shift assay. i) stem bromelain with SDS ii). SB with 30 µM E-64 and SDS iii). SB with 18 µM Congo Red and SDS iv). SB with 25 µM EGCG and SDS. The dye was present in 10X final concentration in each tube. Panel a and b represent the fluorescence and derivative curves respectively. The temperature range was set at 25-95 °C with temperature increment of 0.2 °C, holding time of 2 seconds and the gain held at 7.

3.6. Cytotoxicity assays

The half maximal cytotoxicity concentration (CC_{50}) of Congo Red and EGCG on HEK 293T cells were determined on the MTT assay and the results are shown as sigmoidal curves in Figure 3.16 and 3.17 respectively. Absorbances were taken at 590 nm wavelength and the CC_{50} was determined for each compound in the presence of different MTT solvents (Table 3 and Table 4).

Table 3.4: Data representing the levels of cytotoxicity of Congo Red in HEK 293T cells tested in the presence of different MTT solvents.

Solvents	**CC_{50}**
SDS	20 ± 1.3 µM
SDS/HCL	18 ± 1.25 µM
SDS/HCL/Isopropanol	14 ± 1.13 µM

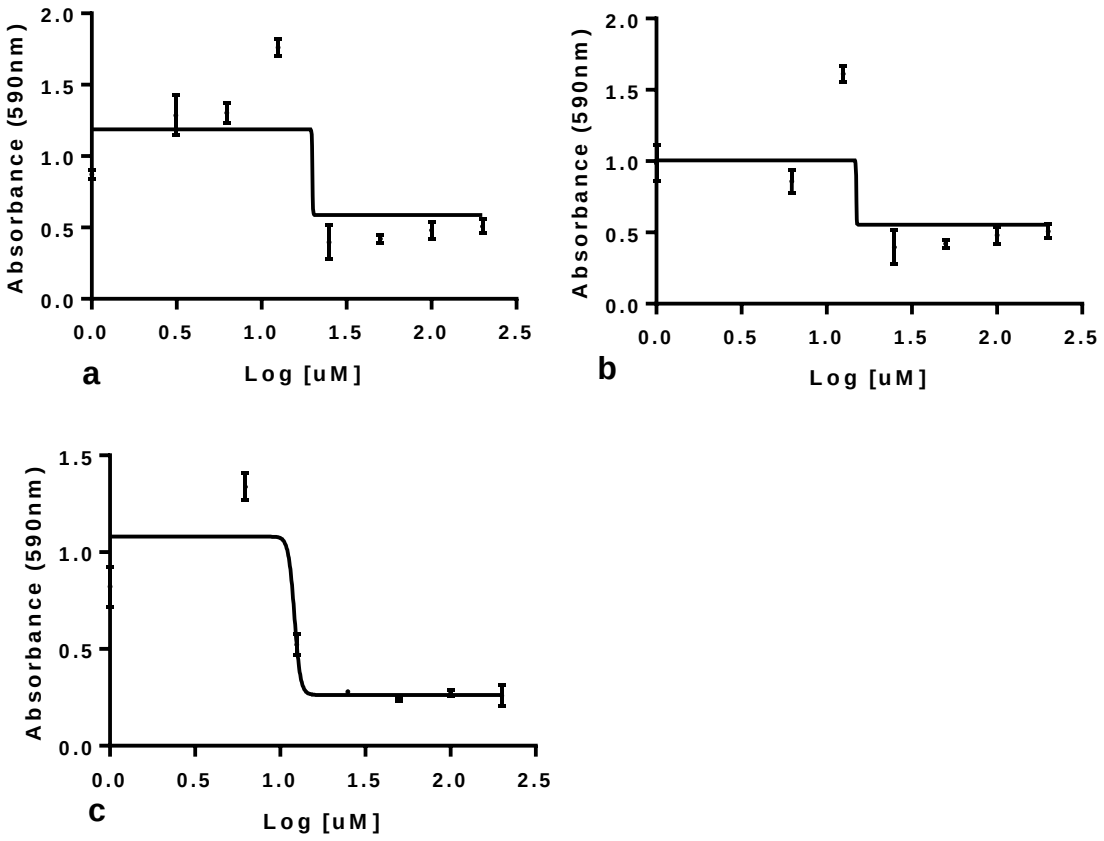


Figure 3.16: Cytotoxicity levels of Congo Red on HEK 293T cells using different solvents. The MTT assay was used to quantify cell viability in the presence of solvents where the absorbance was read at 590 nm wavelength. The experiment was done in triplicates and the averages are plotted as data points in the dose-response curves where the standard deviation is presented as error bars. Panels a (SDS), b (SDS/HCL) and C SDS/HCL/Isopropanol) represent different MTT solvents used for detection.

Comparable cytotoxicity concentrations were observed for both Congo Red and EGCG in the presence of different MTT solvents. These results are in agreement with a study done in 2014 where the CC_{50} of EGCG was reported to be 23 ± 1 µM (Abrahams, 2014). Weisburg and colleagues also reported a similar CC_{50} of 25 ± 4.2 µM on CAL27 cells (Weisburg *et al*., 2004). Conversely, Nance and colleagues reported that EGCG was not toxic to peripheral blood mononuclear cells at concentrations lower than 100 µM (Nance *et al*., 2009).

Table 3.5: Data representing the levels of cytotoxicity of EGCG in HEK 293T cells tested in the presence of different MTT solvents.

Solvents	**CC_{50}**
SDS	25 ± 1.41 µM
SDS/HCL	19 ± 1.27 µM
SDS/HCL/Isopropanol	24 ± 1.39 µM

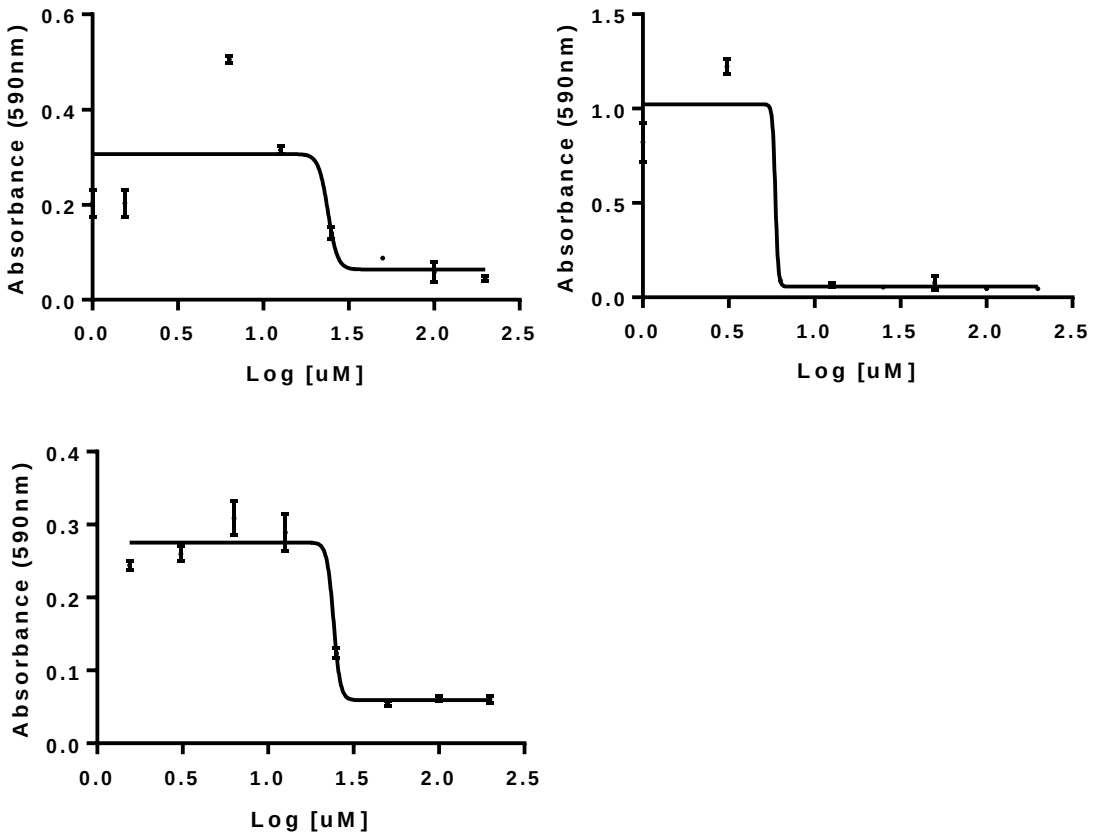


Figure 3.17: Cytotoxicity levels of EGCG on HEK 293T cells using different solvents. The MTT assay was used to quantify cell viability in the presence of solvents where the absorbance was read at 590 nm wavelength. The experiment was done in triplicates and the averages are plotted as data points in the dose- response curves where the standard deviation is presented as error bars. Panels a (SDS), b (SDS/HCL) and C SDS/HCL/Isopropanol) represent different MTT solvents used for detection.

Chapter 4: Discussion

Drugs that specifically target affected cells rather than interfering with the healthy cells present are sought in drug discovery. As such, specificity is a desirable trait in a drug. The drug discovery process is however, challenged with non-selective drug-like compounds that result in false positives. These compounds bind to unrelated targets in a non-specific manner and disturb or confuse the drug screening process. These compounds are called promiscuous inhibitors owing to their non-selective nature. In this study, we sought to develop a novel biochemical assay to identify promiscuous compounds. Briefly, the DSF thermal shift assay was adapted and optimized to study and compare the signatures of aggregate-based promiscuous inhibitors to those of true inhibitors. Fluorescence-based thermal shift assays are commonly used in drug discovery, however, this study presents a specific method to identify both true and promiscuous compounds. Specifically, this study compared true inhibitors of stem bromelain with known promiscuous compounds, however, this assay can potentially apply to other proteins or targets. Importantly, this is the first developed assay to concurrently identify both true and promiscuous inhibitors using a single assay.

4.1. Stem bromelain is an ideal protein to use in the thermal shift assay

Stem bromelain is the most active cysteine proteinase obtained from pineapple and it has various reported benefits (Maurer, 2001; Tochi *et al*., 2008; Pavan *et al*., 2012). Since this protein has not been previously used in the thermal shift assays, bioinformatics was seen a fitting start for this study. This was done to acquaint with the structure and the composition of the protein as both these factors are important for the thermal shift assay. Through bioinformatics, this study showed that stem bromelain is a hydrophobic protein. This was shown by the distribution of non-polar amino acid residues both in the core and surface of the protein (Figure 3.1). Even though it was not clear at this stage whether the hydrophobic surface of stem bromelain would interfere with the findings in the thermal shift assay, the hydrophobic core of this protein encouraged further investigations. The thermal shift assay uses environmentally sensitive fluorescent dyes such as SYPRO orange to track the unfolding transitions of the protein. The fluorescence of SYPRO orange is quenched in the aqueous environments, which validates the general downward trend from 25 - 65 °C observed in the melt curves in this study. However, an increase in temperature unfolds the protein, exposing its core hydrophobic regions. SYPRO orange exhibits an increased quantum yield at lower dielectric environment, such as the hydrophobic regions of the proteins. As such, the dye binds to the hydrophobic regions and becomes

unquenched and this binding results to an increase in fluorescence. In the thermal shift assays conducted in this study, SYPRO orange was unquenched and was observed to bind to the non-polar residues of stem bromelain. This was indicated by an increase in fluorescence in the melt curve of stem bromelain at temperature 65-76 °C which further supported and correlated the hydrophobicity shown in bioinformatics.

The detection of the protein's T_m is the main objective of thermal shift assays. The temperature gradient created by the real-time PCR machine is generally from 25-95 °C, hence, ideal proteins to use in thermal shift assays should have melting temperatures that fall within this range. The theoretical T_m of stem bromelain is reported to be 77 °C at acidic pH (Haq *et al*., 2005), hence, this validates the choice of using stem bromelain as a protein of interest as its T_m fall within the assay's temperature range. These results were further validated in this study where stem bromelain yielded a T_m of ~75-76 °C. Furthermore, the T_m of stem bromelain is maintained in all tested concentrations, where even at low concentrations of 5 µM protein peaks were visible. This observation further confirmed stem bromelain to be a well-behaved enzyme suitable for the thermal shift assay.

Even though commercially obtained as a pure enzyme, isolation through molecular exclusion chromatography and separation by SDS-PAGE revealed that stem bromelain still existed in a mixture of five proteins as per previous reports (El-Gharbawi and Whitaker, 1963). Following this observation, it was expected that in the thermal shift assay stem bromelain would produce five peaks due to the different proteins of different sizes obtained. This expectation was due to the thermal shift results obtained in a previous study were protein-protein interaction resulted in multiple peaks indicating the presence of different proteins (Layton and Hallinga, 2011). However, in the present study a single peak was produced for the bromelain mixture. This could be due to a number of factors, one being the possibility that stem bromelain is in fact pure and the different proteins observed are a result of protein degradation. It could also be possible that the other present proteins are at low concentrations in the mixture and could not be detected in the thermal shift assay. The other proteins present in the mixture may possess melting temperatures that are either below 25 °C or above 95 °C, this would explain why they are not visible. Another possible explanation could be that all the proteins present in the bromelain mixture have a similar T_m of ~75-76 °C, hence the single peak. Stem bromelain was identified as the prominent band at 33.11 kDa (Figure 3.3) which was expected following previous reports (Ritonja *et al*., 1989; Margaretta *et al*., 2015). This size also correlated with the 33 kDa stem bromelain size predicted by ExPASy. The molecular weight of proteins used in thermal shift assays starts from 19 kDa (Ericsson *et al*., 2006). Stem bromelain at a size of 33.11 kDa was therefore confirmed to be suitable for the thermal shift assay as it above this protein size.

In this study, stem bromelain was detected as an active enzyme following the successful degradation of the substrate gelatin in both the titrimetric and the radial diffusion assay. The activity of stem bromelain can be detected in the presence of other substrates such as casein. However, gelatin is favourable due to its availability and broad assay options. In the titrimetric assay, the enzyme yielded 2085.50 and 2024.36 GDU/ mg enzyme for 2 and 4-units enzyme respectively (Table 3.1). A detection of the enzyme activity on the absorbance spectrum showed that stem bromelain is fully able to break down its substrate gelatin into oligopeptides and amino acids. This reaction stopped upon the addition of hydrogen peroxide (H_2O_2) (Figure 3.4) and further titration with sodium hydroxide removed the amino acids (Dubey and Reddy, 2012). Further substrate degradation by stem bromelain was correlated in the radial diffusion assay where degraded wells were not stained by Amido black. This confirmed that stem bromelain is structurally correct to use in the thermal shift assay (Figure 3.6). In summary stem bromelain was an ideal protein to use in the thermal shift assay as it is hydrophobic, structurally correct and well-behaved. Stem bromelain also has both the size and the melting temperature that falls within the range set for the thermal shift assay.

4.2. E-64 stabilizes stem bromelain

E-64 is an irreversible class specific cysteine protease inhibitor (Trinchese *et al*., 2008). In this study, the inhibition and stabilization of stem bromelain by E-64 was observed in the radial diffusion assay and in the thermal shift assay respectively. E-64 and another cysteine protease inhibitors, iodoacetate and TPCK, successfully inhibited the degradation of gelatin by stem bromelain in the radial diffusion assays. These inhibitors form bonds with the cysteine protease. Particularly, bonding with the thiol (-SH) regions of cysteine-containing proteins such as papain and bromelain results in full inhibition. This bond formation alters the structure of the enzymes thereby blocking the active sites which slows the activity of the enzyme. Mercury was also observed to inhibit stem bromelain and even though it is not a true inhibitor of cysteine protease, total inhibition of stem bromelain was expected following previous reports (Masdor, 2013).

In the thermal shift assay, the binding of E-64 to the protein resulted in a more stable complex observed as a shift in the melting temperature of stem bromelain. As an irreversible inhibitor, E-64 interacts covalently with the protein and changes its conformation. This covalent attachment stabilizes the protein which then requires extreme temperatures to unfold, hence, the T_m increases to ~84- 86 °C. The shift observed is visible from interactions of low inhibitor concentrations (5 µM) with protein concentration of 17 µg/ 25 µL.

Interestingly, iodoacetate and TPCK did not cause a shift in the T_m of the protein. This indicated that there was no specific interaction between the inhibitors and the protein. Even though iodoacetate and TPCK are irreversible inhibitors like E-64, they are, however, not class specific to cysteine proteases as they inhibit different protease classes (Otto and Schirmeister, 1997). This could be a possible explanation for the lack of effect observed in the thermal shift assay, nonetheless, shifts were expected in the presence of these inhibitors. In previous studies the presence of irreversible inhibitors demonstrated a shift in T_m and biphasic protein melt curves which was not the case in this study (Rojas *et al*., 2015; Bai *et al*., 2019).

4.3. Aggregation of Stem bromelain by Congo Red was reversible in the presence of a detergent

Promiscuous compounds generally interact with proteins in a non-specific manner, as a result, in their presence, the activity of the protein appears to be suppressed. Therefore, there is a drive to explore purpose specific assays that expose this non-specific inhibition. Congo Red is a known aggregate-based promiscuous compound that interacts with proteins of different or unrelated classes (Khurana *et al*., 2001). Like most promiscuous compounds, Congo Red forms micelle-like colloids and due to such factors, researchers are still not certain about the mechanism of interaction of this compound. In this study, the interaction of Congo Red with stem bromelain was investigated. Radial diffusion assays are typically used to determine enzyme activity where the activity is considered as the degradation of the substrate which is indicated by the cleared zone around the wells. Using this assay, Congo Red was observed to lack interaction with the protein as there was no interference with the protein's activity. This was shown as cleared-zones on gelatin gel, however, at low concentrations of 8 µM Congo Red was observed to have a partially darker well than the other tested concentrations (Figure 3.6). To confirm interference or lack of it, the activity of stem bromelain was investigated in the presence of other compounds that do not interfere with the activity of the protein. The compounds EDTA and soybean trypsin inhibitor are typical inhibitors of metallo and serine protease inhibitors respectively. Therefore, the lack of effect on stem bromelain as a cysteine protease was expected. However, there have been reports suggesting that soybean trypsin inhibitor deactivates through the cleavage of the C-terminal of arginine present in the protein (Ma, 2010; Sabotič *et al*., 2012; Chowdhury *et al*., 2014). The chelating agent EDTA has a great affinity for divalent cations such as Mg^{2+} and Ca^{2+} that are found ubiquitously in proteins. Nevertheless, the activation of stem bromelain by EDTA suggests the need for this chelator to bind to the cations for its activity. Overall, studying Congo Red in the radial diffusion assay was necessary as the partial inhibition observed guided studies in the thermal shift assay to further understand the effect of this aggregator on stem bromelain.

In this study, low concentrations of 5 - 8 µM Congo Red did not alter stem bromelain's T_m in the thermal shift assay. However, the presence of higher concentrations (9 - 18 µM) of the compound aggregated the protein which resulted in the low fluorescence intensity curve. The aggregation of the protein was observed to be concentration dependent, as higher concentrations of Congo Red completely sequestered the protein from the reaction. As a result, the melting temperature of stem bromelain could not be determined. These results supported reports that Congo Red is an aggregate-based promiscuous compound (McGovern *et al.*, 2002; McGovern *et al.*, 2003).

Further investigations involved the use of the detergent since such surfactants have been promoted in assays to detect the presence of aggregate-based promiscuous compounds. The presence of the detergent SDS restored the protein which was indicated by an increase in the fluorescence curve previously aggregated by Congo Red. The presence of the detergent is known to degrade the colloid formed by aggregators, hence, the restored stem bromelain curve has a comparable T_m in the presence and in the absence of Congo Red. This provides further evidence that there is no direct interaction between the protein and Congo Red as predicted in the radial diffusion assay. Furthermore, these results confirmed that inhibition by aggregate-based promiscuous compounds is reversible upon the addition of a detergent. Toxicity is the main cause of late- stage failures in drug discovery where a number of approved drugs have been withdrawn from the market due to toxicities that were not detected in the late-stages of clinical trials (Chen *et al*., 2013). As a result, cytotoxicity assays were conducted to assist in explaining this hypothesis. Promiscuous compounds are generally toxic and as expected, Congo Red had a toxicity of 20 ± 1.3 µM in HEK 293T cells in the cytotoxicity assays and yielded steep sigmoidal curves with steep hill slopes which further correlated the promiscuity of Congo Red.

4.4. EGCG interacts with stem bromelain

The compound (–)-epigallocatechin-3-gallate also known as EGCG belongs to the family of five catechins found in green tea where it is the most active. This polyphenolic natural compound is believed to have therapeutic benefits when consumed in green tea or taken in supplements (Nagle *et al*., 2006). Antioxidant effect, anti-cancer, a boost in cardiovascular health, skin protection from radiation are among the numerous benefits of EGCG (Nagle *et al*., 2006; Khan and Mukhtar, 2007; Forester and Lambert, 2011). Despite these benefits, not much is known about EGCG's mechanism of action. Efforts to investigate this revealed that EGCG exhibits promiscuous binding and inhibits proteins of different origins (Liang *et al*., 1997; Ehrnhoefer *et al*., 2008; Ishii *et al*., 2011). Here, EGCG was used to verify the objectives of this study.

In this study, radial diffusion assays detected no interaction between EGCG and stem bromelain. However, in the presence of 0.8 µM EGCG, the well is partially darker compared to the other concentrations. A similar observation was obtained with Congo Red at this concentration which indicated partial inhibition of stem bromelain. Furthermore, studies on the thermal shift assay correlated the partial interaction between the protein and EGCG. The presence of this compound increased the T_m of the protein in a manner comparable to that of E-64. This behaviour was expected as EGCG is a promiscuous compound and not an inhibitor of stem bromelain. Therefore, it was suspected that EGCG could not have specifically interacted with the protein in its active site. Previous reports supported this observation through suggestions that EGCG undergoes oxidation to form quinone that react with the nucleophilic thiol group of cysteine residues and as a result interact with the protein (Ishii *et a*l., 2008). Furthermore, unlike with Congo Red, EGCG was not affected by the presence of the detergent. This showed that although EGCG non-specifically binds to stem bromelain, it was not through aggregation. Steep dose-response curves and toxicity at 25 ± 1.41 µM in HEK 293T cells in the cytotoxicity assays supported the hypothesis that EGCG is promiscuous as both these factors indicate promiscuity of the compound.

4.5. The DSF thermal shift assay is a potential method for the identification of promiscuous compounds

The DSF thermal shift assay is mostly known for monitoring protein to ligand binding in the presence of fluorescent dyes. Different fluorescent dyes are suitable for the thermal shift assays, however, SYPRO Orange has been successfully used and is favored due to its elevated signal to noise ratio (Lo *et al.*, 2004). Here, the thermal stability of stem bromelain was studied in the presence of increasing concentrations of SYPRO orange dye, at different concentrations of protein and in the presence of increasing amounts of true and promiscuous inhibitors E-64, Congo Red and EGCG. An evaluation of the performance and robustness of T_m determination by DSF showed that the assay is effective with minimal dye and protein concentrations. This was evident during dye optimization assays as at 10X dye concentration, the protein had the highest fluorescence intensity. Furthermore, visible peaks were observed even with low concentrations of 5 µg protein, which is the minimum concentration tested in this study. Drug discovery is an expensive process that involves a series of tests to identify potential leads. During these tests, large amounts of resources are used. Therefore, having an assay that uses minimal resources is desirable. Furthermore, the success of the optimization of concentration-dependent assays showed that DSF assay can be used to screen a wide range of protein and compound concentrations. This is ideal for identifying

promiscuous compounds as their interactions with proteins is concentration dependent, as confirmed in this study. A number of factors contribute to the stability of the protein which in thermal shift assays is shown by an elevated T_m. Examples of such factors include buffer contents such as salts and pH. The sodium acetate buffer containing sodium chloride has been used in stem bromelain assays and have previously shown to maintain the protein's activity (Gautam *et al*., 2010). For thermal shift assays in this study, a buffer library was tested comparing buffers of different pH in the presence and the absence of a salt gradient. Stem bromelain was found to possess a higher T_m in the presence of the sodium acetate buffer containing sodium chloride at pH 4.5. Therefore, the ability to use the thermal shift assay to optimize conditions that maintain the stability of the protein makes this assay ideal, as target stability is a sought trait in drug discovery.

4.5.1. The ability to detect partial unfolding, sequestering and restrain dynamics as mechanisms of action for promiscuous compounds

Evidence that DSF thermal shift assay can be employed to distinguish between the mechanism of action for promiscuous compounds is provided. Two visible changes in the DSF curve were observed in the presence of Congo Red, one being an aggregated peak and the other being a shift to the left. These two observations were suspected to present the methods of interaction between Congo Red and stem bromelain. The three possible mechanisms suspected to have occurred in this study; partial unfolding, sequestering and restrained dynamics are discussed below.

The shift to the left was suspected to be due to partial protein unfolding, which is one of the reported mechanisms of action for the aggregate-based promiscuous compounds (Coan *et al*., 2009). Even though we are fully aware that several other factors could have contributed to the decrease in the T_m, the fact that it occurred in the presence of Congo Red encouraged the partial unfolding suspicions. According to Coan and colleagues, upon interaction with proteins, aggregate-based promiscuous compounds unfold the protein's structure resulting in decreased or no activity (Coan *et al*., 2009). Furthermore, this decrease in T_m was maintained in the recovered peak of stem bromelain in the presence of Congo Red and SDS. Therefore, these results support partial unfolding as the mechanism of interaction between promiscuous compounds and proteins.

The concentration-dependent protein aggregation by Congo Red could possibly present two other mechanisms of action previously determined for aggregate-based promiscuous compounds. The first proposed mechanism is restrained dynamics, here, Congo Red could

have restrained movement of the protein making it rigid. This could have prevented the protein from unfolding at ~75-76 °C as this is where it was expected to melt. This consequently, prevented the dye from binding and hence no increase in fluorescence which accounts for the lack of the T_m determination. The second mechanism of action evident here can be sequestering, where Congo Red could have formed colloids which aggregated and completely sequestered the protein from the reaction. Once sequestered, the protein, as in the restrained dynamics model, could not be accessed and as a result the melting temperature could not be determined.

The investigations of the protein-inhibitor complex in the presence of promiscuous compounds were guided by the efforts to further validate these proposed mechanisms. It was evident in these assays that aggregation in the presence of Congo Red was not specifically for proteins, but aggregation can occur in the presence of a stabilized complex (protein-inhibitor complex) (Figure 3.12). This result was shown as a decreased protein fluorescent peak in the presence and in the absence of the inhibitor (Figure 3.12). It is also evident in Figure 3.12 that although aggregated, Congo Red did not directly interact with the protein-inhibitor complex as the shift is still observed. Furthermore, this showed that partial unfolding in the presence of an aggregate based compound is selective to proteins. This observation was justified by the maintained T_m of the protein-inhibitor complex (~84-86 °C) whereas in the presence of the protein alone, the partial shift was still visible.

The DSF thermal shift assay is influenced by the binding of the SYPRO orange dye, with the principle that the increase in fluorescence intensity is due to the dye binding to unfolded proteins. However, If the protein remains folded the SYPRO orange remains unbound in solution. This was considered the case with aggregation of stem bromelain by Congo Red, as increase in fluorescence was not observed which meant the dye was still accessible. This principle was supported by the recovery of an already aggregated protein-Congo Red peak upon the addition of more protein (Figure 3.13). Additionally, these results supported the principle as the dye was still available in solution to bind to the protein resulting in a partially decreased T_m of 72-73 °C. Furthermore, this observation also supported the reports that increasing protein concentration can gradually reverse or decrease inhibition by promiscuous compounds (McGovern *et al.*, 2002). Overall, these results correlated the three proposed mechanisms; unfolding shown as decrease in T_m in the recovered peak; restrained dynamics and sequestering which resulted in a flattened protein peak. It was finally concluded that restrained dynamics, sequestering and unfolding could possibly occur concurrently.

4.5.2. The ability to use detergents for the detection of aggregate-based promiscuous compounds

Detergents are a widely used tool in drug discovery and have since been rescuing screening assays as they are able to expose the presence of aggregate-based promiscuous compounds. This owes to the detergents' ability to decrease the surface tension of these compounds. However, detergents are among the components that have been predicted to cause potential destabilization and unfolding of proteins in thermal shift assays (Yeh *et al*., 2006; Alexandrov *et al*., 2008). Here, this study supported this finding as in the presence of high SDS concentrations the T_m of the protein was altered. Surprisingly, in the presence of low concentration (0.004%) of SDS the T_m of stem bromelain was maintained. Therefore, this study provides evidence which suggest that this detergent can be applied in thermal shift assays under these specified conditions. Normally, detergent-based assays use non-ionic detergents such as Triton-X100 and Tween 20. However, these latter detergents adversely affected the ability to monitor fluorescence changes of the protein in this study.

The detergent SDS remarkably restored the protein peak previously aggregated by Congo Red, while no change was observed in the presence and the absence of E-64 and EGCG. As proof of concept, this study showed that the DSF thermal shift assay can be used to successfully distinguish between aggregate- based promiscuous inhibitors (Congo Red) and true inhibitor (E-64) in the presence of SDS.

4.6. Conclusion

Commercial stem bromelain was isolated through molecular exclusion chromatography and the protein separation was achieved by SDS-PAGE with the prominent band at 33.11 kDa as indicated by previous studies. The enzyme was confirmed structurally correct in the gelatin titrimetric enzyme activity assay with the activity measured in GDUs. Promiscuous compounds Congo Red and EGCG were investigated in the radial diffusion assays and were predicted to not interfere with the activity of stem bromelain when compared with known activators and inhibitors of the enzyme. DSF thermal shift assays were optimized to compare the effects of E-64 the true-inhibitor of stem bromelain with that of Congo Red and EGCG where the mechanism of action for each compound was predicted. Aggregation of stem bromelain by Congo Red was predicted to occur through three mechanisms; sequestering, restricted dynamics and unfolding which were observed to occur concurrently. Additionally, EGCG was predicted to accomplish non-specific binding, however, it does not achieve it through aggregation. The use of the detergent SDS (at a specific concentration) in the DSF thermal shift assay confirmed that Congo Red was an aggregator, while EGCG and E-64 were not

affected by the presence of SDS. Both Congo Red and EGCG demonstrated cytotoxicity. Overall, this study successfully demonstrated that the DSF thermal shift assay can be used to concurrently distinguish between a true and promiscuous compound, moreover, this assay can be used to determine the mechanism of action of compounds.

4.7. Future works

The results obtained in this study can be advanced in the following ways;

1. Evaluation of a compound library to identify aggregate-based compounds and compare different mechanism on the thermal shift assay. A compound library consisting of both natural compounds and commercial compounds can be screened in the thermal shift assay under the conditions optimized in this study to identify or compare the behaviour of the different compounds.
2. Comparing the effect of other aggregators such as Rottlerin and methylene blue on different proteins and their ligands using the DSF thermal shift assay. The promiscuous compounds can be tested for their effect on proteins of unrelated origins, of different functions and sizes. This will correlate the promiscuity of these compounds.
3. Screening of different inhibitors for different targets to identify which are true inhibitors.
4. Optimizing the detergent-based thermal shift assays with different detergents such as CHAPS and chloric acid to see if their presence can restore the fluorescent peak of the protein without affecting the melting temperature.
5. Validating the interaction of promiscuous compounds with other more sensitive thermal shift assays such as CETSA, DSLS, and DSC.

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