Chapter 1

Introduction

1.1 Background

The utilisation of synthetic drugs as a therapeutic approach is becoming increasingly prevalent in the present days owing to their versatility and effectiveness to treat various diseases (Flick et al., 2021). Nevertheless, approximately 8% of hospital admissions and roughly 100,000 people are annually reported dead in the United States due to the adverse effects and toxicities of synthetic drugs (Karimi et al., 2015). Therefore, many practices have utilised alternative remedies reported to be safer and have fewer adverse effects (Kazemipoor et al., 2012). Therapeutic effects of naturally derived medicines are attributed to the abundance of bioactive compounds present, including bioactive proteins, polysaccharides, antioxidants, to name a few (Rafieian-Kopaei, 2012; Rafieian-Kopaei et al., 2013). Thus, renders them to be suitable for adjuvant use in modern medicines, especially in the immunotherapy field (Sarris et al., 2010).

Fungi, particularly medicinal mushrooms of the higher family of Basidiomycetes, are widely harvested and used as alternative medicine (Lindequist et al., 2014). Numerous studies have identified and isolated bioactive compounds from these mushrooms' medicinal characteristics (Lopez-Gartner & Uribe-Echeverry, 2017). Various bioactive compounds found in fungi include immunomodulatory proteins, lectins, antioxidants, β-glucan, *etc.* (Li et al., 2019; Xu et al., 2011; Zhou et al., 2019), which shows to enhance the body's immune system, reduce inflammation, and inhibit cancer metastasis (Lai et al., 2008). Fungal immunomodulatory proteins (FIPs) are fungal-derived bioactive proteins that have immunomodulatory properties (e.g., promoting lymphocyte proliferation and enhancing cytokines production). To date, 38 FIPs from different mushrooms have been characterised and grouped into five classifications comprising the Fve-type, Cerato-type, PCP-like, TFP-like, and unclassified FIPs (Liu et al., 2020). FIP-glu isolated from *Ganoderma lucidum* and FIP-fve from *Flammulina velutipes*

(classified as Fve-type) have been shown to elicit a robust anti-tumour, immunomodulation, and anti-allergy properties (Cong et al., 2014; Lin et al., 2013). Among the functional properties reported for FIPs include anti-cancer, anti-inflammatory, anti-allergy, and the ability to promote neurite regeneration (Wang et al., 2011).

The research team at the Faculty of Applied Sciences, UCSI University, was the first to isolate and characterise the FIP-Lrh from the Tiger Milk Mushroom (TMM) or *Lignosus rhinocerus* and reported the production of recombinant FIP-Lrh in *E. coli* and *P. pastoris* expression system (Pushparajah et al., 2016, Ejike et al., 2021). In addition, it was shown to elicit immunomodulation and cytotoxicity properties on cervical (HeLa), lung (A549), and breast (MCF-7) cancer cell lines in dose-dependent. Furthermore, FIP-Lrh's 3D structure, which was predicted through homology modelling to FIP-fve, showed that it is a homodimer with a more positively charged CBM-34 binding pocket (strong binding to N-acetylgalactosamine and N-acetylglucosamine) (Pushparajah et al., 2016). However, the biological 3D structure of FIP-Lrh is still unavailable to accurately decipher its binding to ligands and, thus, its biological properties (Gligorijević et al., 2021; Pushparajah et al., 2016).

To date, crystal structures have been reported for rFIP-Glu (PDB: 3F3H), rFIP-fve (PDB: 1OSY), rFIP-gmi (PDB: 3KCW and 7WDM), and rFIP-nha (PDB: 7WDL) (Hsu et al., 2010; Huang et al., 2009; Liu et al., 2021; Liu et al., 2022; Paaventhan et al., 2003; Seow et al., 2003). The limited information on FIP-Lrh's crystal structure hinders further elucidation, which necessitates the protein crystallisation work on FIP-Lrh. Thus, genetic engineering was previously used to produce a soluble rFIP-Lrh protein of substantial yield in the *E. coli* expression systems with less cost and labour for functional studies (Pusparajah et al.2016). The recombinant 6xHis-tagged rFIP-Lrh protein was purified using Ni-NTA affinity chromatography which binds to the 6xHis-tag polyhistidine residues on the recombinant protein.

Protein crystallisation of rFIP-Lrh is required to generate sufficiently refined crystals for subsequent protein X-ray crystallography analysis that will shed light on its biological properties (e.g., functions, binding interaction, thermostability, *etc.*) of FIP-Lrh (Parker, 2003; Liu et al., 2021). However, protein crystallisation is not a one-size-fits-all technique, as different proteins require different optimal conditions to form crystalline structures. Screening and optimisation of conditions (e.g., pH, salt, crystallising agents, buffer, temperature, *etc.*) favouring the crystal formation of FIP-Lrh are required to generate a refined, well-diffracting crystal structure (McPherson & Gavira, 2013; Smyth & Martin, 2000). A preliminary study on FIP-Lrh crystallisation showed two different conditions that produced different crystal growth of FIP-Lrh (Lau et al.). A hexagonal, rod-shaped, irregular crystal was observed under 0.1M Tris-HCl with a pH of 8.5 and 2.0M ammonium sulphate. Microcrystal was observed under 0.1M MES monohydrate with a pH of 6.5 and 1.6M magnesium sulphate heptahydrate. Hence, this research project aimed to crystallise the rFIP-Lrh as well as optimising its crystallisation conditions to obtain sufficiently refined crystals of FIP-Lrh which can then be subjected to protein X-ray crystallography for elucidating its 3D structure.

1.2 Objectives

The objectives of the project are as follows:

1. To express recombinant FIP-Lrh in *E. coli* BL21 expression system and to purify the recombinant protein using Nickel-Nitrilotriacetic Acid (Ni-NTA) affinity chromatography and size exclusion chromatography.
2. To optimise the crystallisation conditions of purified recombinant FIP-Lrh.

1.3 Hypothesis

It was hypothesised that the optimisation of crystallisation conditions, which included protein concentration, buffer, precipitants, pH, temperature, drop to total volume ratio, and other parameters, could influence the effectiveness of FIP-Lrh protein crystallisation.