#### PURIFICATION AND CHARACTERIZATION OF AN IMMUNO-STIMULATORY COMPOUND FROM THE WATER EXTRACT OF *ROYOPORUS BADIUS* (PERS.) A.B. DE

by

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#### Abstract

A novel immuno-stimulatory polysaccharide-protein complex (ISPP-Rb) was isolated from *Royoporus badius* sporocarps, collected in northern BC forests. ISPP-Rb was purified from the water extract of *R. badius* using anion-exchange and size-exclusion chromatography and could significantly activate murine macrophage (RAW 264.7) cells. ISPP-Rb is highly branched and has an average molecular weight of 400 kDa. GC-MS data suggest the polysaccharide component of ISPP-Rb has a backbone of  $(1 \rightarrow 6)$ -Gal/Man and  $(1 \rightarrow 2,6)$ -Glc, with the latter being attached to either glucose, galactose or fucose at its terminal end. The protein component, found to be indispensable for its immuno-stimulatory activity, is proposed to link to a mannose residue via O-linked glycosylation. ISPP-Rb was able to induce various proinflammatory cytokines including TNF- $\alpha$ , IP-10, MIP-2, G-CSF, GM-CSF, MIP-1 $\alpha$ , and MIP-1 $\beta$ . This is the first immuno-stimulatory compound to be reported from *R. badius*, and hence making ISPP-Rb novel.

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#### **Chapter 1**

#### **Introduction and Literature Review**

This chapter will mainly describe (i) a brief history of cancer and how the disease has emerged as a major health concern in recent years, (ii) the importance and value of mushrooms as anti-cancer agents, (iii) the biological activity of some North American mushrooms (iv) a short overview on the mushroom under investigation (*Royoporus badius*), (v) the use of large molecules from natural sources as an alternative for anti-cancer drugs, (vi) the re-emergence of natural products, and (vii) the experimental objectives of this research.

#### **1.1** Cancer and Its Properties

The word "cancer" was first described by Hippocrates, the father of medicine using the Greek word *Karkinos*, which means tumors (Nobili *et al.*, 2009). However, cancer was believed to have been documented even earlier, during the classical periods of the Egyptian Pharaohs' reign (Nobili *et al.*, 2009). Cancer is a deadly disease involving the formation of malignant tumors as a result of uncontrolled cell growth and their ability to invade or metastasize to other regions of the body (Dai *et al.*, 2016). Such a phenomenon is thought to be caused by several factors including exposure to carcinogens, radiation, and viruses, along with genetic inheritance (Sudhakar, 2009). It is believed that a vast majority of cancer cells possess six immortal traits; the ability (a) to bypass cell death (apoptosis), (b) to be self-sustaining in growth signals, (c) to be unresponsive to antiproliferative signals, (d) to metastasize, (e) to form new blood vessels (angiogenesis) sustainably, and (f) to replicate endlessly, allowing them to breach the anti-cancer defense mechanism of cellular tissues (Hanahan & Weinberg, 2000). Today, cancer is documented as a leading cause of deaths globally regardless of economic status, and the numbers are estimated to rise even further due to population growth, age, and the adoption of unhealthy lifestyles (Torre *et al.*, 2016). There

were 14.1 million new cancer cases followed by 8.2 million deaths from the disease worldwide in 2012, whereby lung, breast and colorectal cancer topped the list as the most commonly diagnosed cancers; while lung, liver and stomach cancer were the most prevalent cause of cancer death cases (Ferlay *et al.*, 2014). Interestingly, despite numerous research efforts in search of a cure, cancer remains as one of the leading causes of deaths in the world.

Although there are conventional methods such as chemotherapy, radiotherapy and surgery to treat cancer, these options often come with limitations and drawbacks (Safarzadeh *et al.*, 2014). Among the downsides of the aforementioned treatments are: (i) patients diagnosed with cancer at a late stage are unable to undergo surgery; (ii) a short postoperative survival rate of not more than five years along with a risk of reoccurrence for most cancer patients; (iii) the harmful side effects from chemotherapy and radiotherapy despite it being an efficient treatment option; and (iv) the high resistance and insensitivity of some cancers to both chemotherapy and radiotherapy, rendering the treatment ineffective (Qi *et al.*, 2010). In another report by Chakraborty & Rahman (2012), they described numerous reasons behind the challenges faced in treating cancer, such as troubles targeting cancer stem cells, anti-cancer drug resistant cancer cells, asymptomatic cancer progression, limited number of cancer epigenetic profiling followed by low specificity of current existing epi-drugs, poor diagnosis and prognosis due to lack of effective cancer biomarkers, the limitation of chemotherapeutic agents due to cytotoxic effects, and the metastatic nature of cancer cells.

The use of anti-cancer drugs in chemotherapy, as previously mentioned, often results in much unwanted side effects due to its high toxicity. For instance, in spite of the remarkable potency of platinum-based Cisplatin in cancer treatment, the use of the drug results in an extensive level of cytotoxicity as Cisplatin often interferes with the DNA replication and/or transcription mechanism

and disrupts biochemical reactions and pathways by binding to enzymes, leading to issues involving nervous and renal system toxicity, along with bone marrow suppression, with combinational chemotherapy using Cisplatin being linked to the resistance to the drug itself (Florea & Büsselberg, 2011). Another side effect, the most commonly observed in chemotherapy is hair loss (alopecia). Alopecia can be classified into (i) telogen effluvium, a condition involving no more than 50% hair loss as a result of the use of 5-fluorouracil, methotrexate, and retinoids during 3-4 months of chemotherapy, and (ii) anagen effluvium, a situation where significant to complete hair loss occurs 1-2 months post-treatment with chemotherapeutic agents like cyclophosphamide, etoposide, topotecan, and paclitaxel (Yeager & Olsen, 2011). In addition to that, Docetaxel, an anti-cancer drug used to treat nonsmall cell lung cancers has been reported to induce fluid retention leading to several pulmonary complications not only in single drug chemotherapy, but also with combinational chemotherapy or radiotherapy (De Sanctis *et al.*, 2011). According to Remesh, (2012), oral toxicity involving the use of anti-cancer chemotherapeutic drugs is also a common side effect. Drugs like Dactinomycin and Doxorubicin causes damage to the buccal or orolabial mucosa due to the cytotoxic effects slows down the cells' renewal rate, making it susceptible to various infections including stomatitis. The above described drugs are just a few of the many examples of chemotherapeutic anti-cancer agents that can result in complications during cancer treatments, hence limiting their efficacy. Therefore, many have directed their focus towards a more natural approach, i.e. the use of natural products.

The use of animal-, plant-, microorganism- and marine-based natural products as medicines to treat diseases have dated back to ancient times (Yuan *et al.*, 2016). Today, at least 60% of the anti-cancer drugs approved and clinically used are derived from natural products, as manipulation of the bioactive compounds can be performed through structural modification or transformation,

leading to production of a more potent, yet less toxic derivative (Song *et al.*, 2014). Furthermore, Woosley & Cossman (2007) claimed that despite the long duration, immense effort and huge monetary investment made into drug research and development, only 10% of the drugs that enter clinical testing by the Food and Drug Administration (FDA) will be deemed safe and qualified for public use. Taken together, it is therefore understandable that researchers have re-focused on discovering novel anti-cancer compounds from natural sources.

With the increasing number of cancer cases globally, people are starting to show interest in a healthier approach, cancer chemoprevention, which is cheaper and safer compared to that of the cancer treatment (Gullett et al., 2010). This preventive approach involves the long-term administration of either synthetic, natural or biological agents by patients to decrease or delay the occurrence or process of tumor formation, with early success shown in trials involving breast, colon and prostate cancer (Steward & Brown, 2013). According to Gullett et al. (2010), the simplest way to practice chemoprevention is by consuming a diet that consists of a wide variety of foods. Green plants have benefited mankind for a very long time by serving as a source of medicine and traditional remedies, while fungi were often overshadowed (Chatterjee et al., 2011). However, fungi, especially mushrooms are currently attracting interest worldwide and are expected to contribute enormously to the nutraceutical and pharmaceutical industry in the near future, mainly due to their antineoplastic (tumor inhibition) properties, with mushroom-derived immunostimulants and adaptogens serving as key anti-cancer agents for clinical purposes (Chatterjee *et al.*, 2011). Hence, this provides a platform to further pursue the exploration of mushroom metabolites in search for potential anti-cancer compounds.

#### 1.2 Medicinal Mushrooms and Their Role in Treating Cancer

The historic use of mushrooms as traditional medicine originated in the far east, and is currently attracting a lot of interest worldwide as mushroom metabolites (organic compounds not directly involved for growth, development and reproduction of the organism) are deemed safe and can be incorporated into our diet without the need to undergo lengthy clinical trials (Lull et al. 2005; Seca & Pinto, 2018). The potential of mushrooms as therapeutics is huge, with many known to exhibit myriad biological activities such as anti-cancer, anti-diabetic, anti-hypercholesterolemia, anti-hypertensive, anti-inflammatory, anti-microbial, antioxidant, immuno-modulatory, and liverprotective activities (Zhang et al., 2016). Mushrooms have been used as food and medicines since ancient times (Wasser, 2014). It was documented that mushrooms were consumed by Greek warriors in belief that they act as an energy booster during battle, while mushrooms were highly treasured by Egyptian Pharaohs as a delicacy (Rahi & Malik, 2016). In addition, ancient Romans believed that mushrooms were the "Food of the Gods", the Chinese worshipped mushrooms as the "elixir of life" for centuries, and the Japanese emperors highly valued the mushroom Lentinus edodes (Berk.) Pegler as an aphrodisiac (Chang & Buswell, 1996). According to Wani et al. (2010), in spite of the astonishing health benefits of mushrooms and their long history of medicinal use, it was not until the 1960s before the bioactivity of mushrooms was scientifically evaluated.

One of the pioneer scientific research studies conducted on mushroom bioactivity was by Ikekawa *et al.* (1969), where hot water extracts of seven different mushrooms were tested on tumor-bearing mice for anti-tumor activity, with all extracts showing tumor inhibition of over 40%, with subsequent purification of one of the mushrooms (*L. edodes*) exerting more than 95% tumor inhibition *in vivo*; the inhibitor compound was expected to be a polysaccharide. Today, countless research studies have been performed to evaluate the biological activity of mushrooms, and

according to Ivanova *et al.* (2014), the anti-cancer effect of mushrooms can be derived from (i) large molecules, which mainly comprises of polysaccharides, polysaccharide-protein complexes, proteins and dietary fibers, and (ii) small molecules, such as terpenoids, steroids, phenolics, and more.

Chen et al. (2012) discovered a protein molecule called AAD with DNA cleaving activity. It was purified from Agrocybe aegerita (now known as Cyclocybe aegerita (V. Brig.) Vizzini), and the protein inhibited cell proliferation in liver, cervical and brain cancer cell lines through apoptosis as a result of chromatin condensation and sub-G1 cell cycle arrest in a caspase-dependent pathway. A 32 kDa lectin was isolated from the fruiting body of Russula lepida Fr. and was found to potently inhibit cell proliferation of two cancer cell lines, namely hepatoma (HepG2) cells and human breast cancer (MCF-7) cells with an IC<sub>50</sub> of 1.6  $\mu$ M and 0.9  $\mu$ M respectively, and the growth-inhibitory property of the lectin was also in line with the reduction in tumor size when treated to mice containing S-180 tumor (Zhang et al., 2010). Furthermore, a water-soluble polysaccharide (ISP2A) purified from the fruiting body of *Inonotus obliquus* (Ach. ex Pers.) Pilát was reported to inhibit human gastric cancer cell line (SGC-7901) tumor-bearing nude mice in vivo but showed no significant activity in vitro, and was able to upregulated lymphocyte proliferation along with an increase in the TNF- $\alpha$  secretion in peritoneal macrophages (Fan *et al.*, 2012). Moreover, two water extracts of Cordyceps militaris (L.) Fr. exerted strong immunostimulating activity by promoting the secretion of COX-2, IL-1 $\beta$ , and TNF- $\alpha$  in THP-1 macrophage cells; while the alkaline extract of C. militaris demonstrated potent anti-inflammatory actions with the active compound being a  $(1 \rightarrow 3)$ - $\beta$ -D-glucan (Smiderle *et al.*, 2014).

As aforementioned, mushroom-derived small molecules were also found to possess anticancer properties. Recently, three triterpenes from an ethyl acetate extract of king oyster mushroom, *Pleurotus eryngii* (DC.) Quél, were shown to exhibit strong growth-inhibitory action against breast cancer (MCF-7) cell lines *in vitro* and some immuno-modulatory effects in mice. These compounds were successfully identified and their structures elucidated (Xue *et al.*, 2015a; Xue *et al.*, 2015b). Elsewhere, eight triterpenes were extracted from the fruiting bodies of *Antrodia camphorata* (M. Zhang & C.H. Su) Sheng H. Wu, with all eight exerting their cytotoxic effect in at least one cancer cell line, and three (zhankuic acids) out of the eight were found to induce cell apoptosis in two cancer cell lines, namely HT-29 and SW-480 cells by causing sub-G1 cell cycle arrest, without exhibiting any toxicity in normal non-cancer cell lines (Yeh *et al.*, 2009). Other than that, an anti-cancer ergosterol molecule (ARHPLC-1) purified from *Amauroderma rude* (Berk.) Torrend extracts was reported to upregulate the expression of a tumor suppressor gene (FOXO3) in a breast cancer (MDA-MB-231) cell lines, causing an increase in the downstream cascade signaling events leading to cell apoptosis, and prolong the life span of B16 tumor-bearing mouse (Li *et al.*, 2015).

The above examples are just a few of the many studies describing anti-cancer properties of mushrooms, and the numbers will only increase as more mushrooms are tested. With so much literature citing their medicinal potential, it is clear that mushrooms would serve as a heightened platform for the discovery of a potential anti-cancer drug.

#### 1.3 Mushrooms and Their Bioactivity Profile in North America

Mushrooms belong to the Kingdom Fungi, and are classified alongside with bracket fungi, chanterelles, club and coral fungi, puffballs, stinkhorns, toadstools, as well as plant-parasitic rusts and smuts in the Basidiomycota division (Rahi & Malik, 2016). According to Wasser (2014), there are approximately 150,000 – 160,000 mushroom species across the globe, and with an estimate of 90% which have yet to be explored. Mushrooms have been used as a source of traditional medicine

in Asia for a very long period of time, however, this medicinal field is relatively new to countries in the West (Chatterjee *et al.*, 2011).

In Canada, other than the studies performed by Dr. Chow Lee's research team at the University of Northern British Columbia (UNBC), only a few studies have been conducted on the bioactivity of mushrooms native to Canada. The first study involved the discovery of a small molecule, ganoderma aldehyde from Ganoderma applanatum (Pers.) Pat. collected in the forest of British Columbia, whereby the structure of the compound was elucidated with the aid of both onedimensional (1-D) and two-dimensional (2-D) Nuclear Magnetic Resonance (NMR) along with Mass Spectrometry (MS) analysis; however, the bioactivity of the compound isolated was not described (Ming et al. 2002). Later, Van et al. (2009) demonstrated that I. obliquus collected from Manitoba, exerted anti-inflammatory activity on RAW 264.7 macrophage cells by downregulating its production of both nitric oxide (NO) and several lipopolysaccharide-induced inflammatory cytokines, but with no further studies performed to identify and purify the bioactive compound. Elsewhere in the province of Quebec, a large  $\beta$ -glucan (CDP) with anti-inflammatory activity, with an estimated size of over 1 million Daltons was successfully purified from Collybia dryophila (now known as Gymnopus dryophilus (Bull.) Murrill), was shown to inhibit the production of nitric oxide in activated murine macrophage (RAW 264.7) cells (Pacheco-Sanchez et al., 2006). Interestingly, polysaccharides similar to CDP were also found in L. edodes and 19 other mushrooms collected in Quebec (Pacheco-Sánchez et al., 2007).

Recently, Dr. Lee's research team at UNBC was the first to report the growth-inhibitory and immuno-modulatory activity of 12 mushrooms. This included five wild mushrooms collected from the forest of northern-central BC, namely *Gyromitra esculenta* (Pers.) Fr., *Hericium coralloides* (Scop.) Pers., *Hydnellum sp., Leucocybe connata* (Schumach.) Vizzini, P. Alvarado, G. Moreno & Consiglio, and *Trichaptum abietinum* (Dicks.) Ryvarden, along with seven other well-known medicinal mushrooms (Smith *et al.*, 2017). In addition, a growth-inhibitory polysaccharide (GIPinv) was successfully purified from *Paxillus involutus* (Batsch) Fr., a forest mushroom also found close to urban trees, which exerted potent growth-inhibitory activity against several cancer cell lines by inducing apoptosis in cancer cells (Barad *et al.*, 2018).

With such limited research done on the therapeutic activity of mushrooms in BC and elsewhere in Canada, combined with the high potential of mushrooms to serve as a pharmaceutical and nutraceutical hub, further studies on their bioactivity are therefore warranted.

#### 1.4 The Mushroom *Royoporus badius* (Pers.) A.B. De

*Royoporus badius* is a fairly large polypore (up to 15 cm in width) with a dark central or lateral stipe and a slightly hairy base (Glaeser & Smith, 2010), which can be found distributed in Asia, Australia, Europe and North America (De *et al.*, 1997). The mushroom had undergone several name changes, including *Boletus badius* (1801), *Grifola badia* (1821), *Polyporus badius* (1832), and *Polyporellus badius* (1989), before being recognized today as *Royoporus badius* in 1997 (Razaq & Shahzad, 2016). *R. badius* is also commonly known as the Black-Footed Polypore (Doskocil *et al.*, 2016).

According to Zmitrovich & Kovalenko (2016), polyporoid and lentinoid fungi are important producers of substances having anti-hyperlipidemic, anti-tumoral, anti-viral, and immuno-modulatory effects. Currently, there are only two studies conducted to assess the biological activity of, *R. badius* sporocarps. Recently Doskocil *et al.* (2016) assessed the cytotoxicity and immuno-modulatory activity of *R. badius*, along with 26 other polypore species, with the results demonstrating that the ethanol extracts of *R. badius* had very minimal cytotoxicity towards two colorectal cancer cell lines, but exerted comparable immuno-stimulatory effects to lipopolysaccharide (LPS) and their reference control (*Ganoderma lucidum*), as represented by their similar potential in stimulating neutrophil phagocytosis. The ethanol extract of all the polypores were then evaluated for their respective phenolics content, with *R. badius* possessing a similar amount of phenolics as *G. lucidum*. The results suggest that the phenolics present in *R. badius* could be responsible for its immuno-stimulatory effects. Unfortunately, the amount of βglucan was not determined. Elsewhere, Koch *et al.* (2002) showed that ethanol extracts of *R. badius* were able to reduce LPS-binding to CD14 transfected cells, resulting in an inhibition to the production of T<sub>H</sub>1 proinflammatory cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) but a stimulation in the production of T<sub>H</sub>2 pattern cytokine IL-4 and reactive oxygen species (ROS).

All in all, only the ethanol extracts of *R. badius* have been reported for their immunomodulatory activity, with no solid evidence on the nature (large or small molecule) of the active compound, and hence the need to further explore the immuno-modulatory activity of *R. badius*.

#### **1.5** Immuno-stimulation as an Approach for Cancer Treatment

Immunomodulators are currently considered as important components in modern healthcare due to the fact that the immune system serves as the first barrier of defense against diseases, and can be categorized into immuno-adjuvants, immuno-stimulants and immuno-suppressants (El Enshasy & Hatti-Kaul, 2013). Mushroom metabolites are known to exhibit anti-tumor and immuno-modulating activity which act on immune effecter cells such as dendritic cells, hematopoietic stem cells, lymphocytes, macrophages, natural killer cells, and T cells involved in both innate and adaptive immunity, and hence elevating the production of cytokines (Moradali *et al.*, 2007). These small intracellular protein mediators secreted by immune cells play a role in an organism's immune response and are cross-regulatory, whereby the expression of one cytokine pattern can regulate other patterns of cytokine production (Guggenheim *et al.*, 2014). The types of

cytokine patterns that are deemed crucial in cancer biology are summarized into four groups as in **Table 1**. Other than measuring cytokine production quantities, assessing immune cells' activity is also a good approach in determining the immuno-stimulatory properties of mushrooms.

**Table 1.** Immune response effect with respect to the type of cytokine produced; (modified from Guggenheim *et al.*, 2014)

Pattern	Cytokines Produced	Pattern Effect
T <sub>H</sub> 1	IFN-γ, IL-12, TNF-α	Stimulates immune response to cancer
T <sub>H</sub> 2	IL-4, IL-5, IL-13	Decrease T <sub>H</sub> 1
T <sub>H</sub> 3/T <sub>regulatory</sub>	TGF-β	Modulates T <sub>H</sub> 1
Proinflammatory	IL-1, IL-6, IL-8, TNF-α	Induce Inflammation

#### **Polysaccharides:**

Currently, many mushrooms are being studied for their bioactivity against cancer and were found to exert immuno-stimulatory activity. Recently, a  $\beta$ -1,6-glucan called PCPS was purified from a hot water extract of *Pleurotus cornucopiae* (Paulet) Rolland which exerted potent proinflammatory effect in macrophage cells, both *in vitro* and *in vivo*, by inducing the TNF- $\alpha$  and IL-1 $\beta$  mRNA expression (Minato *et al.*, 2017). Elsewhere, another water-soluble polysaccharide called ISP2a was purified from *I. obliquus*, which expressed its anti-tumor and immunostimulatory activity by inhibiting tumor growth in tumor-bearing mice by over 50% at a dose of 100 µg/g, stimulating lymphocyte proliferation, macrophage phagocytosis and TNF- $\alpha$  production *in vivo* (Fan *et al.*, 2012). According to Zhang *et al.* (2013), *Ganoderma atrum* J.D. Zhao, L.W. Hsu & X.Q. Zhang polysaccharide called PSG-1 expressed profound immuno-stimulatory effects by stimulating macrophage phagocytosis in CT26 tumor-bearing mice, via the upregulation IL-1 $\beta$ , TNF-a and nitric oxide production in a TLR4-dependent signaling pathway. Similarly, another polysaccharide called GSG was isolated from *Ganoderma lucidum* (Curtis) P. Karst, which enhanced the secretion of TNF- $\alpha$  and IL-6 in murine peritoneal macrophages isolated from BALB/c mice, which was in line with the significantly increased Concanavalin A splenocytes proliferation in mice and the reduced tumor weight observed (Guo *et al.*, 2009). In addition, a water-soluble neutral polysaccharide called JP1 was recently purified from the fruiting body of *Cantharellus cibarius* Fr. and the active compound was able to elevate the secretion of IL-6 and nitric oxide in RAW 264.7 cells and mouse peritoneal macrophages respectively, indicating the purified polysaccharide's immuno-stimulating activity (Chen *et al.*, 2017). Moreover, Xu *et al.* (2012) described a novel heteropolysaccharide called L2 isolated from the fruiting body of *L. edodes* capable of elevating the production of TNF- $\alpha$ , IL-6 and nitric oxide in RAW 264.7 cells through the toll-like receptor 2 (TLR2) signaling pathway.

#### **Polysaccharide-Protein Complexes:**

Immuno-stimulating polysaccharide-protein complexes have also been characterized from various mushrooms. Krestin (PSK) and PSP are well-known mushroom-derived immunomodulating and anti-tumor polysaccharide-protein complex; they are classified as biological response modifier that triggers T-cell activation and induces the secretion of IFN- $\gamma$  and IL-2, resulting in an increase in the number of leucocytes (Moradali *et al.*, 2007). A recent study conducted by Liu *et al.* (2016) reported a water-soluble polysaccharide-protein complex (PRW1) obtained and purified from *Polyporus rhinocerus* Cooke (now called *Lignosus rhinoceros* (Cooke) Ryvarden) that managed to upregulate the secretion of a variety of cytokines and the release of nitric oxide in RAW 264.7 cells with minimum cell cytotoxicity. In addition to that, a fucose-containing polysaccharide-protein fraction from *G. lucidum* was shown to enhanced the expression of IL-1, IL-2 and IFN- $\gamma$ , which was in line with increase mouse spleen cell proliferation (Wang *et*) *al.*, 2002). Elsewhere, another polysaccharide-protein complex called GLIS was isolated from *G. lucidum*, which demonstrated its immuno-stimulatory activity by inducing mouse spleen lymphocytes proliferation, resulting in the activation of B-cells, which coincided with the slight increase in IL-2 (Zhang *et al.*, 2002). Mushroom immuno-stimulating polysaccharide-protein complexes were also reported from *Tricholoma lobayense* R. Heim, noted by the improvement of phagocytosis activity of peritoneal exudate cells which was suppressed in tumor-bearing mice, along with the increase in TNF- $\alpha$  production (Liu *et al.*, 1996).

#### **Proteins:**

Besides polysaccharides and polysaccharide-protein complexes, mushroom proteins alone also have the potential to stimulate immune responses. A 9.5 kDa ubiquitin-like protein isolated and purified from the fruiting body of Agrocybe cylindracea (now called Cyclocybe aegerita (V. Brig.) Vizzini) was reported to express anti-proliferative activity on hepatoma (HepG2) and leukemia (M1) cell lines, together with activation of macrophages via nitric oxide induction (Ngai et al., 2003). Elsewhere, a homodimer protein purified from the mushroom Trametes versicolor (L.) Lloyd was claimed to have significantly augmented the splenocyte and human peripheral blood lymphocytes, along with the elevation of nitric oxide and TNF- $\alpha$  levels in LPS-activated macrophages. However, despite being able to stimulate the production of immune cells, the nitric oxide and TNF- $\alpha$  production remains questionable as LPS itself was able to stimulate cells (Li et al., 2011). Mushroom protein FVE isolated from *Flammulina velutipes* (Curtis) Singer, commonly known as Enoki mushroom, was shown to upregulate the expression levels of class I and II major histocompatibility complex (MHC) genes, together with CD80 expression on antigen presenting cells (APCs) in mice. The isolated FVE also suppressed cell growth in murine hepatoma cancer cell line and reduced tumor progression in hepatoma tissues, and the anti-cancer effects was believed to be attributed to the cytokine, IFN- $\gamma$  (Chang *et al.*, 2010). Another fungal immunomodulatory protein (ACA) obtained from the mycelium extract of *A. camphorata* was demonstrated to activate macrophages via the TLR2/MyD88 dependent signaling pathway which results in the activation of the NF- $\kappa$ B gene expression, leading to the secretion of proinflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  together with a variety of other cytokines and chemokines (Sheu *et al.*, 2009). In addition, lectin (VVL) isolated from mushroom, *Volvariella volvacea* (Bul.) Singer potently activated T-lymphocytes compared to well-known immunomodulator concanavalin A (Con A) at the same dosage, and the lymphocyte proliferation was activated with the rapid expression of CD25, CD69, IL-2, NFAT, and PCNA proteins along with the secretion of IFN- $\gamma$  and IL-2 (Sze *et al.*, 2004).

#### **Small Molecules:**

Although there have been reports on mushroom small molecules, such as terpenes and terpenoids having immunomodulatory activities (El Enshasy & Hatti-Kaul, 2013), to the best of my knowledge, there are currently no publications specifying the immuno-stimulatory effect from mushroom small molecules. Hence, there should be efforts not only to search for potent immuno-stimulatory polysaccharides and polysaccharide-protein complexes, but also to screen for novel small molecules that could possibly stimulate the immune system.

In spite of the significant number of publications reporting the immuno-stimulatory effects from mushrooms through cytokine and immune cells regulation, when linked with cancer however, the secretion of proinflammatory cytokines have the tendency to cause undesired inflammatory responses associated with fever symptoms (Guggenheim *et al.*, 2014). Behavioral changes like anxiety, depression, fatigue, labile anger, and psychomotor slowing have also been observed with the use of certain cytokines in other disease treatment (Duivis *et al.*, 2013; Lotrich *et al.*, 2010;

Miller, 2009; Moreira *et al.*, 2015). With all the side effects taken into account, the use of immunostimulatory compounds for cancer treatment might not seem to be ideal. Nevertheless, the high potential of immuno-stimulatory compounds clearly outweighs the minor drawbacks that could arise, and therefore is still a promising approach in the treatment of cancer.

#### 1.6 The Structure and Anti-Cancer Properties of Polysaccharides and Polysaccharide-Protein Complexes

Polysaccharides are macromolecules with large structural diversity as compared to nucleic acids and proteins: the monosaccharide unit in polysaccharides can be linked to one another at numerous points, forming multiple branches as well as linear chains, whereas there is only a oneway linkage systems for nucleotides (sugar phosphate backbone) and amino acids (N- and Cterminus) (Sharon & Lis, 1993). Moreover, the ability of polysaccharides to form secondary structures based on size, sugar residue conformation and inter- and intra-chain hydrogen bonding provides the molecule with a much require plasticity for specific cell regulatory mechanisms (Ooi & Liu, 2000). Today, immune-modulating polysaccharides and polysaccharide-protein complexes in particular, are often the center of attention for scientists due to their effectiveness and low cytotoxicity (Liu et al., 2016). Although the exact mechanism whereby polysaccharides or polysaccharide-protein complexes act against tumors is still not clear, these large molecules have been demonstrated to be biological response modifiers that acts to improve and restore the immune system (Ooi & Liu, 2000). The potency of these immuno-modulatory molecules is often associated with (i) the molecular weight of the molecule, with the larger one generally being more potent than smaller ones, (ii) the degree of branching, with high linearity and little branching generally being more biological active, and (iii) the helical conformation, with single and triple helices being the most commonly reported polysaccharide structure with immuno-modulatory activities (Meng et al., 2016; Ooi & Liu, 2000).

#### 1.7 The Promising Potential of Natural Products in Drug Discovery

Natural products have long been recognized for their traditional role in many drug discoveries and were the foundation of many early medicines (Butler, 2005). However, around two decades ago, there was a massive decline in interest towards the use of natural products as a source for drug discovery by pharmaceutical companies, with many redirecting their attention towards synthetic chemical libraries. This was due to (i) challenges faced in developing and maintaining a high quality natural product library, (ii) the labor intensive and time consuming downstream process involved in the large-scale production of natural products, (iii) the high risk of rediscovering already discovered compound, resulting in replication of data in the database which can be problematic to remove, and (iv) challenges faced during compound modification (designing of second generation molecules) due to the high degree of stereochemistry natural products possess (Lam, 2007).

Interestingly, despite the large commercially available chemical space of synthetic chemical libraries compared to that of the natural product, the use of the former proved to be disappointing, hence highlighting the importance of biologically relevant chemical space compared to library size. With the current advancement and development of modern technology such as improvised functional assays, phenotypic screening processes, and the application of genomics, proteomics and metabolomics into natural product screening, together with the enormous diversity in natural products, it is expected that pharmaceutical companies will turn their focus back to natural products (Harvey *et al.*, 2015). Furthermore, natural products hold numerous advantages which are thought to contribute strongly to the drug discovery industry. It is important to note that natural products exhibit a high degree of stereochemistry and an extensive array of pharmacophores (bioactive molecules with their atoms or chemical groups arranged spatially),

which helps improve screening hits even with the more challenging screening targets, such as protein-protein interactions. Moreover, the fact that natural products are derived from natural origin increases their bioavailability, which is crucial when applied in functional assays such as cell-based and animal model testing. Ultimately, the enormous biodiversity of both eukaryotes and prokaryotes makes the natural product drug discovery research area relatively untapped, branding this field very promising in conjunction with the recent advancement and development of technological tools for screening (Harvey *et al.*, 2015).

Today, natural products are the largest single source of modern anti-cancer agents, with almost 50% of the natural anti-cancer drugs discovered between the years of 1940 and 2010 in Japan and Western countries (Gurnani *et al.*, 2014). Such an accomplishment further clarifies the rationale for using natural products as a hub for the discovery of more novel anti-cancer drugs. However, it is also important to note that natural products too come with some disadvantages, namely (i) the belief that the process of developing natural products usually lacks proper standardization and regulation, (ii) the complexity of the drug discovery process due to irreproducibility, inconsistent supply of material, presence of complex mixtures and cost, and (iii) the ideology that natural products lack pharmacological efficacy (Carmona & Pereira, 2013).

#### **1.8** Research Objectives

At the outset of this research, Dr. Lee's lab has shown that the water extract from BC mushroom *Royoporus badius* has strong immuno-stimulatory activity. The research objectives of this MSc thesis are: (i) to purify the immuno-stimulatory compound(s) from the water extracts of *R. badius*, and (ii) to biologically and chemically characterize the purified immuno-stimulatory compound(s) of *R. badius*.

#### Chapter 2

# Purification of an Immuno-Stimulatory Compound from the Water Extract of *Royoporus badius* (Pers.) A.B. De

Three collections of fresh mushroom specimens of *R. badius* were made by Dr. Keith Egger in the summers of 2016, 2017 and 2018 on rotting wood located in Miworth, BC, Canada and were assigned as #86, #150 and #152 respectively. Mushroom #86 was used as the starting material in the year of 2017 while mushroom #150 was used in the year of 2018 (after mushroom #86 was depleted). Mushroom #152 was also used in 2018. According to the literature, there have been no known immuno-stimulatory compounds identified from *R. badius*. Thus far, only two studies have been performed on the ethanol extracts of *R. badius*, with one exhibiting mild cytotoxic activity against colorectal cancer cell lines (Doskocil *et al.*, 2016), while the other was shown to inhibit the production of proinflammatory cytokines but elicited  $T_H2$  response (Koch *et al.*, 2002). Despite showing the presence of bioactivity in the ethanol extract of *R. badius*, no efforts were done to further purify the bioactive compound.

Several purification techniques were required to separate the active compound from the other inactive compounds. The purification processes were optimized in an attempt to obtain a sufficiently pure active compound. The main focus of this chapter will be the description of protocols established in purifying the immuno-stimulatory compound from the water extract of *R*. *badius*.

#### <u>Methodology</u>

#### 2.1 Identification, Chemical Extraction and Immuno-Stimulatory Activity of *R. badius*.

#### 2.1.1 Genetic Identification of Mushroom #86 and Mushroom #150 as *R. badius*

Fruiting bodies collected were dried in a conventional oven at 55°C for a week until the specimens were completely dried. A small voucher specimen was kept in the UNBC herbarium

for future reference, another small portion of the dried fruiting body was then dissected for genomic DNA (gDNA) identification purposes while the remaining dried mushroom samples were pulverized into powder using a food blender. The powder of the mushroom samples was then stored in a Ziploc<sup>®</sup> bag at room temperature for chemical extraction purposes.

The gDNA of both mushrooms was extracted, amplified, and purified by Dr. Lee and his undergraduate students. The ITS 2 region was used for DNA sequencing analysis, with ITS3 and NLB4 being the primers used for DNA amplification. Polymerase Chain Reaction (PCR) conditions are as shown in **Table 2**. The refined PCR products were then sent for DNA sequencing and the DNA sequences were analyzed followed by comparison using the Basic Local Alignment Search Tool (BLAST). A nucleotide BLAST of the mushroom DNA sequences was used to infer the identity of the mushroom analyzed.

Temperature	Time	Cycle
95°C	5 minutes	1x
95°C	30 seconds	29x
52°C	30 seconds	29x
72°C	30 seconds	29x
72°C	10 minutes	1x
4°C	Until Collected	1x

Table 2. PCR conditions for DNA amplification.

#### 2.1.2 Chemical Extraction of *R. badius* using the DIONEX ASE 50 Speed Extractor

The chemical extraction protocol has been established in the Dr. Chow Lee's lab, namely the pulverized dried mushroom powder was subjected to sequential chemical extraction using 4 different solvents. Samples were subjected to extraction with 80% ethanol followed by 50% methanol which were expected to isolate any low molecular weight compounds. After that, the remains were extracted using water and 5% sodium hydroxide, whereby the water extract was expected to contain water-soluble polysaccharides, while the alkaline extract was expected to contain water-insoluble polysaccharides. All solvent extractions were performed using the Dionex ASE 350 Speed Extractor (Thermo Fisher) at 65°C and at a pressure of 1500 psi except for the sodium hydroxide extract, which was done manually at 65°C as it will corrode the walls of the machine. The outline of the extraction process using the Speed Extractor was as follow:

<b>Dionex ASE 350 Extraction Process</b>	<b>Duration</b>
Static extraction with rinse (80% ethanol)	4 cycles, 15 minutes per cycle
Static extraction with rinse (50% methanol)	4 cycles, 15 minutes per cycle
Static extraction with rinse (water)	4 cycles, 15 minutes per cycle
Final rinse (water)	1 cycle for 90 seconds
	-

<u>Manual Extraction</u> 5% sodium hydroxide extraction **Duration** 6 hours with constant stirring

Mushroom extracts were labeled as E1, E2, E3 and E4 for ethanol, methanol, water, and sodium hydroxide extracts respectively. All extracts were concentrated using the roto-evaporator and pH neutralized prior to lyophilization using FREEZONE freeze dryer (Labconco). Freezedried samples were then stored at 4°C for subsequent experiments. For long term storage, dried samples were stored at -80°C. **Figure 1** outlines the chemical extraction and the downstream steps taken.



Figure 1. Sequential chemical extraction *R. badius* (collections #86 and #150)

### 2.1.3 Assessing the extracts of *R. badius* for Immuno-Stimulatory Activity using Murine Macrophage (RAW 264.7) Cells

In order to assess the immuno-stimulatory activity of the extracts from mushrooms #86 and #150, a stock of 20 mg/mL from each extract was prepared by resuspending 20 mg of crude extract powder in 1 mL of MilliQ water followed by filter sterilization using a 0.22 µm filter (Sarstedt). The filter sterilized extracts were then diluted to 2 mg/mL extract using BioWhitaker<sup>®</sup> Dulbecco's Modified Eagle's Medium (DMEM) without Fetal Bovine Serum (FBS).

Murine macrophage (RAW 264.7) cell line was obtained from American Type Culture Collection (ATCC) and cultured using BioWhitaker<sup>®</sup> Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) until 70-80% confluent prior to trypsinizing adhering cells with Life Technology TrypLE<sup>TM</sup> Express (Thermo Fisher) for 5-10 min. Trypsinized cells were then diluted using BioWhitaker<sup>®</sup> Dulbecco's Modified Eagle's Medium (DMEM) without FBS prior to counting the cell density. A total of 200 µL containing 100,000 cells in DMEM without FBS were seeded in 96-well plate and incubated at 37°C and 5% carbon dioxide (CO<sub>2</sub>) overnight.

After the overnight incubation, the medium was removed from the wells and the cells were washed with 200  $\mu$ L of BioWhitaker<sup>®</sup> Dulbecco's Phosphate Buffer Saline (PBS). The buffer solution was removed and 100  $\mu$ L of DMEM without FBS was pipetted into each well. The cells were treated with 100  $\mu$ L of the crude extracts prepared, with a final well concentration of 1 mg/mL. Controls used in the assay were lipopolysaccharide (LPS) resuspended in DMEM without FBS with a final concentration of 500 ng/mL, along with water and DMEM without FBS, all with a treatment volume of 100  $\mu$ L. The RAW 264.7 cells were left to incubate at 37°C and 5% CO<sub>2</sub> for 6 hours prior to collecting the supernatant. Cell morphology was observed prior to collecting the supernatant was observed prior to collecting the supernatant was then stored at -80°C.

ELISA was performed using the BD OptEIA<sup>TM</sup> Mouse TNF (Mono/Mono) ELISA Set A and Reagent Set B (BD Bioscience). The assay was conducted in accordance to the manufacturer's protocol and using a 96-well Microtiter<sup>TM</sup> polystyrene plates (Thermo Fisher). In short, the microwells were first coated with 100  $\mu$ L of Capture Antibody diluted in Coating Buffer (1:250 dilution) and incubated at 4°C overnight. During the day in which the assay was performed, each well was then washed 3 times with 300  $\mu$ L of 1x wash buffer and blotted on paper towel till dry. The plates were then block with 200  $\mu$ L of assay diluent, sealed and incubated at room temperature for 1 hour. Following that, the plates were again washed 3 times with 300  $\mu$ L of 1x Wash Buffer and blotted on paper towel till dry. The empty wells were then loaded with 100  $\mu$ L of the Recombinant Mouse TNF- $\alpha$  standards resuspended in Assay Diluent (0, 15.6, 31.3, 62.5, 125, 250, 500, 1000 pg/mL) and the supernatant (diluted 10x or 20x with assay diluent) collected from the RAW 264.7 cells treated with mushroom samples. The plate was then sealed and left to incubate at room temperature. After 2 hours, the plates were washed with 5 times with 300  $\mu$ L of 1x Wash Buffer and blotted on paper towel till dry, prior to adding 100  $\mu$ L of working detector, a mixture of Detection Antibody and Streptavidin-Horse Radish Peroxidase reagent diluted in Assay Diluent (1:250 dilution). The plates were then sealed and left to incubate for another hour at room temperature. After one hour, the plates were then washed 7 times with 300  $\mu$ L of 1x Wash Buffer and blotted on paper towel till dry, followed by the addition of 100  $\mu$ L of Substrate Solution, an equal mixture of Substrate Reagent A and Substrate Reagent B. The plates were then incubated in the dark without the sealer for 30 min prior to stopping the reaction with 50  $\mu$ L of Stop Solution into each well. Upon completion of the assay, the microplate was subjected to absorbance reading at 450 nm and 570 nm using SYNERGY 2 (Biotek®) and the wavelength correction was performed by subtracting the absorbance at 570 nm from the absorbance obtained from 450 nm.

## 2.2 Purification of E3 from *R. badius* using Low Resolution Size-Exclusion Chromatography

## 2.2.1 Size-Exclusion Chromatography with a Mini-Drip Column using Sephadex<sup>TM</sup> LH-20 Matrix

The initial choice of matrix to perform the size-exclusion chromatography was Sephadex<sup>TM</sup>

LH-20 (GE Healthcare). A total of 6.5 g of dry Sephadex<sup>™</sup> LH-20 powder swelled in 50 mL of degassed MilliQ water for 2 days to yield approximately 20 mL bed volume (BV) resin. On the second day, excess water was discarded to make a slurry with a resin to medium ratio of 3:1. To construct a 25 mL size column, a 25 mL serological pipette (Sarstedt) was used as the column. The top tip of the 25 mL serological pipette was removed using a pair of scissors and the bottom tip of the 25 mL serological pipette was stuffed with some cotton wool, acting as a filter to prevent the resin from leaking out. The swollen resin was then stirred gently using a spatula to ensure a homogenous mixing with its media and then transferred to the self-constructed column. Upon

loading of the resin into the column, using a Pasteur pipette, the column was packed and equilibrated with 3 BV of degassed MilliQ water. Once the column was successfully packed, a stopper was inserted to the bottom tip of the column to stop the flow of the mobile phase.

A total of 20 mg of E3 from mushroom #86 was measured and resuspended in 1 mL of MilliQ water to make up a 20 mg/mL extract solution. The sample was then filter sterilized using a 0.22  $\mu$ m filter (Sarstedt) to remove any insoluble particulates from the sample. The loading volume was set at 2% of the total bed volume, hence only 500  $\mu$ L of the prepared sample was loaded manually using a Pasteur pipette into the column with the bottom tip closed with a stopper. Once the sample was loaded onto the column, the stopper was removed, MilliQ water was run continuously using a Pasteur pipette into the column, and a total of 26 fractions each consisting of 1 mL volume were collected. The collected fractions were then stored in 4°C until ready to be assessed for their immuno-stimulatory activity.

## 2.2.2 Size-Exclusion Chromatography with a C26/100 Column using Sephadex<sup>TM</sup> LH-20 Matrix

To increase the yield and resolution of the sample separation using Sephadex<sup>TM</sup> LH-20, the column size was scaled up to approximately 400 mL BV of matrix. To achieve such a volume, 100 g of dry Sephadex<sup>TM</sup> LH-20 powder swelled in 1000 mL of degassed MilliQ water for 2 days to ensure complete swelling of the resin. After 48 hours, excess water was discarded to make a slurry with a resin to medium ratio of 3:1. The swollen resin was then stirred gently using a spatula to ensure a homogenous mixing with its media and then transferred to a C26/100 column (GE Healthcare). The matrix was then equilibrated and packed with 2-3 BV of MilliQ water with the aid of a P-50 pump (Pharmacia Biotech). Following that, 200 mg of sample was measured and dissolved in 8 mL of MilliQ water and filtered sterilized using a 0.22 µm filter (Sarstedt) to remove any insoluble particulates. A total of 2% loading volume (8 mL) was manually loaded into the

packed column and eluted with MilliQ water at a flow rate of 1 mL/min which was maintained by the P-50 pump (Pharmacia Biotech).

A total of 50 fractions, each consisting of 10 mL eluted volume was collected using a RediFrac fraction collector (Pharmacia Biotech). The collected fractions were then stored in 4°C until ready to be assessed for their immuno-stimulatory activity.

#### 2.2.3 Assessing the Post LH-20 Fractions for Bioactivity

The collected post LH-20 fractions were used to treat RAW 264.7 cells and the supernatants collected were used in ELISA to quantify the amount of TNF- $\alpha$  produced by the cells. The cell treatment and ELISA protocols are as described previously in section 2.1.3, with modifications to the volume of the sample treated on cells. In general, 100 µL of the samples collected from the fraction collector were used to treat the RAW 264.7 cells. However, should the sample result in cell killing, the assay was repeated by lowering the volume used for cell treatment. This is because the concentration of each fraction collected is unknown. Fractions that exert significant immuno-stimulatory activity were pooled and lyophilized and named BRLI\_86\_E3\_LH20.

#### 2.3 Purification of E3 from *R. badius* using Anion-Exchange Chromatography

## 2.3.1 Determining a Suitable Buffer System for the Anion-Exchange Chromatography with a Poly-Prep® Drip Column using DEAE Sephadex<sup>TM</sup> A-50 Matrix

The choice of matrix to perform the anion-exchange chromatography was the weak anion exchanger, DEAE Sephadex<sup>TM</sup> A-50 (Sigma Aldrich), with an approximate 200 kDa exclusion limit. To determine a suitable buffer system, a range of amine-based buffers were selected as in **Table 3**. All buffers were classified into two categories, namely the running buffer (20 mM buffer) and the elution buffer (1 M NaCl in 20 mM buffer), with the pH all being adjusted to its  $pK_a \pm 0.05$ .
<b>Buffer Substance</b>	pH Interval (pK <sub>a</sub> at 25°C)	pKa at 25°C
Piperazine (PPZ)	4.8-5.8	5.33
Bis Tris	6.0-7.0	6.48
Water	N/A	7.00
Triethanolamine (TETHN)	7.3-8.3	7.76
Propane-1,3-diamino (PDA)	8.4-9.4	8.88
Ethanolamine (ETHN)	9.0-10.0	9.50

**Table 3.** List of buffers selected for an ion-exchange chromatography.

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To determine a suitable buffer system, 150 mg of dry DEAE Sephadex<sup>TM</sup> A-50 powder was measured in a 15 mL Falcon tube (Sarstedt) and 13 mL of the 20 mM running buffer was added to it. The mixture was then gently swirled to ensure uniform mixing and left to swell for 2 days. After 48 hours, excess buffer was removed from each Falcon tube, leaving a matrix to buffer ratio of 3:1. The resulting solution was then gently swirled to form a slurry and was then transferred using a serological pipette into the Poly-Prep® column with a stopper at the bottom of the column.

In order to pack and equilibrate the column, the stopper was unplugged and 3-4 BV of 20 mM running buffer was pipetted manually to the column using a Pasteur pipette. The pH of the eluted buffer should be in the range of  $6.50 \pm 0.1$  before proceeding to sample application. Upon successful packing of the column, the stopper was plugged in to stop the flow of the buffer. A total of 2.5 mg (250 µL of 25 mg/mL) of BRLI\_86\_E3\_LH20 was loaded to the column. Once the samples have been loaded, the stopper was removed and the column was flushed with 2-3 BV of running buffer and the eluted solution was collected and named "Flow Through". Once the Flow Through has been collected, the column was then flushed with 2-3 BV of elution buffer, and the eluted solution collected was named "Elution". The collected flow through and elution from each respective buffer were then dialyzed using 3500 MWCO SnakeSkin<sup>TM</sup> Dialysis Tubing (Thermo Fisher) to remove the buffer and salt, prior to slant freezing and lyophilization.

Due to the immeasurable mass of the product yield from both the flow through and elution, the samples were simply reconstituted in 1 mL of MilliQ water, filtered sterilized and treated on cells. The cell treatment and ELISA protocols are as described previously in section 2.1.3, with modifications to the volume of the sample treated on cells. In general, 100  $\mu$ L of the samples were used to treat the RAW 264.7 cells. However, for any sample that resulted in cell killing, the assay was repeated by lowering the volume used for cell treatment. This was because the concentration of each fraction collected was unknown. Generally, a buffer system was considered to be suitable for purifying the active compound if the flow through did not exhibit significant immunostimulatory activity while the elution did.

# 2.3.2 Optimization of the Anion-Exchange Chromatography with an Intermediate-Sized XK26/40 Column using DEAE Sephadex<sup>TM</sup> A-50 Matrix

#### 2.3.2.1 Loading of Post LH20 Samples from Mushroom #86

With the successful establishment of a suitable buffer system (Bis Tris, pH 6.5) to perform the anion-exchange chromatography, the purification step was extended to an intermediate scale for optimization purposes. To achieve an approximate 75 mL BV matrix, 1.5 mg of dry DEAE Sephadex<sup>TM</sup> A-50 powder was measured and swelled in 150 mL of 20 mM BisTris running buffer for 2 days. Once the swelling process was completed, excess buffer was discarded to achieve a matrix to medium ratio of 3:1. A slurry was formed by gently mixing the swollen matrix with a spatula and transferred to the intermediate-sized XK26/40 column (GE Healthcare). The column was packed and equilibrated with 3-4 BV of running buffer at 1.5 mL/min which was maintained by the P-50 pump (Pharmacia Biotech). The pH of the eluted buffer should be in the range of 6.50  $\pm 0.1$  before proceeding to sample application.

Sample (BRLI\_86\_E3\_LH20) with different amounts (20 mg and 30 mg) were used to investigate the binding capacity of DEAE Sephadex<sup>TM</sup> A-50 matrix. As previously described in

section 2.3.1, the column was then flushed with 2-3 BV of running buffer, but with a flow rate of 1.0 mL/min, and the flow through was collected. Following was the eluting of the column with 2-3 BV of elution buffer, also with a flow rate of 1.0 mL/min, and the elution was collected. Both the flow through and elution collected were then subjected to rota-evaporation to reduce the volume prior to dialyzing using 3500 MWCO SnakeSkin<sup>TM</sup> Dialysis Tubing (Thermo Fisher) to remove the buffer and salt. The dialyzed products were then slant frozen and lyophilized. The active product from this purification step was named BRLI\_86\_E3\_LH20\_DEAE.

#### 2.3.2.2 Loading of Crude E3 Samples from Mushroom #150

With the depletion of mushroom #86 and also the inefficiency of the Sephadex<sup>TM</sup> LH-20 as a first step for purification, it was decided that using DEAE Sephadex<sup>TM</sup> A-50 (GE Healthcare) for anion-exchange chromatography will act as the first purification step for mushroom #150. The protocol was as previously described in section 2.3.2.1. In order to investigate the binding capacity of the matrix, the sample amount loaded was a trial of 40 mg, 60 mg and 100 mg.

# 2.3.2.3 Assessing the Binding Capacity of the DEAE Sephadex<sup>TM</sup> A-50 Matrix and the Specific Activity of BRLI\_#86\_E3\_LH20\_DEAE

A total of 2 mg of the dried flow through and elution from both mushroom #86 and #150 were weighed using an analytical microbalance and reconstituted in 1 mL of MilliQ water and 150 mM NaCl respectively, followed by filter sterilization using a 0.22 µm filter (Sarstedt). A dose-dependent assay with concentrations of 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/mL was performed to assess the binding capacity of the matrix used. Crude E3 of both mushroom #86 and #150 with similar concentrations were also compared. The cell treatment and ELISA protocols are as described previously in section 2.1.3, with modifications to the volume of DMEM without FBS in each well due to the different concentrations applied. Generally, if the flow through did not exhibit significant immuno-stimulatory activity; while the elution did, it can be concluded that the

column was not oversaturated with the amount of sample loaded. This is because excess samples loaded would have been eluted off the column into the flow through, causing it to be immuno-stimulatory,

For the specific activity assay, should the amount of TNF- $\alpha$  produced by the RAW 264.7 treated with BRLI\_#86\_E3\_LH20\_DEAE be equal or higher than that of BRLI\_#86\_E3\_LH20, then it was deemed that the purification process had taken place.

# 2.3.3 Anion-Exchange Chromatography with a Large-Sized XK50/100 Column using DEAE Sephadex<sup>TM</sup> A-50 Matrix

Upon successful optimization of the binding capacity of the matrix, the purification step was scaled up to a large XK50/100 column, with an approximate BV of 750 mL. To achieve the BV, 13 g of dry DEAE Sephadex<sup>TM</sup> A-50 powder was measured and mixed with 1 L of 20 mM Bis Tris running buffer. The resin was allowed to swell in the buffer for 2 days prior to column packing. Once the resin has completely swollen, the resin was gently mixed with a spatula to form a slurry and loaded into the XK50/100 column. The column was packed by pumping 3-4 BV of 20 mM Bis Tris running buffer at a flow rate of 1.5 mL/min. The pH of the eluted buffer should be in the range of  $6.50 \pm 0.1$  before proceeding to sample application.

A total of 860 g of crude sample E3 of mushroom #150 was loaded manually to the column and flushed with 2-3 BV of running buffer with a flow rate of 1.0 mL/min. The flow through was then discarded. The elution process was then resumed by eluting of the column with 2-3 BV of elution buffer (1.0 M NaCl in 20 mM Bis Tris), also with a flow rate of 1.0 mL/min, and the elution was collected. The elution collected was then subjected to rota-evaporation to reduce the volume prior to dialyzing using 3500 MWCO SnakeSkin<sup>TM</sup> Dialysis Tubing (Thermo Fisher) to remove the buffer and salt. The dialyzed products were then slant frozen and lyophilized. The active product was named BRLI\_#150\_E3\_DEAE.

# 2.4 Purification of the Post DEAE Active Fractions from E3 of *R. badius* using High Resolution Size-Exclusion Chromatography

# 2.4.1 Hi-Prep Sephacryl S500 Equipped with ÄKTA Pure Fast Protein Liquid Chromatography (FPLC) System

The pre-packed Hi-Prep Sephacryl S500 (GE Healthcare) was used as the final step to purify BRLI\_#86\_E3\_LH20\_DEAE and BRLI\_#150\_E3\_DEAE. The column was equilibrated with 2-3 column volumes (CV) of 150 mM NaCl solution at a flow rate of 1.3 mL/min prior to loading the sample. Sample amount ranging from 25 to 115 mg reconstituted in 2 mL of 150 mM NaCl were filter sterilized and loaded into the column. The sample was eluted for 1.5 CV at a flow rate of 1.3 mL/min and a total of 50 fractions, each consisting of 10 mL was collected using the F9-R fraction collector (GE Healthcare). The column was then washed with 1.5 CV of 150 mM NaCl before the next sample application. From time to time, the column was subjected to regular cleaning with 0.5 CV of 0.2 M NaOH solution followed immediately by 2 CV of MilliQ water and equilibration with 3 CV of 150 mM NaCl prior to the next sample application, all with a flow rate of 1.3 mL/min.

#### 2.4.2 Assessing the Fractions Collected for Bioactivity

#### 2.4.2.1 Post LH20-DEAE-Sephacryl Fractions of Mushroom #86

All the fractions collected were assessed for immuno-stimulatory activity on RAW 264.7 cells. The supernatants collected after treatment on cells were used in ELISA to quantify the amount of TNF- $\alpha$  produced by the cells. The cell treatment and ELISA protocols are as previously described in section 2.1.3, with modifications to the volume of the sample treated on cells. In general, 100 µL of the samples collected from the fraction collector were used to treat the RAW 264.7 cells. However, should the sample result in cell killing, the assay was repeated by lowering

the volume used for cell treatment. This was because the concentration of each fraction collected was unknown. However, the samples were believed to be contaminated as there was significant detectable immuno-stimulatory activity present in all samples, especially the initial few fractions, and hence the sample was discarded. Details of the issue are discussed in section 2.11.

#### 2.4.2.2 Post DEAE-Sephacryl Fractions of Mushroom #150

Fractions were selected for their immuno-stimulatory activity. The selection was based on the chromatogram produced by the ÄKTA Pure FPLC system, where every subsequent fraction within the peak region were assessed, while only alternative fractions at the beginning or the end were tested. The cell treatment and ELISA protocols are as previously described in section 2.1.3, with modifications to the volume of the sample treated on cells. In general, 100 µL of the samples collected from the fraction collector were used to treat the RAW 264.7 cells. However, should the sample result in cell killing, the assay was repeated by lowering the volume used for cell treatment. This was because the concentration of each fraction collected was unknown. Fractions exerting strong immuno-stimulatory activity as compared to negative controls were pooled, dialyzed using 3500 MWCO SnakeSkin<sup>™</sup> Dialysis Tubing (Thermo Fisher) to remove the salt, slant frozen, and freeze dried. This purified sample was named BRLI\_#150\_E3\_DEAE\_Sephacryl.

# 2.4.3 Assessing the Specific Bioactivity of BRLI\_#150\_E3\_DEAE\_Sephacryl and BRLI\_#150\_E3

A total of 1 mg of the dried BRLI\_#150\_E3\_DEAE\_Sephacryl was weighed using a Cubis® Micro Balance (Sartorius) and reconstituted in 1 mL of MilliQ water and subjected to filter sterilization using a 0.22 µm filter (Sarstedt). A dose-dependent assay with concentrations of 0, 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 mg/mL was used to treat RAW 264.7 cells. Both BRLI\_#150\_E3\_DEAE\_Sephacryl and BRLI\_#150\_E3 were added to cells with a similar concentration. The cell treatment and ELISA protocols are as described previously in section 2.1.3,

with modifications to the volume of DMEM without FBS in each well due to the different concentrations applied. Should the amount of TNF- $\alpha$  produced by the RAW 264.7 treated with BRLI\_#150\_E3\_DEAE\_Sephacryl be equal or higher than that of BRLI\_#150\_E3, then it is a confirmation that the purification process had taken place.

# 2.5 Large Scale Production of Post DEAE-Sephacryl Active Fractions from E3 of Mushroom #152

#### 2.5.1 Bulk Extraction of Mushroom #152 by Manual Extraction

The third batch of *R. badius* (DNA confirmation pending) was collected in the summer of 2018 and was used to produce the material needed for further characterization studies. The amount of mushroom obtained from the third batch was approximately triple the amount of what was collected in both the summers of 2016 and 2017, and hence the use of the Speed Extractor was omitted, but rather a manual extraction was performed. A total of 350 g of pulverized dried powder of Mushroom #152 were subjected to sequential manual extraction, starting with 80% ethanol at 65°C for 3 hours. The ethanol extract (E1) was then filtered and the residue was subjected to manual extraction using 50% methanol at 65°C for 3 hours. The methanol extract (E2) was then filtered and the residue was subjected to manual extraction using water at 65°C for 6 hours. The hot water extract (E3) was then filtered and the residue was discarded. The alkaline extract (E4) was not performed. All three extracts were then subjected to rota-evaporation, slant freezing at -80°C followed by lyophilization. The dried powder of all three extracts, were assessed for their immuno-stimulatory activity to ensure reproducibility as of the previous batches (mushroom #86 and #150).

### 2.5.2 Large Scale Purification of E3 from Mushroom #152 using Anion-Exchange Chromatography with a Large-Sized XK50/100 Column using DEAE Sephadex<sup>TM</sup> A-50 Matrix

The purification protocol was as described in section 2.3.3, but with an increase in loading

amount from 860 mg of crude E3 powder to 1 g of crude E3 powder. A total of 6 column runs

were performed, each with a loading amount of 1 g of crude E3 powder from Mushroom #152.

### 2.5.3 Large Scale Purification of Post DEAE Active Fractions from Mushroom #152 using Hi-Prep Sephacryl S500 Equipped with ÄKTA Pure Fast Protein Liquid Chromatography (FPLC) System

The purification protocol was as described in section 2.4.1. A total of 12 column runs were

performed, each with a loading amount of approximately 120 mg of Post DEAE active fraction

powder from E3 of Mushroom #152. The product was named BRLI\_#152\_E3\_DEAE\_Sephacryl.

#### 2.6 Statistical Analysis

Statistical analysis using One Way ANOVA (Tukey Test) or Student t-Test was performed

in Graph Prism Version 6.0, whereby the data shown are expressed in mean  $\pm$  standard deviation

(SD).

### **Results and Discussion**

### 2.7 Genetic Identity of Mushroom #86 and Mushroom #150

Both mushroom collections #86 and #150 were made by Dr. Keith Egger at Miworth, BC, Canada during the summer of 2016 and 2017 respectively. Based on the method described in section 2.1, the mushrooms were genetically identified as *Royoporus badius* (**Table 4**). The consensus sequence has 98-100% identity to voucher specimens identified as *Royoporus badius* (Pers.) A.B. De or one of its synonyms (e.g. *Polyporus badius* (Pers.) Schwein.) Recently a new name has been proposed, *Picipes badius* (Pers.) Zmitr. & Kovalenko (Zmitrovich & Kovalenko, 2016), but it has not yet been widely accepted so I retained the commonly used name *Royoporus badius*. **Figures 2** and **3** show photos of the mushrooms collected.

Mushroom	Mushroom	Date	Location in	Best GenBank Match
Number	Identity	Collected	BC	(%Similarity/%Coverage)
#86	Royoporus badius	August 2016	Miworth	KM411465.1 (98%/87%)
#150	Royoporus badius	June 2017	Miworth	KM411465.1 (98%/87%)

Table 4. Summary of mushroom #86 and #150 collected in BC, Canada.

### Consensus sequence (CL86 and CL150):



Figure 2. Fresh samples of mushroom #86 collected in Miworth, BC, Canada.



Figure 3. Oven-dried samples of mushroom #150 collected in Miworth, BC, Canada.

### 2.8 Immuno-Stimulatory Activity of Crude Extracts from Mushroom #86 and Mushroom #150

All mushroom samples collected were oven-dried, pulverized to powder and subjected to chemical extraction using the Dionex ASE 350 Speed Extractor (Thermo Fisher) as described in section 2.1.2. **Tables 5** and **6** provide the percentage yield obtained post extraction of mushroom #86 and mushroom #150 respectively. A total of 97.617 g of dried mushroom #86 powder was used for chemical extraction, while 123.657 g of dried mushroom #150 powder was used for the extraction process.

Extract	Mass Yield (g)	Percentage Yield (%)
E1	1.523	1.56
E2	2.997	3.07
E3	2.000	2.05
E4	ND*	ND*

 Table 5. Percentage yield from the sequential extraction of mushroom #86.

\* Due to low immuno-stimulatory activity, bulk extraction of E4 was omitted and hence the yield was not calculated.

Table 6. Percentage yield from the sequential extraction of mushroom #150.

Extract	Mass Yield (g)	Percentage Yield (%)
E1	2.264	1.83
E2	3.442	2.78
E3	3.568	2.89
E4	ND*	ND*

\* Due to low immuno-stimulatory activity, bulk extraction of E4 was omitted and hence the yield was not calculated.

The dried extracts from the two respective mushroom samples were then added to RAW 264.7 cells and their immuno-stimulatory activity assessed as described in section 2.1.3. LPS was used as the positive control, while water and media (DMEM without FBS) was used as the negative control. All extracts treated to RAW 264.7 cells were at a final concentration of 1 mg/mL and all extracts were reconstituted in water. **Figures 4a** and **4b** show the immuno-stimulatory activity profile of mushroom #86 and mushroom #150 respectively.



**Figure 4. (a)** The immuno-stimulatory activity profile of the extracts E1, E2, E3 and E4 (1 mg/mL) of mushroom #86 treated on RAW 264.7 cells. LPS (500 ng/mL) was used as the positive control while water and DMEM without FBS were used as negative controls; **(b)** The immuno-stimulatory activity profile of the extracts E1, E2, and E3 (1 mg/mL) of mushroom #150 treated on RAW 264.7 cells. LPS (500 ng/mL) was used as the positive control while water and DMEM without FBS were used as the positive control while water and DMEM without FBS were used as negative control. Data presented are representatives from two separate experiments. Error bars indicated the SD from the mean. Statistical analysis was assessed using One-Way ANOVA (Tukey Test) with \*\*\* (P < 0.05) compared to negative control water.

Based on the data obtained from **Tables 5** and **6**, it was clear that the yield for the extracts were very low, ranging from 1.56% to 3.07%. In my thesis, the water extract (E3) was selected as the choice for further study. However, it is clear that the limitation of the study was due to the low availability of working material. Despite the low availability of the extract from *R. badius*, the water extract of the mushroom exhibited strong immuno-stimulatory activity as compared to that of the other extracts and negative controls. The activity profile (all E1 - E4) of mushroom #86 was initially screened for bioactivity by Mr. Jatinder Khatra. Upon depletion of the material from mushroom #86, the extracts of mushroom #150 were used, and hence a confirmation of the bioactivity was necessary. Both the data from **Figures 4a** and **4b** confirmed the presence of the immuno-stimulatory activity in the water extracts, E3 of *R. badius* collected in Miworth, BC.

### 2.9 Immuno-Stimulatory Activity of the Post LH20 Fractions of Mushroom #86

Upon confirming the presence of immuno-stimulatory activity in the water extract E3 of *R*. *badius*, the first approach was to estimate the relative molecular size of the bioactive compound by using a size separation technique. LH20 Sephadex<sup>TM</sup> was selected as the matrix for the size-exclusion chromatography. To start, a Mini-Drip column containing LH20 Sephadex<sup>TM</sup> was used with the procedures are as described in section 2.2.1. With the presence of immuno-stimulatory activity peaks obtained from the eluted sample of the Mini-Drip LH20 Sephadex<sup>TM</sup> column (data not shown), I further improved the separation resolution by upscaling the separation technique using a 400 mL LH20 Sephadex<sup>TM</sup> bed volume packed into a C26/100 column. The procedures are as described in section 2.2.2. Figure 5 illustrates the immuno-stimulatory profile of the fractions collected from a 400 mL C26/100 LH20 Sephadex<sup>TM</sup> column.



**Figure 5.** The immuno-stimulatory activity profile of the 400 mL C26/100 LH20 Sephadex<sup>TM</sup> fractions from E3 of mushroom #86. A total of 50 fractions was collected from the column and were used to treat RAW 264.7 cells to assess their bioactivity. Data shown is a representation of three separate experiments.

The LH20 Sephadex<sup>™</sup> matrix is a relatively crude separation matrix, with an exclusion limit of approximately 4-5 kDa. Based on the data from the mini-drip column, three immunostimulatory peaks were observed, starting within an elution volume of 9 mL, fraction 9 to 20 mL, fraction 20 (data not shown). Despite the interesting activity profile, no conclusion can be made as only a small volume (i.e. 20 mL BV) was used as the matrix volume and the entire purification process was based on the flow of gravity. In order to obtain a clearer picture, the separation process was scaled up to a 400 mL BV column together with a controlled flow rate. Interestingly, only one single activity peak was reproducibly observed, which was eluted at approximately between 130mL (fraction 13) to 170 mL (fraction 17) as depicted in **Figure 5**. Between the elution volume 200 mL and 240 mL, minor activity peaks were detected in some experimental replicates but not others (data not shown), and hence was omitted as it was suggested that the compound, if present was very unstable. The data suggests that the active compound is a large molecule due to its early elution volume.

#### 2.10 Immuno-Stimulatory Activity of the Post LH20-DEAE Fractions of Mushroom #86

Upon completion of the first step purification using LH20 Sephadex<sup>TM</sup>, the resulting active products were subjected to a second step purification with Anion-Exchange Chromatography using DEAE Sephadex<sup>TM</sup> A-50. Several buffers as previously mentioned in section 2.3.1 were used for the selection of the buffer suitable for the purification of the active compound of interest. All flow through and elution samples were dialyzed to remove the buffer and salt, followed by lyophilization and reconstituted in water prior to testing on RAW 264.7 cells. **Figure 6** shows the immuno-stimulatory activity profile of the Post LH20-DEAE fractions from E3 of Mushroom #86 eluted with different buffers in a Poly-Prep® Drip Column packed with DEAE Sephadex<sup>TM</sup> A-50 matrix.



**Figure 6.** Determination of a buffer suitable for use in DEAE Sephadex<sup>TM</sup> A-50 to purify the immuno-stimulatory compound from water extract, E3 of *R. badius*. The immuno-stimulatory activity profile of the flow through and elution of the Post LH20 active fractions on the Poly-Prep<sup>®</sup> drip DEAE Sephadex<sup>TM</sup> A-50 column. Samples were run with various buffer systems as shown. The data is a representation of one single experiment.

Based on the data obtained from **Figure 6**, it can be observed that the elution of all the six solvent systems showed significant immuno-stimulatory activity as compared to that of the flow through and the negative controls (water and media), with buffers Bis Tris, Triethanolamine (TETHN) and Ethanolamine (ETHN) showing higher activity. In order to confirm the reproducibility of the buffer systems in eluting out the active compound, the three aforementioned buffers were used in a separate experiment to purify the Post LH20 of E3 from mushroom #86. **Figure 7** shows the immuno-stimulatory activity profile of the Post LH20-DEAE fractions from E3 of Mushroom #86 eluted with Bis Tris, Triethanolamine and Ethanolamine in a Poly-Prep® Drip Column packed with DEAE Sephadex<sup>TM</sup> A-50 matrix.



**Figure 7.** Determination of a buffer suitable for use in DEAE Sephadex<sup>TM</sup> A-50 to purify the immuno-stimulatory compound from water extract, E3 of *R. badius*. The immuno-stimulatory activity profile of the flow through and elution of the Post LH20 active fractions on the Poly-Prep® drip DEAE Sephadex<sup>TM</sup> A-50 column. Samples were run with three buffer systems as shown. The data is a representation of two separate experiments.

Based on **Figure 7**, it was concluded that the three buffers, Bis Tris, Triethanolamine (TETHN) and Ethanolamine (EHTN) were all suitable for the purification of Post LH20 of E3 from mushroom #86. The buffer Bis Tris was selected as the buffer of choice as it takes a shorter amount of time to equilibrate the column due to its relatively lower pH (pKa) value compared to that of Triethanolamine and Ethanolamine. With the establishment of the optimum buffer system, I then proceeded with optimizing the binding capacity of the DEAE Sephadex<sup>TM</sup> A-50 matrix as described in section 2.3.2.1. Because the active compound had previously undergone a first step purification using LH20 Sephadex<sup>TM</sup>, it was believed that the loading capacity would have been lower due to a higher content of the actual active compound relative to the impurities per weight.

**Figures 8a** and **8b** demonstrates the immuno-stimulatory activity profile of the flow through, elution and preload (Post LH20 of E3 from mushroom #86) treated on RAW 264.7 cells in a dose-dependent manner based on different loading amounts into the DEAE intermediate column.



**Figure 8. (a)** The immuno-stimulatory activity profile of the flow through, elution and Post LH20 fractions from Mushroom #86. RAW 264.7 cells were treated in a dose-dependent manner as shown. The samples were from 30 mg of Post LH20 loaded onto the 75 mL intermediate DEAE column; (b) The immuno-stimulatory activity profile of the flow through, elution and Post LH20 fractions from Mushroom #86. The samples were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers from Mushroom #86. The samples were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate DEAE numbers were from 20 mg of Post LH20 loaded onto the 75 mL intermediate

The initial trial involved loading 30 mg of Post LH20 fractions of mushroom #86 onto the 75 mL DEAE column, which resulted in an increase in immuno-stimulatory activity of the flow through compared to the negative control, water (0 mg/mL) as depicted in **Figure 8a**. This suggested that the column was over-saturated by the loaded sample and hence a lower amount should be loaded in the future. In the second trial, a total of 20 mg of Post LH20 fractions of Mushroom #86 was loaded into the 75 mL DEAE column, which resulted in insignificant immuno-stimulatory in the flow through compared to the negative control, elution and preload as illustrated in **Figure 8b**. This confirms the suitable loading capacity and the amount (20 mg) was used for the subsequent purification of the Post LH20 fractions of mushroom #86. Based on **Figures 8a** 

and **8b**, the preload exhibited cytotoxicity to the RAW 264.7 cells and doses greater than 0.1 mg/mL, whereas the eluted fraction did not show any cytotoxicity at concentrations up to 0.25 mg/mL. Such an observation also suggests the removal of the cytotoxic impurities that may have been initially present in the Post LH20 fraction prior to DEAE purification, thus indicating the success in the purification step using DEAE Sephadex<sup>TM</sup> A-50.

# 2.11 Immuno-Stimulatory Activity of the Post LH20-DEAE-Sephacryl Fractions of Mushroom #86

Upon completion of the second step purification using DEAE Sephadex<sup>TM</sup> A50, the resulting active fraction, BRLI\_#86\_E3\_LH20\_DEAE was subjected to a third purification step using Hi-Prep Sephacryl S500 equipped with ÄKTA Pure FPLC system. **Figure 9** shows the immuno-stimulatory activity profile of the Post LH20-DEAE-Sephacryl fractions of E3 from mushroom #86 treated on RAW 264.7 cells.



**Figure 9.** The immuno-stimulatory activity profile of the fractions of Post LH20-DEAE-Sephacryl fractions of E3 from mushroom #86 treated on RAW 264.7 cells. Data presented is from one experiment.

Based on **Figure 9**, there was a significant TNF- $\alpha$  stimulation in RAW 264.7 cells treated with the all fractions collected from Hi-Prep Sephacryl S500 column relative to the negative control, 150 mM NaCl solution (data not showed), especially the first few fractions early in the elution volume. The activity detected is not likely to be from the active compound purified as the highest activity peak was detected in the first fraction, which is illogical due to the fact that the flow rate of the purification process was controlled and it will definitely require time for the samples to pass through the column. Hence, it was concluded that the samples were likely contaminated. Unfortunately, the source of the contamination was unknown. The sample loaded into this column was all of the remaining sample and hence no replicate could not be carried out.

#### 2.12 Immuno-Stimulatory Activity of the Post DEAE Fractions of Mushroom #150

With the depletion of material from mushroom #86, I resumed my studies with the water extracts, E3 from mushroom #150, whereby its immuno-stimulatory activity has been confirmed as demonstrated in Figure 4b. In order to have a more efficient purification process, the use of LH20 Sephadex<sup>TM</sup> was excluded, simply because the data obtained from previous LH20 Sephadex<sup>TM</sup> experiments suggested that the active compound is a mixture of carbohydrate (see section 3.14) and protein (data not shown), which are basically large molecules and are very likely to exceed the exclusion limit (4-5 kDa) of LH20 Sephadex<sup>TM</sup>. Hence, the crude E3 extracts were directly subjected to anion exchange chromatography purification using the DEAE Sephadex<sup>TM</sup> A-50. According to the data obtained in section 2.10, Bis Tris was a suitable buffer for the further purification of BRLI\_#86\_E3\_LH20 and the optimum loading capacity per 75 mL BV DEAE Sephadex<sup>TM</sup> A-50 was 20 mg. However, because the current sample, i.e. E3 from mushroom #150 had yet to undergo any purification step, it was thought that the loading capacity would be higher compared to that of BRLI #86 E3 LH20. As described in section 2.3.2.2, a total of 40, 60 and 100 mg of E3 from mushroom #150 were loaded on a 75 mL BV DEAE Sephadex<sup>TM</sup> A50 column to check for the loading capacity. Figure 10 shows the immuno-stimulatory activity profile of the flow through, elution and preload (E3) of mushroom #150 treated on RAW 264.7 cells in a dosedependent manner when 100 mg of E3 of mushroom #150 was loaded into the 75 mL intermediate DEAE column.



**Figure 10.** Immuno-stimulatory activity profile of the flow through, elution and preload treated on RAW 264.7 cells in a dose-dependent manner when 100 mg of E3 of mushroom #150 was loaded into the 75 mL intermediate DEAE column. The data presented is a representation of two separate experiments.

The data obtained from **Figure 10** shows that even when 100 mg of E3 of mushroom #150 was loaded into the 75 mL DEAE Sephadex<sup>TM</sup> column, the column matrix was not oversaturated, as depicted by the low immuno-stimulatory activity detected in the flow through. Hence, the large-scale purification of E3 of mushroom #150 was performed as described in section 2.3.3 to obtain the semi-purified BRLI #150 E3 DEAE.

# 2.13 Immuno-Stimulatory Activity of the Post DEAE-Sephacryl Fractions of Mushroom #150

Upon the completion of the first step purification of mushroom #150 using DEAE Sephadex<sup>TM</sup> A-50, the product BRLI\_#150\_E3\_DEAE was subjected to a second step purification using Hi-Prep Sephacryl S500 equipped with ÄKTA Pure FPLC system. The purification methods

are as described in section 2.4.1. **Figure 11** shows a representation of the immuno-stimulatory activity profile from the fractions collected from the Sephacryl S500 column.



**Figure 11.** Immuno-stimulatory activity profile of the Sephacryl S500 column eluted fractions. Data presented is a representation of five separate experiments.

The data obtained from Figure 11 is completely different from the data obtained in Figure 9 (suggested be contaminated). The immuno-stimulatory activity of to BRLI #86 E3 LH20 DEAE Sephacryl collected was detected in fractions of the early elution volume, whereas BRLI #150 E3 DEAE Sephacryl showed a peak activity at around an elution volume of 250 mL (fraction 28), and the activity was confirmed using 5 independent experiments (n=5). Hence, it can be concluded that BRLI #86 E3 LH20 DEAE Sephacryl was contaminated and was discarded. The purified BRLI #150 E3 DEAE Sephacryl was then used for subsequent characterization studies as will be discussed in Chapter 3.

# 2.14 Specific Bioactivity of the Purified Immuno-Stimulatory Compound from Mushroom #150

To determine whether the active compound was successfully purified, the specific immuno-stimulatory activity of the purified compound was compared to that of the less pure form, i.e. crude E3 and Post DEAE of mushroom #150. **Figure 12** shows the specific bioactivity of the three samples. Based on the data, it can be observed that at 0.4 mg/mL, the bioactivity of the Post DEAE-Sephacryl compound was more potent as compared to that of the Post DEAE and the crude E3, which indicates a successful purification of the bioactive compound of interest. Unfortunately, due to insufficient material from the crude E3 and semi-purified post DEAE fraction, the experiment was done only once.



**Figure 12.** Specific immuno-stimulatory activity profile of crude extract E3, Post DEAE and Post DEAE-Sephacryl of mushroom #150. Presented data is a representation of one single experiment.

#### 2.15 Reproducibility Check on the Extraction and Purification Product of Mushroom #152

All three extracts obtained from the manual extraction of mushroom #152 as described in section 2.5.1 were lyophilized into powder. **Table 7** shows the percentage yield obtained post extraction of mushroom #152, while **Table 8** outlines the percentage yield after each downstream process of mushroom #152. A total of 350 g of dried mushroom #152 was subjected to manual extraction, yielding a total of 16.368 g (4.68%), 17.314 g (4.95%), and 8.945 g (2.56%) of crude extracts E1, E2 and E3 respectively. From there, a total of 6.00 g of crude E3 was subjected to DEAE Sephadex<sup>TM</sup> purification, yielding 1.652 g (27.53%) of BRLI\_#152\_E3\_DEAE. Following that, a total of 1.595 g of BRLI\_#152\_E3\_DEAE was subjected to Sephacryl S500 column, yielding 206.6 mg (12.95%) of BRLI\_#152\_E3\_DEAE\_Sephacryl.

Extract	Mass Yield (g)	Percentage Yield (%)
E1	16.368	4.68
E2	17.314	4.95
E3	8.945	2.56
E4	ND*	ND*

**Table 7.** Percentage yield from the sequential extraction of mushroom #152.

\* Due to low immuno-stimulatory activity, bulk extraction of E4 was omitted and hence the yield was not calculated.

**Table 8.** Percentage yield from each downstream processing of mushroom #152.

Downstream Process	Starting Amount (g)	Percentage Yield (%)	Final Product
Manual Extraction	350	2.56	BRLI_#152_E3
DEAE Sephadex <sup>TM</sup>	6.00	27.53	BRLI_#152_E3_DEAE
Sephacryl S500	1.595	12.95	BRLI_#152_E3_DEAE_Sepahcryl

Based on **Table 7**, the percentage yield obtained for all the three extracts were less than 5%, which was similar to what was obtained for mushroom #86 and mushroom #152. Despite the low yield, the abundance in the starting material (350 g) compared to approximately 100 g each

from the previous two batches, should provide sufficient material for further downstream purification and characterization studies.

The immuno-stimulatory activity of all three crude extracts were assessed to ensure reproducibility prior to further downstream purification. **Figure 13** shows the immuno-stimulatory activity profile of mushroom #152. Based on **Figure 13**, it was concluded that the activity profile of E1, E2 and E3 of mushroom #152 was similar and reproducible compared to that of mushroom #86 and mushroom #150, with the water extract E3 being the most potent, followed by the methanol extract E2 and the ethanol extract E1.



**Figure 13.** The immuno-stimulatory activity profile of the extracts E1, E2, and E3 (0.5 mg/mL) of mushroom #152 treated on RAW 264.7 cells. LPS (500 ng/mL) was used as the positive control while water and DMEM without FBS were used as negative controls. Data presented is a representation of two separate experiments. Error bars indicated the SD from the mean. Statistical analysis was assessed using One-Way ANOVA (Tukey Test) with \*\*\* (P < 0.05) compared to negative control water.

With the confirmation of the presence of immuno-stimulatory activity in E3 of mushroom #152, the lyophilized powder of the extract was then subjected to large scale DEAE Sephadex<sup>TM</sup> purification. **Figure 14** shows the immuno-stimulatory activity profile of the flow through, elution and preload (E3) of mushroom #152 treated on RAW 264.7 cells in a dose-dependent manner when 1 g of E3 from mushroom #152 was loaded into the 750 mL large DEAE column.



**Figure 14.** Immuno-stimulatory activity profile of the flow through, elution and preload treated on RAW 264.7 cells in a dose-dependent manner when 1 g of E3 from mushroom #152 was loaded into the 750 mL large DEAE column. Data presented is a representation of two separate experiments.

Based on **Figure 14**, it is clear that despite increasing the loading capacity from 860 mg to 1000 mg per 750 mL DEAE Sephadex<sup>TM</sup> column, there was still no significant immuno-stimulatory activity detected in the flow through, and hence confirming that the column was not

oversaturated. On the other hand, the elution exhibited comparable immuno-stimulatory activity as to the crude extract E3 up to 0.5 mg/mL, and hence, the elution was concentrated, dialyzed, and lyophilized for the second purification step using the Hi-Prep Sephacryl S500 column. **Figure 15** shows a representation of the immuno-stimulatory activity profile from the fractions collected from the Sephacryl column.



**Figure 15.** Immuno-stimulatory activity profile of the Sephacryl S500 column eluted fractions. Data presented is a representation of two separate experiments.

The immuno-stimulatory activity of BRLI\_#152\_E3\_DEAE\_Sephacryl showed a peak activity at around an elution volume of 250 mL (fraction 28), which was similar to the activity profile observed with BRLI\_#150\_E3\_DEAE\_Sephacryl. Hence, this confirms the reproducibility

of the immuno-stimulatory activity of E3 from mushroom #152 and will be used for further purification and characterization upon the depletion of BRLI\_#150\_E3\_DEAE\_Sephacryl in Chapter 3 of this thesis.

## **Chapter 3**

# Characterization of an Immuno-Stimulatory Compound from the Water Extract of *Royoporus badius* (Pers.) A.B. De

Mushroom polysaccharides and polysaccharide-protein complexes have often been regarded for their immuno-modulatory potential (Ooi & Liu, 2000). Hence, efforts were made to purify and characterize an immuno-stimulatory compound from E3 of *R. badius*. An understanding of the chemical and biological properties of ISPP-Rb will provide insightful knowledge to future studies conducted to understand the molecular mechanism of ISPP-Rb if it were to be used for pharmacology and drug development studies. This chapter describes the experiments conducted to characterize and understand the chemical and biological nature of the purified bioactive compound (BRLI\_#150\_E3\_DEAE\_Sephacryl, see Chapter 2), which was named as ISPP-Rb (Immuno-Stimulatory Polysaccharide-Protein from *R. badius*).

Carbohydrate, protein and  $\alpha/\beta$ -glucan quantification were performed to determine the chemical constituents of the compound. In addition to that, the size of the large molecule was estimated. Enzyme assays were conducted to understand the essential component of the molecule responsible for its immuno-stimulatory activity. Types of glycosidic linkages and monosaccharide content were determined using gas chromatography-mass spectrometry/flame ionization detector (GC-MS/FID) respectively. A colorimetric assay was also performed to determine if the active compound contain a triple helix structure. Other than that, the protein portion of the proteoglycan was also analyzed for the presence of O-linked glycosidic bonds. A Mouse Cytokine 32-Plex Discovery Assay was used to determine the types of cytokines and chemokines induced by ISPP-Rb, a colorimetric-based phagocytosis assay was attempted in an effort to demonstrate the biochemical action of the purified compound.

### **Methodology**

#### 3.1 Carbohydrate Quantification using Phenol-Sulphuric Acid Method

For both size-exclusion chromatography (Sephadex<sup>TM</sup> LH-20 and Sephacryl S500) and ion-exchange chromatography (DEAE Sephadex<sup>TM</sup> A-50), the fractions collected were assessed for the carbohydrate content using 96-well Microtiter<sup>TM</sup> polystyrene plates (Thermo Fisher). A total of 50  $\mu$ L sample or glucose standard (0, 25, 50, 75, 100, 250, 500, 750, and 1000  $\mu$ g/mL) was aliquot into their designated wells, followed by the addition of 50  $\mu$ L of 6% phenol solution. The resulting solution was subjected to hydrolysis with the addition of 125  $\mu$ L of concentrated sulphuric acid. The 96-well plate was left to incubate at room temperature for 20 min without the lid prior to reading the absorbance at 490 nm using the SYNERGY 2 (BIOTEK®) multiplate reader.

### **3.2** Protein Quantification using Bicinchoninic Acid Assay (BCA assay)

The protein content of the fractions collected from both size-exclusion chromatography (Sephadex<sup>TM</sup> LH-20 and Sephacryl S500) and ion-exchange chromatography (DEAE Sephadex<sup>TM</sup> A-50) was analyzed using Pierce<sup>TM</sup> BCA Protein Assay Kit (Thermo Scientific) in 96-well Microtiter<sup>TM</sup> polystyrene plates (Thermo Fisher). The assay was performed by first putting 10  $\mu$ L of sample or bovine serum albumin (BSA) standard (0, 25, 125, 250, 500, 750, 1000, 1500 and 2000  $\mu$ g/mL) into their designated wells, followed by the addition of 200  $\mu$ L of working reagent (BCA Reagent A:BCA Reagent B working ratio = 50:1). The 96-well plate was vortex at 800 rpm for 30 sec using a Micro Plate Shaker (VWR®) and was left to incubate at 37°C for 30 min prior to reading the absorbance value at 562 nm using the SYNERGY 2 (BIOTEK®) multiplate reader.

### 3.3 Total Carbohydrate and Protein Content Quantification of ISPP-Rb

In order to quantify the total protein content, a total of 1 mg of both ISPP-Rb powder was weighed out using a Cubis® Micro Balance (Sartorius). The sample was then reconstituted in 1

mL of MilliQ water to make a sample concentration of 1 mg/mL. The sample was then subjected to BCA assay to quantify its total carbohydrate content and total protein content respectively. The assay procedures were as previously described in section 3.2. The total carbohydrate content was quantified based on the GC-FID analysis for monosaccharide content analysis (described in more details in section 3.7). The percentage of carbohydrate and protein content with respect to the sample mass was calculated using the formula stated below.

Percentage Carbohydrate (%) =  $\frac{\text{Total Carbohydrate Concentration (µg/mL)}}{\text{Sample Concentration (µg/mL)}}$ 

Percentage Protein (%) =  $\frac{\text{Total Protein Concentration } (\mu g/mL)}{\text{Sample Concentration } (\mu g/mL)}$ 

### 3.4 Size Estimation of ISPP-Rb using Hi-Prep Sephacryl S500 Equipped with ÄKTA Pure Fast Protein Liquid Chromatography (FPLC) System

Dextran T50, T80, T270, T410, T670, and Dextran Blue T2000 (Sigma Aldrich) were used to calibrate the column prior to running both the semi-purified BRLI\_#86\_E3\_LH20\_DEAE and BRLI\_#150\_E3\_DEAE. The flow rate was set to 1.3 mL/min and the buffer used was degassed 150 mM NaCl in MilliQ water. The combination of dextran used in plotting the trendline are indicated in **Table 9**. The retention volumes obtained from each run were plotted using a logarithmic trendline which allows estimation of the molecular weight of the purified immunostimulatory compound.

<b>Run Combination Set</b>	Mass (mg)	Dextran Size (kDa)
	5.000	670
Run 1	5.000	270
	10.000	80
Run 2	5.000	410
	10.000	50
Run 3	0.625	2000

Table 9. Combination of dextran used to calibrate the Hi-Prep Sephacryl S500.

#### **3.5** α/β-Glucan Quantification of ISPP-Rb

The quantification of the  $\alpha/\beta$ -Glucan content of ISPP-Rb was performed using the Mushroom and Yeast Beta-Glucan Assay Kit (Megazyme) with modification to the recommended protocol.

# 3.5.1 Measurement of Total Glucan (α-Glucan + β-Glucan) Plus D-Glucose in Oligosaccharides, Sucrose and Free D-Glucose

A total of approximately 10.0 mg of ISPP-Rb, *Phaeolepiota. aurea* (Matt.) Maire ex Konrad & Maubl polysaccharide sample (provided by Ms. Vicky Myhre, a former graduate student) and yeast control were measured into a Screw Cap Flint Glass Vial, 3.5 mL, 15 mm x 45 mm (VWR International). The vial was then gently tapped to ensure that all of the sample falls to the bottom of the tube. A total of 200  $\mu$ L of ice cold 12 M sulphuric acid was pipetted to each vial. The vials were then capped and the tubes were vigorously stirred on a vortex mixer before placing the vials in an ice-water bath for 2 hours with occasional vortex mixing. After 2 hours, 400  $\mu$ L of MilliQ water was added to each vial, capped, and vigorously stirred on a vortex mixer for 10 sec, followed by the addition of 600  $\mu$ L of MilliQ water. The vial was then capped and vortexed for another 10 sec. The vials were then transferred to the Isotemp Dry Heating Block (Fisher Scientific) set to 100°C with the caps loosened. After 5 min, the caps were tightened and the vials were left to incubate for 2 hours. After the incubation period, the vials were removed from the heating block and cooled the tubes to room temperature before loosening the caps. The contents in the vial were then quantitatively transferred using a 100  $\mu$ L pipette to a 10 mL volumetric flask and the remains in the vial were rinsed with 5 mL of 200 mM sodium acetate buffer (pH 5) prior to transferring to the 10 mL volumetric flask. Then, 600  $\mu$ L of 10 M KOH solution was pipetted to the volumetric flask and the volume was adjusted with 200 mM sodium acetate buffer (pH 5). The contents were well mixed by inversion and the contents all the contents were transferred to a 15 mL Falcon tube (Sarstedt) and centrifuged using the Allegra X-12R Centrifuge (Beckman Coulter) centrifuge at 1,500 g for 10 min.

A total of 10 µL of the supernatant (in duplicates) of the centrifuged extract (ISPP-Rb, *P. aurea* polysaccharide and yeast control) was transferred to a 1.5 mL Eppendorf tube followed by the addition of 10 µL of a mixture of exo-1,3- $\beta$ -glucanase (20 U/mL) plus  $\beta$ -glucosidase (4 U/mL) in 200 mM sodium acetate buffer (pH 5.0). The same procedure was performed with the glucose and negative control, with a total of 10 µL of the 1 mg/mL Glucose Standard solution or 200 mM sodium acetate buffer (pH 5) mixed with 10 µL of a mixture of exo-1,3- $\beta$ -glucanase (20 U/mL) plus  $\beta$ -glucosidase (4 U/mL) in 200 mM sodium acetate buffer (pH 5) mixed with 10 µL of a mixture of exo-1,3- $\beta$ -glucanase (20 U/mL) plus  $\beta$ -glucosidase (4 U/mL) in 200 mM sodium acetate buffer (pH 5.0). The resulting solution was then gently vortexed and incubated using the Isotemp Dry Heating Block (Fisher Scientific) at 40°C for 60 min. After incubation, 300 µL of GOPOD Reagent was pipetted to each tube and further incubated at 40°C for 20 min. Upon completion, 100 µL of the solution was pipetted to a 96-wells UV plate and the absorbance of all solutions (ISPP-Rb, *P. aurea* polysaccharide, yeast control, negative control and glucose control) was measured at 510 nm against the reagent blank using SYNERGY 2 (Biotek®).

# **3.5.2** Measurement of α-Glucan (Phytoglycogen and Starch) Plus D-Glucose in Sucrose and Free D-glucose.

A total of approximately 10.0 mg of ISPP-Rb, P. aurea polysaccharide and yeast control were measured into a Screw Cap Flint Glass Vial, 3.5 mL, 15 mm x 45 mm (VWR International). The vial was then gently tapped to ensure that all of the sample falls to the bottom of the tube, followed by the addition of 200 µL of 2 M potassium hydroxide (KOH) solution. The vials were then capped and hand-shaken on an ice-water bath for 20 min and proceeded with the addition of 800 μL of 1.2 M sodium acetate buffer (pH 3.8) and 20 μL of amyloglucosidase (1,630 U/mL) plus invertase (500 U/mL). The vials were then left to incubate at 40°C for 30 min in the Isotemp Dry Heating Block (Fisher Scientific) with occassional mixing on a vortex stirrer. Later, the contents were then transferred using a 100 µL pipette to a 1.5 mL Eppendorf tube and centrifuged at 1,500 g for 10 min using the bench top Centrifuge 5424 (Eppendorf). A total of 10 µL of the supernatant (in duplicates) were pipetted a separate 1.5 mL Eppendorf tube, followed with the addition of 10 µL of sodium acetate buffer (200 mM, pH 5.0) plus 300 µL of GOPOD reagent and incubate at 40°C for 20 min in the Isotemp Dry Heating Block. The same procedure was performed with the positive control (glucose) and negative control (sodium acetate buffer), with a total of 10 uL of the 1 mg/mL Glucose Standard solution or 200 mM sodium acetate buffer (pH 5) mixed with 300 µL of GOPOD reagent. Upon completion, 100 µL of the solution was pipetted to 96-well UV plate and the absorbance of all solutions (ISPP-Rb, P. aurea polysaccharide, yeast control, negative control and glucose control) were measured at 510 nm against the reagent blank using SYNERGY 2 (Biotek<sup>®</sup>).

#### **3.5.3** Estimation of Total β-Glucan Content in ISPP-Rb

The calculations were performed based on the manufacturer's guidelines with slight modification. The calculation formulas are as listed below and are as provided by the manufacturer.

Total Glucan (% w/w) plus Oligomers =  $\Delta E \times F/W \times 90$ 

 $\alpha$  Glucan (% w/w) plus Oligomers =  $\Delta E \times F/W \times 9.27$ 

 $\beta$  Glucan (% w/w) = Total Glucan plus oligomers –  $\alpha$  Glucan plus Oligomers

where:  $\Delta E = reaction absorbance - blank absorbance.$   $F = \frac{10 \,\mu g \, of \, the \, D - glucose \, standard}{GOPOD \, absorbance \, f \, or \, 10 \mu g \, of \, D - glucose \, standard}$   $W = weight \, of \, sample \, analyzed.$ 90 and 9.27 = Volume correction factor multiplied by a factor to convert from free D-glucose to anhydroglucose, as occurs in  $\beta$ -glucan.

### 3.6 Enzyme Assay Analysis on ISPP-Rb using Enzyme Degradation Assays.

The enzyme assay was performed in two separate stages. The first stage was carried out prior to obtaining the carbohydrate linkage data from GC-MS, while the second stage was carried out after obtaining the carbohydrate linkage data from GC-MS.

#### **3.6.1** Enzyme Degradation Assay using Proteinase K and α-Amyloglucosidase

For the first stage, pooled active fractions of Sephaceyl S500 (ISPP-Rb) from one purification run were used to study the effect of certain enzymes on its immuno-stimulatory activity. Due to the immeasurable mass of the purified product, the freeze-dried powder was simply reconstituted in 3.5 mL of MilliQ water and filter sterilized using a 0.22  $\mu$ m filter (Sarstedt). A total of 7 mg of Krestin (PSK, purchased in a local shop in Hong Kong) was weight out using a Cubis® Micro Balance (Sartorius) and resuspended in 3.5 mL of MilliQ water to make a concentration of 2 mg/mL, and the resulting solution was then filter-sterilized. Enzymes used in this assay were Proteinase K (New England Biolabs) and  $\alpha$ -amyloglucosidase from *Aspergillus niger* (Sigma Aldrich). The enzyme Proteinase K (PK) was a 20 mg/mL stock solution, while the
enzyme α-amyloglucosidase (AMG) was a 5 mg lyophilized powder, with every 1 mg accounting for 30-60 activity units (U). To prepare the enzyme AMG in solution form, all 5 mg were reconstituted in 1 mL of MilliQ water, and it was assumed that every 1 mg of the protein will contribute to 45 activity units (median value). The sample-enzyme mixture set up is as depicted in **Table 10-13**. All samples were incubated at 37°C for 2 hours, followed by heat deactivation at 80°C for 20 min, with the exception of ISPP-Rb\* and PSK\*, which were only subjected to the 2 hours incubation at 37°C, and not heat deactivation. After heat deactivation, the samples will be stored at 4°C prior to addition to RAW 264.7 cells the following day.

The cell treatment and ELISA protocols are as described previously in section 2.1.3, with modifications to the volume of DMEM without FBS in each well due to the different doses applied. For this assay, 100  $\mu$ L of the enzyme-treated mushroom polysaccharide samples were used to treat the RAW 264.7 cells.

**Table 10.** Preparation of the treatment of ISPP-Rb with Proteinase K (PK) and  $\alpha$ -amyloglucosidase (AMG).

Sample/Fnzyme	PK (0.4 mg/mL)	PK (2 mg/mL)	AMG (1.0 U)	AMG (10.0 U)
Sample/Enzyme		Volum	e (µL)	
Purified Compound	300	300	300	300
PSK (2 mg/mL)	NIL	NIL	NIL	NIL
PK (20 mg/mL)	5.0	25.0	NIL	NIL
AMG (45 U/mL)	NIL	NIL	4.50	45.0
MilliQ Water	45.0	25.0	45.5	5.00
Total Volume		350	).0	

\*Only subjected to the 2 hours incubation at 37°C but not heat deactivation.

Sample/Fnzyme	PK (0.4 mg/mL)	PK (2 mg/mL)	AMG (1.0 U)	AMG (10.0 U)
Sample/Enzyme		Volum	Volume (µL)	
Purified Compound	NIL	NIL	NIL	NIL
PSK (2 mg/mL)	300	300	300	300
PK (20 mg/mL)	5.0	25.0	NIL	NIL
AMG (45 U/mL)	NIL	NIL	4.50	45.0
MilliQ Water	45.0	25.0	45.5	5.00
Total Volume		350	).0	

**Table 11.** Preparation of the treatment of positive control, PSK with Proteinase K (PK) and  $\alpha$ -amyloglucosidase (AMG).

\*Only subjected to the 2 hours incubation at 37°C but not heat deactivation.

**Table 12.** Preparation of negative controls, water treated with Proteinase K (PK) and  $\alpha$ -amyloglucosidase (AMG).

Sample/Enzyma	PK (0.4 mg/mL)	PK (2 mg/mL)	AMG (1.0 U)	AMG (10.0 U)
Sample/Enzyme		Volum	e (µL)	
Purified Compound	NIL	NIL	NIL	NIL
PSK (2 mg/mL)	NIL	NIL	NIL	NIL
PK (20 mg/mL)	5.0	25.0	NIL	NIL
AMG (45 U/mL)	NIL	NIL	4.50	45.0
MilliQ Water	345.0	325.0	345.5	305.0
Total Volume		350	0.0	

\*Only subjected to the 2 hours incubation at 37°C but not heat deactivation.

**Table 13.** Preparation of the preloads, ISPP-Rb and PSK without treatment with Proteinase K (PK) and  $\alpha$ -amyloglucosidase (AMG).

Sample/Enzyme	ISPP-Rb*	ISPP-Rb	PSK*	PSK
Sample/Enzyme –		Volum	e (µL)	
Purified Compound	300	300	NIL	NIL
PSK (2 mg/mL)	NIL	NIL	300	300
PK (20 mg/mL)	NIL	NIL	NIL	NIL
AMG (45 U/mL)	NIL	NIL	NIL	NIL
MilliQ Water	50.0	50.0	50.0	50.0
Total Volume		350	0.0	

\*Only subjected to the 2 hours incubation at 37°C but not heat deactivation.

For the second stage of the enzyme degradation experiment, whereby preliminary data from GC-MS analysis was obtained, the enzyme degradation assay was reassessed by repeating the use Proteinase K (PK) but not with  $\alpha$ -amyloglucosidase (AMG). For the PK enzyme

degradation assay, the previously described protocol was used, but this time with a known mushroom sample concentration and a higher PK treatment concentration. **Tables 14-15** depicts preparation of the sample-enzyme mixture for the enzyme degradation assay using PK.

Sample/Engume -	Preload*	Preload	ISPP-Rb	<b>PK Control</b>
Sample/Enzyme	Volume (µL)			
ISPP-Rb (2 mg/mL)	150.0	150.0	150.0	NIL
PK (20 mg/mL)	NIL	NIL	10.0	10.0
MilliQ Water	50.0	50.0	40.0	190.0
Total Volume		20	0.0	

Table 14. Preparation of the treatment of ISPP-Rb, treated with 1 mg/mL of Proteinase K (PK).

\*Only subjected to the 2 hours incubation at 37°C but not heat deactivation.

**Table 15.** Preparation of the treatment of positive control (*P. aurea*) treated with 1 mg/mL of Proteinase K (PK).

Samula/Enzyma -	Preload*	Preload	P. aurea	<b>PK Control</b>
Sample/Enzyme		Volun	ne (μL)	
<i>P. aurea</i> (2 mg/mL)	150.0	150.0	150.0	NIL
PK (20 mg/mL)	NIL	NIL	10.0	10.0
MilliQ Water	50.0	50.0	40.0	190.0
Total Volume		20	0.0	

\*Only subjected to the 2 hours incubation at 37°C but not heat deactivation

## **3.6.2** Enzyme Degradation Assay using α-L-Fucosidase and α-Galactosidase

The second stage involved the use of  $\alpha$ -L-Fucosidase (FCS) from bovine kidney (Sigma Aldrich) and  $\alpha$ -Galactosidase (GAL) from green coffee beans (Sigma Aldrich). During this stage, the activity of PSK which was previously active had been lost and hence could not be used as a positive control. Polysaccharide samples from *P. aurea* were tested along with ISPP-Rb to see if the carbohydrate linkage was important for its immuno-stimulatory activity.

For the FCS and GAL enzyme degradation assay, the experiment was first carried out for 2 and 24 hours in the absence of a buffer, to ensure that the enzyme has sufficient time to react with the polysaccharide samples. The sample-enzyme mixture set up are as depicted in **Tables 16-18**. All samples were incubated at 37°C for 2 or 24 hours, followed by heat deactivation at 80°C

for 20 min, with the exception of ISPP-Rb\* and *P. aurea*\*, which was only subjected to the 2 or 24 hours incubation at 37°C, and not heat deactivation. After heat deactivation, the samples were stored at 4°C prior to addition to RAW 264.7 cells the following day.

**Table 16.** Preparation of the treatment of ISPP-Rb and *P. aurea* with  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL) in the absence or presence of 10 mM piperazine buffer (pH 5.5).

Sample/Enzyme	FCS (0.01 U)	GAL (0.1 U)	
Sample/Enzyme	Volume (µL)		
Purified Compound (2 mg/mL)	150	150	
FCS (7.7 U/mL)	1.3	NIL	
GAL (47.3 U/mL)	NIL	2.1	
MilliQ Water/Buffer (40 mM)	48.7	47.9	
Total Volume	20	0.0	

\*Only subjected to the 2 or 24 hours incubation at 37°C but not heat deactivation.

**Table 17.** Preparation of the negative control, water/buffer treated with  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL).

Samula/Enzyma	FCS (0.01 U)	GAL (0.1 U)	
Sample/Enzyme	Volume (µL)		
Purified Compound (2 mg/mL)	NIL	NIL	
FCS (7.7 U/mL)	1.3	NIL	
GAL (47.3 U/mL)	NIL	2.1	
MilliQ Water/Buffer (40 mM)	198.7	197.9	
Total Volume	20	0.0	

**Table 18.** Preparation of the preloads, ISPP-Rb and *P. aurea* without treated with  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL) in the absence or presence of 10 mM piperazine buffer (pH 5.5).

Sample/Enzyme	ISPP-Rb*	ISPP-Rb	P. aurea*	P. aurea	
Sample/Enzyme	Volume (µL)				
Purified Compound (2 mg/mL)	150	150	150	150	
FCS (7.7 U/mL)	NIL	NIL	NIL	NIL	
GAL (47.3 U/mL)	NIL	NIL	NIL	NIL	
MilliQ Water/ Buffer (40 mM)	50.0	50.0	50.0	50.0	
Total Volume		20	0.00		

\*Only subjected to the 2 or 24 hours incubation at 37°C but not heat deactivation.

# 3.6.3 Glycoprotein Stain using SDS-PAGE Gel on ISPP-Rb Treated with Enzymes α-L-Fucosidase and α-Galactosidase

Due to insignificant changes in immuno-stimulatory activity of the mushroom samples treated with FCS and GAL (see **Figures 24-26** in section 3.21), the mushroom samples treated with both the enzymes were run on a SDS PAGE gel to visualize any changes in band or smearing pattern as compared to their respective preloads, which were not treated by the enzymes. If a different band or smearing pattern was observed amongst the treated and untreated sample, this will confirm that the enzyme was actually active, and not because of the insufficient amount of enzyme used in the experiment. The samples were treated as previously described in **Tables 16-18** but at a lower volume. **Tables 19-20** illustrates the preparation of the sample-enzyme mixture for the enzyme degradation assay using both FCS and GAL.

Sample/Enzyme	Preload	FCS (0.01 U)	GAL (0.1 U)
Sample/Enzyme —	Volume (µL)		
Purified Compound (20 mg/mL)	15.0	15.0	15.0
FCS (7.7 U/mL)	NIL	1.3	NIL
GAL (47.3 U/mL)	NIL	NIL	2.1
MilliQ Water	5.0	3.7	2.9
Total Volume		20.0	

**Table 19.** Preparation of the treatment of ISPP-Rb and *P. aurea* with  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL).

**Table 20.** Preparation of the negative and enzyme controls treated with  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL).

Sample/Enzyme	Water	FCS Control	GAL Control	
Sample/Enzyme	Volume (µL)			
Purified Compound (20 mg/mL)	NIL	NIL	NIL	
FCS (7.7 U/mL)	NIL	1.3	NIL	
GAL (47.3 U/mL)	NIL	NIL	2.1	
MilliQ Water	20.0	18.7	17.9	
Total Volume		20.0		

The above mixtures were incubated at 37°C for 2 hours prior to heat deactivation at 80°C for 20 min and cooling on ice. The samples were then pulsed using a bench top centrifuge to spin down the condensate form at the top of the Eppendorf tubes. A total of 14  $\mu$ L of each sample was mixed with 3  $\mu$ L of dye (95% dye and 5%  $\beta$ -mercaptoethanol) and heated at 95°C for 10 min followed by pulsing with a bench top centrifuge to spin down the condensate formed at the top of the Eppendorf tubes. A total of 15  $\mu$ L (approximately 200  $\mu$ g of mushroom sample) and 5  $\mu$ L of protein ladder were loaded into a 10% SDS PAGE gel which was prepared based on **Table 21** and ran for approximately 45 min at 200 mV.

Table 21. Preparation mixture for 10% SDS PAGE gel.

Components	<b>Resolving Gel</b>	Stacking Gel
30% Polyacrylamide	2.66 mL	0.48 mL
4x SDS Buffer	2.00 mL (pH 8.8)	0.45 mL (pH 6.8)
MilliQ Water	3.34 mL	1.77 mL
TEMED	4.8 μL	3 µL
20% APS	16 µL	7.5 μL

The gel was then stained using Pierce<sup>TM</sup> Glycoprotein Staining Kit (Thermo Scientific) based on the manufacturer's protocol. The gel was first immersed in 100 mL of 50% methanol for 30 min to allow proper fixation. The gel was the washed twice with 100 mL of 3% acetic acid, each for 10 min with gentle shaking. Following that, the gel was then transferred to a 25 mL oxidizing solution and gently agitated over a shaker for 15 min. The gel was then subjected to washing with 100 mL of 3% acetic acid three times, each for 5 min over gently shaking. Next, the gel was then stained with 25 mL of Glycoprotein Staining Reagent and gently agitated. After 15 min, the gel was then transferred to 25 mL of reducing solution and gently shaken for another 15

min prior to extensive washing with 3% acetic acid and MilliQ water. Upon complete washing, the gel was visualized using a gel viewer machine (FluoChem Q, ProteinSimple).

## 3.7 Monosaccharide Content Analysis of ISPP-Rb using GC-FID

The mushroom polysaccharide sample (ISPP-Rb) was sent to Dr. Peter Cheung's lab at the Chinese University of Hong Kong (CUHK) and the monosaccharide contents were analyzed by Dr. Maggie Li.

## 3.8 Carbohydrate Linkage Analysis of ISPP-Rb using GC-MS

Mushroom polysaccharide sample (ISPP-Rb) was sent to Dr. Peter Cheung's lab at the Chinese University of Hong Kong (CUHK) and the carbohydrate linkages were analyzed by Dr. Maggie Li.

# **3.9** Determining the Presence of a Triple Helix Structure in ISPP-Rb Using Congo Red Colorimetric Assay

## 3.9.1 Optimizing a Suitable Congo Red Concentration for Absorbance Visibility

The Congo red colorimetric assay utilizes the ability of triple helix polysaccharides to form a complex with Congo red through hydrogen bonding and/or hydrophobic interactions when mixed with dilute alkaline solutions, which will result in a shift in the complex's maximum absorption compared to the blank, i.e. Congo red solution (Smiderle *et al.*, 2014). The assay was adapted and modified from Wang *et al.* (2013) and Smiderle *et al.* (2014). A 10 mM Congo red stock solution was prepared by dissolving the measured powder in MilliQ water. The stock was further diluted to form a 0.5, 1.0 and 5.0 mM Congo red stock for optimization steps. Four different Congo red concentrations were tested against six NaOH concentrations in order to search for an optimum absorbance profile. **Tables 22-25** shows the preparation of the various Congo red-NaOH mixture. The Congo red concentration with the best absorbance visibility was selected for subsequent experiments.

Final NaOU	Congo R	Final Valuma		
Concentration (M)	1M NaOH (µL)	MilliQ Water (µL)	0.5 mM Congo Red (μL)	μL)
0	180	0		
0.05	170	10	-	
0.10	160	20	- 20	200
0.20	140	40	- 20	200
0.30	120	60	-	
0.40	0	80	-	

Table 22. Preparation of 50  $\mu$ M Congo Red in various NaOH concentrations.

Table 23. Preparation of 100 µM Congo Red in various NaOH concentrations.

Final NoOU	Congo Re	Final Volumo		
Concentration (M)	1M NaOH	MilliQ Water	1 mM Congo	
	(µL)	(µL)	Red (µL)	(μL)
0	180	0		
0.05	170	10		
0.10	160	20	20	200
0.20	140	40	20	200
0.30	120	60		
0.40	0	80		

Table 24. Preparation of 500 µM Congo Red in various NaOH concentrations.

Final NaOU	Congo Re	Final Valuma		
Concentration (M)	1M NaOH	MilliQ Water	5 mM Congo	- rmai volume
	(µL)	(µL)	Red (µL)	(µL)
0	180	0		
0.05	170	10		
0.10	160	20	20	200
0.20	140	40	20	200
0.30	120	60		
0.40	0	80		

Final NaOU	Congo Re	Final Valuma		
Concentration (M)	1M NaOH (µL)	MilliQ Water (µL)	10 mM Congo Red (µL)	- Final Volume (μL)
0	180	0		
0.05	170	10	-	
0.10	160	20	20	200
0.20	140	40	- 20	200
0.30	120	60	-	
0.40	0	80	-	

Table 25. Preparation of 1000 µM Congo Red in various NaOH concentrations.

# 3.9.2 Analysis of the Presence of a Triple Helix Structure in ISPP-Rb

Upon successful determination of the optimum Congo red concentration, the triple helix analysis of the polysaccharide samples was performed. The samples used were ISPP-Rb, *P. aurea* sample and *Echinodontium. tinctorium* (Ellis & Everh.) Ellis & Everh. sample (provided by Ms. Mehreen Zeb, a graduate student in Dr. Lee's Lab). The controls selected were PSK and Dextran (410 kDa). A 2 mg/mL solution of all samples and controls were prepared for analysis. Generally, samples and controls (final concentration 1 mg/mL) will be treated with diluted NaOH solution (0, 0.05, 0.1, 0.2, 0.3, and 0.4M) and allowed to react with 100 µM of Congo red solution prior to reading the absorbance spectrum from 450-550 nm. The reaction mixture is as shown in **Table 26**.

Final NaOH	Congo Red Final Concentration 100 µM			Final	
Concentration	1M NaOH	MilliQ	2 mg/mL	1 mM Congo	- Fillal Volumo (uI.)
(M)	(µL)	Water (µL)	Sample (µL)	Red (µL)	volume (µL)
0	80	0			
0.05	70	10			
0.10	60	20	100	20	200
0.20	40	40	100	20	200
0.30	20	60			
0.40	0	80			

Table 26. Treatment of mushroom samples with various NaOH concentrations and 100  $\mu$ M Congo red solution.

**3.10** Determination of the Presence of O-Glycosidic Bond in the Immuno-Stimulatory Compound of ISPP-Rb using the β-Elimination Assay

The carbohydrate-peptide linkages of ISPP-Rb were analyzed using the  $\beta$ -elimination assay described by Wu *et al.* (2015) with modification. The first step was to optimize the reaction time required. Reaction times of 2, 4, 10, 12 and 16 hours were used on an immuno-stimulatory polysaccharide purified from *P. aurea* due to the abundant material available. Generally, a total of 100 µL of 2 mg/mL *P. aurea* sample were mixed with 100 µL 0.2 mol/L NaOH containing either 2.0 M or 4.0 M NaBH<sub>4</sub> (in duplicates) and incubated in the Accublock<sup>TM</sup> Digital Dry Bath (Labnet) at 45°C for 2 hours. After the incubation period, 100 µL of the sample mixture was transferred to a 96-wells UV plate and the absorbance read from 230 - 300 nm against the blank solutions (0.1 M NaOH with 1.0 M or 2.0 M NaBH<sub>4</sub> and water) using SYNERGY 2 (Biotek®). In the presence of O-glycosidic bonds, an increase in absorbance at 240 nm should be observed. The sample that failed to show an increase in absorbance at 240 nm will serve as the negative control. Upon confirming the optimum reaction time, the experiment proceeded with purified bioactive compounds, i.e. ISPP-Rb, immuno-stimulatory polysaccharide from *E. tinctorium* and a positive control, aggrecan (2 mg/mL).

Another  $\beta$ -elimination assay without the use of the reducing agent NaBH<sub>4</sub> was also carried out based on a protocol established by Yi *et al.* (2011) with modification. A total of 100 µL of 2 mg/mL mushroom samples (ISPP-Rb, *P. aurea*, and *E. tinctorium*) and positive control, Aggrecan (2 mg/mL) were mixed with 100 µL 0.2 mol/L NaOH (in duplicates) and incubated in the Accublock<sup>TM</sup> Digital Dry Bath (Labnet) at 45°C for 2 hours. After the incubation period, 100 µL of the sample mixture was transferred to a 96-wells UV plate and the absorbance was read from 230 - 300 nm against the blank solution using SYNERGY 2 (Biotek®). In the presence of O- glycosidic bonds, an increase in absorbance at 240 nm should be observed. The sample that failed to show an increase in absorbance at 240 nm served as the negative control.

## 3.11 Analysis of Cytokines Induced by ISPP-Rb in RAW 264.7 Cells

The ISPP-Rb fraction that stimulated the highest TNF- $\alpha$  production in RAW 264.7 cells as depicted by the data from ELISA as well with a negative control (RAW 264.7 cells treated with 150 mM NaCl) were selected for further analysis on the types of cytokines and chemokines that could be induced. A total of 100 µL of the supernatant collected post-treatment with the selected fraction or with 150 mM NaCl serving as a blank were each aliquoted into a 0.5 mL Eppendorf Safe-Lock Tubes<sup>TM</sup> (Eppendorf) and sent to Eve Technologies (Calgary, AB) for analysis using the Mouse Cytokine 32-Plex Discovery Assay. Analysis of the cytokine induced will allow for a better understanding on the types of immune responses triggered by the immuno-stimulatory compound from *R. badius*.

# 3.12 Analysis of Phagocytic Activity of RAW 264.7 Cells Treated with ISPP-Rb using Neutral Red Colorimetric Assay

## 3.12.1 Investigating a Suitable Cell Media Requirement and LPS Concentration

The neutral red assay was adopted and modified from Teng *et al.* (2015) and Zheng *et al.* (2017). The first approach was to determine the medium requirement for the assay. A total of 100  $\mu$ L containing 10,000 RAW 264.7 cells in DMEM with and without FBS were seeded in 96-wells plate and left to adhere to the bottom of the wells for 4 hours. The cells were then treated with LPS (100, 250, and 500 ng/mL) and left to incubate at 37°C under 5% CO<sub>2</sub> for 24 hours. The supernatants of the cells were then removed and the cells were washed once with 200  $\mu$ L of PBS prior to adding 100  $\mu$ L of 0.075% Neutral Red dissolved in DMEM with and without FBS. The cells were then left to incubate at 37°C under 5% CO<sub>2</sub>. After 3 hours, the neutral red solution was removed and the cells were washed twice with 200  $\mu$ L of PBS each to remove any excess stains

prior to adding 100 µL of lysis buffer (Acetic Acid:Ethanol, 1:1). The plate was vortexed at 800 rpm for 30 sec using a Micro Plate Shaker (VWR®) prior to reading the absorbance value at 540 nm using SYNERGY 2 (BIOTEK®) machine.

## 3.12.2 Optimizing a Suitable Mushroom Sample Dose for Cell Treatment

The protocol stated in section 3.12.1 was adopted for use, with DMEM without FBS being the media of choice. LPS (250 and 500 ng/mL), along with and ISPP-Rb and *P. aurea* samples (all at 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, and 250 µg/mL) were treated to RAW 264.7 cells.

## 3.12.3 Optimizing the Neutral Red Concentration for Absorbance Visibility

With inconsistencies from the previous optimization steps, the next approach was to modify the Neutral Red dye concentration with the rationale of seeing a higher absorbance reading and also the dissolving solvent. Three concentrations of 0.0375, 0.075 and 0.15% Neutral Red dye dissolved in MilliQ water were tested on cells. The protocol used was similar to that in section 3.12.1, but with modification to the Neutral Red addition step. Generally, 100  $\mu$ L containing 10,000 RAW 264.7 cells in DMEM with and without FBS were seeded in 96-well plate and left to adhere to the bottom of the wells for 4 hours. The cells were then treated with LPS (250 and 500 ng/mL) and left to incubate at 37°C under 5% CO<sub>2</sub> for 24 hours. The supernatants of the cells were then removed and the cells were washed once with 200 µL of PBS prior to adding 100 µL of DMEM without FBS followed by the addition of the aforementioned Neutral Red solution. The cells were then left to incubate at 37°C under 5% CO<sub>2</sub>. After 3 hours, the neutral red solution was removed and the cells were washed twice with 200 µL of PBS each to remove any excess stains prior to adding 100 µL of lysis buffer (Acetic Acid:Ethanol, 1:1). The plate was vortexed at 800 rpm for 30 sec using a Micro Plate Shaker (VWR<sup>®</sup>) prior to reading the absorbance value at 540 nm using SYNERGY 2 (BIOTEK®).

## 3.13 Statistical Analysis

Statistical analysis using One Way ANOVA (Tukey Test) or Student t-Test was performed in Graph Prism Version 6.0, whereby the data shown are expressed in mean ± standard deviation (SD).

# **Results and Discussion**

# 3.14 Relative Carbohydrate and Protein Content of BRLI\_#86\_E3\_LH20, BRLI\_#86\_E3\_LH20\_DEAE and BRLI\_#150\_E3\_DEAE\_Sephacryl (ISPP-Rb)

Carbohydrate content analysis was initially carried out on the Post LH20 fractions of the

water extracts, E3 of mushroom #86. Figure 16 depicts the relative carbohydrate content and their

respective immuno-stimulatory activity of the Post LH20 fractions from the 400 mL LH20 column.



**Figure 16.** Carbohydrate content and TNF- $\alpha$  production of the Post LH20 fractions of E3 from mushroom #86 eluted off the 400 mL LH20 column. Data presented is a representation of three separate experiments.

It can be observed in **Figure 16** that there are two carbohydrate peaks, where the first carbohydrate peak corresponds to the first immuno-stimulatory activity peak. This suggests that the bioactive compound could be a polysaccharide. Unfortunately, the relative protein content for the Post LH20 fractions of E3 from mushroom #86 eluted off the 400 mL LH20 column and was not determined at that time. Upon completion of the purification step using LH20 Sephadex<sup>™</sup>, the semi-purified fractions were then subjected to purification using DEAE Sephadex<sup>™</sup> A-50. The purification optimization data are as presented in section 2.10 in Chapter 2. According to **Figure 16**, it can be observed that there were two carbohydrate peaks eluted from the LH20 columns, however the immuno-stimulatory activity only corresponded with the first carbohydrate peak. Hence, a carbohydrate and protein quantification assay were performed on the flow through and elution collected from the DEAE column. **Figures 17a** and **17b** depict the carbohydrate and protein content of the flow through and elution collected off the DEAE column respectively.



**Figure 17. (a)** Carbohydrate content and **(b)** protein content of the Post LH20-DEAE fractions (flow through and elution) collected when 20 mg of E3 from mushroom #86 was loaded into the 75 mL DEAE column. Data presented is an average from two independent experiments (n=2).

The data above confirms the presence of both carbohydrate and protein components in the active compound, whereby the protein content of the Post LH20 eluted active fractions could not be determined. Furthermore, it also suggests that the DEAE purification step was indeed removing impurities as depicted by the presence of carbohydrate and protein content in the flow through, which could be from the second carbohydrate observed in **Figure 16** which did not contribute to any immuno-stimulatory activity.

Following the completion of the DEAE purification of the water extracts (E3 of mushroom #86), the third purification step was using the Sephacryl S500 column. Unfortunately, due to contamination issues as described in section 2.11 in Chapter 2, the relative carbohydrate and protein contents were not determined. The purification step had to be redone with a new batch of *R. badius* (mushroom #150), whereby the activity in the water extracts, E3 has been confirmed as presented in **Figure 4b** in section 2.8 of Chapter 2. With the rationale that the DEAE Sephadex<sup>TM</sup> A-50 was able to remove the inactive carbohydrates and proteins (see **Figures 17a-b**), and also the inefficiency of the Sephadex<sup>TM</sup> LH20 due to its low exclusion limit of 4-5 kDa, it was decided that the initial first step of purification using Sephadex<sup>TM</sup> LH20 was no longer necessary and hence omitted. Therefore, the purification of the E3 of mushroom #150 was subjected directly to DEAE Sephadex<sup>TM</sup> followed by Sephacryl S500. **Figures 18a** and **18b** shows the relative carbohydrate and protein of the fractions eluted off the Hi-Prep Sephacryl S500 column.



**Figure 18. (a)** Carbohydrate content of the Post DEAE-Sephacryl fractions from mushroom #150 collected using Hi-Prep Sephacryl S500 column; **(b)** Protein content of the Post DEAE-Sephacryl fractions from Mushroom #150 collected using Hi-Prep Sephacryl S500 column. Data presented are each a representation from three separate experiments.

Based on **Figures 18a-b**, in Post-DEAE Sephadex<sup>TM</sup> A-50 column, the second carbohydrate and/or protein peak that were previously seen using the Sephadex<sup>TM</sup> LH20 column was no longer detected. This is consistent with my previous hypothesis that DEAE Sephadex<sup>TM</sup> column was able to remove inactive carbohydrate and protein components. With the higher resolution Hi-Prep Sephacryl S500 column (exclusion limit of 40-2000 kDa), the active compound is expected to be better separated as compared to using the crude Sephadex<sup>TM</sup> LH20 column. Although the active compound, when previously separated using Sephadex<sup>TM</sup> LH20 column, showed that the immuno-stimulatory activity peak corresponded to the carbohydrate peak (see **Figure 16**), it was no longer the case after passing through the high resolution Sephacryl S500 column. Despite that, there was still a small amount of protein and carbohydrate detected within the active region of the fractions collected, which still suggests that the active compound could be a polysaccharide-protein mixture.

## 3.15 Total Carbohydrate and Protein Content of ISPP-Rb

In order to investigate if the active compound has any carbohydrate or protein content, due to their relative low amount as depicted in **Figures 18a-b**, the total carbohydrate and protein content of ISPP-Rb are as stated below in **Table 27**. It was confirmed that the active compound contains both carbohydrate and protein, with a total carbohydrate and protein content of approximately 57.4% and 16.7% respectively, with the remaining 25.9% being unknown. This could possibly be due to the presence of impurities or the possibility of any inorganic binding metals or organic matter bound to the active compound.

**Table 27**. Total carbohydrate and protein content of the purified bioactive compound from the water extracts, E3 of mushroom #150. Data presented are of two independent experiments (n=2).

Mushroom Sample	Total Carbohydrate	<b>Total Protein Content</b>
	Content (% w/w)	(% w/w)
ISPP-Rb	$57.4 \pm 2.1$	$16.7 \pm 1.7$

Note: The carbohydrate content as determined using GC-FID while the protein content was determined using BCA assay.

## 3.16 Size Estimation of ISPP-Rb

Since the immuno-stimulatory compound was suggested to be a polysaccharide-protein complex, it was important to estimate its approximate size. With the use of dextran standards, the molecular size of the ISPP-Rb was estimated to be approximately 400 kDa. **Figure 19** depicts the dextran standards and immuno-stimulatory activity of the eluted fractions from Sephacryl S500.



**Figure 19.** Immuno-stimulatory activity and size of the Post DEAE-Sephacryl fractions of E3 from mushroom #150 eluted off the Hi-Prep Sephacryl S500 column. Data presented is a representation from three separate experiments. The numbers indicate the dextran standards in kDa.

## **3.17** Total β-Glucan Content of ISPP-Rb

With the data in **Table 27** from section 3.15 confirming the presence of carbohydrate in ISPP-Rb, it was thought that the active compound could possibly be a  $\beta$ -glucan.  $\beta$ -D-glucans are made up of linear unbranched polysaccharides of  $\beta$ -D-glucose which have been reported to possess immuno-modulating properties and have attracted a wide interest especially after the discovery of glucan receptors responsible for the innate immune response on the cell surface of various immune cells (Vannucci *et al.*, 2013). Most importantly, fungi are the main source of  $\beta$ -D-glucans, which usually originates from the fungal cell wall (Novak & Vetvicka, 2008). Taken together, I decided to quantify the amount of  $\beta$ -glucan present in my immuno-stimulatory compound as described in section 3.5. **Table 28** depicts the amount of glucan present in ISPP-Rb.

Mushroom Sample	Total Glucan (% w/w)	Total α-Glucan (% w/w)	Total β-Glucan (% w/w)
ISPP-Rb	$0.48 \pm 0.03$	$0.20 \pm 0.05$	$0.28 \pm 0.08$
P. aurea Polysaccharide	$22.0 \pm 0.4$	$3.1 \pm 1.1$	$18.9 \pm 0.8$
Yeast Control	$45.9 \pm 2.7$	$0.5 \pm 0.0$	$45.5 \pm 2.7$

**Table 28**. Glucan content of the purified active compound from the water extracts (E3) of mushroom #150. Data presented are from two independent experiments (n=2).

Interestingly, extremely low level or no  $\beta$ -glucan was detected in ISPP-Rb, despite reports claiming how frequent these sugar polymers are found in fungal tissues. Hence, it can be concluded that the immuno-stimulatory activity of ISPP-Rb is not due to a  $\beta$ -glucan polymer.

# 3.18 Monosaccharide Content of ISPP-Rb

With the absence or extremely low abundance of  $\beta$ -glucans in ISPP-Rb, it was thought that a further investigation in the monosaccharides content present in the active compound would provide a better understanding regarding its chemical characteristic. The monosaccharide content analysis of ISPP-Rb was performed by Dr. Maggie Li in Dr. Peter Cheung's lab at CUHK using GC-FID (Gas Chromatography-Flame Ionization Detector). According to the preliminary data obtained from GC-FID analysis, it was determined that approximately 45% of the active compound was made up of galactose, while glucose accounted for nearly 40% of the total monosaccharides present, with mannose, xylose and fucose each accounting for less than 10% each. **Table 29** depicts the types of monosaccharide present in the polysaccharide of the immunostimulatory compound from mushroom #150.

Monosaccharide	Percentage (% w/w)
Fucose	$3.8 \pm 0.3$
Xylose	$1.3 \pm 0.1$
Mannose	$9.4 \pm 1.8$
Glucose	$39.8 \pm 0.1$
Galactose	$45.8 \pm 1.6$
Total	100

**Table 29.** Monosaccharide composition of the purified active compound from the water extracts (E3) of mushroom #150. Data presented are an average from two independent experiments (n=2).

Interestingly, despite the lack of  $\beta$ -glucan present in ISPP-Rb, around 40% of glucose was detected in GC-FID analysis. This indicates the absence of 1,3-Glucose and 1,6-Glucose, which are the carbohydrate linkages present in  $\beta$ -glucan, but also suggests the possible presence of other glucose linkages.

# 3.19 Carbohydrate Linkages of ISPP-Rb

The carbohydrate linkages of ISPP-Rb was performed by Dr. Maggie Li in Dr. Peter Cheung's lab at CUHK using GC-MS. **Table 30** depicts the types of carbohydrate linkages present

in the polysaccharide of the immuno-stimulatory compound of mushroom #150.

**Table 30**. Types of carbohydrate linkages of the purified active compound from the water extracts (E3) of Mushroom #150. Degree of branching (DB)\* =0.71. \*DB =  $(N_T + N_B)/(N_T + N_B + N_L)$ , where  $N_T$ ,  $N_B$ ,  $N_L$  are the total numbers of the terminal residues, branching residues, and linear residues respectively. Data presented is a representation of one single experiment.

Carbohydrate Linkages	Percentage (% w/w)
Terminal Fucose	8.2
Terminal Glucose	6.0
Terminal Galactose	16.8
1,6-Mannose	16.3
1,6-Galactose	13.0
1,2,6-Glucose	39.7
Total	100

According to the preliminary data obtained from GC-MS, the 1,2,6-Glucose linkage was found to make up approximately 40% of all the linkages, which resulted in the high degree of branching (0.71). This was followed by terminal galactose,  $(1 \rightarrow 6)$ -mannose (Man) and  $(1 \rightarrow 6)$ galactose (Gal), each accounting for 16.8%, 16.3% and 13.0% respectively. Both terminal glucose and fucose only accounted for less than 10% each. This result is consistent with the data obtained in **Tables 28** and **29** (section 3.17 and section 3.18). As previously mentioned, ISPP-Rb did not contain any  $\beta$ -glucan, however glucose was detected, which can now be explained as the glucose detected was derived from the presence of terminal glucose and the  $(1 \rightarrow 2,6)$ -glucose (Glc) linkages. By comparing **Tables 29** and **30**, it can be seen that all the monosaccharides detected were all part of the carbohydrate linkages resolved, except for xylose, which is likely due to its relatively low amount, making it undetectable during the linkage analysis using GC-MS.

## 3.20 Helical Conformational Analysis of ISPP-Rb

It has been reported that the helical conformation of polysaccharides play an important role in determining its immuno-stimulatory potency, generally with triple helix structures being the more potent compared to that of single helices (Meng *et al.*, 2016). Hence, the attempt was to investigate the possible helical conformation of ISPP-Rb as described in section 3.9. **Figure 20a** illustrates the results of an experiment to optimize the concentration of Congo Red. **Figure 20b** shows the result on the effect of NaOH on the binding of polysaccharide samples to Congo red. According to Smiderle *et al.* (2014), triple helical polysaccharides will form a complex with Congo red in diluted NaOH solutions as a result of the stabilization between the polysaccharide and the dye molecule via strong hydrogen bonding along with possible hydrophobic interactions. Such a phenomenon can be detected by observing a change in the wavelength where absorbance is at maximum ( $\lambda_{Max}$ ) of the Congo red spectrum.



**Figure 20**. UV spectra (400-600 nm) at an interval of 10 nm of (a) Congo red solution at 50, 100, 500, and 1000  $\mu$ M; (b) the polysaccharide sample treated with 0.1 M NaOH and 100  $\mu$ M Congo red. Data shown is from (a) one experiment, or (b) an average of two independent experiments (n=2).

Based on **Figure 20a**, 100  $\mu$ M Congo red showed a peak absorbance reading at approximately 1.0, which allows for visible changes during absorbance changes (increase or decrease). On the other hand, Congo red concentrations of 500 and 1000  $\mu$ M experienced an overexpression of absorbance reading which resembles too high a concentration for the assay and was hence omitted. Furthermore, the concentration of Congo red tested in this particular study have been reported to range from 10 – 100  $\mu$ M (Palacios *et al.*, 2012; Smiderle *et al.*, 2014). Hence, selecting 100  $\mu$ M Congo red as the working concentration is therefore a good rationale. Unfortunately, despite obtaining a suitable working Congo red concentration, none of the samples and controls tested had any visible shift in the maximum absorbance at 0.1 M NaOH (**Figure 20b**) and the other NaOH concentration tested (data not shown). Based on Ogawa *et al.* (1972), the Congo red had only previously been reported to form a complex with  $\beta$ -1,3- and  $\beta$ -1,4- glucans

under dilute NaOH conditions. Surprisingly then, PSK, a protein bound  $\beta$ -1,3- and 1,6-glucan failed to show any shift in maximum absorbance. This could be due to the interference of the protein molecule attached to it. Chan *et al.* (2009) explained that despite PSK having  $\beta$ -glucan, the fact that a protein molecule is bounded to it makes it different from a pure  $\beta$ -glucan. Without a working positive control, the results obtained were inconclusive.

## 3.21 Effect of Enzyme Treated ISPP-Rb on Its Bioactivity

The enzyme assays were carried out in two phases, with the first phase being done prior to the linkage analysis, while the second phase was performed after obtaining the carbohydrate linkage data. The rationale of this study is to treat the active compound with certain enzymes to see if the enzyme was capable of affecting the active compounds immuno-stimulatory activity. If the immuno-stimulatory activity of the compound is decreased after enzyme digestion, that would be indicative of the significant role played by the digested portion (i.e. protein or polysaccharide). The first phase of the enzyme digestion experiment was carried out on ISPP-Rb using Proteinase K (PK) and  $\alpha$ -amyloglucosidase (AMG). **Figures 21a** and **21b** show the PK treatment on ISPP-Rb and PSK respectively; while **Figures 22a** and **22b** illustrate the AMG treatment on ISPP-Rb and PSK respectively.



**Figure 21**. The effect of Proteinase K (PK) treatment on the immuno-stimulatory activity of (a) ISPP-Rb and (b) positive control, PSK. The symbol (\*) indicates heated at 80°C for 20 min. Data presented are a representative of two separate experiments. Statistical analysis was assessed using One-Way ANOVA (Tukey Test) with <sup>N.S</sup> (P > 0.05), <sup>\*\*\*</sup> (P < 0.05).



**Figure 22**. The effect of  $\alpha$ -amyloglucosidase (AMG) treatment on the immuno-stimulatory activity of **(a)** ISPP-Rb and **(b)** positive control, PSK. The symbol (\*) indicates heated at 80°C for 20 min. Data presented are a representative of two separate experiments. Statistical analysis was assessed using One-Way ANOVA (Tukey Test) with <sup>N.S</sup> (P > 0.05), <sup>\*\*\*</sup> (P < 0.05).

Based on **Figures 21a-b**, it can be seen that at 0.4 mg/mL of PK treatment, the immunostimulatory activity of ISPP-Rb and positive control PSK was lost as compared to the initial preload (unheated ISPP-Rb and PSK), indicating that the protein component is important for their bioactivity. However, treatment of PK at 2 mg/mL on PSK had no effect against its immunostimulatory activity, which is likely due to the high concentration of glycerol present in the PK stock solution, inhibiting the enzyme activity of PK. On the other hand, treatment with AMG as depicted in **Figures 22a-b**, resulted in an increase in immuno-stimulatory activity as compared to their respective preloads (unheated ISPP-Rb and PSK). AMG functions by cleaving  $\alpha$ -1,3-, 1,4and 1,6-glucosidic linkages, and despite ISPP-Rb not having any detectable quantities of the aforementioned three linkages, these linkages could have been of very minute quantities derived from the presence of the terminal glucose, which could be directly linked to a glucose from the 1,2,6-glucose linkage present in high amounts. Hence, the stimulation observed could be a consequence of the digestion of the compound to a more potent derivative of the previous active compound. The possibility that AMG itself being immuno-stimulatory is less likely because the enzyme alone (control) did not show any significant immuno-stimulation. Other than the effect of enzymes, it can also be deduced that the active compound is heat sensitive, as depicted by the decrease in activity upon heat treatment during enzyme deactivation.

To further confirm the role of the protein component in the bioactivity of ISPP-Rb and its sensitivity towards heat, the experiment was repeated. The results shown in **Figures 23a-b** included a different positive control (*P. aurea* sample) and a known concentration of ISPP-Rb as compared to the previously unknown concentration which was the reason behind the lower immuno-stimulatory activity observed. The final concentration of both mushroom samples was 1.5 mg/mL and the treatment was 1 mg/mL of the enzyme PK. Enzyme treated mushroom samples were then treated on RAW 264.7 cells at a final concentration of 0.375 mg/mL.



**Figure 23**. The effect of Proteinase K (PK) treatment on the immuno-stimulatory activity of (a) ISPP-Rb and (b) positive control, *P. aurea* polysaccharide sample. The final concentration of both samples was 1.5 mg/mL and treated with 1 mg/mL of the enzyme PK. Enzyme treated mushroom samples were then treated on RAW 264.7 cells at a final concentration of 0.375 mg/mL. The symbol (\*) indicates heated at 80°C for 20 minutes. Data presented is the mean  $\pm$  SD from three independent experiments (n=3). Statistical analysis was assessed using One-Way ANOVA (Tukey Test) with <sup>N.S</sup> (P > 0.05), <sup>\*\*\*</sup> (P < 0.05).

Unfortunately, at the time of this experiment the immuno-stimulatory activity of Krestin (PSK) could not be observed, possibly due to degradation, and hence the results were not included in **Figure 23**. However, previous experiments done by Ms. Vicky Myhre in Dr. Lee's lab have shown that the immuno-stimulatory activity of her *P. aurea* samples were also reduced by PK treatment (data not shown), and hence could serve as a positive control. With the above confirmation, the deduction that ISPP-Rb is relatively heat stable and the requirement of the protein component for its immuno-stimulatory activity was confirmed and validated.

The second phase involved the use of  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL). **Figures 24a** and **24b** depict the FCS and GAL treatment respectively on *P.aurea* sample and ISPP-Rb for 2 hours, while **Figures 25a** and **25b** illustrate the FCS and GAL treatment on *P.aurea* sample and ISPP-Rb for 24 hours respectively.



**Figure 24**. The effect of  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL) treatment on the immuno-stimulatory activity of (a) *P. aurea* and (b) ISPP-Rb for 2 hours in the absence of a buffering system. The symbol (\*) indicates heated at 80°C for 20 minutes. Data presented is the mean  $\pm$  SD from a single experiment. Statistical analysis was assessed using One-Way ANOVA (Tukey Test) with <sup>N.S</sup> (P > 0.05), \*\*\* (P < 0.05).



**Figure 25**. The effect of  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL) treatment on the immuno-stimulatory activity of (a) *P. aurea* and (b) ISPP-Rb for 24 hours in the absence of a buffering system. The symbol (\*) indicates heated at 80°C for 20 minutes. Data presented is the mean  $\pm$  SD from a single experiment. Statistical analysis was assessed using One-Way ANOVA (Tukey Test) with <sup>N.S</sup> (P > 0.05), <sup>\*\*\*</sup> (P < 0.05).

Based on **Figures 24a-b** and **25a-b**, there was no significant change in the immunostimulatory activity of the active compounds treated with the enzymes FCS and GAL at both 2 and 24 hours. This could be due to the absence of a proper buffering system as the manufacturer (Sigma Aldrich) states that the optimum pH for both enzymes should be within a pH range of 5-6. Hence, a second assay was performed in the presence of a 10 mM piperazine buffer at pH 5.5 for only 24 hours as there was no effect observed on the preloads when incubated at 37°C for 2 or 24 hours in the absence of a buffer. Unfortunately, with the addition of a buffer system, there was also no significant change observed on the immuno-stimulatory activity of both polysaccharide samples when treated with FCS and GAL (**Figures 26a-b**).



**Figure 26**. The effect of  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL) treatment on the immuno-stimulatory activity of (a) *P. aurea* and (b) ISPP-Rb for 24 hours in the presence of 10 mM Piperazine buffer at pH 5.5. The symbol (\*) indicates heated at 80°C for 20 minutes. Data presented is the mean ± SD from a single experiment. Statistical analysis was assessed using One-Way ANOVA (Tukey Test) with <sup>N.S</sup> (P > 0.05), <sup>\*\*\*</sup> (P < 0.05).

It is unlikely that the enzymes used were insufficient as they were calculated to be in excess relative to the amount of samples used. In addition to the above experiments, the mushroom polysaccharide samples treated/not treated with enzymes FCS and GAL were run on a SDS PAGE

gel and visualized using a glycoprotein staining kit. The rationale behind this experiment was intended to check if the enzyme had already cleaved the polysaccharide, in hope that a difference in the band or smearing pattern will be observed between the enzyme-treated and non-treated mushroom samples. Such a difference will suggest that the absence of suppression in the immuno-stimulatory activity of the mushroom samples treated with enzymes as compared to their respective preloads (enzyme-untreated samples) were not due to the lack of enzyme concentration, but due to the non-importance of the specific carbohydrate bonds towards the active compound's immuno-stimulatory activity. **Figure 27** depicts the band images of *P. aurea* sample and my ISPP-Rb on the SDS PAGE gel stained with a glycoprotein staining kit respectively.



**Figure 27.** The effect of  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL) treatment on the relative size of *P. aurea* sample and ISPP-Rb for 2 hours in the absence of a buffering system. Data presented is a representative of two separate experiments.

Based on Figure 27, it can be clearly observed that the treatment of FCS and GAL on P. aurea resulted in a change in the band intensity at the loading wells (stacking gel) and at the end of the resolving gel (MW  $\leq$  10 kDa). This result suggested that both the enzymes, FCS and GAL were most likely to be functioning by cleaving the carbohydrate linkages of P. aurea. Hence, this further suggested that the  $\alpha$ -fucose and  $\alpha$ -galactose linkages in the polysaccharide from *P. aurea* are not important for its immuno-stimulatory activity. As for ISPP-Rb, upon treatment with both enzymes, a smear pattern with less visible distinct bands at around or above 250 kDa and around 45-70 kDa were observed. I reasoned that both enzymes were also active against ISPP-Rb. This is because (i) equal amounts of enzymes (FCS and GAL) were added in excess to the polysaccharide, (ii) both the polysaccharides had fucose and galactose linkages, and (iii) both the enzymes were shown to be active against polysaccharide from *P. aurea* (Figure 27). Nevertheless, it remains a possibility that ISPP-Rb did not have any  $\alpha$ -fucose and  $\alpha$ -galactose linkages but rather  $\beta$ -fucose and  $\beta$ -galactose. This requires confirmation by structural elucidation using NMR analysis. With the above rationale, it was concluded that the  $\alpha$ -fucose and  $\alpha$ -galactose linkages, if truly present in ISPP-Rb, are not important for its immuno-stimulatory activity.

## 3.22 Glycosidic Bond Analysis of ISPP-Rb

Enzyme assays using Proteinase K to digest ISPP-Rb showed that the protein portion of the polysaccharide-protein mixture is crucial for the compound's immuno-stimulatory activity. Hence, it is important to know whether there exist covalent interactions (O- or N-glycosidic bond) between the protein and polysaccharide. To investigate the possibility of an O-linked bond between the polysaccharide and the protein, we used a method called  $\beta$ -elimination assay which employs a reducing agent, Sodium Borohydride (NaBH<sub>4</sub>). The methodology was as described in

section 3.10. Figure 28a and 28b illustrate a one-time  $\beta$ -elimination assay performed on *P. aurea* polysaccharide-protein complex with the presence of NaBH<sub>4</sub> for 2 hours and 16 hours respectively.



**Figure 28. (a)** UV spectra (230-300 nm) of *P. aurea* polysaccharide-protein complex treated/not treated with 0.1 M NaOH and NaBH<sub>4</sub> for 2 hours; **(b)** UV spectra (230-300 nm) of *P. aurea* sample treated/not treated with 0.1 M NaOH and NaBH<sub>4</sub> for 16 hours Data presented is a representation of one single experiment.

Based on the data from the above optimization step, it can be observed that the pattern (trend) and the absorbance values from both times were similar. Hence, the data suggests that reaction time can take place any time between 2 - 16 hours without a change in absorbance values. With that, subsequent  $\beta$ -elimination assays were conducted using a positive control, aggrecan and ISPP-Rb. **Figure 29a** and **Figure 29b** illustrate the effect of their UV spectrum upon treatment with 0.1 M NaOH and 2 M NaBH<sub>4</sub> each for 4 hours.



**Figure 29.** UV spectrum (230-300 nm) of (a) positive control, aggrecan; (b) the polysaccharide protein complex of *R. badius*, ISPP-Rb treated/not treated with 0.1 M NaOH and NaBH<sub>4</sub> for 4 hours. Data presented is a representation of one single experiment.

Based on **Figures 29a-b**, it can be noted that the positive control (aggrecan) demonstrated an increase in absorbance at 240 nm, while ISPP-Rb showed a decrease in absorbance. Based on the results from this experiment, I decided to experiment on another  $\beta$ -elimination assay without the use of NaBH<sub>4</sub>, with the methodology also described in section 3.10. **Figures 30a-b** illustrate the effect of the UV spectrum of the samples upon treatment with 0.1 M NaOH each for 2 hours.



**Figure 30.** UV spectra (230-300 nm) of (a) positive control, aggrecan and (b) polysaccharide protein complex of *R. badius*, ISPP-Rb treated/not treated with 0.1 M NaOH 2 hours. Data presented is the mean  $\pm$  SD from two independent experiments (n=2). Statistical analysis was assessed using Multiple Student t-Test with <sup>N.S</sup> (P > 0.05), <sup>\*\*\*</sup> (P < 0.05) on treated samples compared to non-treated samples at their respective wavelengths.

According to **Figures 30a-b**, it can be concluded that the positive control (aggrecan) was valid with an increase in absorbance value at 240 nm. ISPP-Rb also showed a small increase in absorbance at 240 nm, suggesting a relatively small amount of O-linked bonds present. On the other hand, a purified sample from *E. tinctorium* sample showed a decrease in absorbance at 240 nm (data not shown), indicating the absence of any O-linked bonds, and hence was served as a negative control in this assay.

According to O'Neill (1996) and Roth *et al.* (2012), the alkaline degradation of the Olinked polysaccharides with the presence of NaBH<sub>4</sub> prevents the degradation of the released glycans by reducing the aldehyde ends, which might be necessary for downstream analysis purposes on the glycans. However, the use of the reducing agent (NaBH<sub>4</sub>) was postulated to have affected the main compound necessary for the visualization of the increase absorbance at 240nm. It was reported that during the  $\beta$ -elimination process,  $\alpha$ -aminoacrylic acid and  $\alpha$ -aminobutenoic acid are produced as a result of alkaline hydrolysis of the O-linked glycosidic bond attached between a serine or threonine group to the glycan, which gives the increased absorbance at 240 nm (Yi *et al.*, 2011). The use of the reducing agent, NaBH<sub>4</sub> was reported to reduce both  $\alpha$ aminoacrylic acid and  $\alpha$ -aminobutenoic acid into alanine and  $\alpha$ -aminobutyric acid respectively (Wu *et al.*, 2015). Such a reaction could have resulted in the decreased of  $\alpha$ -aminoacrylic acid and  $\alpha$ -aminobutenoic acid, and hence resulting in a less significant increase in the absorbance at 240 nm. This could be the reason why there was a greater increase in absorbance in the absence of NaBH<sub>4</sub> (**Figure 29a**) compared to that of in the presence of NaBH<sub>4</sub> (**Figure 30a**). With the above rationale, it is safe to say that the data obtained from the  $\beta$ -elimination in the absence of NaBH<sub>4</sub> is more reliable, which allows for the conclusion that ISPP-Rb contains a small portion of O-linked glycosidic bonds.

## 3.23 Cytokine Analysis of ISPP-Rb

Throughout the entire study of the immuno-stimulatory activity of the compound from the water extracts, E3 of *R. badius*, the validation of its bioactivity was entirely dependent on detection of TNF- $\alpha$  produced by RAW 264.7 cells. This suggests that the active compound functions through a proinflammatory response. Hence, the supernatants of the ISPP-Rb treated RAW 264.7 cells were collected and sent to Eve Technologies to screen for other cytokines/chemokines induced using the Mouse Cytokine 32-Plex Discovery Assay. **Table 31** shows the cytokines and chemokines that were significantly induced by the active compound, while **Table 32** depicts the abbreviations and full names of the cytokines listed in **Table 31**.

Immuno		Replicate 1		Replicate 2	
Response	Cytokine	Blank	ISPP-Rb	Blank	ISPP-Rb
T <sub>H</sub> 1	ΤΝΓ-α	2.07	363.25	3.89	445.33
T 17	IL-17	< 0.10	2.01	< 0.10	2.07
1 H1 /	IL-6	< 0.67	2.28	< 0.67	27.63
	IP-10	< 0.05	43.51	1.52	87.66
	MIP-2	7.27	1528.48	23.08	5493.2
	G-CSF	< 0.41	54.62	< 0.41	705.67
	<b>GM-CSF</b>	< 0.77	19.78	0.77	192.76
Other	IL-1a	<1.89	6.46	<1.89	8.41
Chemokines	1L-1β	4.96	8.94	1.88	5.4
	LIF	< 0.17	8.19	< 0.17	32.97
	MIP-1a	108.71	6564.67	340.68	6463.46
	MIP-1β	96.1	35133.11	337.21	177428.68
	RANTES	< 0.60	3.43	<0.60	6.29

**Table 31**. Major cytokines and chemokines induced with the treatment of the purified active compound, ISPP-Rb. Data presented are from two separate experiments.

**Table 32**. Major cytokines and chemokines induced with the treatment of the purified active compound, ISPP-Rb.

Abbreviation	Full Name
TNF-α	Tumor-Necrosis Factor
IL-17	Interleukin-17
IL-6	Interleukin-6
IP-10	Interferon Gamma-Induced Protein 10
MIP-2	Macrophage Inflammatory Protein 2
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
IL-1α	Interleukin-1a
1L-1β	Interleukin-1 <sup>β</sup>
LIF	Leukemia Inhibitory Factor
MIP-1a	Macrophage Inflammatory Protein 1α
MIP-1β	Macrophage Inflammatory Protein 1β
RANTES	N/A

Based on **Table 31**, it can be seen that the major cytokines induced by ISPP-Rb were mainly proinflammatory such as are TNF- $\alpha$ , IP-10, MIP-2, G-CSF, GM-CSF, MIP-1 $\alpha$ , and MIP-1 $\beta$ . The data shows that the active compound mainly stimulates the T<sub>H</sub>1 and/or T<sub>H</sub>17 response. Because the cells were treated with an unknown dose of the bioactive compound directly eluted from the Hi Prep Sephacryl S500 column, the average values were not able to be estimated and hence are presented as two separate experimental data.

## 3.24 Effect of ISPP-Rb on the Phagocytic Activity of RAW 264.7 Cells

With the cytokine data depicting the induction of proinflammatory response, it was decided to see if the bioactive compound would be able to results in cell phagocytosis. The optimization protocols are as described in section 3.12. Initial results suggested that the assay should be carried out in DMEM without FBS due to the fact the FBS resulted in untreated RAW 264.7 cells to continue to proliferate. Such a phenomenon resulted in an increase in cell density in control wells compared to those treated with LPS (data not shown), making the experimental condition of the controls different from the treated group. The subsequent experiments were resumed using DMEM without FBS as the media of choice, however, due to inconsistencies obtained from the absorbance reading after several trials, the experiment was abandoned.
# **Chapter 4**

# **General Discussion and Future Directions**

This chapter aims to compare and contrast the immuno-stimulatory polysaccharide-protein complex isolated from *Royoporus badius* (ISPP-Rb) with immuno-stimulatory mushroom polysaccharide/polysaccharide-protein complex reported in the literature. Recommendations and suggestions will also be given to further improve the future execution of this research study.

#### 4.1 Bioactivity Studies on the Mushroom Genus *Royoporus*

To the best of my knowledge, there are currently only 3 species categorized in the genus *Royoporus*, namely *Royoporus badius*, *Royoporus spathulatus* (Jungh.) De and *Royoporus pseudobetulinus* (Murashk. Ex Pilát) A.B. De (Doskocil *et al.*, 2016; Karun & Sridhar, 2017; Sotome *et al.*, 2011). However, out of the three species, only *R. badius* has been studied for its bioactivity, but with very limited information available. One study showed that the ethanol extract of *R. badius* exhibited little cytotoxic activity towards colorectal cancer cell lines and had some phenolics present in the extract (Doskocil *et al.*, 2016). Elsewhere, the ethanol extracts of *R. badius* successfully inhibited the production of proinflammatory cytokines but elicited T<sub>H</sub>2 response (Koch *et al.*, 2002). In the two studies, there were no reports on possible compounds responsible for the bioactivity. The general lack of bioactivity studies described for the genus *Royoporus*, and the absence of any report on immuno-stimulatory compound from *R. badius* lends support for the novelty of the research described in my study.

# 4.2 General Project Overview

The crude extracts of mushroom *R. badius* was initially screened for immuno-stimulatory activity by an undergraduate student (Mr. Jatinder Khatra) in Dr. Lee's lab, and it was discovered that the water extract E3 of the polypore exhibited potent immuno-stimulatory activity on mouse

macrophage RAW 264.7 cells. With that initial finding, I started my research study on *R. badius*, which involved purifying and characterizing the immuno-stimulatory compound from E3 of the mushroom. LH20 Sephadex<sup>TM</sup> size-exclusion chromatography was first used in the purification step. Based on the elution volume from LH20 Sephadex<sup>TM</sup>, the bioactive compound was predicted to be relatively large and was subjected to purification using DEAE Sephadex<sup>TM</sup> anion-exchange chromatography, followed by a high-resolution size-exclusion chromatography (SEC) using Sephacryl S500 as the second purification step (Chapter 2). The purified immuno-stimulatory compound, ISPP-Rb was then subjected to various biological and chemical characterization (Chapter 3).

#### 4.3 **Purification of the Water Extract E3 from** *R. badius*

# 4.3.1 Using LH20 Sephadex<sup>TM</sup> to Purify the Water Extract E3 from *R. badius*

LH20 Sephadex<sup>TM</sup> is a separation matrix used in SEC, with an exclusion limit of approximately 4-5 kDa. The water extract E3 of *R. badius*, when subjected to the LH20 Sephadex<sup>TM</sup> column, eluted the immuno-stimulatory compound at an early elution volume, suggesting that the immuno-stimulatory compound is relatively large. Carbohydrate analysis using the Phenol-Sulphuric assay showed that the compound was likely to be a polysaccharide. The estimated large size of the immuno-stimulatory compound and the possibility of it being a polysaccharide suggested that LH20 Sephadex<sup>TM</sup> might not be a suitable separation matrix for the purification of the immuno-stimulatory compound.

# 4.3.2 Using DEAE Sephadex<sup>TM</sup> A-50 to Purify the Water Extract E3 from *R. badius*

Various amine buffers were assessed for possible use as the buffer system with DEAE Sephadex<sup>™</sup> A-50 purification. All 6 buffers, Piperazine (pH 5.33), Bis Tris (pH 6.48), water (pH 7.00), Triethanolamine (pH 7.76), Propane-1,2-Diamino (pH 8.88) and Ethanolamine (pH 9.50) were shown to be suitable for the elution of the immuno-stimulatory compound using 1 M NaCl

dissolved in their respective buffers (see **Figure 6**). Such a phenomenon can be explained by the fact that the immuno-stimulatory compound is likely to have an isoelectric point (pI) lower than pH 5.33. Amongst all buffers experimented, Bis Tris (pH 6.48) was selected based on the fact that it took little time to equilibrate. Despite water being widely used in DEAE purification of polysaccharides (Chen *et al.*, 2017; Yuan *et al.*, 2015), the immuno-stimulatory compound eluted from the water-buffered DEAE column gave the least immuno-stimulatory activity as compared to the buffer systems experimented (see **Figure 6**). The fact that water being a poor buffer would result in the possibility of pH fluctuations occurring during column runs; hence, water was not used for DEAE purification.

One of the major advantages offered by DEAE as the first purification step was the matrix's high loading capacity as compared to SEC. The loading capacity of size-exclusion columns are restricted by the matrix's loading capacity which results in less amount being able to be loaded per column run. Challenges encountered in DEAE are the long column run hours and the need to rota-evaporate and dialyze eluted fractions containing high amounts of salt.

### 4.3.3 Using Hi-Prep Sephacryl S500 to Purify the Water Extract E3 from *R. badius*

SEC, otherwise known as gel-permeation chromatography equipped with detection system units is by far the most technologically advanced and easily accessible equipment available for proper separation and size estimation of polysaccharides (Vilaplana & Gilbert, 2010). The Hi-Prep Sephacryl S500 is a high-resolution SEC with a separation capacity of 40-2000 kDa and was used as the second step purification. The buffer solution used was degassed 150 mM NaCl in MilliQ water to reduce interaction between the compound(s) with the matrix of the column. The fractions were collected and assessed for immuno-stimulatory activity on RAW 264.7 cells. Active fractions were then pooled, dialyzed, lyophilized and named ISPP-Rb. Prior to pooling the active fractions, each individual fraction was also assessed for their respective carbohydrate and protein contents. Despite the presence of both carbohydrates and protein detected in the peak active fraction, the carbohydrate and protein peak were detected later in the subsequent fractions (see **Figure 18a-b**). This suggested the possibility that unwanted carbohydrates and proteins were co-eluted with the immuno-stimulatory compound. The use of HPLC will allow for purity confirmation using the refractive index detector (RID). Based on Sephacryl S500, the size of the immuno-stimulatory compound was estimated to be approximately 400 kDa using dextran standards (see **Figure 19**).

The advantage of using the Hi-Prep Sephacryl S500 was that the column was equipped with the ÄKTA FPLC System allowing visualization of the chromatogram. Furthermore, the preparative column allowed for an increase in loading capacity, which is often limited in sizeexclusion columns and analytical columns.

# 4.4 Characterization of the Water Extract E3 from *R. badius*

#### 4.4.1 Chemical Composition of ISPP-Rb

Carbohydrate and protein analysis using GC-MS and BCA assay showed that ISPP-Rb contained 57.4% carbohydrate and 16.7% protein, with the remaining 25.9% still unknown. A similar pattern was observed elsewhere in the literature (Chen *et al.*, 2008), whereby a polysaccharide (AAP) extracted from *Auricularia auricula* (Bull.) J.Schröt. was reported to contain 42.5% of carbohydrate, 19.6% uronic acids, with the remaining made up of sulfate groups, nitrogen, and ash. Hence, it is possible that the unknown 25.9% of ISPP-Rb are a mixture of other inorganic components.

 $\beta$ -glucans are naturally occurring heterogenous polysaccharides. The immuno-stimulatory effect of mushroom  $\beta$ -glucans have been extensively reported, which includes but is not limited to

β-glucans from *L. edodes*, *Schizophyllum commune* Fries, and *Sclerotium glucanicum* Halleck (Kim *et al.*, 2011). This led to the interest in investigating the carbohydrate portion of ISPP-Rb for the presence of β-glucans. However, it was discovered that ISPP-Rb contain none or very little amount of β-glucan, which is surprising due to the fact that the production of β-glucans are mainly found in fungi as compared to seaweeds, bacteria and cereal (Novak & Vetvicka, 2008). The results were interesting considering that not only are β-glucans found naturally in fungi, but also due to the presence of approximately 40% of glucose monosaccharides detected using GC-FID analysis on ISPP-Rb. However, it eventually became clear upon the results from GC-MS analysis; these results showed that the source of the glucose monosaccharides was derived from the presence of  $(1 \rightarrow 3)$ -and/or  $(1 \rightarrow 6)$ -Glc linkages further confirm the absence or extremely low amount of β-glucan in ISPP-Rb.

The preliminary monosaccharide content data from GC-FID showed that ISPP-Rb were made up mainly of galactose (45.8%) and glucose (39.8%), with only 10% of mannose and less than 5% each of xylose and fucose (see **Table 29**). The data was consistent with the linkage analysis from GC-MS (see **Table 30**), with glucose and galactose linkages accounting for the majority of all the carbohydrate linkages. The backbone of the polysaccharide contains a linkage mixture of  $(1 \rightarrow 6)$ -Gal (13.0%) and  $(1 \rightarrow 6)$ -Man (16.3%), with a branching point of  $(1 \rightarrow 2, 6)$ -Glc (39.7%) having unsubstituted, fucose (8.2%), glucose (6.0%) and galactose (16.8%). However, xylose linkages were not detected, which is likely due to the low amounts (1.3%) present. The carbohydrate linkage analysis data showed that ISPP-Rb is a highly branched polysaccharide, with a degree of branching (DB) of 0.71. The high level of branching originated from the presence of the branching point at  $(1 \rightarrow 2, 6)$ -Glc. To the best of my knowledge, only one immuno-modulatory proteoglycan containing such a linkage was only reported. Ye *et al.* (2011) reported an immunoregulative proteoglycan extracted from the fruiting body of *G. lucidum*, capable of enhancing the proliferation of mouse spleen lymphocyte resulting in the activation of B-cells. Elsewhere, the (1  $\rightarrow$  2,6)-Glc linkage was also reported but not for the compounds with immuno-modulatory activities. Pan *et al.* (2012) identified a neutral polysaccharide with anti-hyperglycemic activity isolated from *G. lucidum*, while Scarpari *et al.* (2017) reported a possible polysaccharide containing such a linkage with pro-antioxidant properties. **Table 33** provides a literature list of some non- $\beta$ -glucan immuno-modulatory mushroom polysaccharide/polysaccharide-protein complexes along with their carbohydrate linkages.

Mushroom	Active Compound	Carbohydrate Linkages	Reference
<i>Phellinus</i> <i>igniarius</i> (L.: Fr) Quel	Polysaccharide (PPIP60-1)	<b>Backbone</b> consists of $(1 \rightarrow 6)$ -Gal, $(1 \rightarrow 2,6)$ -Gal, $(1 \rightarrow 2,4)$ -Gal, $(1 \rightarrow 2,6)$ -Man;	(Yang <i>et al.</i> , 2007)
		Side Chain consists of terminal glucose and fucose	
	Polysaccharide (PISP1)	<b>Backbone</b> consisting of $(1 \rightarrow 6)$ -Gal, $(1 \rightarrow 3,6)$ -Man, $(1 \rightarrow 4)$ -Gal, and $(1 \rightarrow 2)$ -Gal;	(Yang <i>et al.</i> , 2009)
		<b>Side Chain</b> contains terminal fucose	
Polyporus rhinocerus	Polysaccharide-Protein Complex (PRW1)	<b>Backbone</b> consist $(1 \rightarrow 4)$ -Glc, $(1 \rightarrow 6)$ -Glc and $(1 \rightarrow 2)$ -Man;	(Liu <i>et al.</i> , 2016)
		<b>Branching/Side chains</b> were (1 $\rightarrow$ 3,6)-Glc, (1, $\rightarrow$ 4,6)-Glc and of terminal glucose	

Table 33. Carbohydrate linkages of some non- $\beta$ -glucan immuno-modulatory mushroom polysaccharide/polysaccharide-protein complexes.

	Polysaccharide (PRW)	<b>Backbone</b> consist of $(1 \rightarrow 2)$ - Man, $(1 \rightarrow 6)$ -Glc, and $(1 \rightarrow 4)$ - Glc; <b>Branching/Side Chains</b> consists of terminal glucose, $(1 \rightarrow 3,6)$ - Glc and $(1 \rightarrow 4,6)$ -Glc	(Liu <i>et al.</i> , 2018)
Ganoderma lucidum	Proteoglycan (LZ-D-7)	<b>Backbone</b> consists of $(1 \rightarrow 4)$ - Glc, and $(1 \rightarrow 2,6)$ -Glc; <b>Side Chain</b> consists of terminal glucose	(Ye <i>et al.</i> , 2011)
	Polysaccharide (FMS)	<b>Backbone</b> consists of $(1 \rightarrow 4)$ - Man and $\alpha$ - $(1 \rightarrow 6)$ -Gal; <b>Side Chains</b> consists of terminal fucose attached to $(1 \rightarrow 2)$ -Gal, $(1 \rightarrow 3/4)$ -Man, $(1 \rightarrow 4)$ -Xyl, and $(1 \rightarrow 2)$ -Fuc	(Liao <i>et al.</i> , 2013)
Hypsizygus marmoreus (Peck) H.E. Bigelow	Proteoglycan (F1) Proteoglycan (F2)	<b>Backbone</b> consists of $\alpha$ - $(1 \rightarrow 3)$ - Glc, $\alpha$ - $(1 \rightarrow 4)$ -Glc, and $\beta$ - $(1 \rightarrow 6)$ -Glc; <b>Branching Chain</b> consists of $(1 \rightarrow 3,6)$ -Glc, $(1 \rightarrow 4,6)$ -Glc, and terminal glucose	(Bao <i>et al.</i> , 2012)
Flammulina velutipes	Polysaccharide (FVPB2)	<b>Backbone</b> consists of $\alpha$ - $(1 \rightarrow 2)$ - Gal, and $\alpha$ - $(1 \rightarrow 6)$ -Glc; <b>Branching Chain</b> consists of (1 $\rightarrow$ 3,6)-Glc, (1 $\rightarrow$ 3,4)-Gal linked with terminal fucose and mannose respectively	(Wang <i>et al.</i> , 2018)

Based on **Table 33**, and to the best of my knowledge, there have not been reports of an immuno-stimulatory mushroom compound containing terminal galactose. Common terminal sugars found are either glucose or fucose. According to the GC-MS data obtained, ISPP-Rb

contained 16.8% of terminal galactose, along with 8.2 % and 6.0 % of terminal fucose and terminal glucose respectively. Other than that, ISPP-Rb is by far the second immuno-stimulatory mushroom molecule that contains the  $(1 \rightarrow 2,6)$ -Glc linkage. Taken together, this could prove that ISPP-Rb is indeed a unique and novel immuno-stimulatory compound.

Reports on mushroom polysaccharides (mainly  $\beta$ -glucans) with high degree of branching exhibiting immuno-modulatory activity are rare. A hyper-branched β-glucans (mPRSon) with a DB of 0.85 was purified from the sclerotia of P. rhinocerus and was shown to exhibit immunomodulatory activity (Liu et al., 2018). Elsewhere, a water-soluble β-glucan (TM3b) isolated from *Pleurotus tuber -rigium* (Rumph. ex Fr.) Singer was found to be extensively branched (DB = 0.58). However, the bioactivity of the polysaccharide was not described (Tao & Zhang, 2006). On the other hand, mushroom polysaccharides ( $\beta$ -glucan) with lower degree of branching (DB  $\leq 0.50$ ) reported are pachymaran, lentinan, pleuran, grifolan, scleroglucan, schizophyllan, and SSG (Novak & Vetvicka, 2008). The only mushroom non-β-glucan, i.e. mushroom polysaccharideprotein complex with a high degree of branching reported was PRW, which was isolated from the sclerotia of *P. rhinocerus*, having a DB of 0.60 and exhibiting potent immuno-stimulatory activity (Liu et al., 2016). From the literature, it is clear that most of the immuno-modulatory polysaccharides are  $\beta$ -glucan, with very limited polysaccharide-protein complexes being immunostimulatory. Hence, the fact that ISPP-Rb is suggested to be a non- $\beta$ -glucan polysaccharide-protein complex containing a high DB of 0.71, suggests that it is a novel immuno-stimulatory compound.

# 4.4.2 Enzyme Degradation Assay on the Immuno-Stimulatory Activity of ISPP-Rb

Experiments were designed to further understand the significance of the carbohydrate and/or protein portion of the purified polysaccharide-protein complex in contributing to the immuno-stimulatory activity. The basis of this study was to treat the active compound with specific

enzymes and see whether its immuno-stimulatory activity is reduced. Any decrease in immunostimulatory activity would suggest the importance of the digested portion (polysaccharide and/or protein) of the compound. Based on the data obtained from section 3.21, I concluded that the protein portion of ISPP-Rb was important for its immuno-stimulatory activity. The treatment of Proteinase K (1 mg/mL) on ISPP-Rb (1.5 mg/mL) for 2 hours at 37°C completely abolished the immuno-stimulatory activity of the compound (see **Figure 23**), indicating that the protein component is indispensable for its immuno-stimulatory activity.

Enzymes that cleave carbohydrate linkages such as  $\alpha$ -amyloglucosidase (AMG),  $\alpha$ -L-Fucosidase (FCS) and  $\alpha$ -Galactosidase (GAL) were also used in the enzyme degradation assay. AMG was selected during the first phase, when the carbohydrate linkage analysis was still unknown. The rationale was the suspicion that the immuno-stimulatory activity of ISPP-Rb was due to the presence of glucans and the fact that AMG is capable of hydrolyzing  $\alpha$ -1,3-,  $\alpha$ -1,4-, and  $\alpha$ -1,6-glucosidic bonds. The use of GAL was because the galactose-linkages (excluding 1,2,6-Glucose) and monosaccharide content were the highest, and it was thought that the high amount of galactose would play a role in the active compound's immuno-stimulatory activity. The use of FCS was based on study reporting that mice immunized with fucose-rich polysaccharides extracted from *G. lucidum* had induced antibodies against Lewis lung carcinoma cells (Liao *et al.*, 2013). However, based on the overall data obtained (see **Figures 22, 24-26**), it was suggested that the carbohydrate portion of ISPP-Rb may not be important for its immuno-stimulatory activity.

To the best of my knowledge, this experiment was the first of its kind as there have been no reports in the literature citing the use of enzymes as an indirect method in assessing the importance of the protein and polysaccharide components in bioactive compounds derived from mushrooms. The advantage of this experiment is the relatively short time required, which includes enzyme treatment followed by cell-based experiment. However, this assay proved difficult due to the lack of suitable positive controls, i.e. polysaccharide/polysaccharide-protein complex with immuno-stimulatory activity. This is because of the differences in carbohydrate and/or protein linkages present in each individual compound. Issues were encountered during the treatment of FCS and GAL on the polysaccharide isolated from *P. aurea* (positive control) and ISPP-Rb, where neither the positive control nor ISPP-Rb showed a change in immuno-stimulatory activity after being treated with FCS and GAL (see **Figures 24-26**). The solution to this was by running the polysaccharide samples treated/not treated with enzymes FCS and GAL on a SDS PAGE gel, which were then visualized using a glycoprotein staining kit to observe any changes in band/smear patterns (see **Figure 27**).

# 4.4.3 Helical Confirmation of ISPP-Rb using Congo Red Assay

The Congo red assays allow for the visualization of the presence of a triple helix structure via an observation of a shift in the maximum absorption of the polysaccharide-Congo red complex as compared to the Congo Red solution (blank) when treated with mild NaOH solution (Smiderle *et al.*, 2014). Ogawa et al. (1972) reported that  $\beta$ -1,3- and  $\beta$ -1,4- glucans will form a complex with Congo red under diluted NaOH conditions. This was consistent with studies involving the use of  $\beta$ -glucans, with  $\alpha$ -glucans failing to form the complex with Congo red (Palacios *et al.*, 2012; Wang *et al.*, 2013). Surprisingly, despite *P. aurea* polysaccharide containing significant  $\beta$ -glucans (see **Table 28**), it failed to bind with Congo red, as indicated by the absence in shift in the maximum absorption (see **Figure 20b**). This phenomenon could be explained by the presence of a protein component on *P. aurea* polysaccharide, which could have hindered the binding process, as was observed with PSK, which is a protein bound  $\beta$ -1,3- and 1,6-glucan but failed to show any shift in maximum absorbance. It was explained that despite both PSK and PSP containing  $\beta$ -glucans, the

presence of a protein molecule attached to the glucan makes them different from pure  $\beta$ -glucans (Chan *et al.*, 2009). The same situation was observed for ISPP-Rb, which also contain a protein portion. Therefore, without a suitable positive control, the results from these experiments remained inconclusive.

#### 4.4.4 Glycosidic Linkages of ISPP-Rb

The  $\beta$ -elimination assay carried out in the absence of the reducing agent, NaBH<sub>4</sub> suggested that there is a small amount of O-linked glycosidic bond present in ISPP-Rb. However, statistical analysis showed that the difference observed was not significant. Despite this, the presence of the O-linked glycosidic bond could indeed be biologically significant and should not be assumed as absent. It is important to point out that fungal glycoproteins containing fucosylated O-glycans (mainly O-mannans), despite being rare, have been reported in chanterelles and *Boletus edulis* (Grass *et al.*, 2011). This further supports the possible presence of the O-linked glycosidic bond in ISPP-Rb and the likelihood that fucosylated O-linked mannans is present in ISPP-Rb.

#### 4.4.5 Cytokine, Chemokine and Phagocytosis Induction in RAW 264.7 Cells by ISPP-Rb

ISPP-Rb was found to mainly induced proinflammatory responses by stimulating the production of TNF- $\alpha$ , IP-10, MIP-2, G-CSF, GM-CSF, MIP-1 $\alpha$ , and MIP-1 $\beta$ . The data shows that the active compound mainly stimulates the T<sub>H</sub>1 and/or T<sub>H</sub>17 response, which led to the rationale that ISPP-Rb would be able to induce phagocytosis in RAW 264.7 cells. Unfortunately, the Neutral Red assay which was designed to visualize macrophage phagocytosis through colorimetry observations were inconsistent and irreproducible. The use of 96-well plates in this experiment increases the chances of error due to the multiple pipetting steps involved. It is thought that with every pipetting step, the adherent cells were disturbed resulting in the fluctuation of cell densities across the wells, which could explain the irreproducible results. A more reliable but much more

sophisticated assay to measure phagocytic activity in RAW 264.7 cells will be to use the flow cytometer. Several studies using the flow cytometer to study macrophage phagocytosis involved the use of biodegradable microspheres/latex beads conjugated to a fluorescence label, fluorescein isothiocyanate (FITC) (Im *et al.*, 2010; Razali *et al.*, 2014).

# 4.5 Proposed Structure for ISPP-Rb

Based on the carbohydrate linkages obtained from GC-MS, the possible presence of the Olinked glycosidic bond, and information from the literature, I proposed a general structure of ISPP-Rb as shown in **Figure 31**.



**Figure 31**. A proposed structure of the polysaccharide component of ISPP-Rb. Fuc*p*, Gal*p*, Glc*p*, and Man*p* represents fucose-pyranose, galactose-pyranose, glucose-pyranose, and mannose-pyranose respectively.

ISPP-Rb contain similar amounts of  $(1 \rightarrow 6)$ -Man and  $(1 \rightarrow 6)$ -Gal linkages, with each accounting for 16.3% and 13.0% respectively. On the other hand, the  $(1 \rightarrow 2,6)$ -Glc linkage was 2-3 times more abundant compared to the prior. With that data, it is very likely that there should be near equal amounts of  $(1 \rightarrow 6)$ -Man and  $(1 \rightarrow 6)$ -Gal linkages, with the  $(1 \rightarrow 2,6)$ -Glc linkage being at least twice as much as compared to the previous two. The presence of terminal fucose, glucose and galactose would suggest that they will be linked by a Fuc/Gal/Glc- $(1 \rightarrow 2)$ -Glc linkage. In this study, I reported the importance of the protein component for the immuno-stimulatory activity of ISPP-Rb. It is suggested that the protein component of ISPP-Rb is being O-linked to the mannose sugar of the polysaccharide. This is because mannoproteins are commonly found in the cell walls of fungi (Klis *et al.*, 2006), with O-mannans being reported to be found in chanterelles and *Boletus edulis* (Grass *et al.*, 2011). In addition, an immuno-stimulatory polysaccharide-protein complex linked to a mannose residue have been previously reported (Liu *et al.*, 2016). To confirm the structure of ISPP-Rb, high resolution NMR studies will be required.

#### 4.6 Conclusion and Future Directions

An immuno-stimulatory polysaccharide-protein complex was successfully purified from the water extract E3 of *R. badius* and was named ISPP-Rb. The immuno-stimulatory compound is water soluble and has a molecular weight of approximately 400 kDa. The active compound is relatively heat stable and definitely requires the protein portion for its immuno-stimulatory activity. Further detailed studies are needed to determine whether the polysaccharide portion of ISPP-Rb is critical for its immuno-stimulatory activity. ISPP-Rb has a highly branched polysaccharide and likely linked to its protein component at least through O-glycosidic bonds. ISPP-Rb induces proinflammatory cytokines and chemokines. This is the first study to report on a large immunostimulatory polysaccharide-protein complex isolated from the mushroom *R. badius*.

The chemical characterization studies conducted on ISPP-Rb is still only preliminary and requires further investigation. HPLC is required to check for purity of the isolated compound. The pure sample should be sent for high-resolution NMR analysis to elucidate the structure of its polysaccharide component. N-glycosidase enzyme PNGase F could be used to determine the possible presence of N-linked glycosidic bond. If N-linked glycosidic bonds are present and the protein component is released and detected on a SDS-PAGE gel upon PNGase F treatment, the protein gel slice could be sent for N-terminus amino acid sequencing analysis. With the N-terminus

amino acid sequence information, the identity of the active protein molecule, if any, could be determined using the National Center for Biotechnology Information (NCBI) database.

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