DIGESTION OF AGRICULTURAL PRODUCTS BY MICROWAVE FACILITATED HYDROLYSIS

BY

MS. SOMRUTHAI PHOTHIPHIPHIT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (ENGINEERING AND TECHNOLOGY)

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was approved as partial fulfillment of the requirements for
the degree of Master of Science (Engineering and Technology)

on August 17, 2021

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ABSTRACT

The thesis covers the experimentation done on microwave assisted digestions of coconut copra meal, grey oyster mushroom and jew's ear mushroom. Jew's ear mushroom *(Auricularia A. auricula-judae)* and grey oyster mushroom *(Pleurotus sajor-caju)* are edible basidiomycetous fungi, and well-known as healthy food in East Asian countries. The work focuses on the application of microwave radiation heating with HCl as a catalyst to hydrolyze the agricultural products into polysaccharides, oligosaccharides and monosaccharides. The study was carried out to screen for the reaction temperature and HCl concentration that gives the highest digestion yield of CCM, grey oyster mushroom and jew's ear mushroom to obtain the desirable oligosaccharides and soluble polysaccharides. The size determination of the hydrolyzed samples was carried out by size exclusion chromatography (SEC). The highest yield of oligosaccharides obtained by the hydrolysis of CCM is 66.71% of oligosaccharides containing 2 to 18 units of monosaccharide from the hydrolysis conditions at 110 °C, the reaction time of 15 min, with 0.2 M HCl solution, and 80:1 (mL/g) ratio of acid solution to CCM. The reaction conditions at 170 °C, 15 min, 0.05 M HCl, and 10:1 (mL/g) ratio of acid solution to CCM provides the highest yield of monosaccharides (45.68%).

The method to hydrolyze glucan polymers from grey oyster mushroom and jew's ear mushroom using HCl as a catalyst and microwave radiation was investigated to obtain the highest yield of glucan-oligosaccharides. The best conditions for the hydrolysis of grey oyster mushroom which resulted in oligosaccharides is at 120 °C, the reaction time of 15 min with 0.6 M HCl solution provide the maximum yield of 2.24%. The oligosaccharides obtained at these conditions have around 2-5 units. The best conditions for the hydrolysis on jew's ear mushroom, which result in oligosaccharides is at 130 °C, the reaction time of 15 min, and with 0.6 M HCl solution provide the maximum yield of 1.28%. The oligosaccharides obtained at these conditions have around 2-20 units. The products were evaluated as a potential immune immunomodulatory agent to induce the immune response against the antigen keyhole limpet hemocyanin (KLH).

Keywords: Jew's ear mushroom, *Auricularia (A.) auricula-judae*, Grey oyster mushroom, *Pleurotus sajor-caju*, Coconut meal, Microwave radiation, Hydrolysis, Beta-glucans

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Ms. Somruthai Phothiphiphit

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LIST OF SYMBOLS/ABBREVIATIONS

Symbols/Abbreviations	Terms
CCM	Coconut Copra Meal
GP	Grey Oyster Mushroom
EP	Jew's Ear Mushroom
HPLC	High Performance Liquid Chromatography
SEC	Size Exclusion Chromatography
KLH	Keyhole Limpet Hemocyanin
NMR	Nuclear Magnetic Resonance
PS	Pure Polysaccharide
SL	Solid Loss
TC	Total Carbohydrate
RS	Reducing Sugar
RO	Reverse Osmosis
HCl	Hydrochloric acid

CHAPTER 1
INTRODUCTION

Recently, finding potential and affordable prebiotic sources in the food industry has drawn much attention in food research. One of the most interesting prebiotics is mushrooms because they contain carbohydrates such as β- and α-glucans, mannan, xylan, galactan, hemicellulose, and chitin (Roberfroid, 2007). The mixture of lignin, polysaccharides, and other plant cell wall constitutes dietary fiber, which is resistant to hydrolysis by human enzymes and has interesting properties. It speeds up the transit of bowel contents, increases frequency and faucal bulk, and protects from irritable bowel syndromes, diverticular diseases, and colon cancer. Additionally, the levels of cholesterol in the blood can also be reduced and against coronary disease can also be prevented (Roberfroid, 2007; Macfarlane et al., 2008).

The different types of glycosidic linkages in most mushroom polysaccharides are linear and branched glucan such as (1,3), (1,6) -β-glucan and (1,3) -α-glucans. The β-glucan form is the most abundant type of glucan. Some polysaccharides present are heteroglycans, including fructose, arabinose, mannose, galactose, xylose and glucose as main side-chain components present in different combinations (Roberfroid, 2007; Macfarlane et al., 2008; Tuohy et al., 2001). The beneficial properties of mushrooms have an important role in the hypothesis, such as enhancement of macrophage function and host resistance to many viral, bacterial, parasitic infections and fungal including activation of the reduction of blood cholesterol, blood glucose levels, and non-specific immune stimulation (Roberfroid, 2007; Macfarlane et al., 2008; Ooi & Liong, 2010).

For the productions of oligosaccharides, the synthesis of oligosaccharides is more difficult than other polymers including nucleic acids and peptides. The reactions need to be controlled specifically to prevent degradation of oligosaccharides. The synthesis process is more expensive, difficult to reproduce on a large scale, with low yields (Brownawell et al., 2012). Extractions of oligosaccharides from abundantly available non-cellulosic agricultural products can efficiently provide the valuable saccharides for consumptions. The ideal extraction method should simplify the oligosaccharide production process, lower the cost, reduce oligosaccharide degradation, and give high yields. The efficient oligosaccharide extraction method would make commercial-scale production feasible.

1.1 Prebiotics

Certain oligosaccharides which are not-digestible by humans are considered as prebiotics. Prebiotics are ingested by the bacteria present in human and animal digestive systems (Tuohy et al., 2001; Slavin, 2013). Thus, prebiotics remain in their original state when they reach the colon because it cannot be digested and absorbed by the upper gastrointestinal tract (Langen et al., 2009). Microflora in human colons, such as *Bifidobacteria* and *Lactobacilli,* are stimulated by consuming prebiotics.

Only certain types of fiber are considered as a prebiotic. Mammalian enzymes and gastric acid present in the stomach cannot hydrolyze plant fibers, thus the upper part of the gastrointestinal tract cannot absorb fibers. Fiber passes through the gastrointestinal tract, and it is fermented by the beneficial microflora, *Bifidobacteria* and *Lactobacilli*, in the colon. *Bifidobacteria* and *Lactobacilli* were reported to inhibit the pathogen growth in the gut, decrease the cholesterol level, improve the immune system, and produce vitamins (Gibson, 1998; Holzapfel et al., 1998; Vanderhoof & Young, 1998). *Bifidobacteria* and *Lactobacilli* provide various health benefits to the host (Slavin, 2013; Langen et al., 2009).

Benefits of prebiotics include anti-cancer and anti-inflammatory activities, and anti-hypercholesterolemia properties. Prebiotics promote bone stability, the lipid metabolism, and the absorption of minerals, such as iron, magnesium, and calcium. In addition, prebiotics can prevent obesity, and prevent diarrhea. The host gains significant benefit from a symbiotic effect of probiotics and prebiotics (Ziemer & Gibson, 1998; Hui et al., 2006).

Prebiotics can be incorporated into many foodstuffs, such as drinks, biscuits, yoghurts, spreads, breakfast cereals, beverages, dairy products, bakery products, infant foods, pet foods, and animal feeds (Tuohy et al., 2001). The Dietary Guidelines for Americans 2010 Committee (DGAC) suggests that an important key to the human health is the gut microflora, and thus, human should consume food with high prebiotic content (Ziemer & Gibson, 1998). The Europeans and The Americans consume large amount of prebiotics each day. The recommendation on the prebiotic intake depends on functions of prebiotics, for example, the Korean Food and Drug Admission (FDA) suggested that consumption of 9 to 10 grams of inulin per day helps maintaining a

healthy blood cholesterol and a postprandial glucose level (Ziemer & Gibson, 1998). In the study of Binns (2013), it was reported that the target level for general consumption of prebiotics in foods are 2 to 20 grams per day.

1.2 Sources of prebiotics

1.2.1 Coconut meal (CCM)

A coconut is an important versatile crop in Asian and South America whose parts can be utilized to create a variety of products. Products from coconut, such as milk, juice, oil, and meat are sources that feed and nourish populations around the world. As a food source one-third of the population around the world rely on coconut plants (Ramaswamy, 2014).

Coconut meal (coconut residue, copra meal, coconut dregs, coconut flour) is a by-product from the production of coconut milk (Khuwijitjaru et al, 2014). Coconut meal is rich in fibers, minerals, and vitamins. It is believed to be functional food because it provides many health benefits beyond its nutritional contents. Coconut meal is naturally low in digestible carbohydrates (Khuwijitjaru et al., 2012). The component of coconut meal depends on coconut milk or oil extraction methods, including wet and dried methods in Table 1.1.

Even though human enzymes cannot digest coconut meal and utilize the available proteins (Khuwijitjaru et al., 2012), coconut meal provides many health benefits. It can improve digestion, protect against diabetes, help regulate blood sugar, aid in weight loss, and help prevent cancer and heart disease. Utilization of coconut meal as a food ingredient provides dietary fiber to human diets.

Table 1.1 Proximate components of coconut meal.

Composition	Dry process (%)	Wet process (%)
Moisture	4.5	6.7
Fat	10.7	10.9
Crude fiber	40.7	60.9
Protein	17.5	10.8
Ash	5.5	3.16
Carbohydrate	61.8	68.5

Coconut meal contains 45–70% of carbohydrate components depending on the source of the coconut meal (Ramaswamy, 2014; Khuwijitjaru et al., 2012; Khuwijitjaru et al, 2014). The carbohydrate content in the coconut meal composes of 61% mannan polysaccharide along with other polysaccharides, including galactoglucomannan, galactomannan, arabinomannogalactan, arabinoxylogalactan, and cellulose. Figure 1.1 shows structure of various types of monosaccharides found in coconut meal (Khuwijitjaru et al., 2012), monosaccharides found in coconut meal are approximately 1.3% arabinose, 6.1% galactose, 12.8% glucose, and 79.8% mannose. The glycosidic linkage found in these polysaccharides is β-1,4 glycosidic bond (Figure 1.2) With this specific linkage, coconut meal cannot be hydrolyzed in the digestive system of human and animals (Kusakabe et al., 1983).

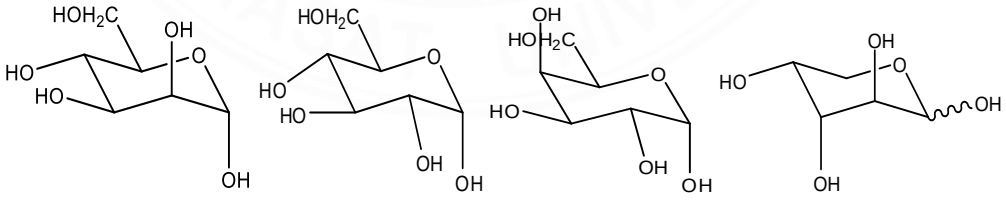

Figure 1.1 Structures of monosaccharides found in coconut meal; mannose, glucose, galactose, and arabinose (Khuwijitjaru et al., 2012).

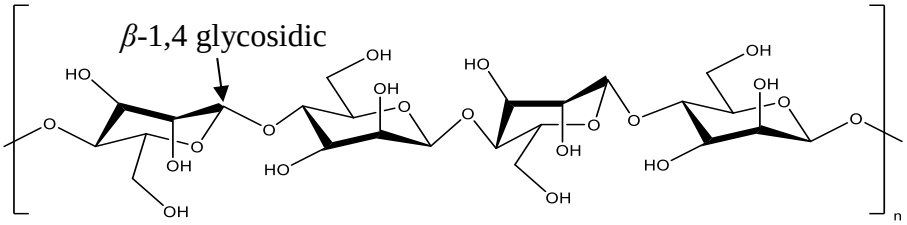

Figure 1.2 The *β*-1,4 glycosidic linkage in mannan polysaccharide (Kusakabe et al., 1983).

1.2.2 Grey oyster mushroom and jew's ear mushroom

Recent demands of prebiotics in the food industry have increased the need to search for a new potential source of prebiotics. Mushrooms can be a potential candidate for prebiotics because it contains carbohydrates such as β- and α-glucans, hemicellulose, chitin, galactans, xylans and mannans (Aida et al., 2009).

Most of mushrooms polysaccharides are present as linear and branched glucans with different types of glycosidic linkages such as (1,3)-α-glucans and (1,3), (1,6)-β-glucans. The most abundant type of glucan present is in beta-glucan form. Other than glucans, some polysaccharides are heteroglycans containing glucuronic acids, glucose, mannose, arabinose, galactose, fructose, and xylose as main side chain components present in different combinations (Aida et al., 2009; Synytsya et al., 2009; Shuqin et al., 20212). These compounds have important roles in beneficial properties of mushrooms, such as enhancement of host resistance to many viral, bacterial, parasitic infections, and fungal and macrophage function; activation of the non-specific immune stimulation; and reduction of blood glucose levels and blood cholesterol (Shuqin et al., 20212; Zhaocheng et al., 2008).

The most cultivated mushrooms worldwide are oyster mushrooms *(Pleurotus* spp), wood ear mushroom *(Auricula auricular),* button mushroom *(A. bisporus*) followed by Lentinus edodes (shiitake), straw mushroom *(Volvariella volvacea*), and winter mushroom *(Flamulina velutipe*) (Jantaramanant et al., 2014; Kadnikova et al., 2015).

The structure of mushroom consists of three tissue layers. The outer tissue contains the soluble-glucan (mucilage), the second part contains the alkaline-soluble glucan (α-1,3-glucan), and inner tissue contains the alkaline-insoluble glucan (β-1,3-

glucan) and chitin. About 50-90% of mushroom polysaccharides found in the cell walls are beta-glucan (Synytsya et al., 2009; Jantaramanant et al., 2014; Kadnikova et al., 2015). Other polysaccharides present in the cell wall are glycogen, chitin, xylan and cellulose. The structures of polysaccharides found in mushroom include (1,3), (1,6)-β-glucans and (1,3)-α-glucans. The *β*-1,3-D-glucan and *β*-1,6-D-glucan forms the backbone chain of the polysaccharides. The health benefits of mushroom polysaccharides include reduction of blood cholesterol level, reduction of hyperglycemia and hyperinsulinemia; control of diabetes mellitus; reduction of risk factors for degenerative diseases such as cardiovascular diseases, cancer, hypertension; and promotion of the beneficial gut microflora growth (Aida et al., 2009).

Oyster mushrooms *(Pleurotus sajor-caju)* are easy to cultivate and commonly found worldwide. This edible fungus can be beneficial to the body and break down toxic chemicals. Oyster mushroom polysaccharides can act as a prebiotic and stimulate the growth of colon microorganisms (probiotics). Mushrooms extract of P. ostreatus and P. eryngii were able to stimulate the growth of probitics such as *Lactobacillus* spp., *Bifidobacterium* ssp. and *Enterococcus faecium*. The water-soluble portion of grey oyster mushroom consists of protein, mannan, galactose, and glucose. The main components of polysaccharides are glucan and mannan (Synytsya et al., 2009; Maftoun et al., 2015; Jantaramanant et al., 2014).

Figure 1.3 Fresh grey oyster mushroom.

Jew's ear mushroom *(Auricularia auricula-judae),* an ear-like shaped edible fungus, belongs to the family of Heterobasidiae. It mainly contains of β-glucan with potential antitumor activities. The *Auricularia* mushroom species is the fourth most

cultivated mushroom used by humans worldwide. *Auricularia* (A.) *auricula-judae* is an edible basidiomycetous fungus, and is well-known as a tonic and health food in East Asian countries than other *Tremella* (T.) species such as *T. f uciformis*, *T. aurantia*, and *T. mesenterica*, which belongs to the jelly mushroom group that contains gelatinous fruiting bodies (Shuqin et al., 2012; Zhaocheng et al., 2008).

Figure 1.4 Fresh jew's ear mushroom.

Beta-glucan is a type of carbohydrates consisting of linked glucose molecules in mushroom. They are major cell wall structural components of some bacteria fungi, and yeast. Some cereals such as oat and barley contain β-glucans as part of their endosperm cell wall (Hui et al., 2006). The carbohydrate structure and glycosidic bonds vary depending on the sources of beta-glucans as shown in Figure 1.5

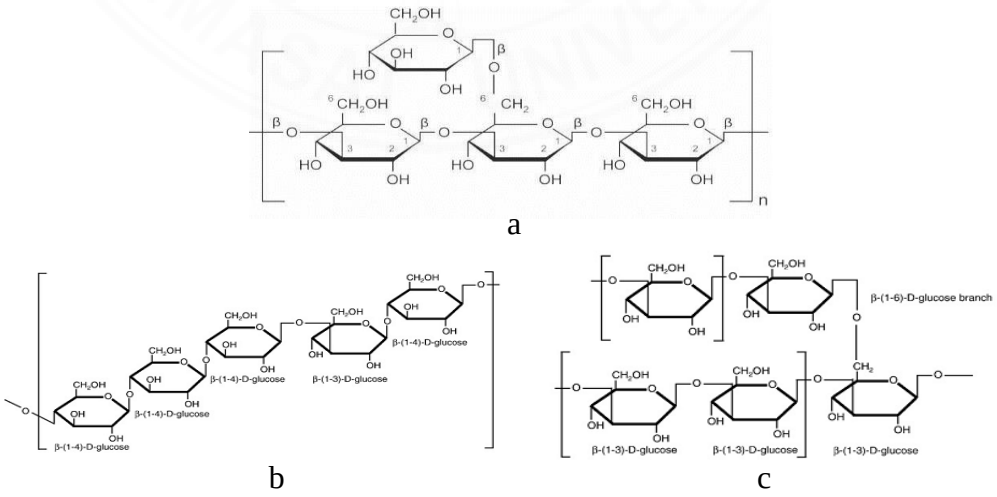

Figure 1.5 Chemical structures of beta-glucans from; a) fungi; b) cereal; and c) yeast (Hui et al., 2006).

The polysaccharides that are commonly found in cereals are β-1,3-D-glucan and β-1,4-D-glucan (Synytsya et al., 2009; Jantaramanant et al., 2014). The molecule mainly consists of cellotriose and cellotetraose blocks separated by β-1,3 linkage.

The most abundant polysaccharides found in mushroom are β- and α-glucans, galactans, xylans, mannans, hemicelluloses, and chitin. Polysaccharides found in mushroom are present mostly as linear and branched glucans with different types of glycosidic linkage such as β-1,3,/ β-1,6- glucans and α-1,3-glucans (Synytsya et al., 2009; Jantaramanant et al., 2014; Hui et al., 2006; Shuqin et al., 2012). The β-glucan oligosaccharides has been produced by using lichenase and β-glucosidase.

1.3 Microwave extraction

A new technique needs to be developed for the extraction of polysaccharides and oligosaccharides. It should aim towards increasing the extraction yield. The extraction can be done by modifying the structure of the biomass or removing unwanted compounds which occur during the traditional extraction step. The traditional extraction methods which are cold or hot water treatment will use changes in temperature of water and time of extraction to increase the productivity of extraction. Currently, the ultrasonic extraction and microwave are used to recover the polysaccharides from different plant materials, mainly because of their capacity to increase disruption of cell, solvent penetration, and mass transfer (Xie et al, 2010).

Microwave radiation is surveyed for utilization as a heat source. It has been explored for its promising applications in many industries, such as food drying, food processing, polymer syntheses, and organic synthesis (Kappe, 2004). When compared to other heating methods, microwave radiation heating has more advantages in many aspects, including non-contact heating, energy transfer as a substitute for heat transfer, rapid heating, short reaction time, energy savings because of volumetric heating, homogeneous heating from the interior of a material, and quick start-up and stopping mechanisms (Kappe, 2009; Menéndez, 2010; Haque, 1999). Furthermore, the amount of acid catalyst required for biomass hydrolysis reaction with microwave radiation heating is reduced by twenty-folds. In addition, the structure and quality of desirable products are not affected by microwave radiation heating (Tanaka et al, 2013).

In this study, we investigated the combination of microwave heating and acid catalysis to promote the hydrolysis of polysaccharides in CCM, grey oyster mushrooms and jew's ear mushroom into possibly more biologically active oligosaccharides. The limitations of hydrolysis reaction done previously are 1) long reaction time, 2) low yields, and 3) high cost (Shaheen, 2012; Ahmed, 2004; Zhang, 2006). The hydrolysis may take a long time to complete, so microwave irradiation is also used in the hydrolysis reaction to speed up the process. The microwave facilitated hydrolysis would constitute a simple, cost-effective, and reproducible protocol on an industrial scale (Thostenson, 1999).

The method may contribute to solving the complications involved in the scale-up of oligosaccharide extraction in the food industry. We investigated the effect of acid concentrations, reaction temperatures, and optimization conditions on the yield of oligosaccharides by microwave facilitated acid hydrolysis of CCM, grey oyster mushroom and jew's ear mushroom**.** The obtained oligosaccharide products from CCM, grey oyster mushroom and jew's ear mushroom were characterized for their molecular weight by using size exclusion chromatography (SEC) analysis method. ^{1}H NMR was used to confirm product structure and the product was tested the adjuvant activity.

CHAPTER 2
REVIEW OF LITERATURE

Hydrolysis is the process that breaks down the biomass into smaller oligosaccharide by the addition of a water molecule (Kusakabe et al, 1983). Hydrolysis can be done by using physical method, enzymatic, or chemical methods. Biomass treatment should be first step before hydrolysis to remove lignin and cellulose. It allows the acid catalyst to access the hydrolysis sites better.

2.1 Biomass treatment methods

2.1.1 Mechanical size reduction

Mechanical size reducing includes shredding, grinding, milling, chipping, and coarse size reduction. The purpose of mechanical reducing size is to increase the specific surface area of biomass and reduce both polymerization and crystallinity of cellulose. The optimal size of biomass for good rate and high yield of biomass hydrolysis is 0.4 mm. Mechanical size reduction is the first step in the biomass treatment (Agbor et al., 2011; Kumar et al., 2009; Zheng et al., 2009).

2.1.2 High energy radiation

UV radiation, γ-ray, electron beam, electrical field, microwave heating, and ultrasound are high energy radiation used to treat biomass. They decrease the crystallinity of cellulose and degree of polymerization, hydrolyze hemicellulose, and partially de-polymerize lignin (Kumar et al., 2009). However, disadvantages of high energy radiation are that they are energy-intensive, and expensive. Moreover, they may raise environmental and safety concerns (Zheng et al., 2009).

2.1.3 Biological treatment

Biological treatment uses fungus, such as brown-rot, white-rot and soft-rot fungus, and bacteria as enzymes to treat biomass. Fungus is more common than bacteria in the treatment for biomass. White-rot fungus is widely used compared to the soft-rot and brown-rot fungus. The fungus produces enzymes that degrade lignin, hemi-cellulose, and poly-phenols (Agbor et al., 2011; Zheng et al., 2009). Brown-rot fungi

primarily attack cellulose whereas the white rot and the soft-rot fungi attack both lignin and cellulose (Kumar et al., 2009; Zheng et al., 2009).

The disadvantages of biological treatment include being too slow for industrial purposes. Normally, the biological treatment requires 10–14 days to effectively complete the treatment. Therefore, large space and carbohydrate fractions are required to perform the treatment industrially (Agbor et al., 2011; Zheng et al., 2009). Advantage of the method is that it is safe and environmental friendly (Agbor et al., 2011; Kumar et al., 2009; Zheng et al., 2009).

2.2 Current Methods of hydrolyzing coconut meal, Jerusalem artichoke, and β-glucans

2.2.1 Acid hydrolysis

Sulfuric acid, phosphoric acid, hydrochloric acid, and nitric acid with different concentrations are used to hydrolyze biomass (Kumar et al., 2009). Higher temperature hydrolyzes biomass more efficientl. Two ranges of temperatures are typically used: a high-temperature range at T>160 °C and a low-temperature range at T<160 °C. Acid hydrolysis degrades lignin and hydrolyzes hemi-cellulose to their monomer units. The advantage of this method is that it is powerful enough to hydrolyze biomass. However, it requires the use of an alkali solution to neutralize the batch afterward (Agbor et al., 2011; Kumar et al., 2009).

The advantage of acid hydrolysis is that it is less expensive than enzymatic hydrolysis (Agbor et al., 2011; Kumar et al., 2009). It is simple, fast and easy to control. However, acid hydrolysis may lead to further degradation of monosaccharides. When monosaccharides are degraded, they form toxic substances such as furfural and 5-hydroxymethylfurfural and result in lower yield of oligosaccharides. The chain length of oligosaccharides cannot be controlled precisely.

2.2.2 Alkaline hydrolysis

Alkaline hydrolysis showed higher yield with the lowest proportion of the lignin in biomass (Agbor et al., 2011; Kumar et al., 2009). Alkaline causes biomass to swell, leading to an increase in an internal surface area of biomass and a decrease in both degree of polymerization and the crystallinity of cellulose. In addition, cellulose and hemicellulose in the hetero matrix are more accessible because the alkaline breaks the

bonds between lignin and other biopolymers and disrupts the lignin structure. Calcium hydroxide is need for this purpose because it is inexpensive and can be recovered afterward (Agbor et al., 2011). The advantage of this method is that it can be done at ambient condition. However, the time required for this method is very long, leading to high alkaline consumption. The need of using water to wash out residual alkaline is also its disadvantage.

2.2.3 Coconut meal

The hydrolysis of coconut meal has been carried out by different methods, including biochemical, chemical, physicochemical, and physical methods.

Khanongnuch et al. (2006) used enzymes from *Bacillus subtilis 5H* to hydrolyze coconut meal. The result showed that the nutritions, metabolizable energy, and the digestibility values of the treated coconut meal were improved. Specifically, the percentage of the fiber decreased from 45.35% to 26.62% of the initial value. Recently, Rungrassamee *et al*. used crude mannanase from *Bacillus subtilis* CAe24 to hydrolyze coconut meal for 6 hours. The digested products from the coconut meal contains 6.08 % of mannose, 8.81 % of mannobiose, 10.41 % of mannotriose, 11.87 % of mannotetraose, 1.21 % of mannopentaose, 0.21 % mannohexaose.

Saitagaroon et al. (1983) hydrolyzed coconut meal with hydrochloric acid as a catalyst. Coconut meal was defatted by 36% HCl solution for 3.5 hours with a ratio between acid and coconut meal of 5:3. After that the mixture was diluted to 1 M of hydrochloric acid, and boiled with reflux system for 5 hours. The result indicated that the efficiency of the hydrolysis in terms of the solid loss was 94%, and the reducing sugar content was 51%.

When hydrolyzing coconut meal with sulfuric acid as a catalyst, Bujang et al. (2013) demonstrated that nearly 1.95 % of glucose could be obtained from the coconut meal using the reaction conditions of 1% H_2SO_4, 60 minutes, and at 130 °C. In this research, by using thermogravimetric infrared, they determined the degradation temperature of lignin, hemicellulose, cellulose. The degradation of different biomass vary at different ranges:from room temperaure to 900 °C for lignin, from 150 °C to 315 °C for hemicellulose, from 315 °C to 400 °C for cellulose.

Hydrolyzing coconut meal by subcritial water in non-isothermal conditions was carried out by Khuwijitjaru et al. (2014). The results showed that the amount of carbohydrate content depends largely on temperature. Specifically, an increase from 3.5 to 9.6 g carbohydrate/100 g of dried coconut meal was corresponded to a temperature increase from 100 °C to 175 °C. However, increasing the temperature from 175 °C to 200 °C led to a decrease in the amount of carbohydrate content from 10.6 to 6.1 g/100 g of dried coconut meal after 30-40 minutes of the treatment. The monosaccharides obtained from the treatment were mannose, glucose, galactose, and arabinose.

2.2.4 Jerusalem artichoke

Inulin from Jerusalem artichoke tuber (JAT) is a major polysaccharide source to produce fructo-oligosaccharide prebiotics (Wolfrom et al, 1974). Fructo-oligosaccharides can be synthesized by enzymatic methods using inulinases for a hydrolysis reaction. Enzymatic methods are limited by low substrate solubility, longer reaction time, complicated processes, high operation cost, and narrow range of temperature activity of enzymes.

Jeruselem artichoke and chicory root are the main sources of inulin and oligofructose that are used in the food industry (Cuong et al, 2016). Jeruselem artichoke tuber powder (JATP) is inulin that contain chain of β-1,2-D-fructose with a glucose terminal. The oligofructose and inulin are considered to be the functional food ingredients since they affect biochemical and physiological processes in human. They can enhance better health and reduce the risk of many diseases. Extraction of inulin in the form of oligosaccharides from Jeruselem artichoke tuber powder (JATP) has applied as prebiotics and a sugar substitute.

2.2.5 Beta glucan

A major component of the yeast glucan is *β*-l,3-glucan (Shuqin et al, 2012). Chemical structure of the yeast glucan possesses low degree of branching which may be selectively hydrolyzed by bacterial, endo-*β*-l,3-glucanase. Alternatively, minor component (mannan and protein) may be preferentially degraded either enzymatically by a fungus, *β*-1,6-glucanase, or chemically by the Smith-degradation method.

The composition of polysaccharides and fractions is usually hydrolysis with trifluoroacetic acid (TFA), sulfuric acid (H_2SO_4), and hydrochloric acid (HCl) at high temperature (Zhaocheng et al, 2008). In the human stomach, hydrolysis of acids is usually dissolved, sugars may be destroyed and incomplete hydrolysis at temperatures of 37 °C and pH 1–1.5. After the HCl and TFA hydrolysis, some amounts of the sample may remain unhydrolyzed, with structural change. On the other hand when using H_2SO_4, the hydrolysis to neutral sugars is completed, but some of the monomer units may be degraded.

2.3 Microwave radiation

Microwave heating has been used in chemistry for decades. In conventional heat, a reaction will be heated by convection from outside to inside and it will take a long time for the whole flask to be heated. In the contrary, microwave heating produces a more localized heating, and acts on all parts of the vessel as shown in Figure 2.1. It has been suggested that as the material more rapidly and directly absorbs the heat, the high temperature can activate a larger percentage of molecules higher than the required activation energy for reaction (Boonyarattanakain, 2013).

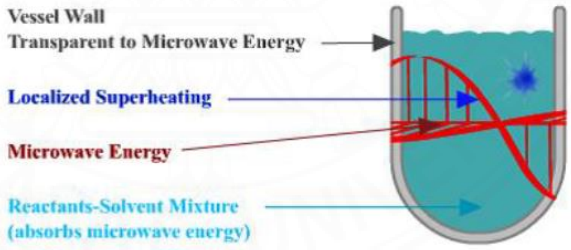

Figure 2.1 Sample heating by microwave (Richard, 2012).

Microwave radiation has proved to be a new heat source technology that greatly assists organic syntheses in an environment friendly fashion (Joshi, 2013). For example, Xiaobing Liu *et al*. reported that microwave irradiation worked efficiently in assisting the synthesis of 2,4,5-trisubstituted imidazoles under the catalysis of nonchloroaluminate ionic liquids (Xiaobing Liu, 2011). Microwave radiation increases reaction rates and reduces reaction time, which is required for the alkaline hydrothermal technique (Ameta et al., 2014; Sharifi et al., 1999). Additionally, microwave radiation

provides uniform heating to the reaction batch. Microwave radiation's qualities make it the most promising heat source for lactic acid production from the alkaline hydrothermal reaction of biomass (Ameta et al., 2014; Epane et al., 2010). Thanks to the qualification of microwave radiation on chemical reaction, CEM has been designed for development of reaction to obtain high yield product. A safety system associates with software that the condition of reaction can be controlled and set up. A scale up reaction can be done with this machine also.

Microwave-assisted extraction is similar to ultrasound-assisted extraction except that microwave irradiation is used instead of ultrasound. Xie (2010) used microwave to extract polysaccharides from a plant (Chen et al. 2008). Microwave-assisted extraction was done in closed vessel system where powdered biomass and solvent is added. The vessel is sealed closed by using a molded plastic lid. The oven power, specified time and temperature were controlled depending on the reaction. After the reaction, the vessel was cooled to room temperature before further analysis. The optimum conditions for the biomass was 20 minutes, solvent/material ratio was 20:1 and the temperature was 100°C. Increasing the time to 20 minutes increases the polysaccharide yield to 5.06%. Increasing the solvent/material ratio from 5:1 to 20:1 increases the polysaccharide yield to from 3.46% to 5.03%. Increasing the temperature from 60°C to 100°C increases the polysaccharide yield from 3.62% to 5.07%. When compared to ultrasound-assisted extraction yield of 4.82%, microwave-assisted extraction gives a higher yield of 5.07% (Chen et al. 2008).

The advantage is that microwave radiation can reduce the extraction time significantly. Microwave can heat up a sample from inside to outside in a very short amount of time. This gives fast and uniform heating of the samples. It can be used with thermal instable substances due to its rapid extraction. The disadvantage is that microwave can only be applied to samples that contain dipolar materials or microwave absorbents, and it cannot be applied to a flow system due to its closed system.

2.4 Biological test for adjuvant activities

The analyses of gray oyster mushroom (Sermwittayawong et al, 2020) show that the maximum yield fraction, 7S1-1, is different sizes and mainly consists of glucose and mannose. The 7S1-1 sample stimulates the breakdown of fat or lipolysis but does

not inhibit the creation of fat or adipogenesis. The laminarins also produced similar results with polysaccharide mushroom samples, although the effects were less clear. These results suggest the anti-obesity potential of polysaccharides and receptors on the surface of 3T3-L1 adipocytes for recognition of β-glucan.

To test for the adjuvant properties (Wattanasiri, 2017), the adjuvant properties of the unmasked mannopyranans were evaluated for the IL-1β, IL-2, IL-6, IL-12, and TNF-α activation capacities in the RAW 264.7 murine macrophage cells. 1.0 g/mL of mannopyranan 3 in a cell culture medium (10% fetal bovine serum) was mixed with lipopolysaccharide (LPS) at a concentration of 100 ng/mL. LPS was applied as an antigen to define an LPS costimulation sample. RAW 264.7 murine macrophage cells were treated with the costimulation sample in a comparison with LPS alone to evaluate the adjuvant activity of synthetic glycans of different sizes. The fetal bovine serum culture medium was collected at 3.5 hr, 24 hr, and 48 hr. In addition, the secreted IL-1β IL-2,, IL-6, IL-12, and TNF-α from the macrophages were evaluated. The results are shown in figure 3.

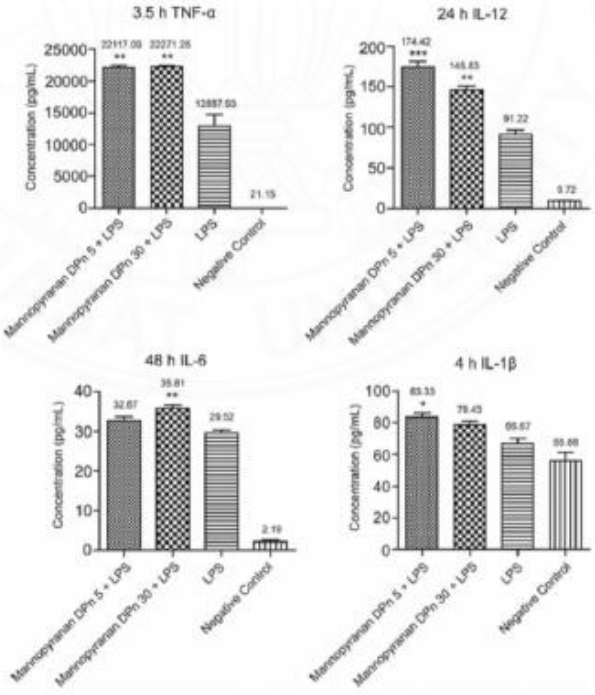

Figure 2.2 Adjuvant activities of the synthetic lipomamnan (LM) glycans (Wattanasiri, 2017).

Wattanasiri, 2017 observed that TNF-α secretion after 3.5 hours of stimulation was found to be higher in all cultures of costimulation when compared to cultures treated with LPS alone. The costsimulation samples had an approximately two-fold increase in the TNF-α response when compared to that of LPS alone. At 24 hours of treatment, all cell cultures with LPS and synthetic LM glycans secreted significantly higher levels of IL-12 at 70 pg/mL more than that of the treatment with LPS alone. The secretion of IL-6 in cultures costimulated at 30 degree of polymerization was significantly higher at 48 hr of the treatment when compared with LPS alone. In addition, IL-1β secretion is a marker for inflammation activation. The result found that the secretion of IL-1β in cultures costimulated with 5 degree of polymerization was significantly higher when compared with LPS alone. Moreover, the supernatant was tested for the presence of IL-2, it was found that there is no difference in secretions from untreated or LPS-treated cells with or without simulations with LM glycans. The research found that the costsimulations by synthetic LM glycans and LPS increased the cytokine productions of IL-2, IL-1β, IL-12, IL-6, and TNF-α by macrophages. Thus, the synthetic LM glycans present a reinforcement in macrophages cells.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 CCM, grey oyster mushroom and jew's ear mushroom pretreatment

CCM was collected from Tropicana oil Ltd., Nakhon Pathom, Thailand after cold press to get oil from coconut meat. Before boiling with reserve osmosis (RO) water (6-7times), CCM was ground in a household blender to reduce the particle size. Afterward, CCM was dried in a hot air oven at 60 °C for 3 days, and then it was soaked overnight in 0.02 M HCl. The treated CCM was washed by RO water until reachingneutral pH. The treated CCM was collected and dried for 3 days at 60 °C to remove free moisture, and it was ground and sieved to obtain the particle sizes less than 0.25 µm. The treated CCM was stored in an air-tight container for further usage

Grey oyster mushroom and jew's ear mushroom were purchased from Talad Thai market, Pathum Thani, Thailand. Grey oyster mushroom and jew's ear mushroom were cut to small pieces, and washed with water three times. After washing, grey oyster mushroom and jew's ear mushroom were dried at 60 °C for 3 days in a hot air oven to remove moisture. Afterward, it was ground in a household blender and sieved to obtain a particle size of less than 0.25 µm. The dried powder of grey oyster mushroom and jew's ear mushroom were stored in an air-tight container for subsequent usage (Figure 3.1).

Beta-glucans were obtained from Dr. Decha Sermwittayawong, Prince Songkla University, PSU (Lot007 S1-1, Lot005 AS2-1-2, Lot005 S1-1, Lot005 SG1-1). Commercially available beta-glycan from baker's yeast (*S. cerevisiae*) was purchased from Transfer Point (USA).

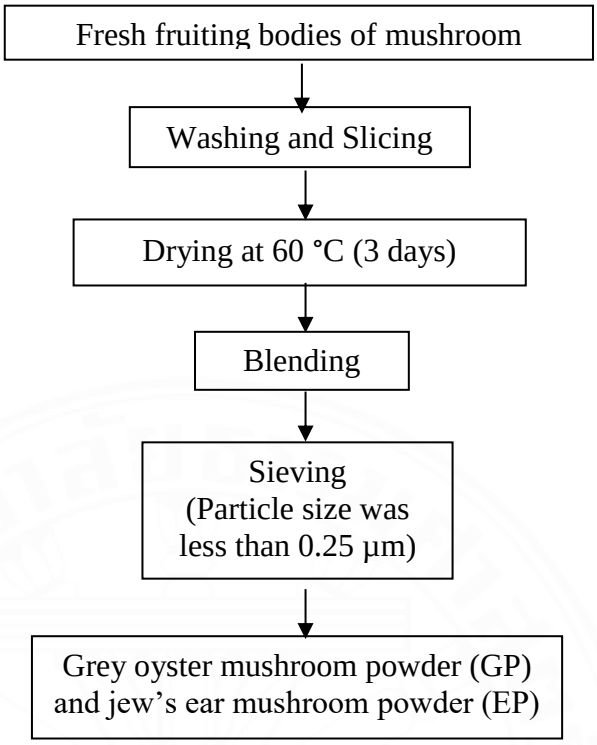

Figure 3.1 Preparation diagram of mushroom sample.

3.1.2 Reagents

Hydrochloric acid, 95-97% sulfuric acid, phenol, Coomassie Brilliant Blue G-250, ethanol (99.8%) and dihydroxyacetone (DHA) were purchased from Merck (Germany). Sodium hydroxide, potassium sodium tartrate and 3, 5-dinitrosalicylic acid were purchased from Sigma-Aldrich (USA). Pullulan polysaccharide calibration kits were purchase from Agilent. Mannose and arabinose were purchased from Senn Chemicals (Switzerland). Glucose and galactose were purchased from Fluka (USA). All chemicals purchased were analytical grade.

3.1.3 Equipment

Microwave reactor (CEM, USA, Discover SP 909155) was used for hydrolysis. High Performance Liquid Chromatography (HPLC) equipped with carbohydrate column (Transgenomic CARBOSEP CHO682, LEAD column, CHO-99-9854, USA) and PL aquagel-OH 5µm column with PL aquagel-OH guard (PL aquagel - OH 20 SEC columns, 5 µm, 7.5 x300 mm) were used for sugar composition analysis and size analysis respectively.

3.2 Microwave radiation hydrolysis

The hydrolysis experiments were carried out in a microwave reactor (CEM, USA, Discover SP 909155). Mushroom was hydrolyzed by microwave radiation with acid catalyst. Microwave radiation was done in a 10 mL batch-type reactor vessel in a closed-system. The reactions were done in the following conditions: maximum pressure at 290 psi, power of 150 watt, and ramping time was 5 min. 0.1g of mushroom was mixed with 1 mL of hydrochloric acid (HCl) as a catalyst in the microwave reactor for 15 min.

After the hydrolysis reaction, the samples were cooled to room temperature. 8 mL of RO water was added and stirred for 1 hr to separate the soluble and insoluble portions. The diluted samples were neutralized and centrifuged at 14000 rpm at 4 °C for 15 min, and the residual solid was collected by filtering through a Whatman paper No.93 on Buchner filter equipped with a vacuum pump. The residual solid was dried at 60 °C overnight. The crude product was kept in the refrigerator at 4 °C for further analysis.

3.3 Methods

The digestion methodology of grey oyster mushroom and jew's ear mushroom are shown in Figure 3.2.

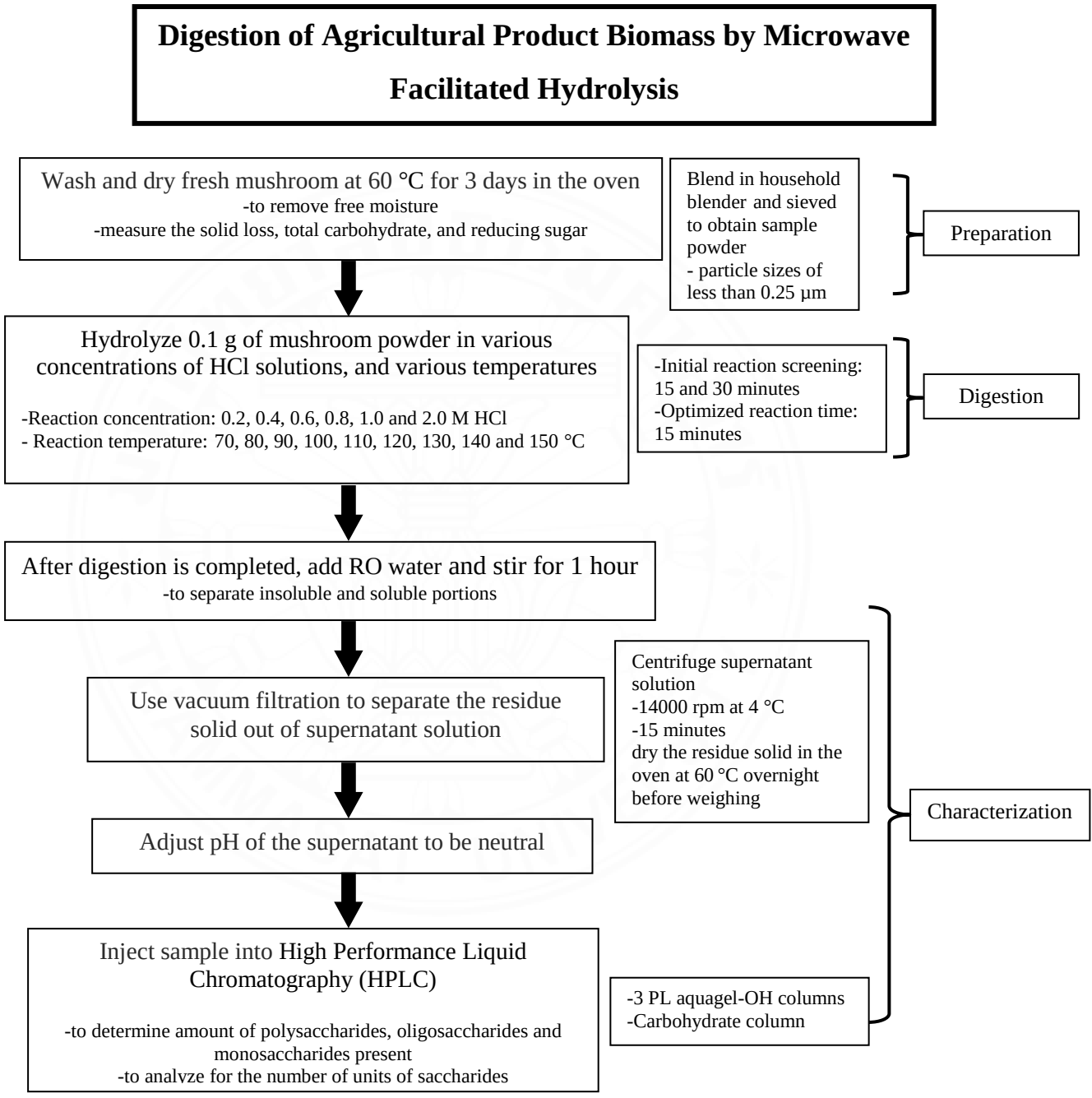

Figure 3.2 Flowchart for digestion methodology.

3.3.1 Physicochemical hydrolysis of mushroom by microwave

Grey oyster mushroom and jew's ear mushroom were hydrolyzed by using microwave radiation and acid catalyst. Microwave radiation was done in a 10 mL batch-type reactor vessel in a closed-system. 1 mL of HCl solution was mixed with 0.1g of sample in the microwave reactor for 15 minutes. The reactions were done in the following conditions: maximum pressure at 290 psi, power of 150 watt, and ramping time of 5 minutes.

After the hydrolysis reaction, the samples were cooled down to room temperature. 8 mL of RO water was added, and then stirred for 1 hour to separate the insoluble and soluble portions. The diluted samples were neutralized and centrifuged at 14000 rpm at 4^{o}C for 15 minutes, and the residual solid is collected by filtering through a Whatman paper No.1 on Buchner filter equipped with a vacuum pump. The residual solid is dried at 60 °C overnight. The crude product is kept in the refrigerator at 4 °C for further analysis.

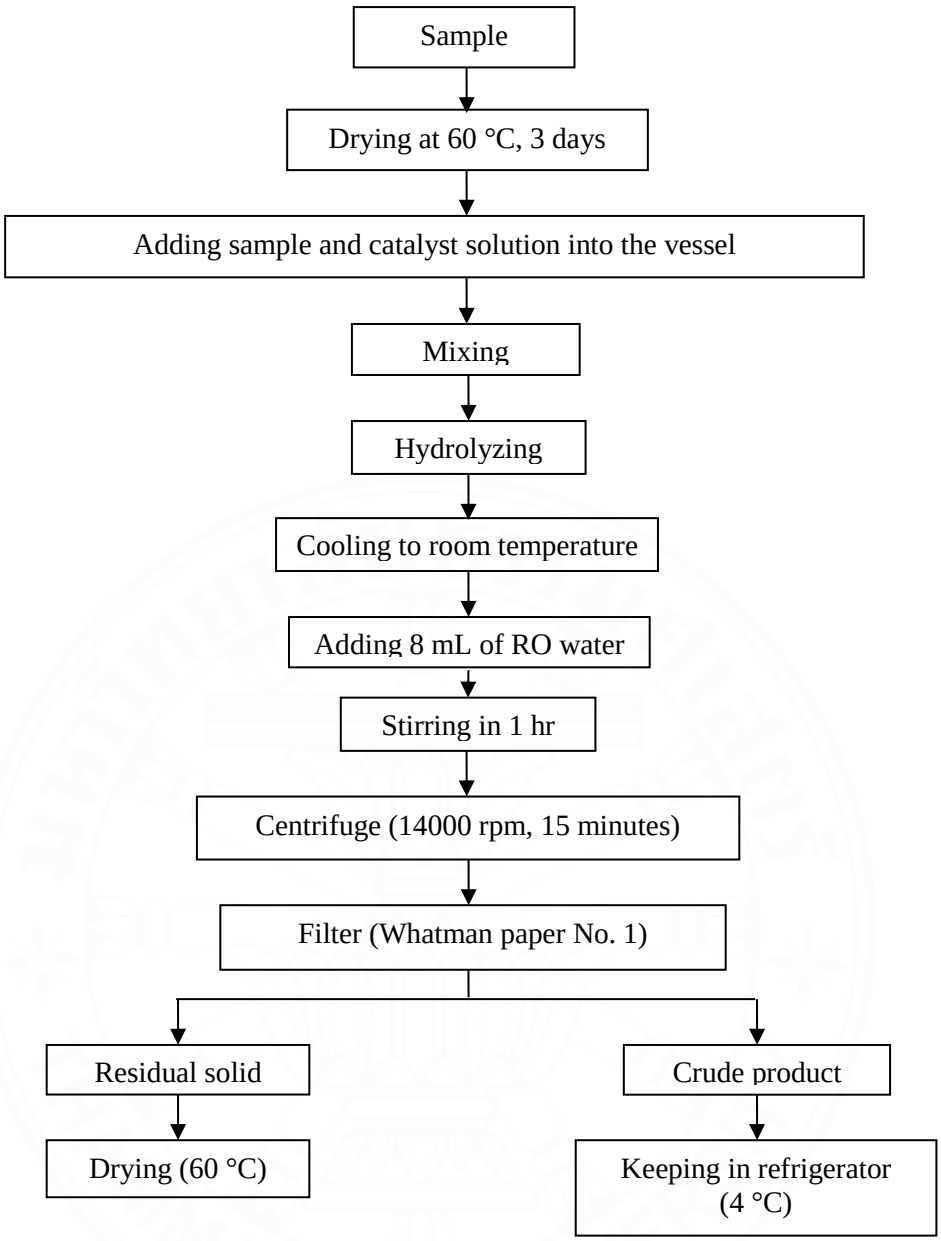

Figure 3.3 Hydrolysis diagram of mushroom.

The sample was prepared to a concentration of 20 mg/mL aqueous solution by DI water. The hydrolysis of the sample was done in a closed vessel (10 mL) in the MCR reactor (CEM, Discover SP 909155, USA). 1 mL of 0.05 M HCl solution was mixed with 0.1 g of sample in a vessel. The reaction was carried out under the set maximal pressure of 290 psi, maximal power of 150 watt, and ramping time of 5 minutes.

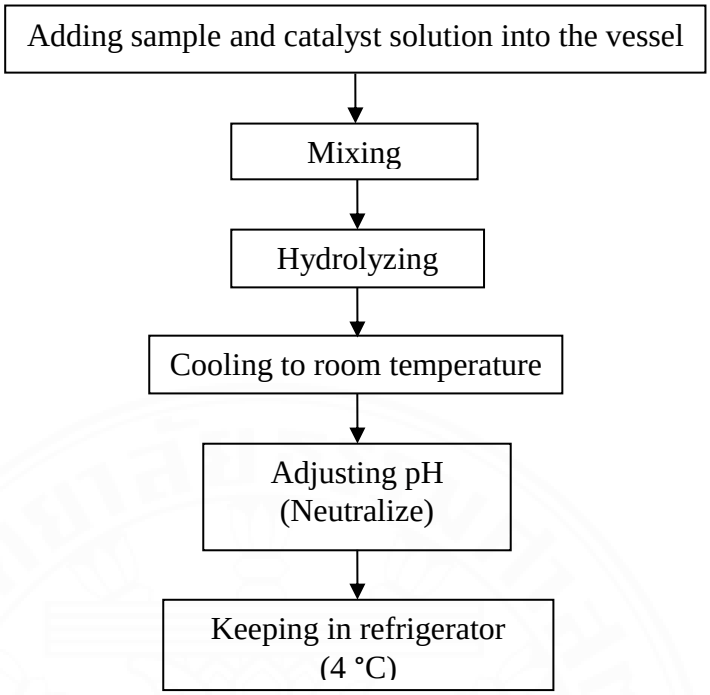

Figure 3.4 Hydrolysis diagram of β–glucan samples from PSU.

3.3.2 Proximate composition analysis of mushroom powder

The proximate constituents of mushrooms were analyzed by using the standard method set by Association of Official Analytical Chemists (A.O.A.C., 2000). The moisture content was obtained by heating 2.0 g of fresh mushroom at 105 °C until a constant weight is obtained. Crude protein is determined using Kjeldahl method which involves finding the percent of total nitrogen in 2.0 g of sample, then multiply the number by 6.25 (Barbano et al., 1991). Crude fat is determined by using petroleum ether to extract fat from 5.0 g of sample in a Soxhlet apparatus. Ash is determined by taking 10.0 g of sample and incinerating it at 550 °C for 5 hr. Crude fiber is obtained by using sulfuric acid and sodium hydroxide to digest 2.0 g of sample, then incinerating the residue in a furnace at 550 °C for 5 hr. (Ranganna et al., 1986)

3.3.3 Determination of solid loss

Solid loss is one way to determine the degree of hydrolysis. More solid loss indicates more hydrolysis that has occurred. The main focus is to hydrolyze the carbohydrate portions of the biomass, but protein may be hydrolyzed as a side reaction. The residual solid was collected by filtering through a Whatman paper No.93 on a

Buchner filter equipped with a vacuum pump. The residual solid was dried at 60 °C overnight. The solid loss was calculated by the equation shown below (A.O.A.C., 2000; Ranganna et al., 1986),

$$\mathrm{SL} = \frac{\mathrm{IS} - \mathrm{RS}}{\mathrm{IS}} \times 100\ \%$$

SL: Solid loss (%, based on the dried weight of mushroom)

IS: Weight of starting materials (Initial dried solid, g)

RS: Weight of dried remain mass (Residual dried solid, g)

3.3.4 Determination of total carbohydrate (TC)

TC was determined by the phenol-sulfuric acid assay method (A.O.A.C., 2000; Ranganna et al., 1986). 0.2 mL of the mushroom solution was diluted to 10 mL using RO water in a volumetric flask. 1 mL of diluted sample solution was mixed with 1 mL of 5% aqueous phenol solution in test tube. After that, 5 mL of sulfuric acid (95 - 97%) was added to the mixture. The mixture was kept in a water bath at 25 °C for 20 min. The absorbance of the sample was measured at 490 nm using a UV-VIS spectrophotometer (Thermo Fisher Scientific, G10S UV-VIS, USA). A mixture of RO water, aqueous phenol solution (5%), and 95 - 97% sulfuric acid (1:1:5, v/v/v) was set as blank. Glucose solutions at different appropriate concentrations were used to create a standard curve for TC determination of sample solution of mushroom. TC was reported in % (gram of TC in 100 g of the dried weight of mushroom).

3.3.5 Determination of reducing sugar (RS)

RS was measured by the dinitrosalicylic acid assay (A.O.A.C., 2000; Ranganna et al., 1986). To prepare the dinitrosalicylic acid solution, 5.0 g of 3,5-dinitrosalicylic acid and 150 g of potassium sodium tartrate were mixed together in 100 mL of 2.0 M NaOH. The mixture was adjusted to 500 mL by using RO water. 0.2 mL of the sample solution and 2 mL of the dinitrosalicylic acid solution were mixed in a test tube. The mixture was soaked in boiling water for 10 minutes before speedily cooled to room temperature using ice water. A UV-VIS spectrophotometer (Thermo Fisher Scientific, G10S UV-VIS, and USA) was used to measure the UV absorbance at 570 nm. A mixture of RO water and dinitrosalicylic acid solution (0.2:2, v/v) was set as a blank.

Glucose solutions at different appropriate concentrations were used to create a standard curve for RS determination of sample solution of mushroom. RS was reported in % (gram of RS in 100g of the dried weight of mushroom).

3.3.6 Monosaccharide analysis

The saccharide compositions of the hydrolyzed sample were analyzed using HPLC (Agilent 1260 Infinity, G1329B, Germany) equipped with a carbohydrate column (Transgenomic CARBOSEP CHO682, LEAD column, CHO-99-9854, USA). The sample was diluted and neutralized. Then, it was filtered through a 0.2 µm membrane. The sample at 20 µL was injected into HPLC. The mobile phase is DI water with a flow rate of 0.4 mL/min and the column is temperature controlled at 80 ℃. The oligosaccharides and monosaccharides were monitored using a refractive index detector.

3.3.7 HPLC size exclusion chromatography (SEC) analysis

The SEC analysis was done by using HPLC (Agilent 1260 Infinity, G1329B, Germany) equipped with a PL aquagel guard and 3 of PL aquagel-OH columns (PL aquagel-OH 20 SEC columns, 5 µm, 7.5x300 mm). The sample was diluted and neutralized. Then, it was filtered through a 0.2 µm membrane. The sample at 100 µL was injected into HPLC. DI water was used as the mobile phase with a flow rate of 0.9 mL/min and the column's temperature at 36 ℃. A refractive index detector on the HPLC was used to monitor the molecular weights of monosaccharides, oligosaccharides, and polysaccharides in samples.

3.3.8 Purification

The sample was evaporated at 40 ℃ until the solution became viscous to obtain saccharide solution. 4.5 mL of propanol was added to 0.5 mL of the saccharide solution and incubate for 16 hr at 40 ℃ with orbital shaking at 60 rpm. The sample was centrifuged at 13,000 rpm for 3 min at 4 ℃ to separate any precipitated solids. The solid material was kept in hot air oven at 60 ℃ for 2 hr and the supernatant was kept in test tube.

3.3.9 NMR spectroscopy

The purified polysaccharide was characterized by ^{1}H nuclear magnetic resonance (NMR). NMR spectra were recorded at 298 K with a Bruker Ascend TM 600 spectrometer operating at 600 MHz. Chemical shifts are expressed in ppm. Deuterium oxide (D_2O) was used as a solvent for NMR analyses. The purified polysaccharide was dissolved in D_2O at 30 ℃ and kept at that temperature during the measurement.

3.3.10 Biological test for adjuvant activities

To test for their adjuvant properties, the extracted polysaccharides were evaluated for the IL-1β, IL-2, IL-6, IL-12, and TNF-α activation capacities in RAW 264.7 murine macrophage cells. The extracted polysaccharides at a concentration of 20 μg/mL in complete medium (3 mL/well) were mixed with 1.5 mL of keyhole limpet hemocyanin (KLH) and 1.5 mL of *lipopolysaccharide* (LPS) which use as an antigen to formulate an LPS costimulation sample. RAW 264.7 murine macrophage cells were treated with the costimulation sample compared with KLH alone to evaluate the adjuvant activity of synthetic glycans with different sizes. The complete medium was collected at 4, 12, 24, and 48 hr and the sample was kept in 4 ℃. Moreover, the secreted IL-1β, IL-2, IL-6, IL-12, and TNF-α from the macrophages were evaluated.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Saccharides standard curves on HPLC

HPLC chromatogram of standard solutions with three columns of PL aquagel-OH for monosaccharides (M_W 180 g/mol), oligosaccharides (M_W 6100, 9600, 21100, and 47100 g/mol), polysaccharides (M_W 342, 667 g/mol) were obtained at 36 °C and the analysis time of 40 minutes.

The number of units of saccharides in the sample was assessed by using size exclusion chromatography (SEC) with three columns. Calibration curve of saccharides standards by SEC (Figure 4.1) revealed a linear relationship between logarithm of molecular weight of saccharides and logarithm of retention time. For example, the highest molecular weight (M_w = 47100 g/mol) with 261 repeated units while lowest molecular weight (M_w = 180 g/mol) has 1 monosaccharide unit. The oligosaccharides and polysaccharides have 2-20 units and more than 30 units, respectively.

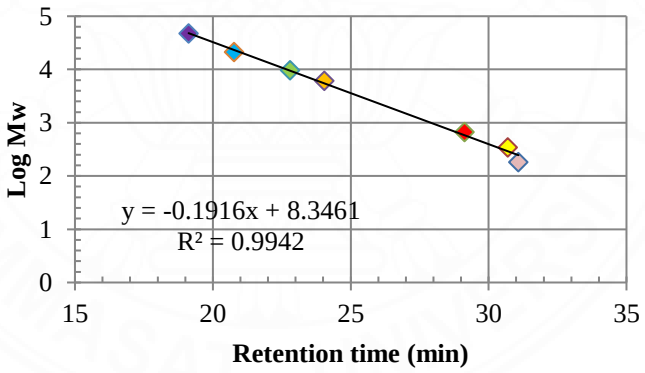

Figure 4.1 Calibration curve of saccharides standard.

Calibration curve of saccharides standards between the logarithm of molecular weight (M_w) and retention time shows linear relationship. The number of units of saccharides in the samples were calculated by the equation y = -0.1916x + 8.3461 where y is the logarithm of molecular weight, and x is the retention time. The molecular weight range of the saccharides standard is 180 (monosaccharides) to 47100 (polysaccharides) g/mol.

4.1.1 Coconut Copra Meal (CCM)

The optimized hydrolysis conditions developed previously by Cuong et al (2016) involves using acid as a catalyst and microwave radiation. The reaction conditions to obtain the highest yield of monosaccharides from the hydrolysis of coconut meal (CCM) is at 170 °C , the reaction time of 15 min, 0.05 M HCl solution, and 1:10 (g/mL) ratio of CCM to acid solution volume. The conditions which gave in the highest yield of oligosaccharides is at 110 °C , the reaction time of 15 min, 0.2 M HCl solution, and 1:80 (g/mL) ratio of CCM to acid solution volume in Figure 4.2. The chromatogram was analyzed by integrating the range of retention time that covers one unit. For example, for monosaccharide with 1 unit, the retention time is from 30.87 minutes to 33.36 minutes, for oligosaccharide with 5 units, the retention time is from 27.93 minutes to 28.38 minutes. The yield of oligosaccharide in the range of units 2 to 18 units is 66.71%, yield of monosaccharides 30.38%, and theyield of polysaccharides with 36 repeated units is 2.91%. Higher temperature and lower reaction volume to CCM ratio results in higher yield of monosaccharides. The reaction conditions of CCM at 170 °C, the reaction time of 15 min, 0.05 M HCl solution, 1:10 (g/mL) ratio of CCM to acid volume (Figure 4.3) generated the yield of polysaccharides (21 to 630 units) to be 50.12%, oligosaccharide with 2 to 20 units to be 4.20%, and highest yield of monosaccharides to be 45.68% with 1 unit.

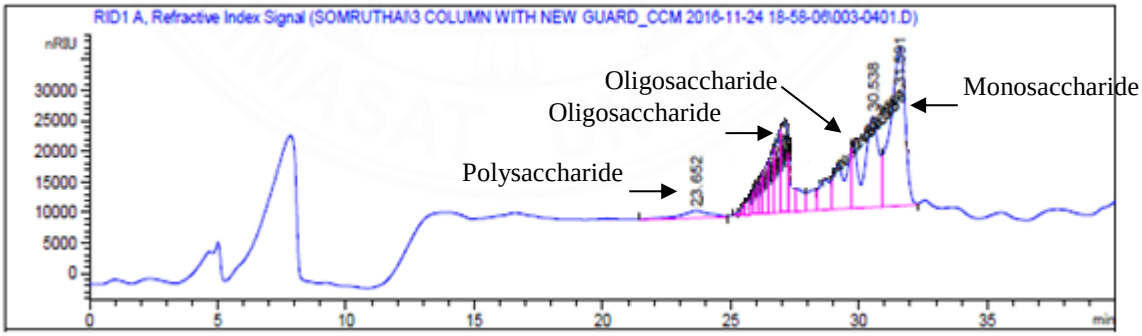

Figure 4.2 SEC chromatogram of the reaction condition at 110 °C, 15 min, 0.2 M HCl, ratio of CCM mass to reaction volume of 1:80 (w/v) with PL aquagel guard and 3 columns of PL aquagel-OH.

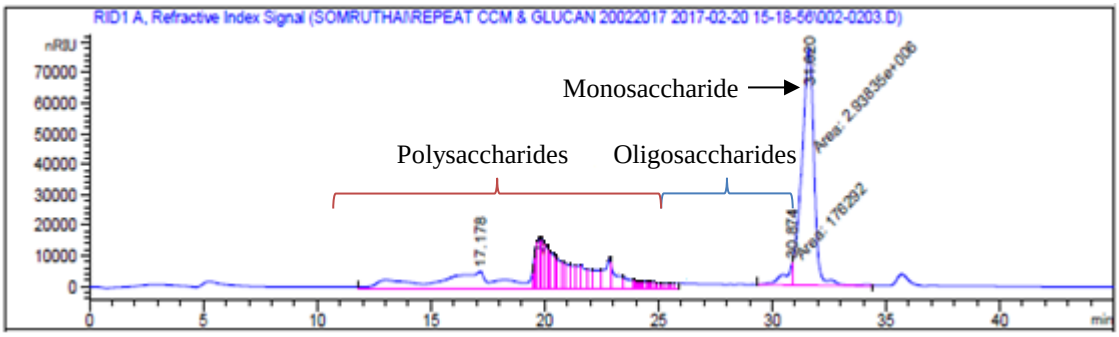

Figure 4.3 SEC chromatogram of the reaction condition at 170 °C, 15 min, 0.05 M HCl, ratio of CCM mass to reaction volume of 1:10 (w/v) with PL aquagel guard and 3 columns of PL aquagel-OH.

Table 4.1 Summary of reaction condition and size of CCM obtained.

The Reaction condition of CCM	**Size of polysaccharides**	**Size of oligosaccharides**	**Size of monosaccharides**
110°C, 0.2 M HCl, 80:1, 15 min	36 units up (2.91%)	18 units (0.09%) 16 units (0.22%) 15 units (0.33%) 14 units (0.76%) 13 units (1.08%) 12 units (1.38%) 11 units (2.12%) 10 units (3.15%) 9 units (4.17%) 8 units (5.25%) 7 units (10.25%) 6 units (2.38%) 4 units (2.44%) 3 units (4.58%) 2 units (28.51%)	1 unit (30.38%)
170°C, 0.05 M HCl, 10:1, 15 min	21 to 630 units (50.12%)	20 units (0.21%) 19 units (0.17%) 18 units (0.19%) 17 units (0.23%) 16 units (0.17%)	1 unit (45.68%)

		15 units (0.23%) 14 units (0.25%) 2 units (2.74%)	

4.1.2 Beta-glucan from PSU

Beta-glucan samples were obtained from Dr. Decha Sermwittayawong, Prince of Songkla University, PSU. The samples were used to verify the size of beta-glucan. The samples included Lot007 S1-1, Lot005 S1-1, Lot005 SG1-1, and Lot005 AS2-1-2.

Polysaccharides in the samples were hydrolyzed using microwave radiation and 0.05 M HCl as a catalyst to obtain monosaccharides. Monosaccharides can be observed at the retention time of 31 minutes. After the hydrolysis, both oligosaccharides and monosaccharides were observed. The predominant monosaccharide observed in all of the samples at a reaction temperature of 150 °C was glucose, with mannose as a minor component. The highest yield of monosaccharide was achieved at 150 °C with 0.05 M HCl, and 15-minute reaction time (Figure 4.4-4.7, Table 4.2).

The type of oligosaccharides and monosaccharides present in the samples were determined by HPLC equipped with the carbohydrate column. The retention times for the common monosaccharides such as mannose, arabinose, galactose, and glucose were 29.5, 27.1, 24.5, and 20.5 minutes, respectively. The retention times of oligosaccharides were found to be less than 20 minutes.

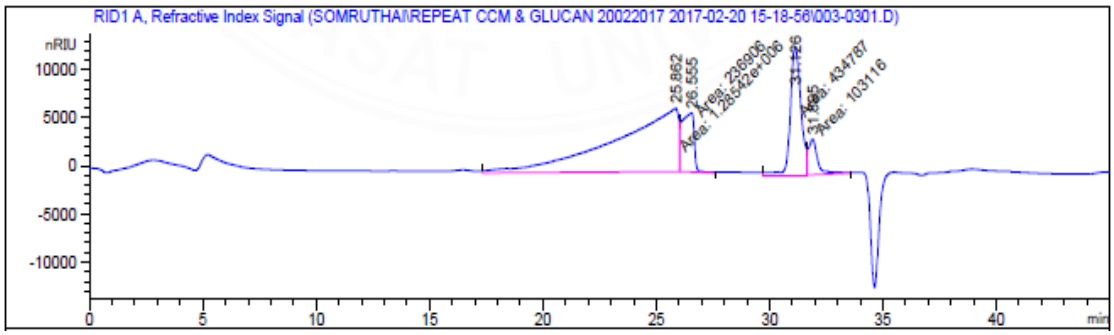

Figure 4.4 SEC chromatogram of the reaction conditions at 150 °C, 15 min, 0.05 M HCl of beta-glucan (Lot005 S1-1), after size exclusion column purifications with 3 columns of PL aquagel-OH.

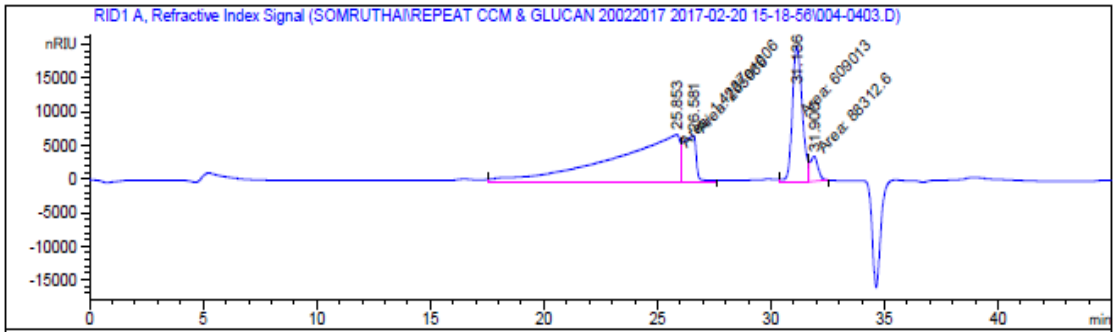

Figure 4.5 SEC chromatogram of the reaction conditions at 150 °C, 15 min, 0.05 M HCl of beta-glucan (Lot005 SG1-1), after size exclusion column purifications with 3 columns of PL aquagel-OH.

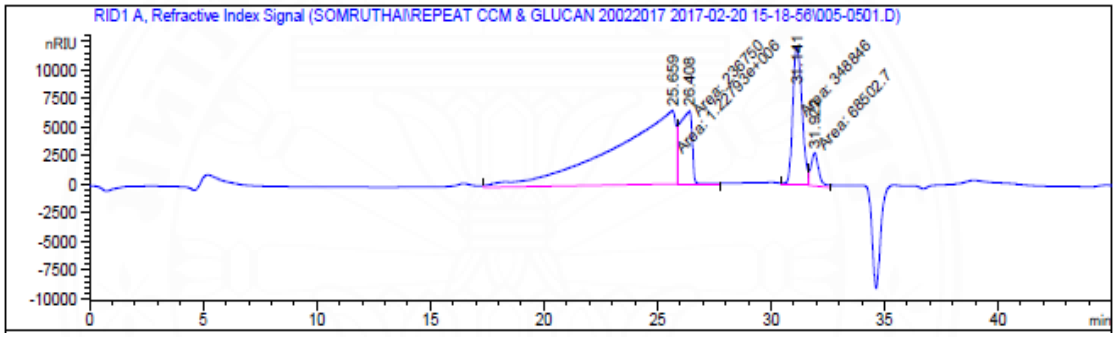

Figure 4.6 SEC chromatogram of the reaction conditions at 150 °C, 15 min, 0.05 M HCl of beta-glucan (Lot005 AS2-1-2), after size exclusion column purifications with 3 columns of PL aquagel-OH.

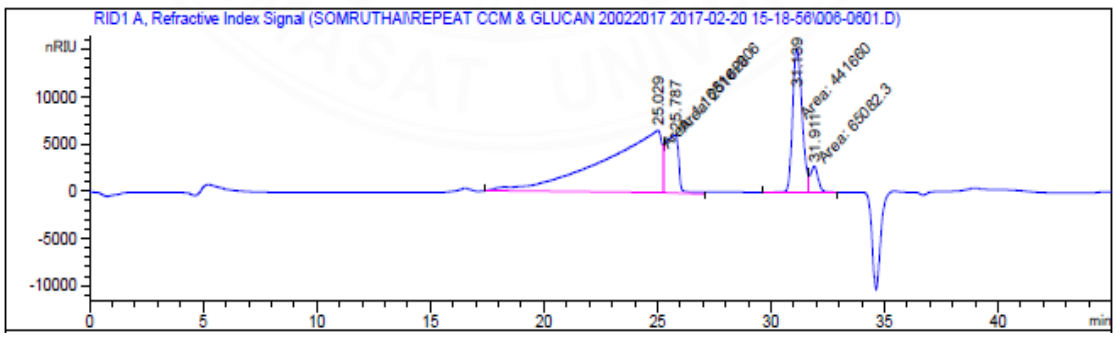

Figure 4.7 SEC chromatogram of the reaction conditions at 150 °C, 15 min, 0.05 M HCl of beta-glucan (Lot007 S1-1), after size exclusion column purifications with 3 columns of PL aquagel-OH.

Table 4.2 Summary the reaction conditions for the beta-glucan samples from PSU to evaluate the number of repeated units.

Sample	Condition	Result	
		Before hydrolysis	**After hydrolysis**
Lot005 S1-1	150 °C, 0.05M HCl, 15 min	3437 units (50.93%), 2928 units (28.84%), 2233 units (20.23%)	1 unit (26.11%), 10 units (11.50%), 13 units (62.39%)
Lot05 SG1-1	150 °C, 0.05M HCl, 15 min	3487 units (49.84%), 2651 units (30.19%), 2088 units (11.61%), 1776 units (5.61%), 1429 units (2.74%)	1 unit (29.22%), 10 units (11.11%), 13 units (59.67%)
Lot005 AS2-1-2	150 °C, 0.05M HCl, 15 min	2980 units (68.20%), 2751 units (21.64%), 1899 units (10.16%)	1 unit (22.18%), 10 units (12.58%), 14 units (65.25%)
Lot007 S1-1	150 °C, 0.05M HCl, 15 min	2740 units (51.10%), 2427 units (26.00%), 1772 units (12.87%), 1286 units (10.02%)	1 unit (26.3%), 14 units (13.06%), 19 units (60.63%)

The samples Lot005 S1-1, Lot005 SG1-1, Lot005 AS2-1-2, and Lot007 S1-1 were hydrolyzed by using microwave radiation with HCl solution as a catalyst at different conditions to obtain the optimal condition to obtain the highest oligosaccharides yield. The reaction conditions at 150 °C, 0.05M HCl, and 15 min reaction time were found to degrade polysaccharides to form oligosaccharides and monosaccharides. In general, the yield of monosaccharides increased gradually with increasing temperature, while the oligosaccharides steadily decreased. The predominant monosaccharides present in all samples was glucose, which were maximized at a reaction temperature of 150 °C. Oligosaccharides were observed at the retention time around 10-18 minutes while glucose and mannose were observed at the retention times of 20 minutes and 29 minutes, respectively (Figure 4.8-4.11).

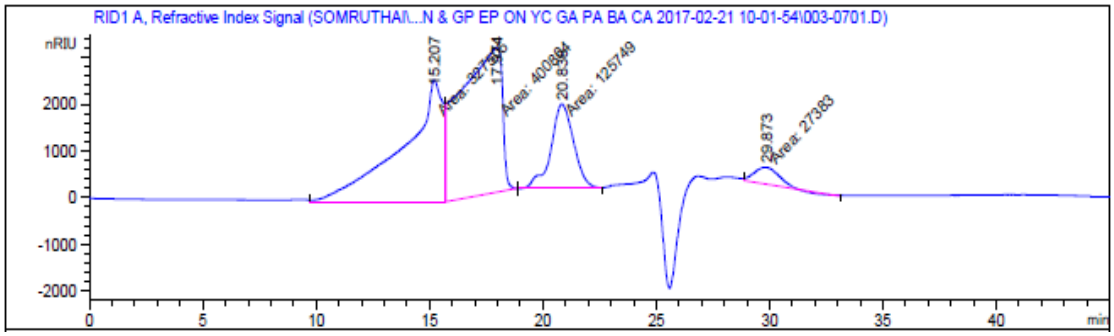

Figure 4.8 Lot005 S1-1 sample after the hydrolysis at under the reaction conditions at 150 °C, 0.05 M HCl solution, 15 minutes.

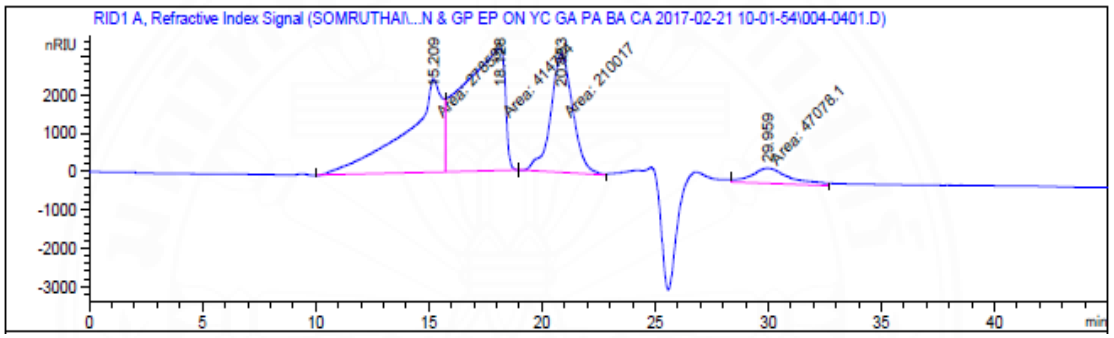

Figure 4.9 Lot005 SG1-1 sample after the hydrolysis at under the reaction conditions at 150 °C, 0.05 M HCl solution, 15 minutes.

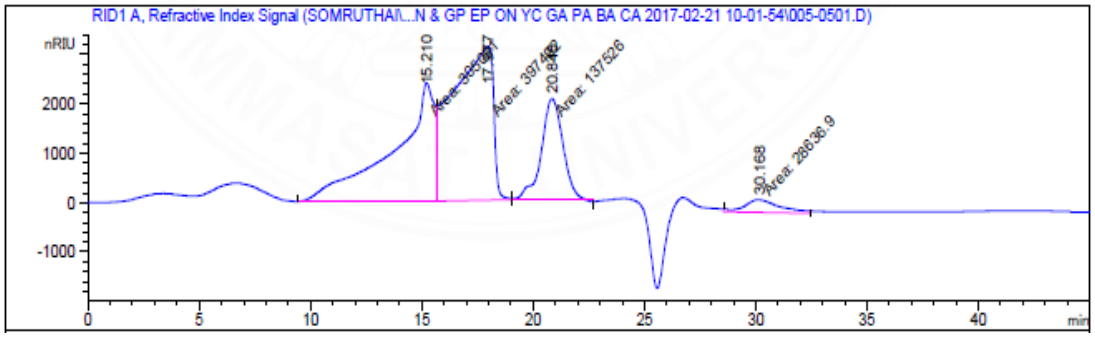

Figure 4.10 Lot005 AS2-1-2 sample after the hydrolysis at under the reaction conditions at 150 °C, 0.05 M HCl solution, 15 minutes.

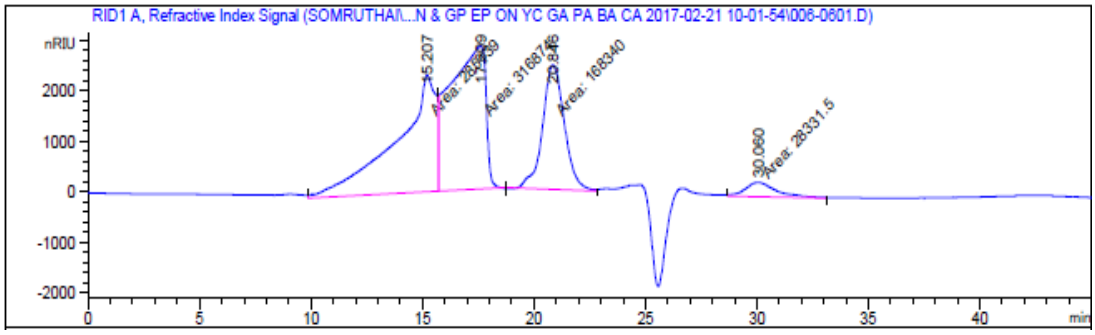

Figure 4.11 Lot007 S1-1 sample after the hydrolysis at under the reaction conditions at 150 °C, 0.05 M HCl solution, 15 minutes.

This study shows that temperature, HCl concentration, and reaction time results in higher selectivity of monosaccharides and/or oligosaccharides from the samples. The highest amount of glucose was extracted out of samples at low HCl concentration and high temperature. In this study for SEC analysis found that polysaccharides were completely hydrolyzed to monosaccharides under the reaction conditions at high temperature (150 °C), low concentration (0.05 M HCl), and reaction time 15 min. The summary of the reaction conditions for the PSU samples are given in Table 4.3.

Table 4.3 Summary the reaction conditions of samples from PSU for oligosaccharides and type of monosaccharides.

Sample	**Condition**	**Result**	
		Before hydrolysis	**After hydrolysis**
Lot005 S1-1	150°C, 0.05M HCl, 15 min	Polysaccharides (100%)	Oligosaccharides 73.90%, Glucose 21.43%, Mannose 4.67%
Lot05 SG1-1	150°C, 0.05M HCl, 15 min	Polysaccharides (100%)	Oligosaccharides 72.95%, Glucose 22.10%, Mannose 4.95%
Lot005 AS2-1-2	150°C, 0.05M HCl, 15 min	Polysaccharides (100%)	Oligosaccharides 82.11%, Glucose 14.81%, Mannose 3.08%
Lot007 S1-1	150°C, 0.05M HCl, 15 min	Polysaccharides (100%)	Oligosaccharides 75.38%, Glucose 21.07%, Mannose 3.55%

4.2 Proximate composition analysis of grey oyster mushroom and jew's ear mushroom

The results of the proximate composition analyses of the mushrooms are summarized in Table 4.4. Carbohydrate, moisture, lipid, and crude fiber content from jew's ear mushroom was higher than that of grey oyster mushroom *(Pleurotus sajor-caju)*. The ash content, and protein of jew's ear mushroom were lower than that of grey oyster mushroom.

Table 4.4 Proximate composition of mushrooms.

	Grey oyster mushroom	**Jew's ear mushroom**
Moisture (%)	1.26 ± 0.03	1.48 ± 0.01
Lipid (%)	1.30 ± 0.03	1.60 ± 0.06
Protein (%)	24.59 ± 0.21	11.54 ± 0.14
Crude fiber (%)	0.97 ± 0.00	1.25 ± 0.02
Ash (%)	6.94 ± 0.09	3.03 ± 0.02
Carbohydrate (%)	64.94 ± 0.26	81.10 ± 0.15

Based on dried weights, carbohydrates contribute 65% and 81% in grey oyster and jew's ear mushrooms, respectively. Carbohydrates are mainly present in mushroom as polysaccharides in the forms of glycogen, indigestible fibers (α- and β-glucans, dietary fibers, chitin, and cellulose) and other hemicelluloses (galactans, xylans and mannans).

4.3 Digestion of grey oyster mushrooms

The first step is digestion of grey oyster mushroom under the conditions according to Cuong et al., 2016. The conditions that provided the highest amount of monosaccharide (glucose) from konjac is 100 °C, 15 minutes, and 1.2 M HCl. The samples were digested at various reaction temperatures and HCl concentrations. The hydrolyzed mushroom crudes were analyzed by HPLC methods such as SEC analysis and saccharide composition analysis.

4.3.1 Effects of the extracting temperature

The reaction temperature plays an important role in the hydrolysis reaction of grey oyster mushroom. Generally, solid loss (SL), total carbohydrate (TC) and reducing sugar (RS) of the hydrolyzed grey oyster mushroom increased with increasing temperature. SL reached the maximum value of 93.63% under 150 °C, 15 min and 0.2 M HCl (Fig. 4.12a). TC reached the maximum value of 65.61% at 130 °C for 15 min with 0.2 M HCl (Fig. 4.12b). RS reached the maximum value of 50.92% at 140 °C for 15 min with 0.4 M HCl (Fig. 4.12c). The maximum values of SL, TC, and RS are in the reaction temperature range of 130 – 150 °C. Intermediate Decomposition Interaction of Maillard Browning and Caramelization (MBCR) is generated from the degradation of proteins and carbohydrates (Namiki, 1988). Beyond this reaction temperature, SL, TC, and RS decreased because some of the carbohydrates and proteins in the hydrolyzed mushroom were converted to a residual black solid at higher temperatures.

The high yield of glucan obtained in this study can be attributed due to the utilization of a combination of HCl as a catalyst and microwave radiation heating.

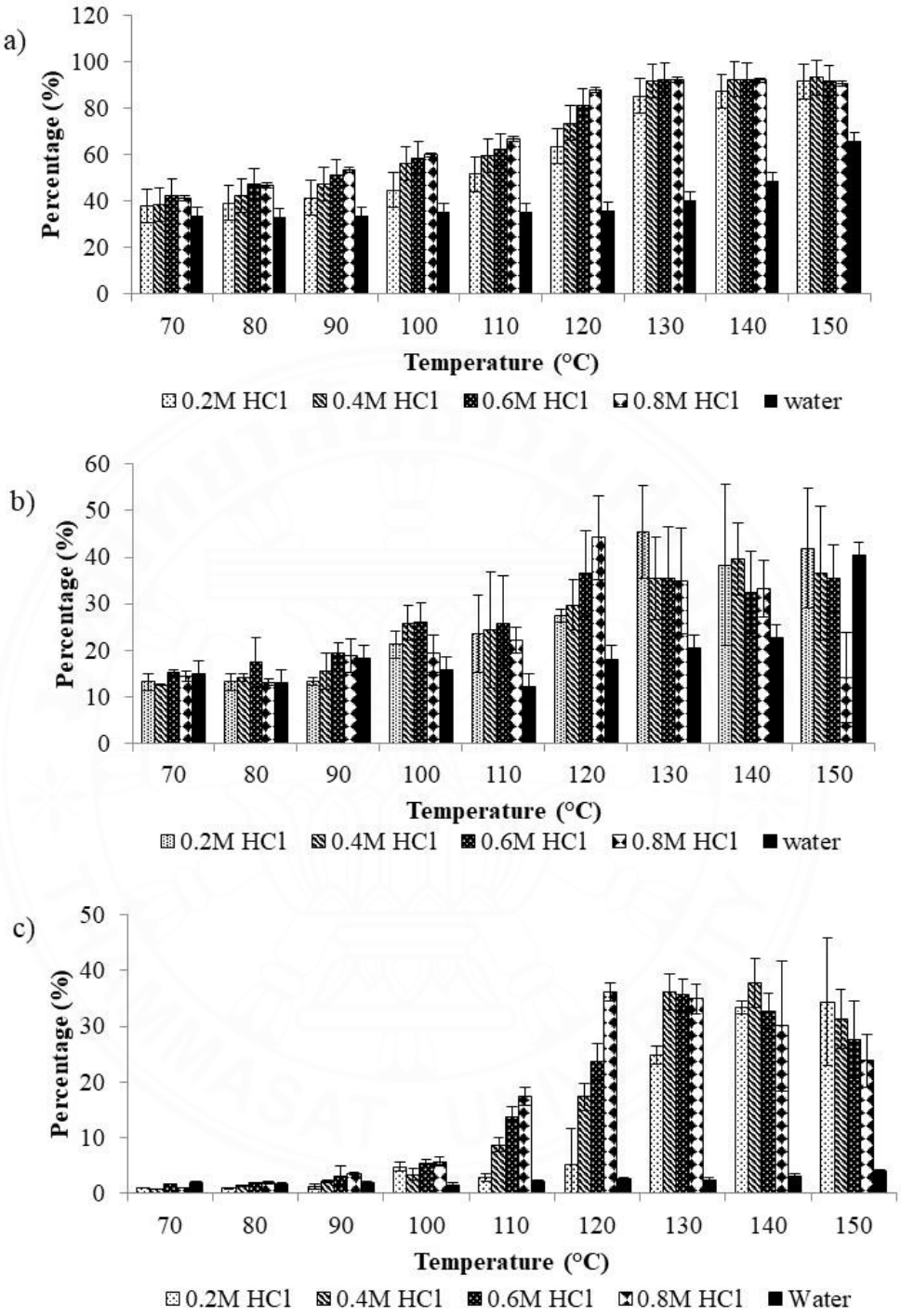

Figure 4.12 The effect of reaction temperature on the hydrolysis of grey oyster mushroom: a) solid loss, b) total carbohydrates, and c) reducing sugars at reaction time 15 min, ratio of grey oyster mushroom mass to reaction volume of 1:10 w/v and repeat 3 times.

Table 4.5 The effect of reaction temperature on solid loss (SL), total carbohydrate (TC), and reducing sugar (RS) of hydrolyzed grey oyster mushroom.

	0.2M HCl			**0.4M HCl**			**0.6M HCl**			**0.8M HCl**		
Temperature (°C)	SL	TC	RS	SL	TC	RS	SL	TC	RS	SL	TC	RS
	(%, based on dried weight of mushroom)											
70	39.24	14.38	1.31	40.63	13.25	1.06	50.85	21.12	3.51	41.35	14.55	1.35
80	36.96	12.42	1.75	41.77	18.46	2.56	44.08	18.81	2.70	46.21	14.85	2.92
90	40.08	16.09	1.97	47.88	19.46	3.54	51.18	24.02	5.22	52.51	22.78	5.44
100	74.60	30.53	12.24	56.92	40.29	6.21	60.31	37.74	9.24	62.13	20.47	7.49
110	47.49	23.84	4.71	55.28	30.29	12.31	58.58	34.85	16.91	61.09	31.35	20.56
120	61.60	38.93	6.54	74.20	39.70	22.35	77.01	48.57	25.97	88.28	55.43	48.84
130	87.96	65.61	36.85	91.75	43.07	41.35	93.52	42.77	43.50	92.53	39.52	40.40
140	87.39	53.01	45.80	91.92	54.72	50.92	91.72	40.76	41.13	91.57	44.31	39.38
150	93.63	51.11	42.70	92.75	45.49	42.92	92.49	44.07	37.11	90.55	32.48	32.80

Note: *at reaction time 15 min, ratio of mushroom mass to reaction volume of 1:10 (w/v)*

4.3.2 Effects of HCl concentration

The concentration level of HCl has an important role in the hydrolysis of grey oyster mushroom. SL, TC, and RS significantly increased with increasing HCl concentrations. SL, TC, and RS increased dramatically when the HCl concentration was increased from 0.2 to 0.8 M. SL of grey oyster mushroom reached a maximum value of 93.63% at 150 °C for 15 min with 0.2 M HCl (Fig. 4.13a). TC of grey oyster mushroom reached a maximum value of 65.61% at 130 °C for 15 min with 0.2 M HCl (Fig. 4.13b). RS of grey oyster mushroom reached a maximum value of 50.92% at 140 °C for 15 min with 0.4 M HCl (Fig. 4.13c). Intermediate products of MBCR is created when the extracted carbohydrates are degraded by high concentrations of HCl (Namiki, 1988).

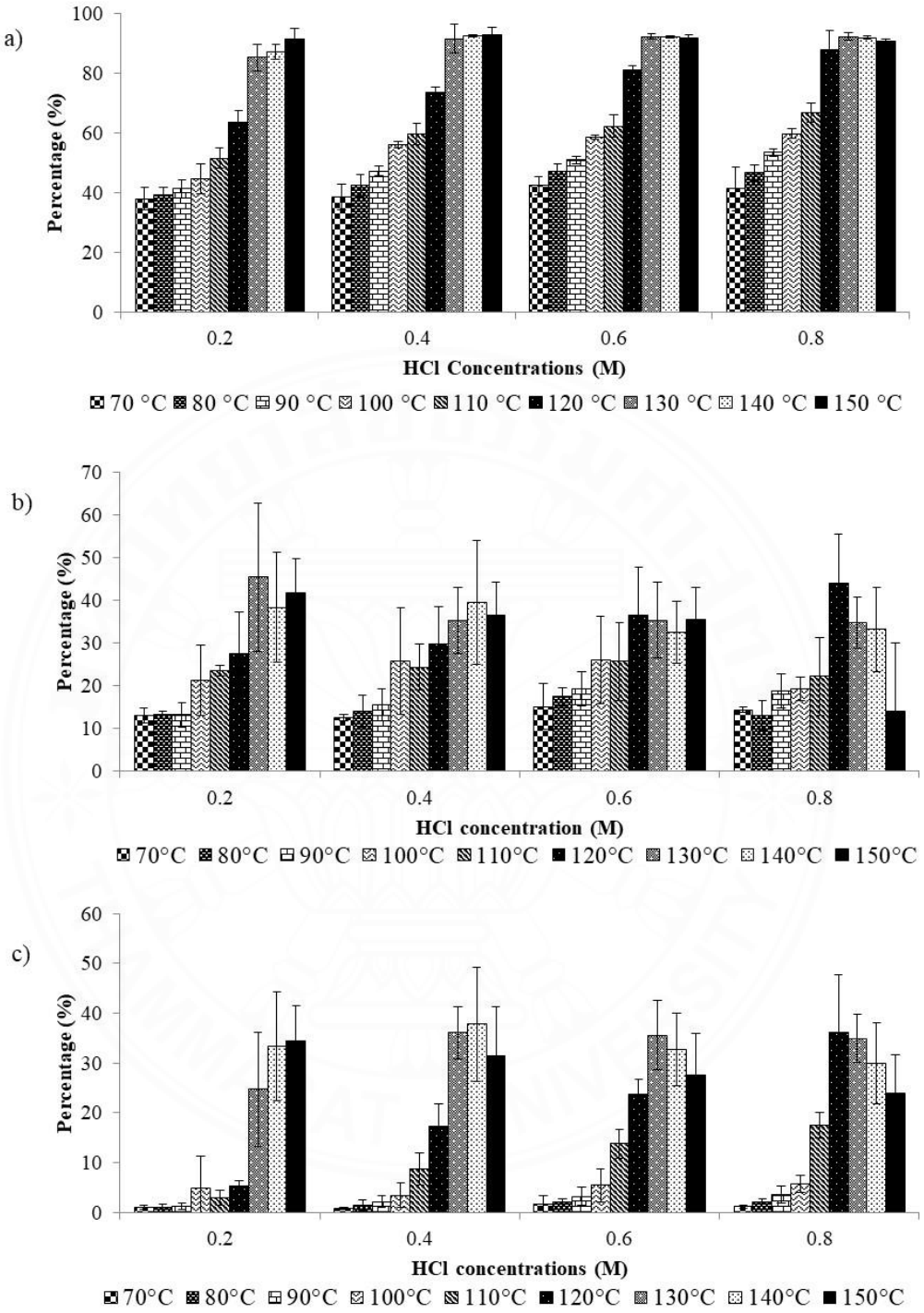

Figure 4.13 The effect of HCl concentration on the hydrolysis of grey oyster mushroom: a) solid loss, b) total carbohydrates, and c) reducing sugars at the reaction time15 min, ratio of grey oyster mushroom mass to reaction volume of 1:10 w/v and repeat 3 times.

4.3.3 Size Exclusion Chromatography (SEC) analysis

To see the effects of the reaction conditions on the oligosaccharides' sizes, the products were analyzed by size exclusion chromatography. The retention time of oligosaccharides with 2-20 is 29 min. Polysaccharide shows up at less than 25 min, while the retention time of monosaccharides is 31 min. After the hydrolysis reaction, the retention times of polysaccharides (short-chain) and oligosaccharides were less than 29 min while the retention time for monosaccharides was around 31 min. Monosaccharides were obtained at higher reaction temperatures of 140-150 ℃. The reaction temperature around 120-130 ℃ provides oligosaccharides, while lower temperatures result in higher polysaccharide portions. The length of shorter polysaccharides obtained was more than 36 units with a yield of 74.25% at 90 ℃, 15 min with 0.2 M HCl. Oligosaccharides with 2-5 units were obtained under the conditions at 120 ℃ for 15 min, and 0.6 M HCl solution. Monosaccharides were obtained at 150 ℃ for 15 min, and 0.6 M HCl solution.

4.3.4 HPLC analyses for types of monosaccharides

Polysaccharides in grey oyster mushroom were hydrolyzed by a combination of the heat generated by microwave radiation at the temperature range of 70 ℃ to 150 ℃, HCl concentration of 0.2 M to 0.8 M, and reaction time of 15 min, to produce oligosaccharides and monosaccharides. After the hydrolysis reaction, the monosaccharides glucose, arabinose, and mannose were observed by using carbohydrate column, with the retention times at 20, 25 and 30 min, respectively (Figure 4.14). As expected, glucose is the major monomer building block of the polysaccharides in grey oyster mushroom.

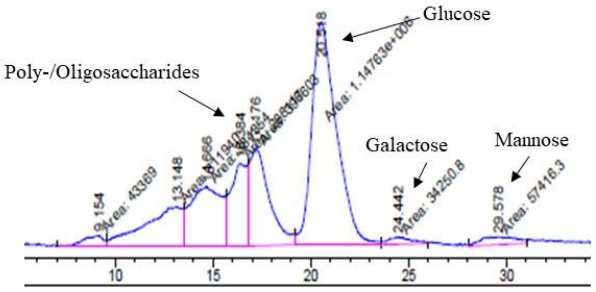

Figure 4.14 The HPLC chromatogram of the grey oyster mushroom after hydrolysis by using carbohydrate column.

4.3.5 ^{1}H NMR analysis

The ^{1}H NMR spectrum of the extracted grey oyster mushroom (at the conditions of 120 °C, 0.6 M HCl solution, 15 min) in D_2O is shown in Figure 4.15. The NMR spectrum shows a good agreement with the previously reported data (Zhaocheng et al., 2008). The signal of the spectrum is reported in chemical shifts, δ (ppm). The signal at δ 4.42 - 4.43 ppm corresponds to the proton signal (H-1) of β – anomeric protons. The signal at δ 4.5 - 4.8 ppm corresponds to the internal H-1 resonances of (1,3) backbone chain of grey oyster mushroom. The signal at δ 3.09 - 4.5 ppm corresponds to the internal H-1 of (1,3) - β - linked backbone chain and (1,6) - β - linked side chain. The ^{1}H NMR signals described above confirm the hydrolysis of grey oyster mushroom from polysaccharides into oligosaccharides with the repeating units of (1,3) and (1,6) - β-glucan form.

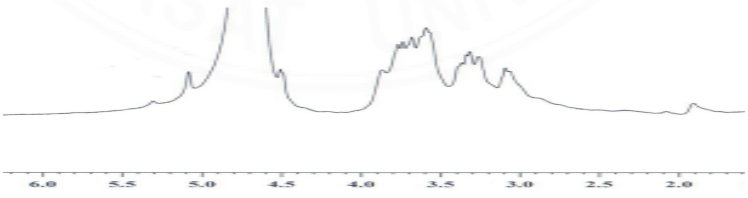

Figure 4.15 ^{1}H NMR spectrum of extracted glucans from grey oyster mushroom in D_2O at 30 °C.

4.4 Digestion of jew's ear mushroom

From the optimum conditions, the samples were digested at various reaction temperatures and HCl concentration. Mushroom samples were analyzed by HPLC methods such as SEC analysis and saccharide composition analysis.

4.4.1 Effects of the extracting temperature

The reaction temperature has an important role in the hydrolysis reaction of Jew's ear mushroom. Generally, solid loss (SL), total carbohydrate (TC) and reducing sugar (RS) of the hydrolyzed mushroom increased with increasing temperature. SL reached the maximum value of 98.27% under 130 °C, 15 min, and 0.8 M HCl (Figure 4.16a). TC reached the maximum value of 83.47% under 150 °C, 15 min, 0.2 M HCl (Figure 4.16b). RS reached the maximum value of 55.76% at 150 °C for 15 min with 0.2 M HCl (Figure 4.16c). The maximum values of SL, TC, and RS are in the reaction temperature range of 130 – 150 °C. Beyond this reaction temperature, SL, TC and RS decreased because some the carbohydrates and proteins in the hydrolyzed mushroom may be decomposed into a residual black solid at higher temperatures.

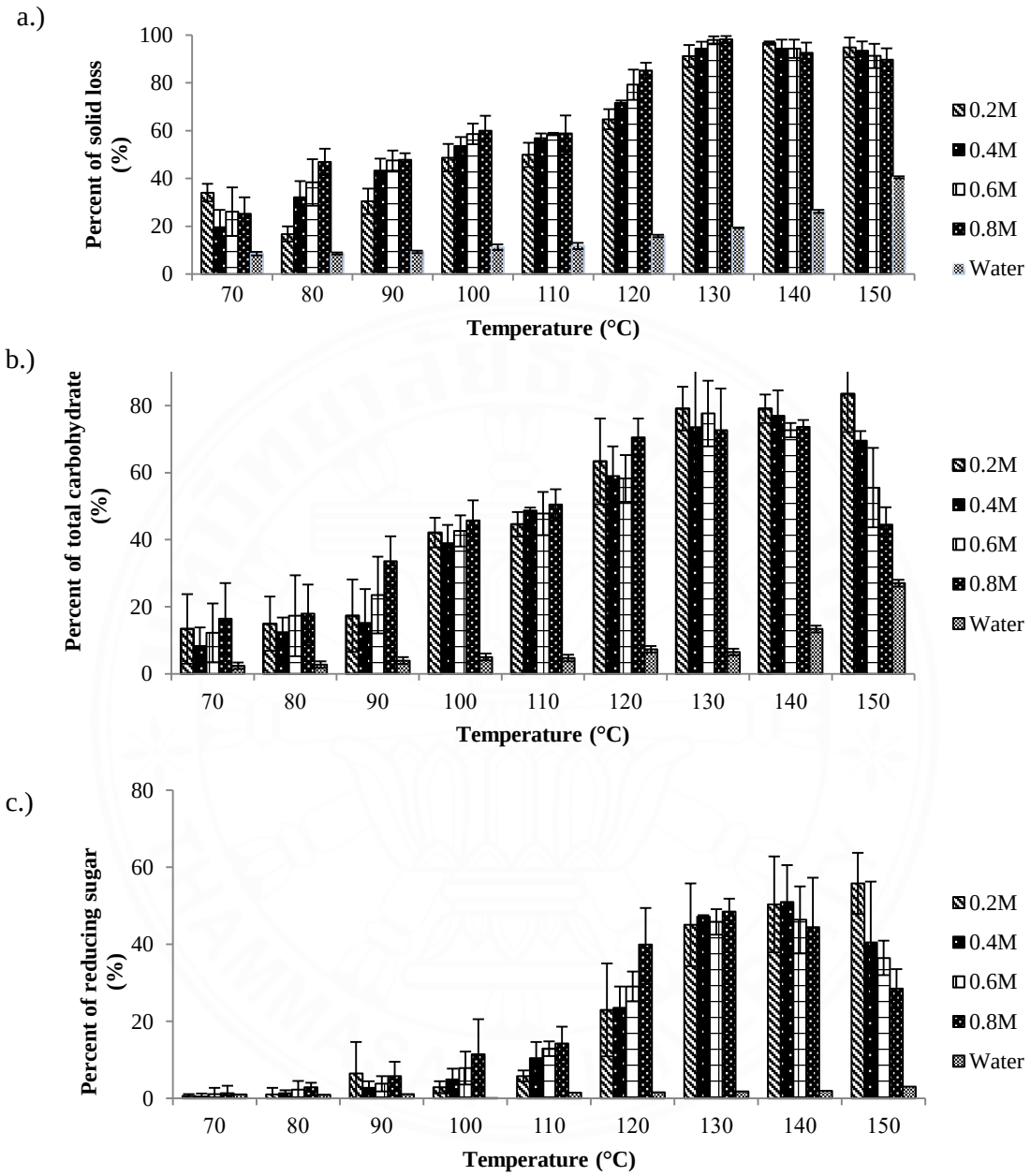

Figure 4.16 The effect of reaction temperature on the hydrolysis of jew's ear mushroom: a) solid loss, b) total carbohydrates, and c) reducing sugars at reaction time 15 min, ratio of jew's ear mushroom mass to reaction volume of 1:10, w/v and repeat 3 times.

4.4.2 Effects of HCl concentration

Jew's ear mushroom was hydrolyzed by a combination of the heat generated by microwave radiation with HCl as a catalyst, to produce polysaccharides and oligosaccharides. In general, the total amount of monosaccharides increased gradually with increasing temperature while the polysaccharides and oligosaccharides steadily decreased. Glucan and mannan are the two main polysaccharides of the cell wall of jew's ear mushroom, which was in agreement with literature (Synytsya et al. 2009). Polysaccharides that are subjected to high heat will result in hydrolysis to monosaccharides. The monosaccharides decreased when the reaction temperature was higher than 160 °C because the monosaccharides degraded to form intermediate degradation products of Maillard browning and caramelization reactions, and some residual black solid. Since the objective is to obtain oligosaccharides, the reaction temperature must not be too high. High extraction temperatures cause saccharides to break down faster. Higher temperatures also lead to more solvent loss and increased cost of the extraction processes. The high yield of glucan obtained in this study can be attributed due to the utilization of a combination of HCl as a catalyst and microwave radiation heating.

Table 4.6 The effect of reaction temperature on solid loss (SL), total carbohydrate (TC), and reducing sugar (RS) of hydrolyzed jew's ear mushroom.

	0.2M HCl			**0.4M HCl**			**0.6M HCl**			**0.8M HCl**		
Temperature (°C)	SL	TC	RS	SL	TC	RS	SL	TC	RS	SL	TC	RS
	(%, based on dried weight of mushroom)											
70	31.61	15.20	0.95	23.05	10.41	1.39	34.39	20.29	3.00	38.54	25.38	3.62
80	35.62	23.31	3.03	31.45	17.39	2.19	43.95	28.99	4.86	44.29	27.81	4.27
90	34.36	24.14	3.03	44.42	26.80	4.71	49.61	28.22	5.95	44.74	41.77	10.04
100	44.40	22.01	4.64	49.90	32.66	8.11	55.88	37.92	12.71	62.96	47.51	21.77
110	45.97	40.41	7.27	56.50	48.33	15.30	58.35	41.18	15.01	55.03	45.67	18.45
120	61.45	50.88	24.58	72.60	48.81	29.84	72.52	53.89	31.92	82.58	65.85	50.15
130	86.71	72.59	43.72	96.70	52.00	47.34	98.32	69.34	49.24	98.01	58.39	52.31
140	96.34	74.30	64.10	96.50	74.13	61.95	96.34	70.52	56.29	94.20	75.43	59.21
150	96.01	70.70	64.87	94.94	66.32	56.43	91.04	43.48	41.49	90.15	38.75	34.30

Note: *at reaction time 15 min, ratio of mushroom mass to reaction volume of 1:10, w/v)*

The concentration of HCl has an important role in the hydrolysis of mushroom. SL, TC, and RS significantly increased with increasing HCl concentrations. SL, TC, and RS increased dramatically when the HCl concentrations increased from 0.2 to 0.8 M. SL gradually increased and reached the maximum value of 98.27% under 130 °C, 15 min, and 0.8 M HCl (Figure 4.17). TC gradually increased and reached the maximum values of 83.47% under 150 °C, 15 min, and 0.2 M HCl. RS gradually increased and reached the maximum value of 55.76% under the reaction conditions of 150 °C, the reaction time of 15 min, and 0.2 M HCl solution, respectively.

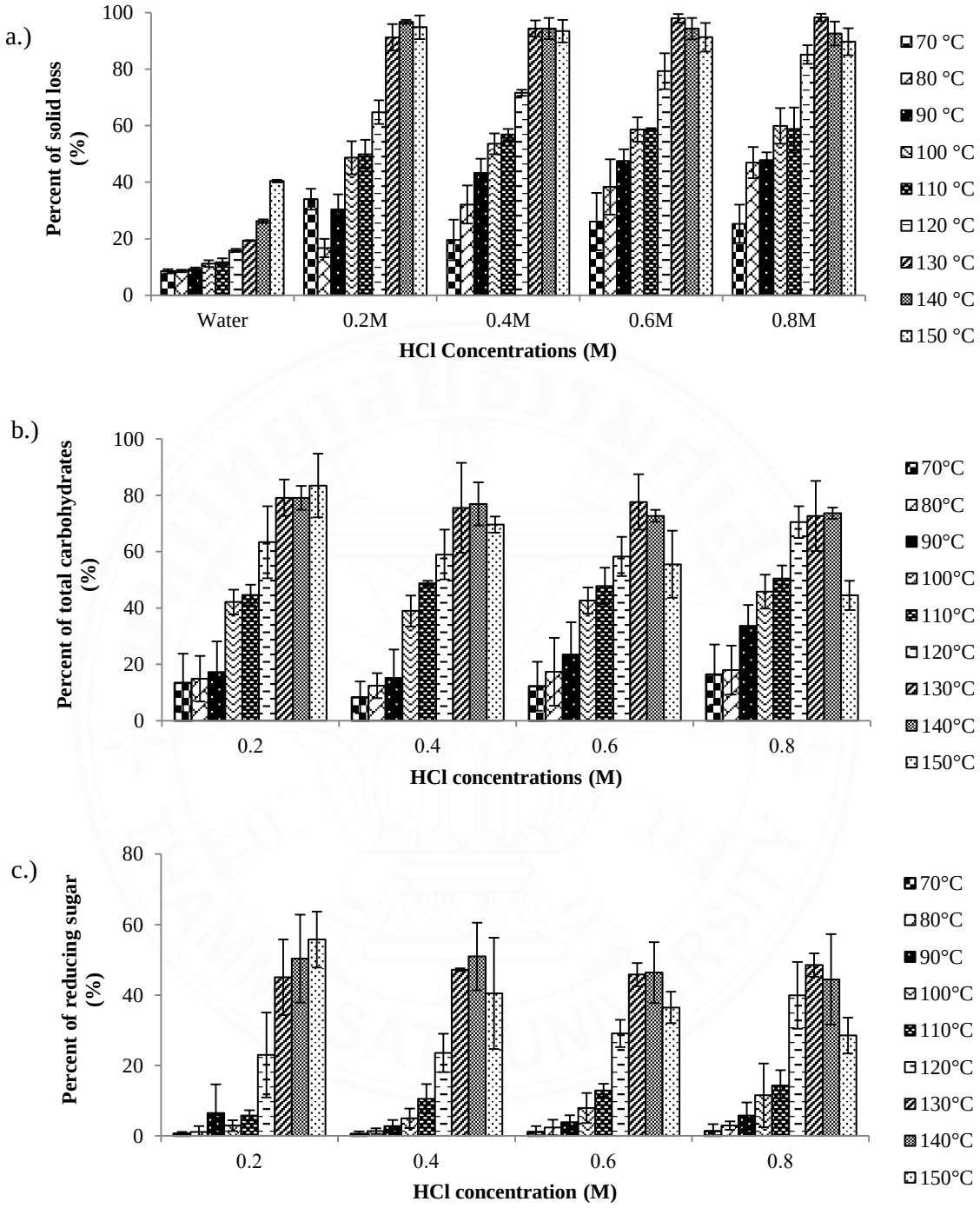

Figure 4.17 The effect of HCl concentration on the hydrolysis of jew's ear mushroom: a) solid loss, b) total carbohydrates, and c) reducing sugars at the reaction time15 min, ratio of jew's ear mushroom mass to reaction volume of 1:10, w/v and repeat 3 times.

4.4.3 Size Exclusion Chromatography (SEC) analysis

To see the effects of the reaction conditions on the oligosaccharides' sizes, the products were analyzed by size exclusion chromatography. Oligosaccharides with 2-20 units had retention times at 29 min, and polysaccharides had reaction times less than 25 min. Monosaccharides had reaction times of 31 min. After the hydrolysis reaction, monosaccharides were obtained at higher reaction temperatures of 140-150 ℃. The reaction conditions with the extraction temperatures around 120-130 ℃ provided oligosaccharides while the low temperatures resulted in higher polysaccharides. The length of shorter polysaccharides obtained were 883-1037 units with the yield of 61.82% at 70 ℃, 15 min, and 0.6 M HCl. Oligosaccharides with 2-20 units were obtained under 130 ℃, 15 min, and 0.6 M HCl. Monosaccharides were obtained under 120 ℃, 15 min, and 0.8 M HCl.

4.4.4 HPLC analyses for types of monosaccharides

Polysaccharides in jew's ear mushroom were hydrolyzed by a combination of the heat generated by microwave radiation at the temperature range of 70 ℃ to 150 ℃, HCl concentration of 0.2 M to 0.8 M, and reaction time of 15 min to produce oligosaccharides and monosaccharides. After the hydrolysis reaction, polysaccharides and oligosaccharides were present at the retention time of less than 20 minutes. The monosaccharides that were present were glucose, arabinose, and mannose with the retention times of 20, 25 and 30 min, respectively (Figure 4.18).

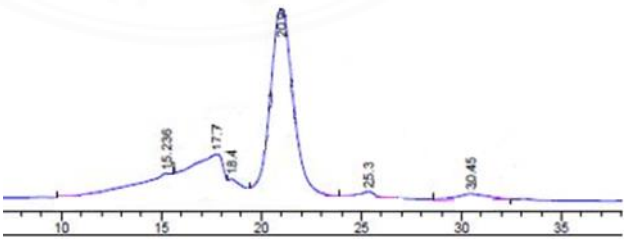

Figure 4.18 The HPLC chromatogram of the jew's ear mushroom after hydrolysis by using carbohydrate column.

4.4.5 ^{1}H NMR analysis

The ^{1}H NMR spectrum of the extracted jew's ear mushroom (at the reaction conditions of 130 °C, 0.6 M HCl solution, 15 min) in D_2O is shown in Figure 4.19. The NMR spectrum shows a good agreement with the previously reported data (Zhaocheng et al., 2008). The signal of the spectrum is reported in chemical shifts, δ (ppm). The signal at δ 4.42 - 4.43 ppm corresponds to the proton signal (H-1) of β – anomeric protons. The signal at δ 4.5 - 4.8 ppm corresponds to the internal H-1 resonances of (1,3) backbone chain of grey oyster mushroom. The signal at δ 3.09 - 4.5 ppm corresponds to the internal H-1 of (1,3) - β - linked backbone chain and (1,6) - β - linked side chain. The ^{1}H NMR signals described above confirm the hydrolysis of grey oyster mushroom from polysaccharides into oligosaccharides with the repeating units of (1,3) and (1,6) - β-glucan form.

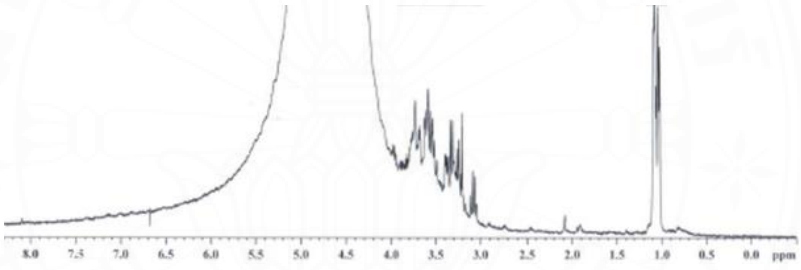

Figure 4.19 ^{1}H NMR spectrum of extracted glucans from jew's ear mushroom in D_2O at 30 °C.

4.5 Biological test for adjuvant activities

The extracted oligosaccharides (with 2-20 units) from grey oyster mushroom and jew's ear mushroom solution at the conditions of 120 °C, 15 min, 0.6 M HCl solution (G7) and 130 °C, 15 min, 0.6 M HCl solution (E15) was purified by precipitations with propanol. Each extracted oligosaccharide sample was mixed with Keyhole Limpet Hemocyanin (KLH) antigen to observe the immunomodulatory effects of the extracted oligosaccharide when compared with KLH alone. KLH can be used to enhance the immune response against small peptides and it is used extensively as a carrier protein in the production of antibodies. It is a cytokine signaling molecule in the immune system that regulates the activities of white blood cells (leukocytes, often lymphocytes) that are responsible for immunity.

Raw 264.7 macrophages were treated with 10 µg/mL of KLH antigen with or without the reagents (E is jew's ear mushroom and G is grey oyster mushroom) at a concentration of 10.0 µg/mL in a 6-well plate with a density of 500,000 cells per well. 200 µL of supernatants without adding the medium were collected for each indicated times. After that, the supernatant was analyzed by ELISA to measure the level of IL-2. Data are presented as mean±SEM (***p < 0.0001; ** p < 0.01; * p < 0.05; all relative to the KLH control).

Grey oyster mushroom (G7 and G16) showed an inflammatory property by significantly increase the production of Interleukin-2 (IL-2), a type of cytokine signaling molecule at 4 hr and 12 hr of the treatment when compared with KLH alone. The oligosaccharides (G7 and G16) enhanced the KLH stimulation of raw white blood cells 264.7 macrophages to secrete cytokines called IL-2 show in Figure 4.20.

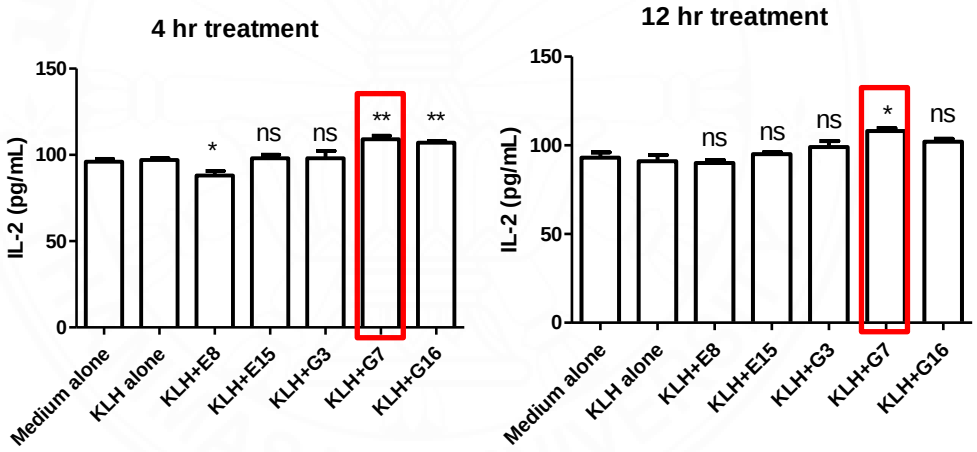

Figure 4.20 β-glucan activities from grey oyster mushroom solution after treatment with antigen (KLH); KLH+G7 is the grey oyster mushroom solution at the reaction condition 120 °C, 0.6 M HCl, 15 min mixed with KLH antigen.

For jew's ear mushroom, at 4 hr, E15 and E8 did not significantly enhanced the KLH stimulation of raw white blood cells 264.7 macrophages to secrete cytokines called IL-2 when compared with KLH alone in Figure 4.21. On the other hand, E8 showed an anti-inflammatory property by significantly decrease the production of IL-

2. At 24h of the treatment, the IL-2 productions were decreased by the presence of E8 and E15.

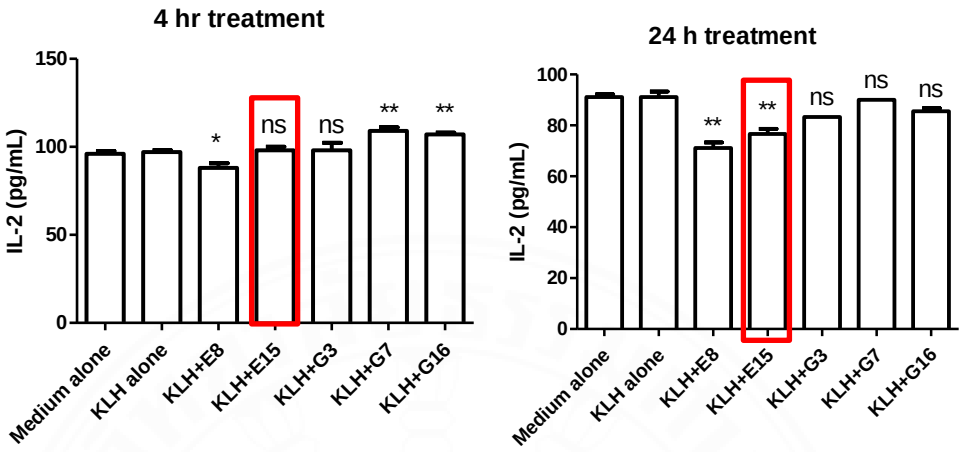

Figure 4.21 β-glucan activities from jew's ear mushroom solution after treatment with antigen (KLH); KLH+E15 is the jew's ear mushroom solution at the reaction condition 130 °C, 0.6 M HCl, 15 min mixed with KLH antigen.

The adjuvant activity of different mushrooms showed different responses, depending on the saccharide structures in each mushroom, the number of saccharide's unit and the concentration of the samples. The result showed that the oligosaccharides from grey oyster mushroom showed an inflammatory property by significantly increase the production of IL-2 better than jew's ear mushroom. The result from SEC analysis showed the oligosaccharides obtained from grey oyster mushroom have a smaller number of saccharide's repeated unit than those obtained from jew's ear mushroom. Therefore, the oligosaccharides from grey oyster mushroom have higher adjuvant activity than the oligosaccharides from jew's ear mushroom. A similar trend was observed by Sermwittayawong et al. (2020). The study showed that a small β-glucan can stimulate the breakdown of fat or lipolysis in the fat cells, suggesting a potential anti-obesity effect (Sermwittayawong et al., 2020).

CHAPTER 5
CONCLUSIONS

In this study, we successfully developed an efficient method to extract oligosaccharides from CCM, grey oyster mushroom and jew's ear mushroom by a combination of microwave and acid hydrolysis to obtain mannan and glucan.

The optimized hydrolysis conditions of CCM using acid as a catalyst and microwave radiation to obtain the highest yield of monosaccharides from the hydrolysis of CCM is at 170 ℃, the reaction time of 15 min, 0.05 M HCl solution, and 1:10 (g /mL) ratio of CCM mass to acid solution. The highest yield of oligosaccharides in the range of units 2 to 18 units was obtained at 110 ℃, the reaction time 15 min, 0.2 M HCl solution, and 1:80 (g/mL) ratio of CCM mass to acid solution. Higher temperature and lower reaction volume to CCM ratio results in higher yield of monosaccharides.

The major saccharide compositions of grey oyster mushroom and jew's ear mushroom are glucan. The effects of the extraction temperature and acid concentration were investigated to determine the optimized conditions which provide the highest oligosaccharide yield. The best conditions for the hydrolysis of grey oyster mushroom which resulted in oligosaccharides is at 120 ℃, the reaction time of 15 min with 0.6 M HCl solution provide the maximum yield of 2.24%. The oligosaccharides obtained at these conditions have around 2-5 units. The best conditions for the hydrolysis on jew's ear mushroom, which result in oligosaccharides is at 130 ℃, the reaction time of 15 min, and with 0.6 M HCl solution provide the maximum yield of 1.28%. The oligosaccharides obtained at these conditions have around 2-20 units. The present findings emphasize the importance of microwave radiation to obtain the oligosaccharides in shorter extraction times when compared to enzyme hydrolysis procedures. It shows that temperature and HCl concentration have considerable effects on glucan oligosaccharides selective production from grey oyster mushroom and jew's ear mushroom. High temperature effectively promotes the hydrolysis of grey oyster mushroom and jew's ear mushroom, resulting in higher monosaccharide production, but not many oligosaccharides were obtained because oligomers were hydrolyzed to monomers. HCl at a high concentration effectively catalyzed the hydrolysis of grey oyster mushroom and jew's ear mushroom at a medium temperature. The highest amount of carbohydrates were extracted out of grey oyster mushroom and jew's ear

mushroom by high HCl concentration, while medium temperature slowed down the possible hydrolysis of oligosaccharides to monosaccharides.

The purified polysaccharide was characterized using ^{1}H nuclear magnetic resonance (NMR) to confirms that the hydrolysis of grey oyster mushroom and jew's ear mushroom from polysaccharides into oligosaccharides has repeat units of (1,3) and (1,6) - β-glucan form.

In addition, the extracted oligosaccharide from grey oyster mushroom and jew's ear mushroom solution after purification by precipitations with propanol was as mixed with KLH antigen to observe the immunomodulatory effects when compared with KLH alone. The IL-2 productions were significantly decreased by the presence of the products from the reaction conditions of jew's ear mushroom at 130 °C, 15 min, 0.6 M HCl solution at 24 hr of the treatment. On the other hand, the IL-2 productions were significantly increased by the presence of the products from the reaction conditions of grey oyster mushroom at 120 °C, 15 min, 0.6 M HCl solution at 4 hr and 12 hr of the treatment. The adjuvant activity of the grey oyster mushroom components shows an inflammatory property by significantly increase the production of IL-2 better than jew's ear mushroom.

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APPENDICES

APPENDIX A
STANDARD CURVE

Total carbohydrate standard curve

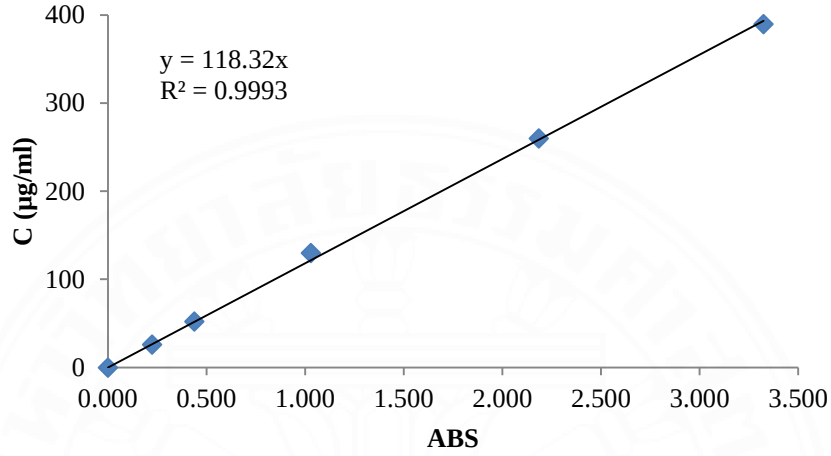

Figure 1 Standard curve for TC determination of mushrooms

Reducing sugar standard curve

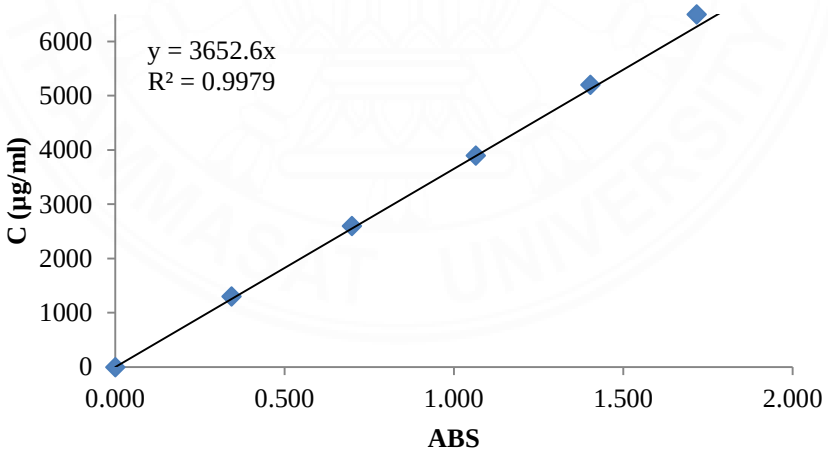

Figure 2 Standard curve for RS determination of mushrooms

Saccharides standard curves on High Performance Liquid Chromatography

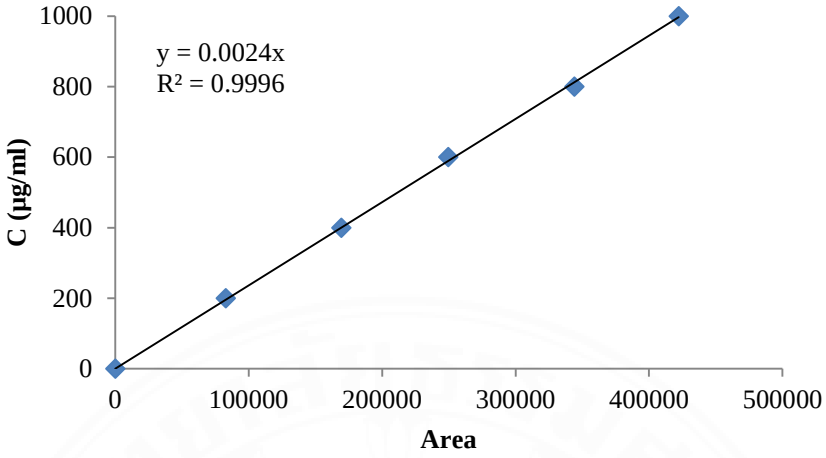

Figure 3 Glucose standard curve

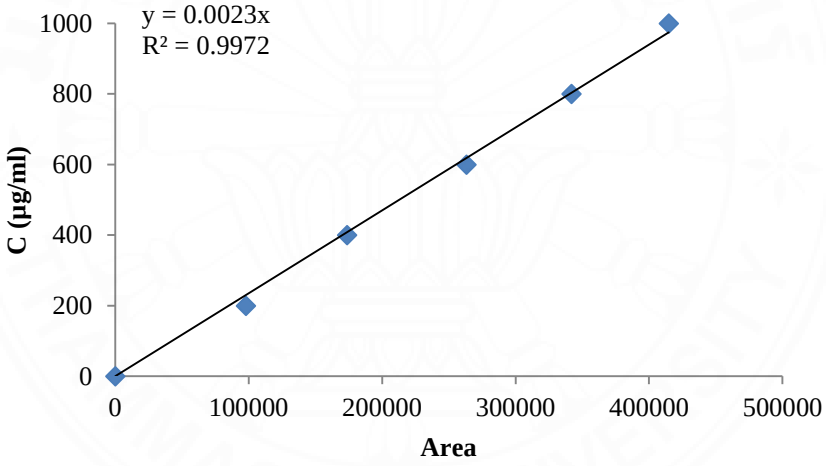

Figure 4 Galactose standard curve

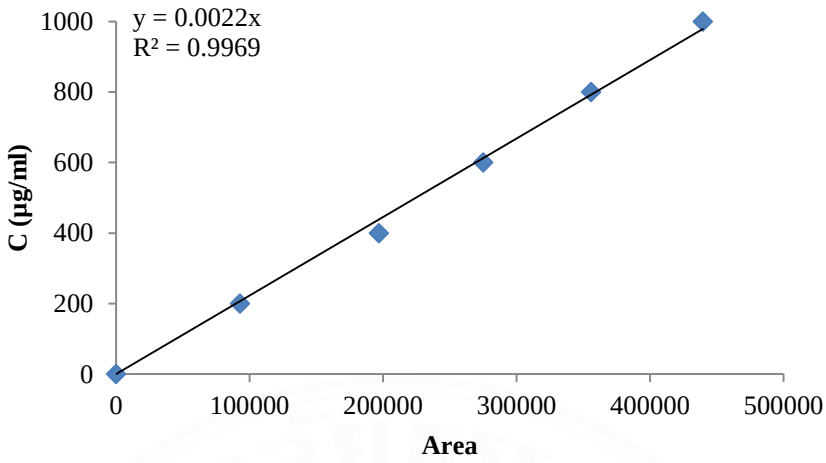

Figure 5 Arabinose standard curve

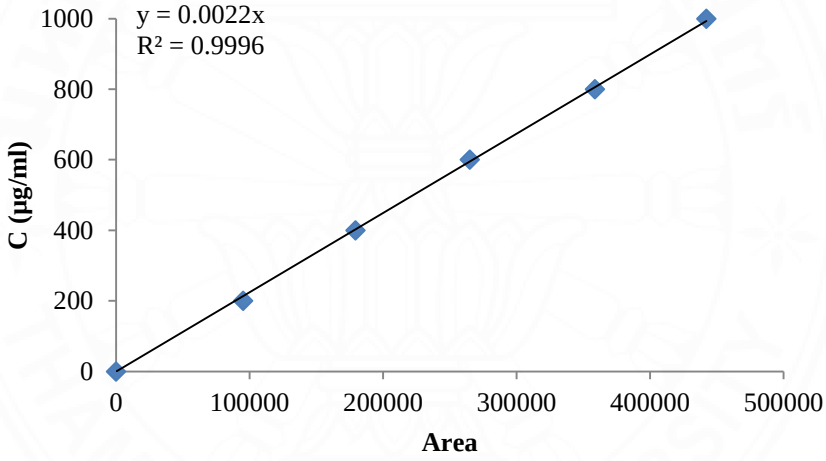

Figure 6 Mannose standard curve

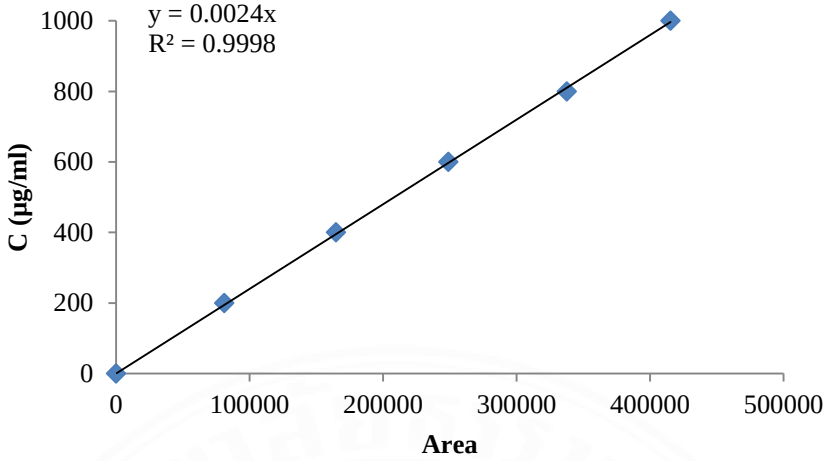

Figure 7 Fructose standard curve

High Performance Liquid Chromatography chromatogram of standard saccharides

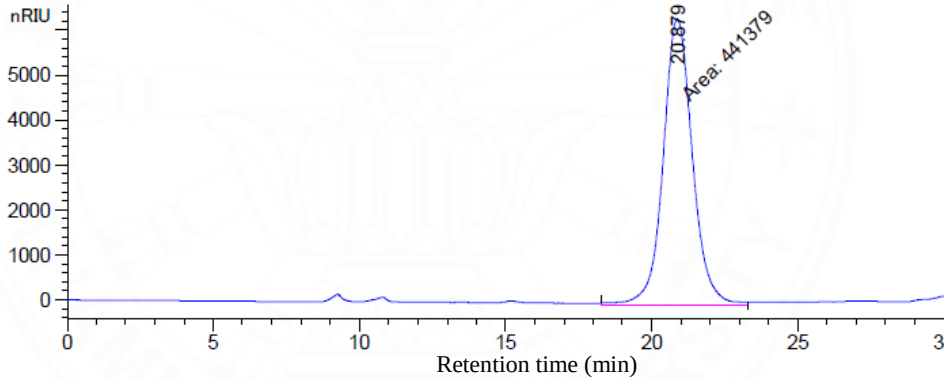

Figure 8 HPLC chromatogram of standard monosaccharide (glucose)

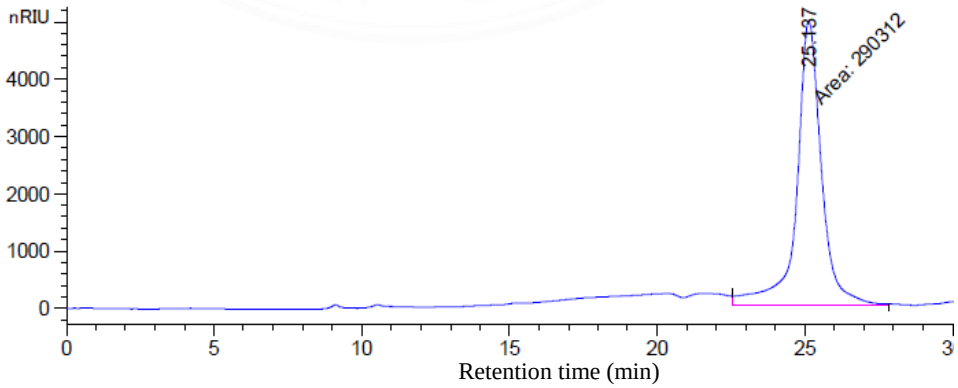

Figure 9 HPLC chromatogram of standard monosaccharide (galactose)

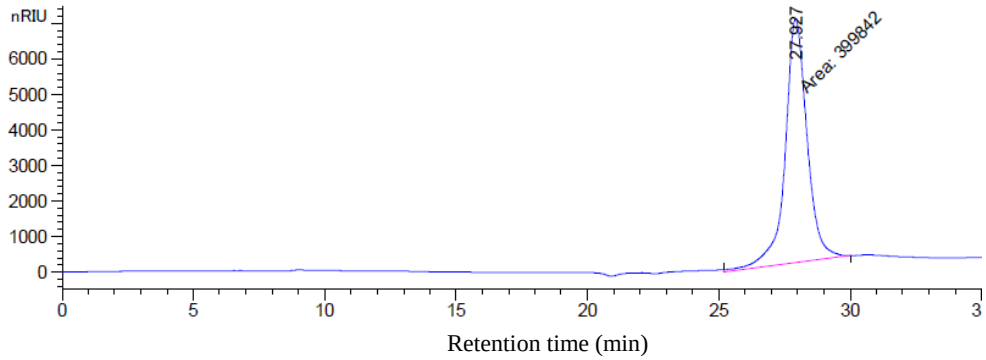

Figure 10 HPLC chromatogram of standard monosaccharide (arabinose)

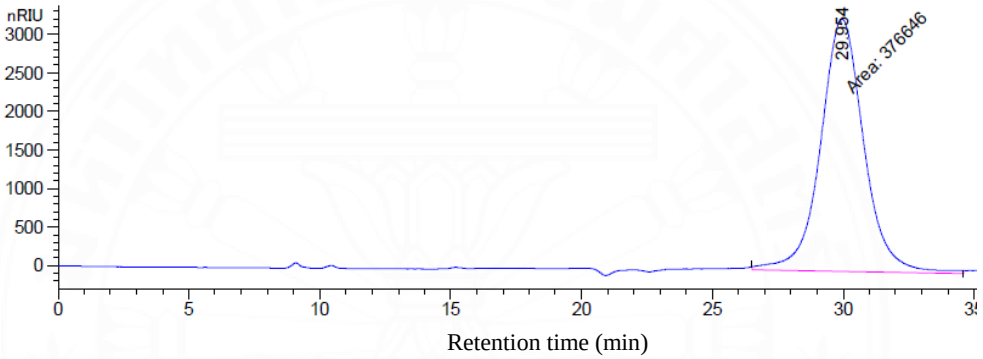

Figure 11 HPLC chromatogram of standard monosaccharide (mannose)

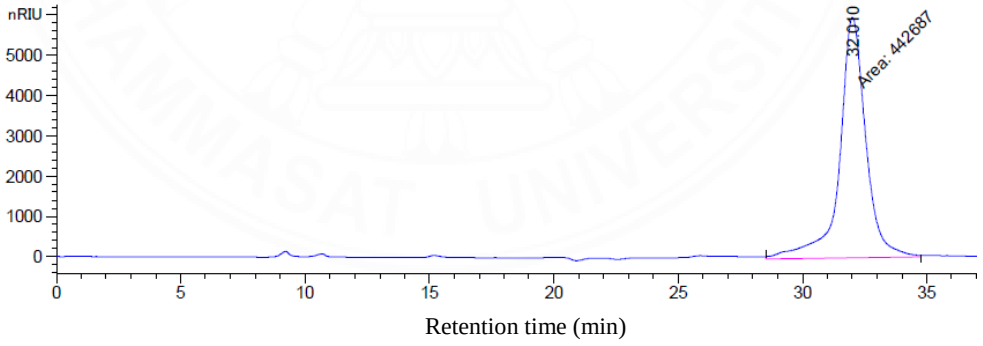

Figure 12 HPLC chromatogram of standard monosaccharide (fructose)

Table 1 The range of retention time of interest for oligosaccharides with 2 to more than 1000 units.

No. of sugar unit	Molecular Weight	Retention time (min)
2-10	342-1,638	29.4072 - 26.0806
11-20	1,800-3,258	25.8803 - 24.6203
21-50	3,420-8,118	24.5172 - 22.6814
51-100	8,280-16,218	22.6395 - 21.2117
101-1,000	16,380-162,018	21.1906 - 16.3239
>1,001	>162,180	<16.3218

APPENDIX B
COMMERCIALLY AVAILABLE BETA-GLUCAN FROM BAKER'S YEAST (S. CEREVISIAE)

The commercially available beta-glucan from baker's yeast (*S. cerevisiae*) was used as a control sample. The beta-glucan from baker's yeast (*S. cerevisiae*) purchased from Transfer Point, Inc is the best immunity product selling in USA with the highest purity of beta-glucan. The structure of the commercially available sample consists of 1,3-linked glucose (86.6%), 1,6-linked glucose (2.1%), 2,3-linked glucose (5.8%), 3,6-linked glucose (1.9%), and terminal glucose (3.6%). The structure of beta-glucan from mushroom and yeast linkage has the same *β*-1,6-D glucopyranose branching on the poly *β*-1,3-glucopyranose backbone.

The reaction conditions used for size analysis of beta-glucan from baker's yeast (*S. cerevisiae*) was the same conditions as those used for grey oyster mushroom and jew's ear mushroom. The reaction temperature range of 70°C to 150°C and HCl concentration range of 0.2 to 0.8M was used to hydrolyze the product. By using three columns of PL aquagel-OH equipped with PL aquagel-OH guard, monosaccharides with 1 unit were obtained at the retention time of 30 minutes while the retention time for polysaccharides and oligosaccharides were at less than 29 minutes. Polysaccharides in baker's yeast (*S. cerevisiae*) were converted to monosaccharides at high temperatures of 110- 150°C. Before hydrolysis of beta-glucan from baker's yeast (*S. cerevisiae*) has a peak at the retention time around 16-17 minutes which shows most of the sugars present were 600 to 900 units (Figure 1).

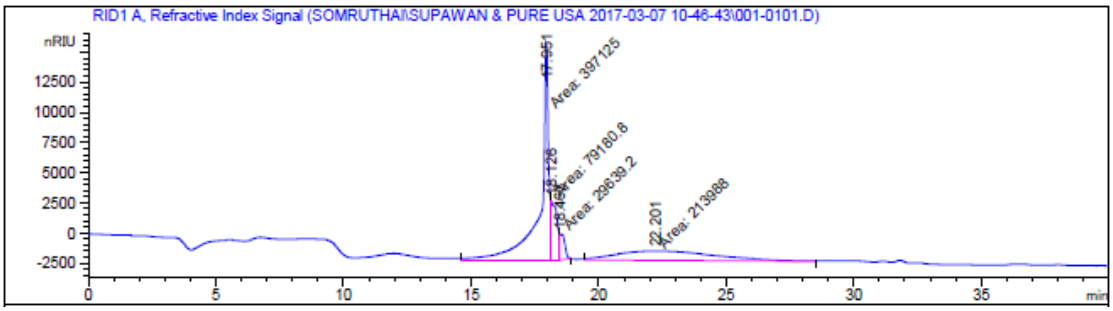

Figure 1 SEC analyzed chromatogram of beta-glucan from baker's yeast (S. cerevisiae) before hydrolyzed

Polysaccharides in baker's yeast (*S. cerevisiae*) were hydrolyzed by using microwave radiation and HCl as catalyst to produce polysaccharides (short chain) and oligosaccharides which have the retention time of less than 20 minutes. The predominant monosaccharide present in baker's yeast (S. cerevisiae) is glucose. Glucose was obtained at reaction temperatures of 110 °C to 150 °C for screening purposes. The HPLC chromatogram for beta-glucan from baker's yeast (*S. cerevisiae*) before the hydrolysis reaction is shown in Figure 35. The peaks shown are at retention time around 9-10 minutes which indicates polysaccharides (69.63%), 19 minutes which indicates oligosaccharides (7.03%), 26 minutes which indicates arabinose (2.71%), and 30 minutes which indicates mannose (20.64%) as shown in Figure 2.

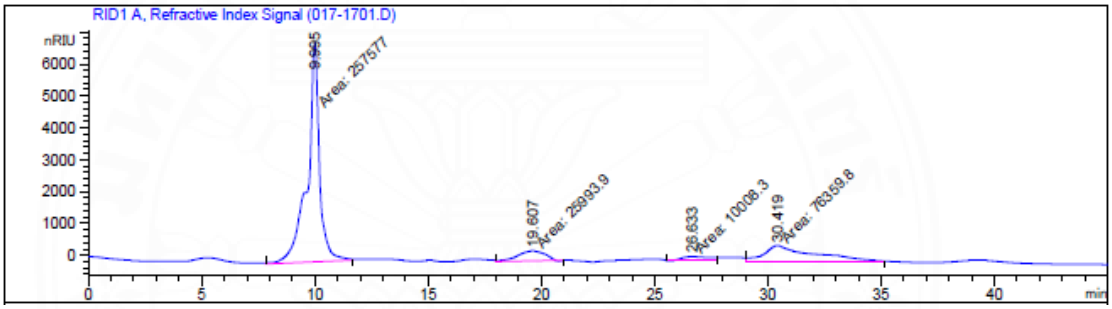

Figure 2 Saccharides HPLC chromatogram of beta-glucan from baker's yeast (S. cerevisiae) before hydrolysis with carbohydrate column

APPENDIX C
BIOLOGICAL ANALYSIS

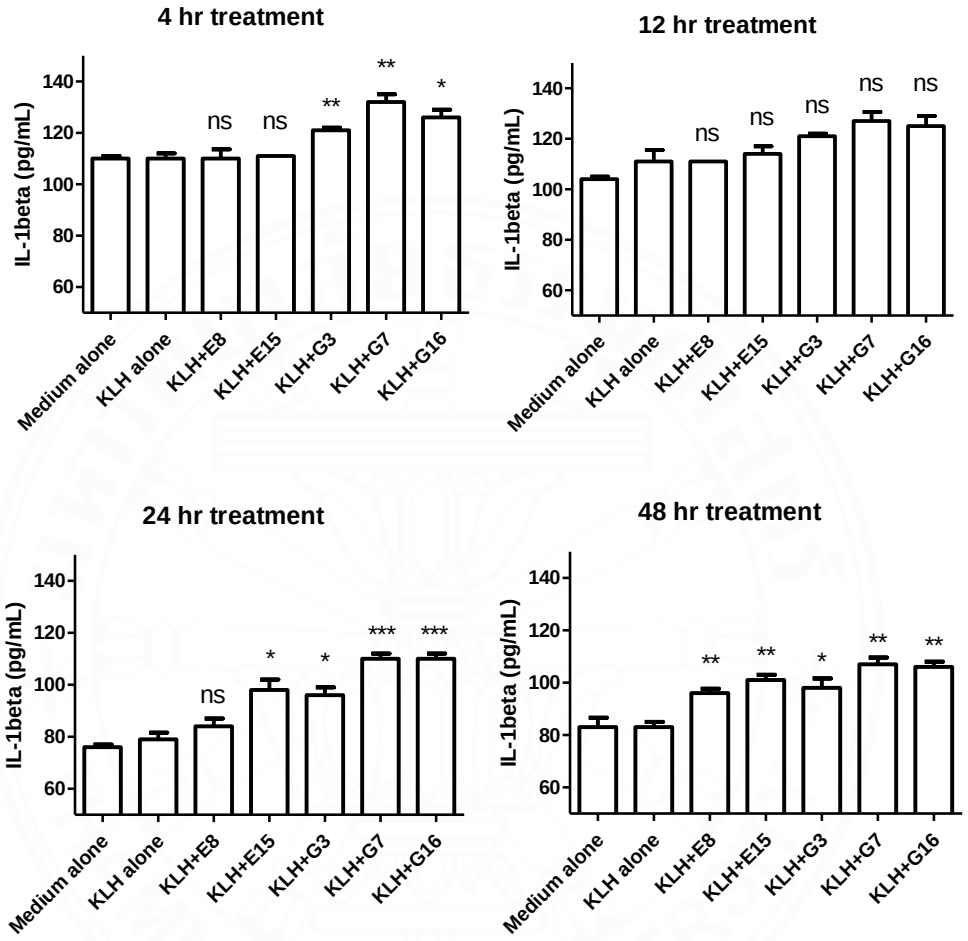

Figure 1 The supernatant was analyzed by ELISA to measure the level of IL-1beta. Data are presented as mean±SEM (***p < 0.0001; ** p < 0.01; * p < 0.05; all relative to the KLH control).

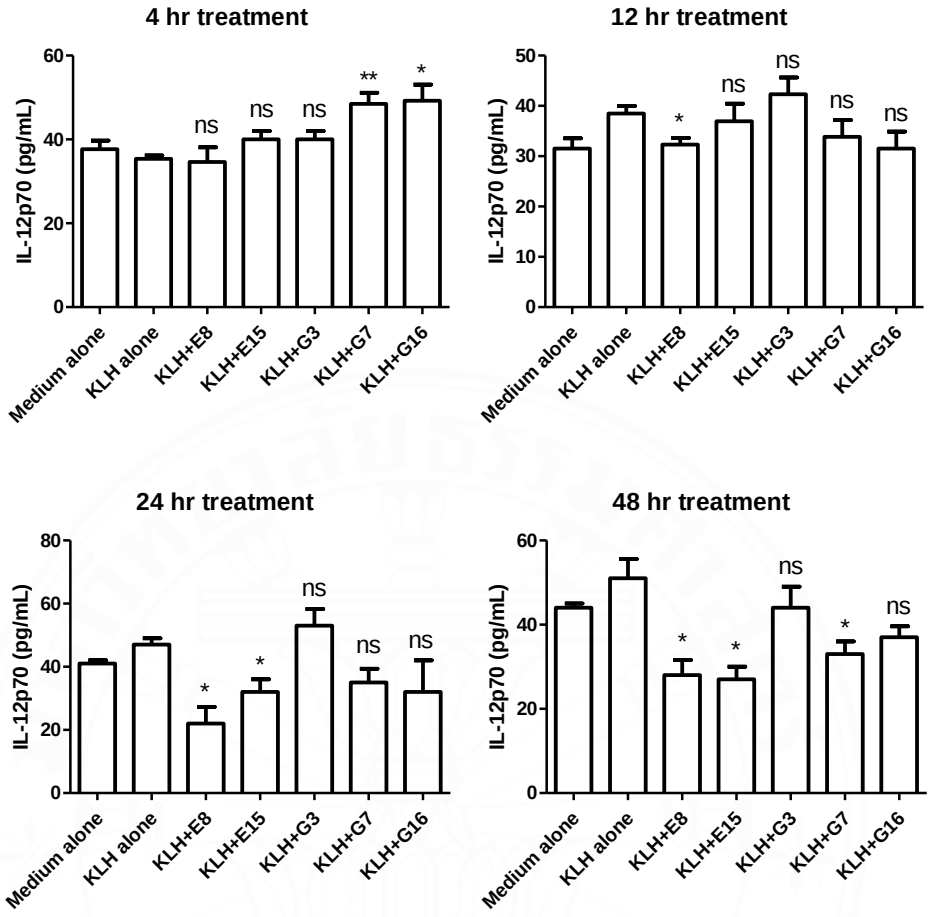

Figure 2 The supernatant was analyzed by ELISA to measure the level of IL-12. Data are presented as mean±SEM (***p < 0.0001; ** p < 0.01; * p < 0.05; all relative to the KLH control).

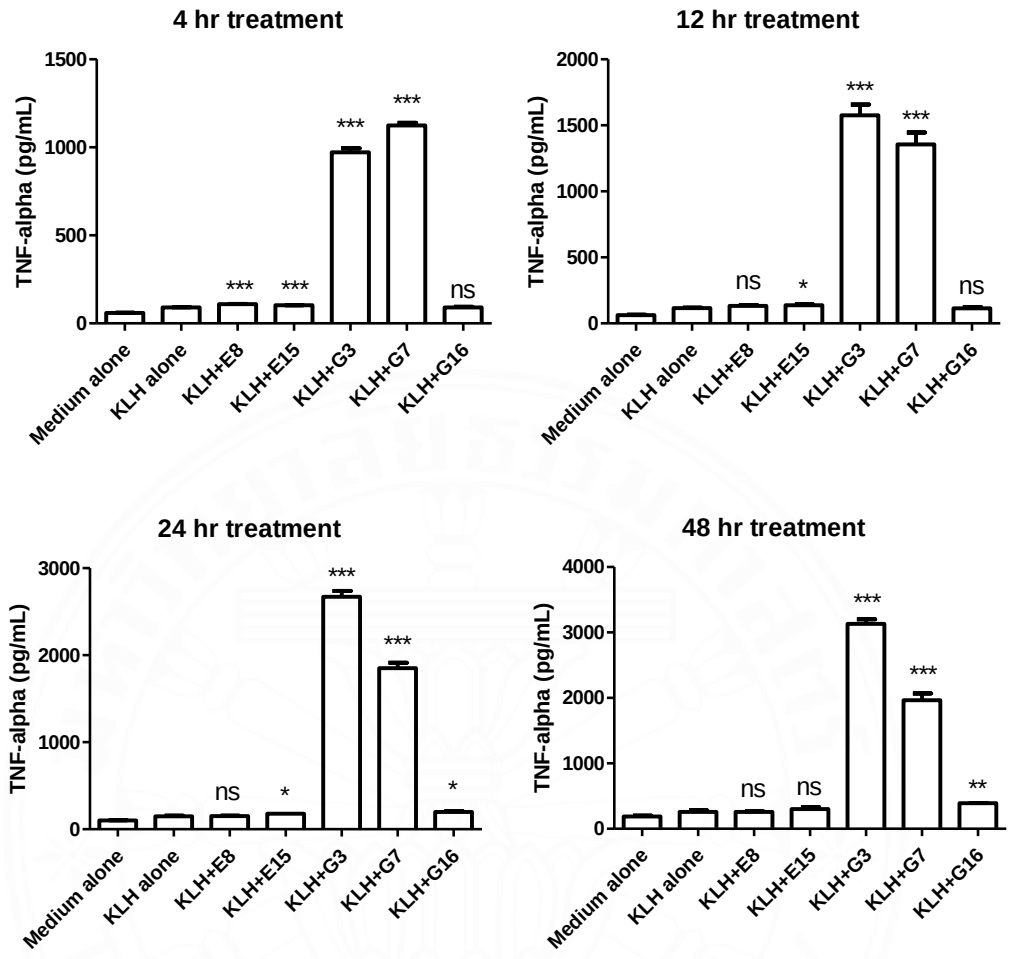

Figure 3 The supernatant was analyzed by ELISA to measure the level of TNF-α. Data are presented as mean±SEM (***p < 0.0001; ** p < 0.01; * p < 0.05; all relative to the KLH control).

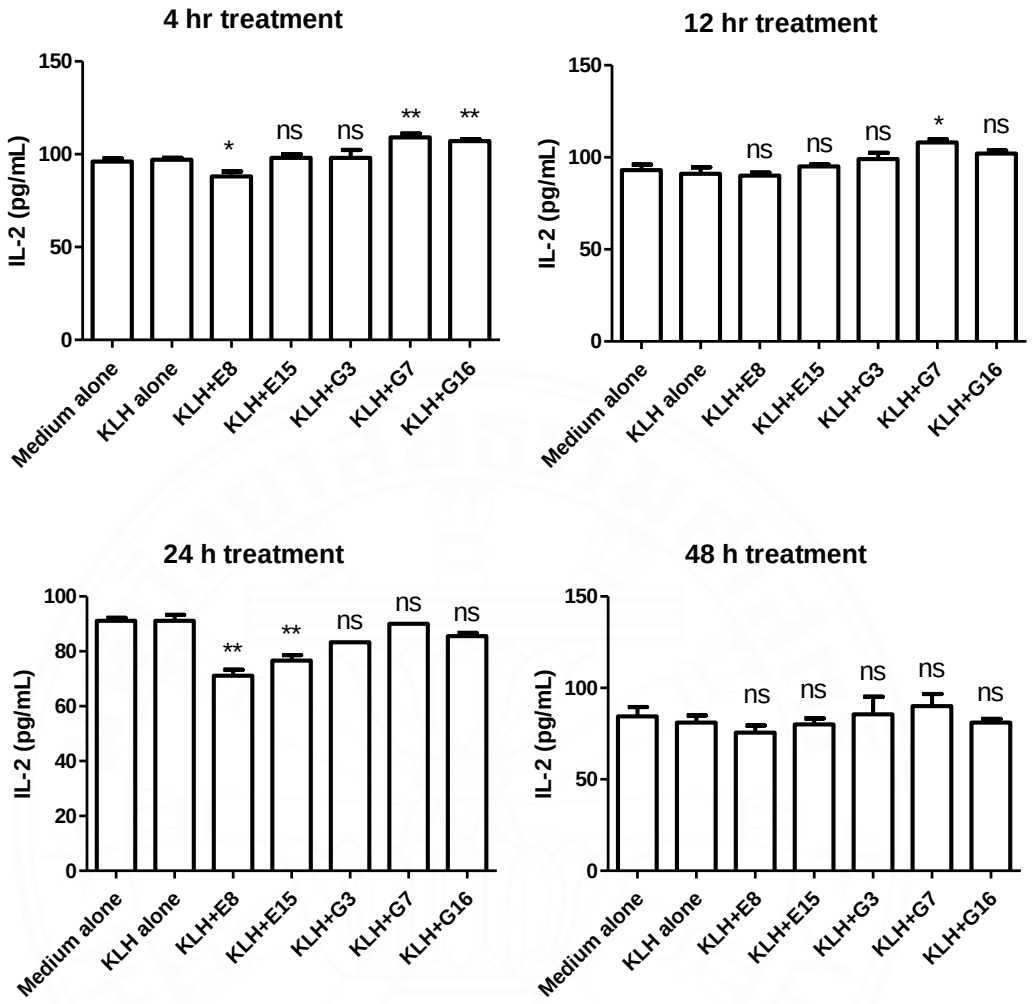

Figure 4 The supernatant was analyzed by ELISA to measure the level of IL-2. Data are presented as mean±SEM (***p < 0.0001; ** p < 0.01; * p < 0.05; all relative to the KLH control).

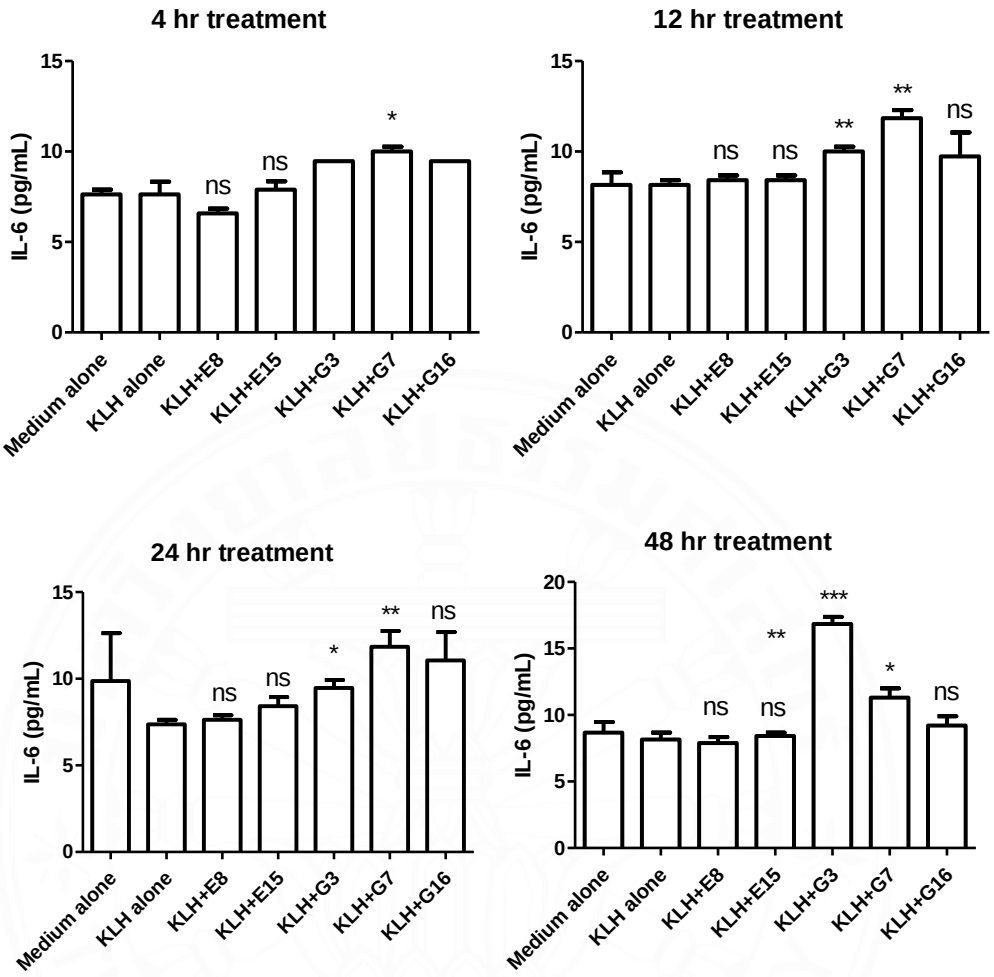

Figure 5 The supernatant was analyzed by ELISA to measure the level of IL-6. Data are presented as mean±SEM (***p < 0.0001; ** p < 0.01; * p < 0.05; all relative to the KLH control).

BIOGRAPHY

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Publications

Sermwittayawong, D., Patninan, K., Jakkawanpitak, C., Phothiphiphit, S., Boonyarattanakalin, S., Inafuku, M., Oku, H., Noipha, K., & Hutadilok-Towatana, N. (2020). Effect of Purified Soluble Polysaccharides Extracted from Gray Oyster Mushroom [Pleurotus sajor-caju (Fr.) Sing.] on 3t3-L1 Adipocytes. *Sains Malaysiana*, 49(1), 103-112.