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Microwave Extraction of Oligosaccharides from Grey Oyster Mushroom by Microwave Facilitated Hydrolysis

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ABSTRACT

The main carbohydrate component of grey oyster mushroom (*Pleurotus sajor-caju* (Fr.) Sing.) is β-glucan, a polysaccharide of glucose linked by β-(1-3), β-(1-6), and α-(1-3) glycosidic bonds. The β-glucan in the form of oligosaccharides has been recognized as an adjuvant and exhibits prebiotic properties. In this report, oligosaccharides were extracted from a fine powder of grey oyster mushroom by a combination of microwave radiation heating and acid hydrolysis. The use of microwave radiation provides more heating efficiency and shortens reaction time. The hydrolysis product was purified by precipitation with propanol. The effects of reaction temperature (70 to 150 °C), a reaction time of 15 min, and HCl concentration (0.2, 0.4, 0.6, and 0.8 M) on the degree of polymerization (DP) of the extracted saccharides were determined. The extracted saccharides were characterized by using size exclusion chromatography and NMR. The analysis showed smaller sizes of extracted saccharides (DP = 2 to 5) when using the higher HCl concentration of 0.6 M. The maximum yield (2.24%) of extracted oligosaccharides was obtained at the conditions of 120 °C, 15 min with 0.6 M HCl as a catalyst.

Keywords: β-glucans; Grey oyster mushroom; Hydrolysis; Immunomodulatory; Microwave radiation; *Pleurotus sajor-caju* (Fr.) Sing.

1. Introduction

Recently, growing demand for prebiotics has prompted the finding of potential and affordable prebiotic sources for the food industry. One of the potential abundant prebiotic sources is mushrooms because they contain carbohydrates such as chitin, hemicellulose, β- and α-glucans, mannan, xylan, and galactan [1].

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The mixture of non-digestible polysaccharides, lignin, and other plant cell wall constituents is dietary fiber. The polysaccharide prebiotics are resistant to hydrolysis by human enzymes, but maybe digested by probiotic bacteria and have health benefits. Prebiotics speed up the transit of bowel contents, increase faucal bulk and frequency, and thus protect the body from colon cancer, diverticular diseases, and irritable bowel syndrome. Additionally, the levels of cholesterol in the blood can also be reduced and coronary disease may also be prevented [1-2].

The different types of glycosidic linkages in most mushroom polysaccharides are linear and branched glucan such as (1,3) - β - glucans, (1,6) - β - glucans and (1,3) - α - glucans. The β - glucan form is the most abundant type of glucans. Some other polysaccharides are heteroglycans containing fructose, arabinose, mannose, galactose, xylose, and glucose in different combinations [1-2, 6]. The potential health benefits of the polysaccharides are from an enhancement of macrophage function and host resistance to many bacterial, fungal, viral, and parasitic infections [1-3, 6-8]. In addition, activation of the non-specific immune stimulation and reduction of blood glucose levels and blood cholesterol [1-3, 6-8] are the health benefits of prebiotic consumption.

Button mushroom (*A. bisporus*) is the most cultivated mushroom around the world, followed by shiitake (*Lentinus edodes*), oyster mushrooms (*Pleurotus* spp), wood ear mushroom (*Auricula auricula*), winter mushroom (*Flamulina velutipes*), and straw mushroom (*Volvariella volvacea*) [4-5].

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 the degradation of oligosaccharides. The synthesis process is more expensive, difficult to reproduce on a large scale, and low yielding [9]. Extractions of oligosaccharides from abundantly available non- cellulosic agricultural products would efficiently provide the valuable saccharides for consumption for various health benefits. An ideal extraction method should simplify the oligosaccharide production process, lower the cost, reduce oligosaccharide degradation, and give a high yield. The efficient oligosaccharide extraction method would make commercial-scale production feasible.

In this study, we investigated the combination of microwave heating and acid catalysis to promote the hydrolysis of polysaccharides in grey oyster mushrooms 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 [11-12]. The hydrolysis may take a long time to complete, therefore microwave irradiation may be applied in the hydrolysis reaction to speed up the process. The microwave facilitated hydrolysis would constitute the simple, cost- effective, and reproducible protocol on an industrial scale [9].

The developed method may contribute to solving the complications involved in the scale-up of oligosaccharide extraction in the food industry. We investigated the effects of acid concentrations, and reaction temperatures on the yields of oligosaccharides by microwave facilitated acid hydrolysis of grey oyster mushroom. The optimal extraction conditions were determined. The obtained oligosaccharide products from grey oyster mushrooms were characterized for their molecular weight by using the size exclusion chromatography (SEC) analysis method.

2. Materials and Methods

2.1 Preparation of grey oyster mushroom powder

Grey oyster mushrooms (*Pleurotus sajor-caju*) were purchased from Prangtong (No. VG2Q-006) at Talad Thai market,

Pathum Thani, Thailand. The mushrooms were cut into small pieces and washed with water three times. After washing, the mushrooms were dried at 60 °C for 3 days in a hot air oven. Afterward, they were blended in a household blender and sieved to obtain a particle size of less than 0.25 µm. The dried powder of grey oyster mushroom was stored in an air-tight container for subsequent usage.

2.2 Reagents

Hydrochloric acid, sulfuric acid (95-97%), 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). Galactose and glucose were purchased from Fluka (USA). All chemicals purchased were analytical grade.

2.3 Microwave radiation hydrolysis

The schematic description of the extraction process and analyses is shown in Fig. 1. The experiments were carried out in a microwave digestion instrument (CEM, USA, Discover SP 909155) for hydrolysis. Grey oyster mushroom powder was dried at 60 °C for 3 days to remove moisture before being hydrolyzed by microwave radiation and an 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, ramping time of 5 min, and power of 150 watts. 0.1 g of grey oyster mushroom was mixed with 1 mL of an HCl solution as a catalyst in the microwave reactor for 15 min.

After the hydrolysis reaction, the samples were allowed to cool to room temperature. 8 mL of reverse osmosis (RO) water was added and stirred for 1 hr to separate the soluble and insoluble portions. The diluted samples were neutralized and centrifuged at 14,000 rpm, 4 °C, for 15 min, and 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 and the crude product was kept in the refrigerator at 4 °C for further analysis.

2.4 Proximate composition analysis of grey oyster mushroom powder

The proximate constituents of grey oyster mushrooms were analyzed by using the standard method set by AOAC [13]. The moisture content was obtained by heating 2.0 g of a fresh sample at 105 °C until a constant weight is obtained. Crude protein was determined by using the Kjeldahl method which involves finding the % total nitrogen in 2.0 g of sample, then multiplying the number by 6.25. Crude fat was determined by using petroleum ether to extract the fat from 5.0 g of sample in a Soxhlet apparatus. Ash was 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. Carbohydrate content was found by subtracting 100 by all numbers [10].

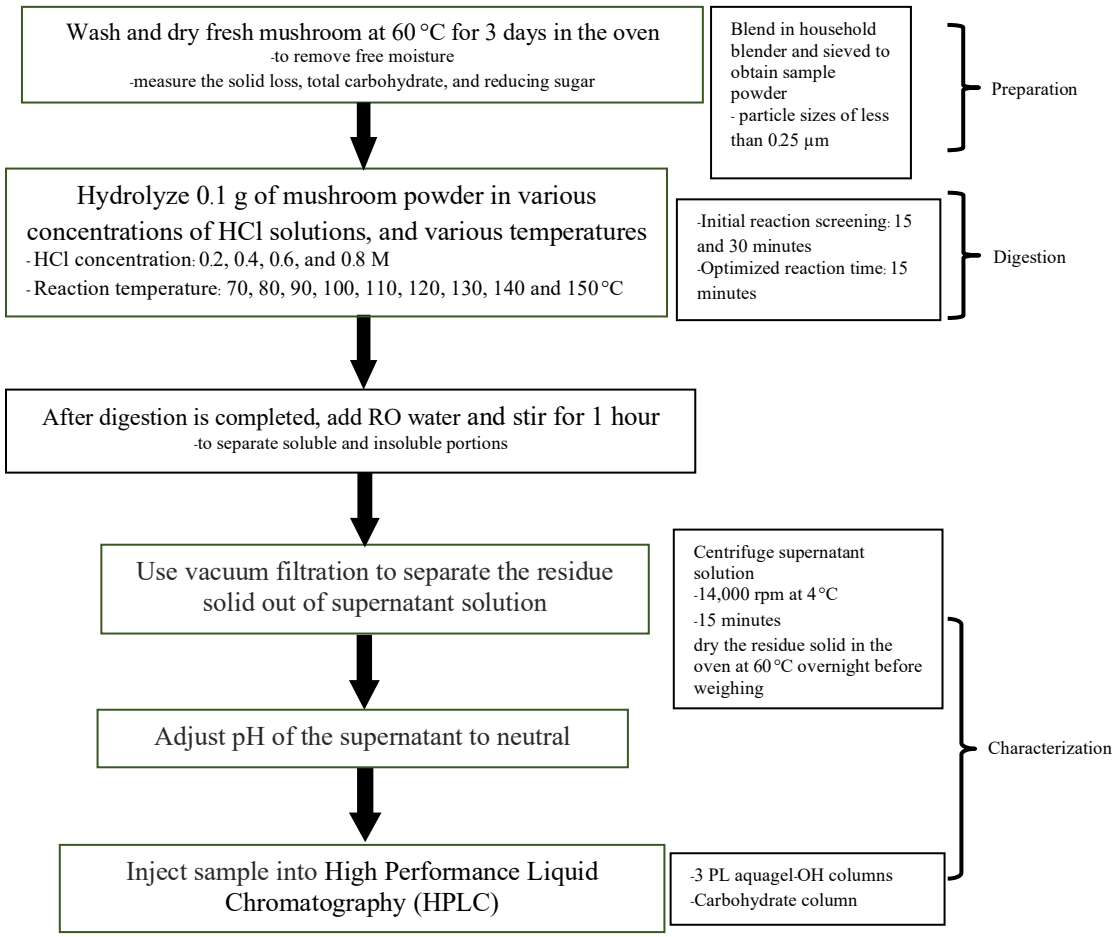

Fig. 1. Schematic descriptions for the extraction and hydrolysis of polysaccharides from grey oyster mushroom and the sample analyses.

2.5 Determination of solid loss

The main focus is to hydrolyze the carbohydrate portions of the biomass. 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 [14-15],

$$SL = \frac{IS - RS}{IS} \times 100\%, \qquad (2.1)$$

where SL = % solid loss based on the dried weight of grey oyster mushroom.

IS = the weight of the initial dried solid (g).

RS = the weight of residual dried solid (g).

2.6 Determination of total carbohydrate (TC)

Total carbohydrate was measured by the phenol-sulfuric acid assay method [18]. 0.2 mL of the grey oyster mushroom solution was diluted to 10 mL of RO water in a volumetric flask. 1 mL of 5% aqueous phenol solution was mixed with 1 mL of the diluted sample solution in a closed test tube, and then 5 mL of sulfuric acid (95-97%) was added to the mixture. The mixture was completely mixed and kept in a water bath at 25 °C for

20 min. A Thermo Fisher Scientific UV-VIS spectrophotometer system (G10S UV-VIS, USA) was used to measure the absorbance of the mixture at 490 nm. A blank was prepared by 5% aqueous phenol solution mixed with RO water, and 95% sulfuric acid at 1:1:5 by v/v/v. The standard curve for TC determination of sample solution of mushroom was made by using aqueous solutions of glucose at different concentrations. TC was reported in % (gram of TC in 100 g of the dried weight of grey oyster mushroom).

2.7 Determination of reducing sugar (RS)

RS was measured by the dinitrosalicylic acid assay [19]. To prepare the dinitrosalicylic acid solution, 5.0 g of 3,5-dinitrosalicylic acid and 150 g of potassium sodium tartrate were mixed 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 completely in a closed test tube. The mixture was immersed in boiling water for 10 min before rapidly being cooled to room temperature by ice water. The UV absorbance of the mixture was measured at 570 nm by using a Thermo Fisher Scientific UV-VIS spectrophotometer system (G10S UV-VIS, USA). A blank was prepared by a mixture of dinitrosalicylic acid solution and RO water (2:0.2, v/v). The standard curve for RS determination was made by using aqueous solutions of glucose at different concentrations. RS was reported in % (gram of RS in 100g of the dried weight of grey oyster mushroom).

2.8 Monosaccharide analysis

The saccharide composition analysis of hydrolyzed samples was carried out with an Agilent 1260 Infinity HPLC system, (G1329B, Germany) equipped with a carbohydrate column (LEAD column, Transgenomic CARBOSEP CHO682, CHO-99- 9854, USA). The hydrolyzed samples were diluted and neutralized followed by filtration through membrane filters (0.2 μm). After filtration, 20 μL of the sample was injected into the HPLC. The mobile phase used was deionized water (DI) and the flow rate was set at 0.4 mL/min. The temperature of the column was kept at 80 °C. Monosaccharides and oligosaccharides were monitors by refractive index detector.

2.9 HPLC size exclusion chromatography (SEC) analysis

The SEC analysis was done by using an Agilent 1260 Infinity HPLC system, (G1329B, Germany) equipped with a PL aquagel guard and 3 PL aquagel-OH columns (PL aquagel-OH 20 SEC columns, 5 μm, 7.5x300 mm). The diluted sample was neutralized and filtered through a 0.2 μm membrane. 100 μL of the sample was injected into HPLC. The mobile phase is DI water with a flow rate of 0.9 mL/min. During the analysis process, the column's temperature was maintained at 36 °C. A refractive index detector on the HPLC was used to monitor the types of monosaccharides, oligosaccharides, and polysaccharides based on retention times.

2.10 NMR spectroscopy

The extracted polysaccharide (PS) was dried *in vacuo*. The structure of the extracted polysaccharide was identified 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 extracted polysaccharide was dissolved in D_2O at 30 °C and kept at that temperature during the measurement.

3. Results and Discussion

3.1 Proximate compositions analysis

The results of the proximate composition analyses of the grey oyster mushrooms are summarized in Table 1. The carbohydrate, moisture, lipid, ash, protein,

and crude fiber contents of grey oyster mushrooms are found to be at similar levels with those of *Fan et al* [16]. Carbohydrate is the only major component of the dry mass of grey oyster mushroom.

Based on dried weights, carbohydrates contribute to 64.94% of grey oyster mushrooms. The carbohydrates consist of various compounds such as monosaccharides, oligosaccharides, and polysaccharides. Carbohydrates are mainly present in the grey oyster mushrooms as polysaccharides in the forms of glycogen, indigestible fibers (cellulose, dietary fibers, chitin, α- and β-glucans), and other hemicelluloses (mannans, xylans, and galactans).

Table 1. Proximate composition of grey oyster mushroom (percent based on dried weights), the data presented as mean±0.06 SEM.

Moisture (%)	1.26 ± 0.03
Lipid (%)	1.30 ± 0.03
Crude Protein (%)	24.59 ± 0.21
Crude fiber (%)	0.97 ± 0.00
Ash (%)	6.94 ± 0.09
Carbohydrate (%)	64.94 ± 0.26

3.2 Effects of the extracting temperature

The reaction temperature has 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 the conditions of 150 °C, 15 min, and 0.2 M HCl solution (Fig. 2a). TC reached the maximum value of 65.61% under the conditions at 130 °C for 15 min with 0.2 M HCl solution (Fig. 2b). RS reached the maximum value of 50. 92% under the conditions at 140 °C for 15 min with 0.4 M HCl solution (Fig. 2c). The maximum values of SL, TC, and RS are in the reaction temperature range of 130 °C-150 °C. Some intermediate decomposition products of Maillard Browning and Caramelization (MBCR) were generated from the degradation of proteins and carbohydrates [20-22]. Beyond this reaction temperature (>150 °C), 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 to the utilization of a combination of microwave radiation heating and HCl as a catalyst.

3.3 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% under the conditions at 150 °C for 15 min with 0.2 M HCl solution (Fig. 3a). TC of grey oyster mushroom reached a maximum value of 65. 61% under the conditions at 130 °C for 15 min with 0.2 M HCl solution (Fig. 3b). RS of grey oyster mushroom reached a maximum value of 50.92% under the conditions at 140 °C for 15 min with 0.4 M HCl solution (Fig. 3c). The higher HCl concentrations tend to generate more MBCR products [20-22].

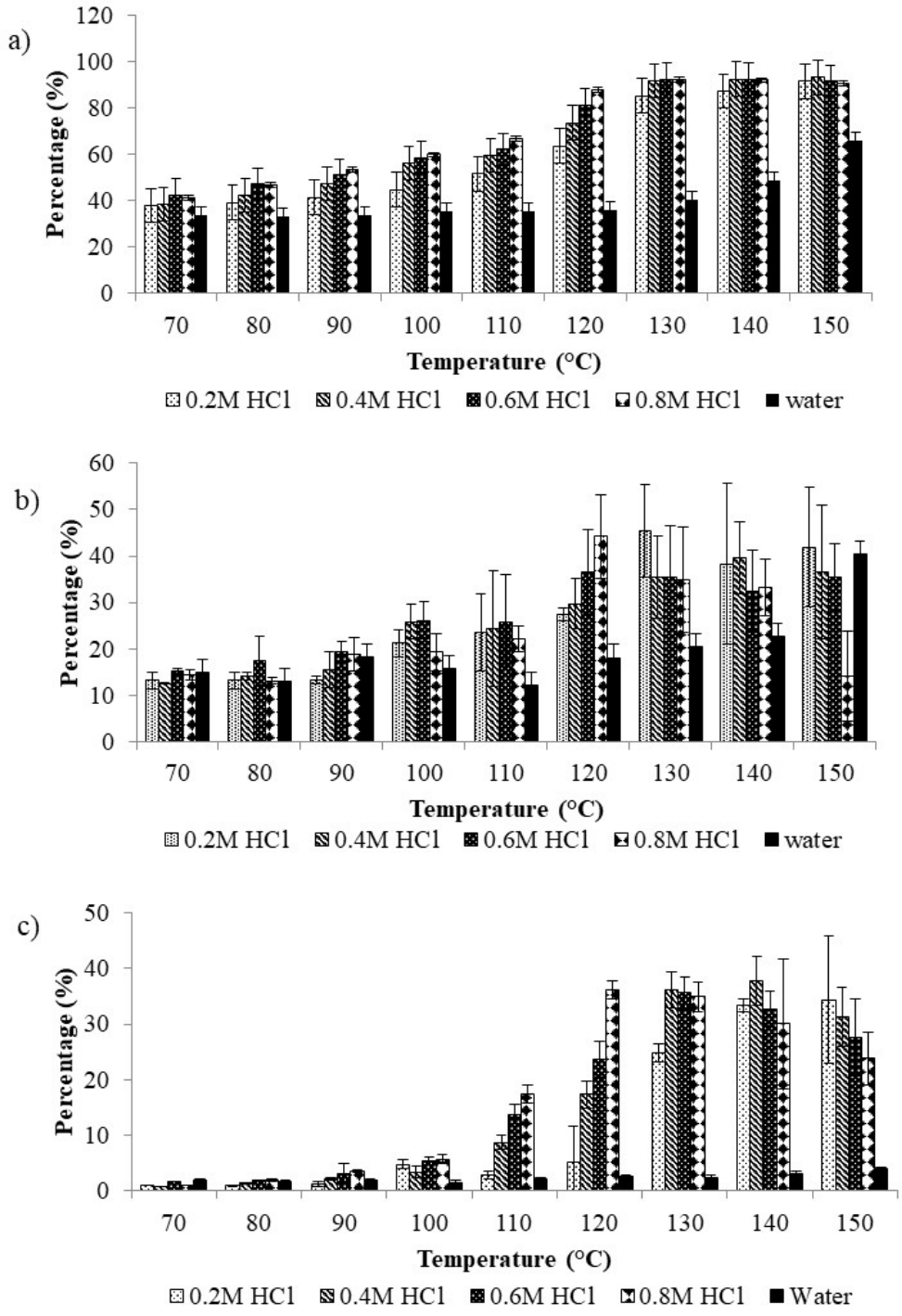

Fig. 2. The effect of reaction temperature on the hydrolysis of grey oyster mushroom: a) solid loss, b) total carbohydrates, and c) reducing sugars at reaction time 15 min, ratio of reaction volume to grey oyster mushroom mass of 10:1 v/w and repeat 3 times.

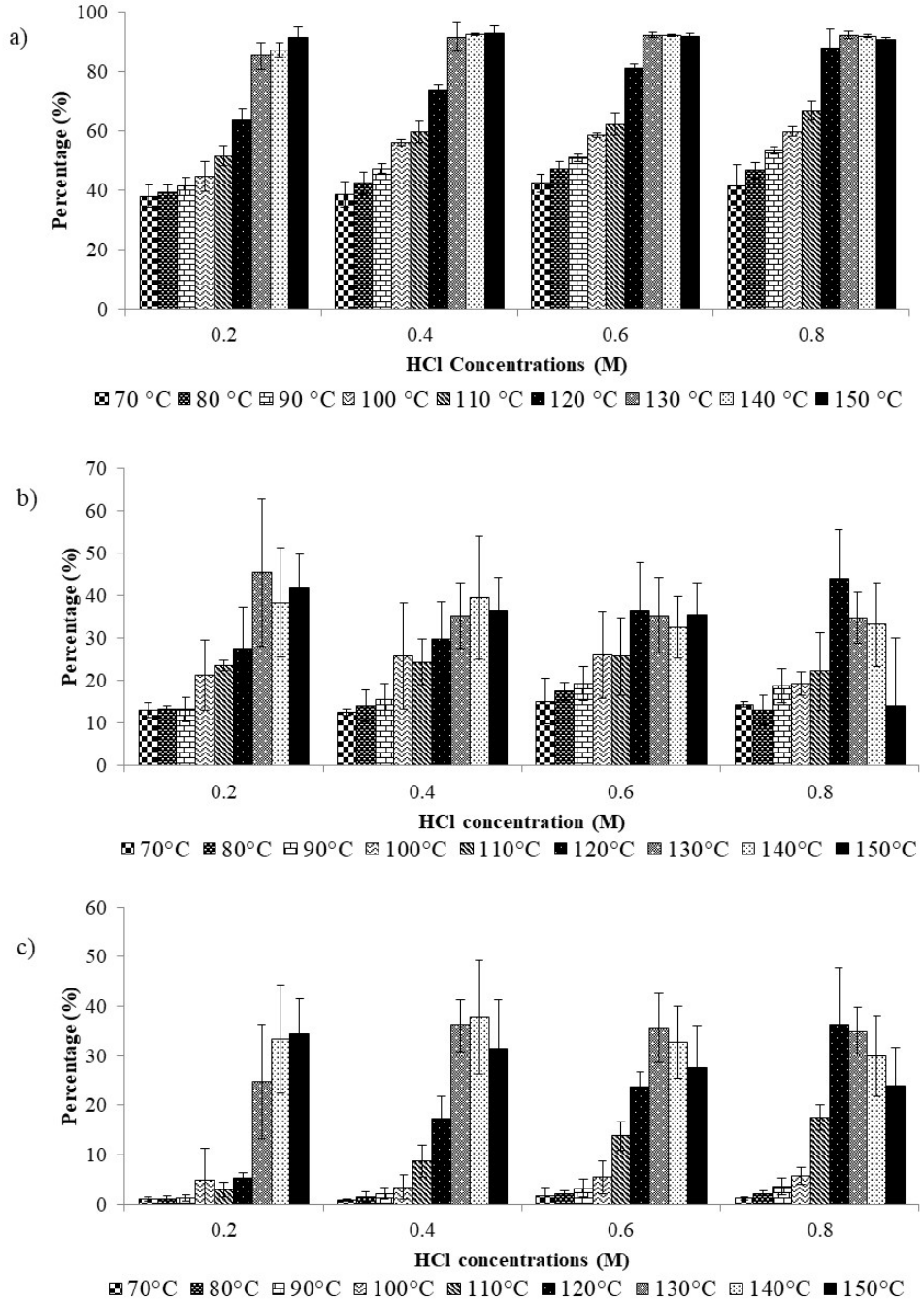

Fig. 3. The effect of HCl concentration on the hydrolysis of grey oyster mushroom: a) solid loss, b) total carbohydrates, and c) reducing sugars at the reaction time15 min, ratio of reaction volume to grey oyster mushroom mass of 10:1 v/w and repeat 3 times.

3.4 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 calibration curve demonstrates a linear relationship between the log of saccharide molecular weights and retention times (Fig. 4). The retention times of oligosaccharides with 2- 20 units is between 25 to 29 min. The polysaccharide peaks are at less than 25 min. The retention time of monosaccharides is around 31 min. After the hydrolysis reaction, the retention times of polysaccharides (short-chain)) and oligosaccharides were less than 29 min. Monosaccharides were obtained at higher reaction temperatures of 140- 150 °C. The reaction temperature around 120- 130 °C provides oligosaccharides, while the lower temperatures result in higher polysaccharide portions. The length of shorter polysaccharides obtained was more than 36 units with the obtained product of 74.25% at the conditions of 90 °C, 15 min with 0.2 M HCl. Oligosaccharides with 2-5 units (93.70%) were obtained under the conditions at 120 °C for 15 min, and 0.6 M HCl solution.

Monosaccharides were obtained with the obtained product of about 76.70% under the conditions at 150 °C for 15 min, and 0.6 M HCl solution.

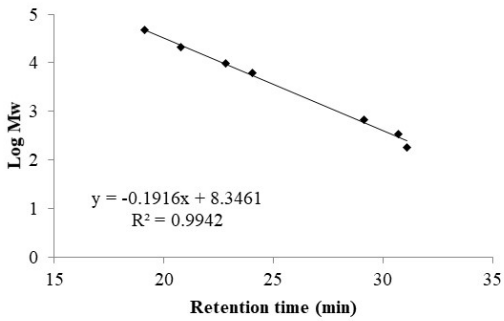

Fig. 4. Calibration curve of saccharides standard used to calculate the number of units of hydrolyzed samples from the analysis by HPLC equipped with size exclusion column.

3.5 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 °C to 150 °C, 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 (Fig. 5). As expected, glucose is the major monomer building block of the polysaccharides in grey oyster mushroom.

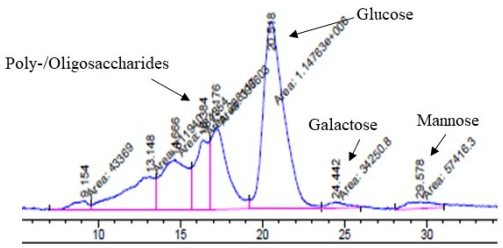

Fig. 5. The HPLC chromatogram of the grey oyster mushroom after hydrolysis by using carbohydrate column.

3.6 ^{1}H NMR analysis

The ^{1}H NMR spectrum of the extracted grey oyster mushroom (at the reaction conditions of 120 °C, 15 min, 0.6 M HCl) in D_2O is shown in Fig. 5. The NMR spectrum shows a good agreement with the previously reported data [8]. 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 1H 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.

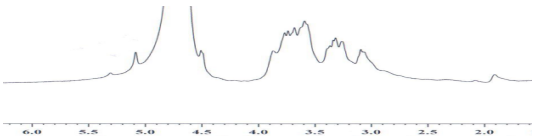

Fig. 5. ^{1}H NMR spectrum of extracted glucans from grey oyster mushroom in D_2O at 30 °C.

4. Conclusion

In this study, we successfully developed an efficient method to extract oligosaccharides from grey oyster mushroom by a combination of microwave and acid hydrolysis to obtain glucanoligosaccharides. The major saccharide composition of grey oyster mushroom is glucose, while mannose is a minor component. 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 °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 and a number average molecular weight of 384-3842 Da. The present findings emphasize the importance of microwave radiation to obtain the oligosaccharides in shorter extraction times when compared to enzyme hydrolysis procedures.

Acknowledgements

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Abbreviations

Solid loss	SL
Total carbohtdrate	TC
Reducing sugar	RS
Degree of polymerization	DP
Size exclusion chromatography	SEC
Nuclear magnetic resonance	NMR

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