A comparison between Hydrochloric acid and Trifluoroacetic acid in hydrolysis method of exopolysaccharide from Ophiocordyceps sinensis in Monosaccharide composition analysis by GC-FID

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ABSTRACT

The monosaccharide composition is one of the crucial factors affecting the bioactivity of exopolysaccharide (EPS) in *Cordyceps* species. Therefore, many scientists have studied, analyzed monosaccharide composition and structure of EPS from *Cordyceps* species, especially *Ophiocordyceps sinensis (O. sinensis).* This study aimed to compare hydrochloric acid (HCl) with trifluoroacetic acid (TFA) in the EPS hydrolysis process in monosaccharide composition analysis by Gas Chromatography with Flame-Ionization Detection (GC-FID). The hydrolysis is a crucial step in forming the acetyl derivative, which helps the GC-FID technique to have good results in monosaccharide composition analysis. The results showed that hydrolysis with HCl gave a higher hydrolysis efficiency and was more suitable than hydrolysis by TFA in pretreatment to EPS for GC-FID. Hydrolysis results were analyzed through thin-layer chromatography and high-performance liquid chromatography (HPLC), then Acetyl derivatives were produced and finally analyzed by GC-FID to determine the monosaccharide composition of EPS. For EPS hydrolyzed by HCl, the analytical results presented that this sample had 6 kinds of monosaccharides, including rhamnose, arabinose, xylose, mannose, glucose, and galactose; the most monosaccharide was glucose. The EPS hydrolyzed by TFA only detected three kinds of monosaccharides,

including mannose, arabinose, and galactose, mainly mannose. The study has set a foundation for further analysis of monosaccharide composition and structure of EPS from *O. sinensis*.

1. Introduction

Ophiocordyceps sinensis (O. sinensis), or cordyceps mushroom, is an insect parasitic fungus (Lo, Hsieh, Lin, & Hsu, 2013). It has been found in abundance on the Qinghai - Tibet Plateau, used to enhance health and supported the treatment of many conditions and diseases (Nie, Cui, & Xie, 2018). Exopolysaccharide (EPS), a natural source of pharmaceutical substances, has possessed promising activities such as immunomodulation, inhibition of tumor formation, and metastases that help support cancer treatment. These biological activities have been studied and applied widely in fields such as pharmaceuticals, nutraceuticals, functional foods, and cosmeceuticals (Yan, Wang, & Wu, 2014). Antioxidants of EPS can support the prevention of related diseases such as diabetes, atherosclerosis, nephritis, Alzheimer's disease. The precious bioactivities of EPS have been proven to relate to the ratio of monosaccharides (Soltani, Kamyab, & El-Enshasy, 2013). Therefore, determining the composition of monosaccharides is a necessary activity to predict and improve the efficiency of EPS from *O. sinensis*.

To determine the composition of monosaccharides, EPS is usually hydrolyzed to simple sugar molecules by acid (Selvendran, March, & Ring, 1979). Monosaccharide molecules will continue to be modified to suit analytical techniques such as gas-liquid chromatography or Gas chromatograph-mass spectrometry (Merkle & Poppe, 1994). The effect of each acid on the same EPS sample is complex so that the analysis results are different in quality (Hon, 2001; Merkle & Poppe, 1994). Therefore, the present study investigated comparing HCl and TFA to consider which acid performed better on hydrolyzing EPS. The results of the study might create a premise for further studies on the chemical structure of EPS.

2. Materials and methods

2.1. Removing protein and obtaining EPS

Added 20 UI Flavourzyme solution in phosphate buffer ($Na_2HPO_4.7H_2O$, $NaH_2PO_4.H_2O$, pH 6.5) to concentrated *O. sinensis* culture at a ratio of 1: 5 (v / v) at 50°C for 2 hours and stirred every 15 minutes. Added a Sevag (chloroform: n-butanol = 2: 1 (v / v)) mixture to the above solution at a ratio of 1: 1 (v / v), shook well for 15 minutes, and left for 15 minutes. Then centrifuged at 5000 rpm water for 10 minutes and collected the supernatant. Repeated 5 times. Added 96^o ethanol to the supernatant in a 3: 1 ratio (v / v), refrigerated at 4°C for 24 hours, collected precipitate and dried (Vo, 2018).

2.2. Hydrolyzing EPS with acid hydrochloric (HCl)

The reaction was carried out as described by Yu et al. (2002). Added 250mg of EPS to 6mL of 10% HCl (the number of moles of acid is 40 times the number of moles of EPS converted to glucose) in a closed container of inert argon gas at 70°C - 80°C for three hours.

After the reaction, allowed to cool to room temperature, neutralized with 1M NaOH, and spin to remove water, then checked the product by thin-layer chromatography and high-performance liquid chromatography.

2.3. Hydrolyzing EPS with acid trifluoroacetic (TFA)

The reaction was carried out as described by Yang et al. (2009). Added 35mg of EPS to 0.8mL of TFA (50 times the number of moles of acid EPS converted to glucose) diluted with 1.7mL of distilled water in a closed container of inert argon gas at a temperature of 70°C - 80°C for 3 hours. After the reaction, cooled to room temperature, neutralized to pH 7 with 1 M NaOH, evaporated to remove water, and then checked hydrolysis products with thin-layer chromatography and high-performance liquid chromatography.

2.4. Acetylation of hydrolyzed EPS

Hydrolysis product was added to anhydrous sodium acetate (moles 5 times the number of moles EPS converted to glucose) and acetic hydride (moles 12 times the number of moles EPS converted to glucose) in the closed system at 80°C - 90°C for 2 hours, then added 20ml of ethyl acetate (repeated 3 times), collected and concentrated the ethyl acetate phase. Finally, the derivatives were checked by thin-layer chromatography and gas chromatography.

2.5. HPLC

HPLC was performed as described in BP 2014 standard. The sample was filtered through a filter paper with a pore size of 0.22µM. The conditions of performing HPLC: mobile phase: purified water; column: sugar CMP, particle size 9µM, 7.8 x 300mM; flow rate: 0.5mL/min; column temperature: 80^{o}C; injection volume: 20µL. Performed with sucrose, glucose, mannose, xylose at a time.

2.6. GC-FID

GC-FID was performed as described by Yuan et al. (2016). After acetylation, the product was analyzed by GC-FID with the condition: The temperature of the injection chamber, probe temperature: 230^{o}C. The starting temperature of the column: 110^{o}C and stayed for 1 minute before rising to 180°C. Finally, it would be increased to 280°C at 20°C/min and kept for 10 minutes.

3. Results and discussion

3.1. Results of the thin-layer chromatography and HPLC of EPS after hydrolysis

Under the effect of acid, polysaccharide hydrolyzed into smaller carbohydrate molecules by breaking down these Glycosidic bonds. The results from the chromatogram showed that the pre-hydrolyzed EPS sample has no streaks (Figure 1, track 1).

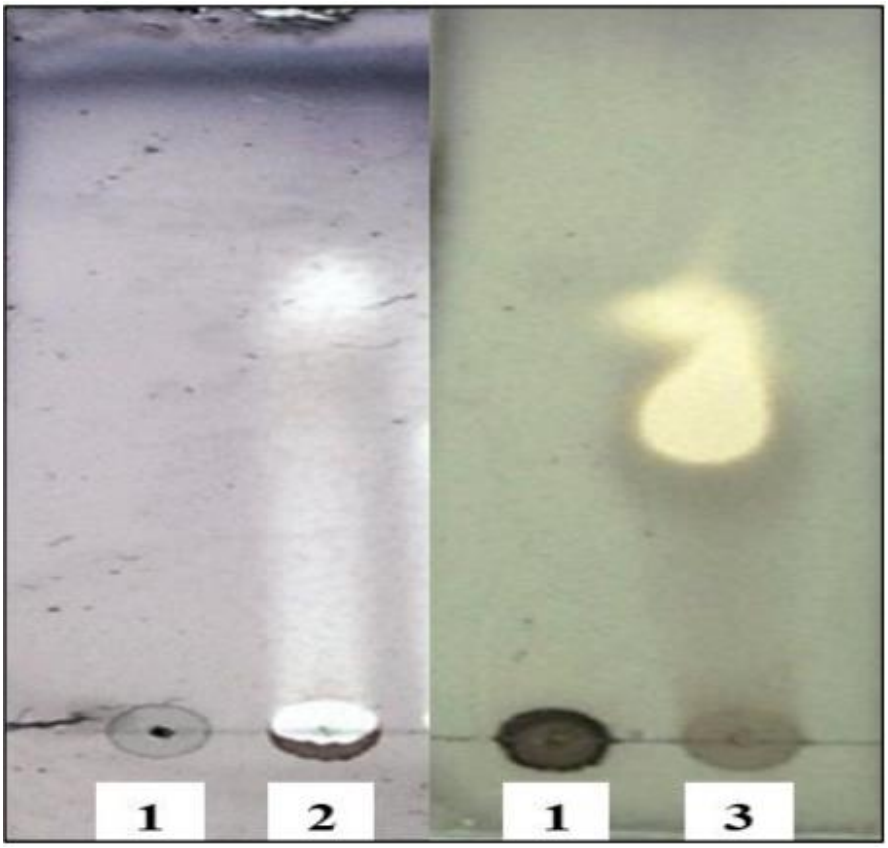


Figure 1. Results of the Thin-layer chromatogram of samples: 1. EPS before hydrolysis, 2. EPS hydrolyzed with HCl, 3. EPS hydrolyzed with TFA

For hydrolyzed samples with HCl (Figure 1, track 2), the thin-layer chromatography result showed that a long dark stain was found on the track; therefore, the EPS sample was hydrolyzed. However, the hydrolysis process with this HCl occurred incompletely, so that the stain was prolonged. The EPS sample after hydrolysis with TFA (Figure 1, track 3) showed a dark stain and a dark spot above the track, which indicated that the EPS sample was hydrolyzed by TFA. The solvent system of Chloroform: methanol: water at the ratio of 10:9:1 has a high degree of polarization so that the streaks on track 2 and track 3 could be the presence of mono-, di-, and oligosaccharide which were the product from hydrolysis of EPS samples.

The results of the thin - layer chromatography and HPLC of post-hydrolyzed samples also showed that HCl exhibited better hydrolysis ability than TFA during EPS hydrolysis.

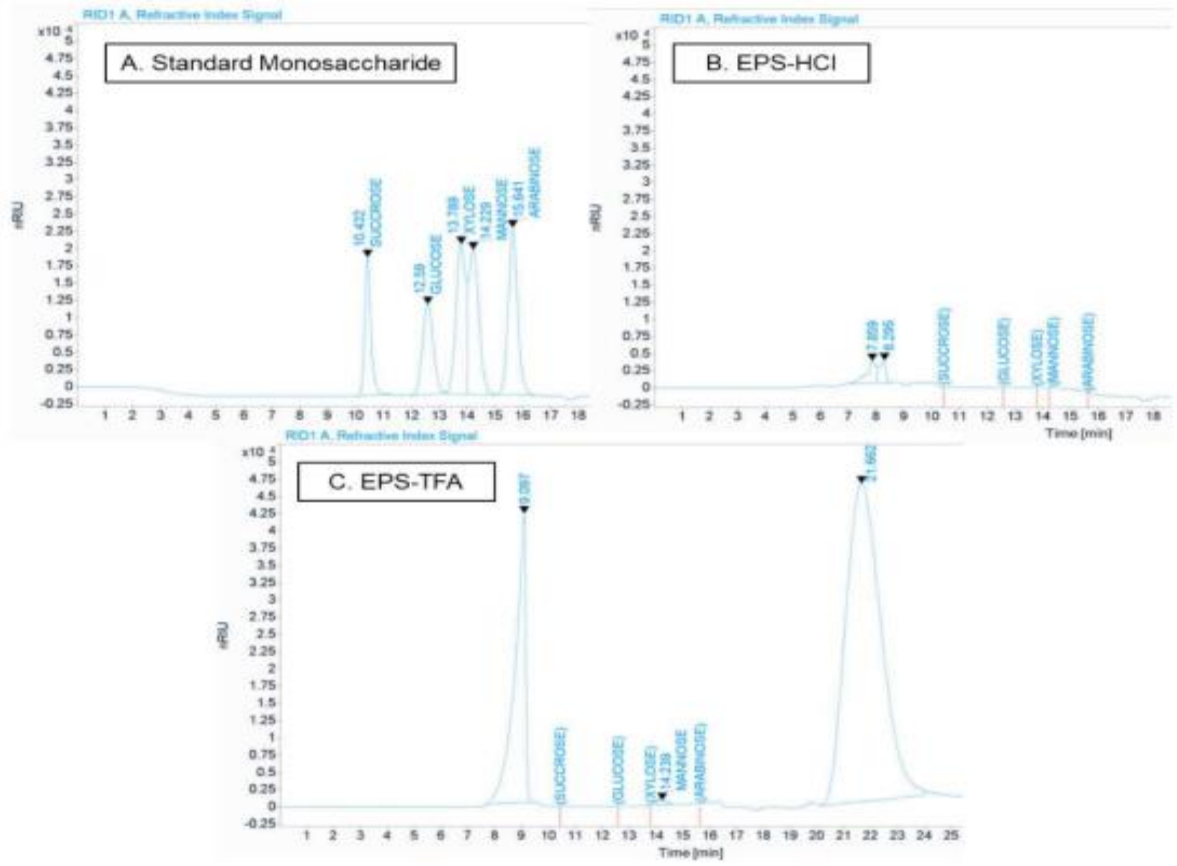


Figure 2. HPLC graphs of samples: A. Standard substance, B. EPS hydrolyzed with HCl, C. EPS hydrolyzed with TFA

HPLC graphs of EPS hydrolyzed with HCl showed that there were two peaks corresponding to the retention time of 7.859 minutes (59.14%) and 8.95 minutes (40.86%), none of these peaks matched 5 standard substances (saccharose, glucose, xylose, mannose, and arabinose) (Figure 2B). However, the retention time of these two peaks was very close to Sucrose's retention time. Two signal peaks contributed to prove that HCl had hydrolyzed EPS.

For EPS samples after hydrolysis by TFA, HPLC graph presented 3 peaks with the retention time of 9.097 minutes (for 21.56%), 14.239 minutes (for mannose - 0.40%) and 21.662 minutes (for 78.04%) which had the retention time longer than that of standard monosaccharide (Figure 2C). The signal peak located near the retention time of mannose peak indicated that the hydrolysis reaction could separate polysaccharide to monosaccharide levels. Most substances were at the peak of 9,097 minutes and 21.662 minutes, which have retention times smaller and longer than the retention time of the five standard sugars. This could happen that a portion of carbohydrate still existed at multi-sugar forms which had not been fully cleaved, whereas a large amount of monosaccharide separated from EPS might have worked with acid to form other substances.

3.2. Results of thin-layer chromatography of EPS samples after acetylated hydrolysis

The results of the thin-layer chromatography showed that the EPS hydrolyzed by HCl was acetylated. The thin-layer chromatogram showed a trace on track 2 (Figure 3), compared

to the hydrolyzed EPS sample but not acetylated (track 1, no signal of the streak). The solvent system of hexane: chloroform at the ratio of 7:3 has low polarity so that there were the streaks on track 2 possibly indicating the presence of acetylated monosaccharides which were various low-polarity derivatives.

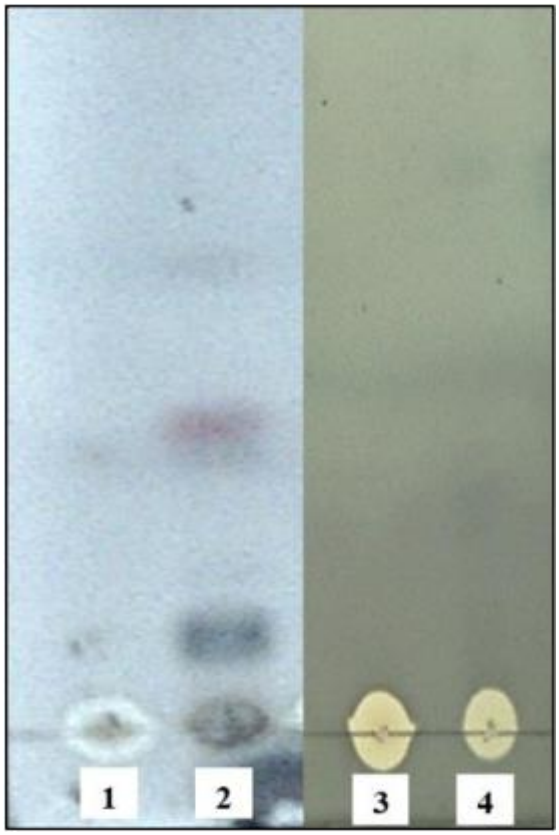


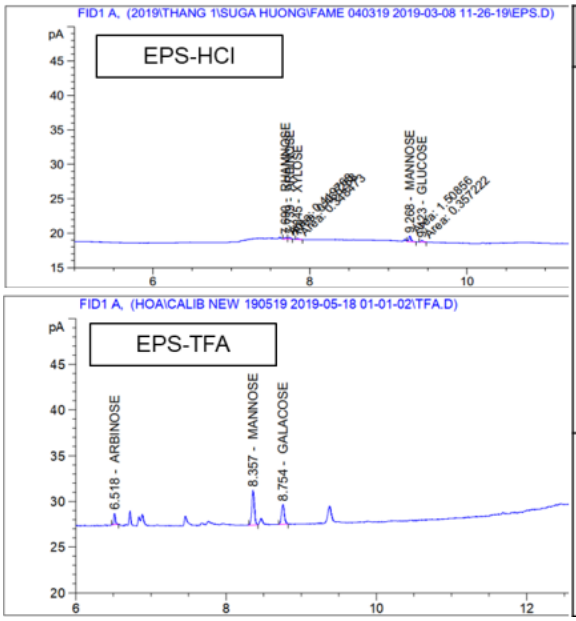
Figure 3. Thin-layer chromatography of samples: 1. HCl-hydrolyzed EPS non-acetylated, 2. HCl-hydrolyzed EPS acetylated, 3. TFA-hydrolyzed EPS non-acetylated, 4. TFA-hydrolyzed EPS acetylated

For EPS hydrolyzed by TFA, the thin-layer chromatographic (Figure 3, track 4) showed very light streaks, the streak at the baseline was still very dark, including the appearance of the white spot. This result suggested that TFA might not completely hydrolyze the EPS sample. The white spots on the baseline presented that there were at least two different substances in the EPS sample after acetylation, in particular, the white spot substance was non-acetylated which had high polarity; therefore, it stayed at the baseline at the end of acetylation.

The results of thin-layer chromatography of samples after acetylation presented that the EPS treated by HCl was well hydrolyzed and facilitated the great acetylation reaction.

3.3. Results of GC-FID

For EPS hydrolyzed by HCl, the analytical results presented that this sample had 6 kinds of monosaccharides, including rhamnose, arabinose, xylose, mannose, glucose, and galactose; the most monosaccharide was glucose with 3.5-fold mannose and 3.1-fold galactose. The EPS hydrolyzed by TFA only detected three kinds of monosaccharides, including mannose, arabinose, and galactose, mainly mannose (Figure 4).



Samples	Monosaccharide	Mg/g	Ratios
EPS-HCl	RHAMNOSE	17.20	1.04
	ARABINOSE	23.70	1.45
	XYLOSE	16.30	1.00
	MANOSE	18.70	1.15
	GLUCOSE	65.70	4.03
	GALACTOSE	20.80	1.28
EPS-TFA	ARABINOSE	0.55	1.00
	MANNOSE	2.83	5.15
	GALACTOSE	2.09	3.80

Figure 4. GC-FID graph and monosaccharide composition results of samples: A. EPS hydrolyzed with HCl, C. EPS hydrolyzed with TFA

Many kinds of EPSs have been detected and collected, the majorities of their monosaccharide composition are generally glucose, mannose, and galactose in various proportions, in which glucose has been the most monosaccharide (Zhang, Yang, Chen, Hou, & Han, 2005). In the results of the present study, the result of the monosaccharide composition of EPS hydrolyzed by HCl was also consistent with the results of Cha et al. (2007) when analyzing the simple sugars of exo-biopolymer from *O. sinensis* 16 mainly including glucose, mannose, and galactose at the ratio of 61.5: 18.1: 9.4; in which glucose was 3-fold mannose and galactose. A study by Vo (2018) on analyzing the monosaccharide composition of EPS segments from *O. sinensis* by HPLC also had the parallel results which showed that the monosaccharides in the EPS segments were mainly mannose, galactose, and glucose, in which glucose was the most monosaccharide.

4. Conclusions

EPS hydrolyzed by HCl had 6 kinds of monosaccharides, including rhamnose, arabinose, xylose, mannose, glucose, and galactose; the most monosaccharide was glucose with 3.5-fold mannose and 3.1-fold galactose. The EPS hydrolyzed by TFA only detected three kinds of monosaccharides, including mannose, arabinose, and galactose, mainly mannose.

The results from the current study indicated that HCl was suitable for *O. sinensis* exopolysaccharide hydrolysis, pre-treatment before analyzing the monosaccharide composition by GC-FID method, compare with TFA.

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