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A survey on acid hydrolysis in analyzing the monosaccharide composition of exopolysaccharide from *Ophiocordyceps sinensis*

Hang, L. T. T.[1,2*], Chau, H. B.[3], Nam, T. V. H.[3], Phu, D. H.[4], Hiep, D. M.[5] and Thang, N. T.[2]

[1]Faculty of Food Technology, Ho Chi Minh City University of Food Industry, 140 Le Trong Tan, Ho Chi Minh City, Vietnam; [2]Institute of Tropical Biology, Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 9/621 Hanoi highway, Linh Trung Ward, Thu Duc District, HCM city; [3]Faculty of Biology &Biotechnology, VNUHCM-University of Science 227, Nguyen Van Cu St., Ho Chi Minh City, Vietnam; [4]Department of Organic Chemistry, Faculty of Chemistry, VNUHCM-University of Science, Ho Chi Minh, Vietnam; [5]Ho Chi Minh City Agricultural Hi-Tech Park, Ho Chi Minh City, 214-214A, D5 St., Ho Chi Minh City.

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Abstract: Exopolysaccharide (EPS) is a heterogeneous group of polysaccharides which has diversed biological activities; in which monosaccharide composition is one of the crucial factors determining the biological activity of EPS. In the analysis of monosaccharide composition by GC-FID technique, the determination of proper acid is a significant step to help cut the glycosidic bond which is less stable in an acidic environment, great hydrolysis of polysaccharide chain to monosaccharide level, to form Acetyl derivatives. The hydrolysis ability of 3 acids: H_2SO_4, HCl, and Triflic acid for hydrolyzing EPS of *Ophiocordyceps sinensis* (*O. sinensis*). Hydrolysis ability was assessed by thin-layer chromatography and high-performance liquid chromatography (HPLC), then the product was treated by acetylation and finally analyzed by GC-FID to determine the monosaccharide composition of the EPS. The results showed that hydrolysis by H_2SO_4 gave higher hydrolysis efficiency and was more suitable than hydrolysis by HCl and Triflic acid in the pretreatment of EPS sample for monosaccharide composition analysis by GC-FID. H_2SO_4-treated EPS detected 5 types of monosaccharides: rhamnose, arabinose, mannose, glucose, and galactose; mainly mannose, galactose, and glucose; mannose portioned the highest percentage. The study has set the fundamental for further analysis of the chemical structure and biological activities of EPS.

Keywords: EPS, Hydrolysis, GC-FID, HPLC, Monosaccharide, *Ophiocordyceps sinensis*

Introduction

Extracellular polysaccharide is also called extracellular polysaccharide, a high molecular weight polysaccharide group, excreted by the microorganisms in the surrounding environment (Radchenkova *et al.*, 2011). The EPS of *O. sinensis* possesses promising activities such as immunomodulatory regulation,

[*] **Corresponding Author:** Hang, L. T. T.; **Email:** hangltt@hufi.edu.vn

inhibition of tumor formation, metastasis that helps support cancer treatment and resistance to oxidative agents and free radicals - The beginning of many dangerous diseases like Alzheimer's, Parkinson's, atherosclerosis, cancer, and aging (Belwal *et al*., 2019). The monosaccharide component is one of the most important factors affecting the biological activity of exopolysaccharide (EPS) of Cordyceps species, especially *O. sinensis* (Soltani *et al.*, 2013). However, little attention has been paid to research this subject. In addition, information on the relationship between monosaccharide composition, molecular structure and biological activity of *O. sinensis*-derived EPS is still very limited (Yan *et al.*, 2014). The purpose of this study is to find the EPS hydrolysis process suitable for the analysis of EPS monosaccharide composition by gas chromatography–flame ionization detection (GC-FID). Acid hydrolysis is one of the earliest chemical methods to hydrolyze the complex carbohydrates into oligosaccharide fragments. Commonly used acids are trifluoroacetic acid (TFA), sulfuric acid (H_2SO_4), hydrofluoric acid (HF) (Selvendran *et al.*, 1979). The hydrolysis process is a crucial step in creating the acetyl derivative, which helps the GC-FID technique achieve good results in monosaccharide component analysis. The study has set the foundation for further analysis of monosaccharide composition and EPS structure of *O. sinensis*.

Materials and Methods

Isolation of EPS from the culture

The strain of *Ophiocordyceps sinensis* is provided by Dr. Truong Binh Nguyen (Dalat University, Vietnam). *O. sinensis* was cultured by liquid medium. After 40 days, the culture was treated by Flavourzyme combined with Sevag (Zha *et al.*, 2012). 20 UI/ml Flavourzymes (mixed in pH 6.5 phosphate buffer) was added into the culture at a rate of 5: 1 (v / v), incubated at 50 ℃ for 2 hours, stirred gently every 15 minutes, then added the mixture of Sevag (chloroform: n-butanol = 2: 1 (v / v)) to the solution at a ratio of 1: 1 (v / v) and shook it continuously for 15 minutes, left in 15 minutes. Centrifugation was done at 5000 rpm for 10 minutes and collected the supernatant fraction. The process was repeated 3-5 times. Finally, ethanol 96% was added to the collected solution at a ratio of 3: 1 (v / v), cooled at 4 ℃ for 24 hours, collected the precipitated EPS and dried at 55 ℃.

Hydrolysis of EPS using sulfuric acid (H_2SO_4)

35 mg of EPS was cooled at 5 ℃ for 15 minutes, added 0.35 ml of sulfuric acid 77 %, incubated at -5.0 ℃ for 14 hours, then added 0.35 ml of 25 % sulfuric acid, stirred well, covered tightly, left to room temperature and

incubated at 55 ℃. After 10-15 minutes, the mixture was stirred quickly and covered tightly, incubated in two hours. The mixture was cooled to room temperature, added 3.5 ml of cold distilled water and heated it in the circulation system at 95 ℃ for one hour. After that, the mixture was left to room temperature, filtered through a porous glass funnel, washed several times with distilled water. The amount of acid in the mixture was neutralized by $Ca(OH)_2$. The neutralized solution was filtered in an 80-85 ℃ water bath, in turn, added four times of 5 ml $Ca(OH)_2$ into the EPS solution, each time stirred for 2 minutes, then adjusted to pH 7 by 0,05 M $Ca(OH)_2$. The mixture was left for at least one hour to precipitate, then removed precipitate with a porous glass funnel. Finally, thin-layer chromatography and high-performance liquid chromatography were conducted (UÇAR and Balaban, 2004).

Hydrolysis of EPS using hydrochloric acid (HCl)

6 ml of 10 % hydrochloric acid was added to 250 mg of EPS in a sealed container containing argon inert gas, at 70-80 ℃ for three hours. After the reaction, the mixture was cooled to room temperature, and neutralize to pH 7 with 1 M NaOH and then evaporated to remove water. Finally, thin-layer chromatography and high-performance liquid chromatography were conducted to preliminary assess the ability of acid hydrolysis (Yu *et al.*, 2002).

Hydrolysis of EPS using trifluoromethanesulfonic acid (triflic acid)

30 mg EPS was reacted with 1 ml of triflic acid in a closed container containing argon gas, at 70^oC-80^oC in one hour. After the reaction, the mixture was cooled to room temperature, neutralized with 1 M NaOH, then removed water by rotary evaporator (Perepelov *et al.*, 2001). Finally, thin-layer chromatography and high-performance liquid chromatography were conducted.

Acetylation of hydrolyzed EPS

Anhydrous sodium acetate (the number of moles was 5 times the number of moles of EPS converted to glucose) was added into acetic hydride (the number of moles was 12 times the number of moles of EPS converted to glucose), then slowly added the hydrolyzed EPS. The reaction conducted in closed systems at 80-90 ℃ for 2 hours. After the reaction, 20 ml of ethyl acetate was added to the solution, then collected the ethyl acetate phase (repeated 3 times). Residual water phase in the ethyl acetate phase was eliminated with anhydrous sodium sulfate, then evaporated to collect acetylated.

Finally, thin-layer chromatography and high-performance liquid chromatography were conducted.

HPLC

After hydrolyzed, the sample was filtered through a filter paper with a pore size of 0.22 μm. The produce was conducted following the conditions: mobile phase: purified water; column: sugar CMP, particle size 9 μm, 7.8 x 300 mm; flow rate: 0.5 ml/min; column temperature: 80 ^{o}C; injection volume: 20 μL.

GC-FID

Set the program to run GC according to the condition: 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. (Yuan *et al.*, 2016).

Results

Results of thin-layer chromatography and HPLC of EPS after hydrolysis

For HCl-hydrolyzed EPS (figure 1A), the thin-layer chromatography result presented a long dark stain on the track (track A2 and A3); therefore, this EPS and starch sample were hydrolyzed; the pre-hydrolyzed EPS sample had no streaks (Track A1). For EPS and starch which were hydrolyzed by H_2SO_4 (Figure 1B), the results showed that there were thick spots on the track, and no stretching (track B2 and B3). The thin-layer chromatography of EPS sample hydrolyzed with Triflic acid (Figure 1C) showed dark and smeared stains (track C2 and C3), similar to EPS sample hydrolyzed with HCl result.

For EPS samples after hydrolysis with HCl, HPLC graphs had two peaks corresponding to the retention time of 7.859 minutes (for 59.14 %) and 8.95 minutes (for 40.86 %) (figure 2B). HPLC graph of this HCl-hydrolyzed EPS had no peaks corresponding to 5 standard substances (saccharose, glucose, xylose, mannose, and arabinose).

For EPS samples after hydrolysis by Triflic acid, HPLC graph presented 3 peaks with the retention time of 8,469 minutes (99,16%) và 9,458 minutes (0,84%) (Figure 2D).

HPLC results showed that the EPS hydrolyzed by H_2SO_4 (Figure 2, graph B) had seven different signal peaks. In particular, there were two peaks near glucose of 12.560 minutes (for 0.12 %) and mannose 14.241 minutes (for 2.66 %). Most of the substances in this hydrolysis EPS were at the peak of 19.788 minutes (for 88.49 %) (figure 2C).

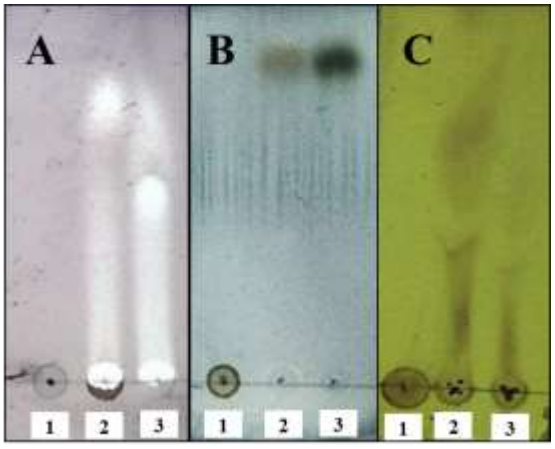


Figure 1. Results of Thin-layer chromatogram of samples hydrolyzed with: HCl (A), H_2SO_4 (B), and Triflic acid (C) samples 1. EPS before hydrolysis, 2. EPS after hydrolysis, 3. Starch after hydrolysis

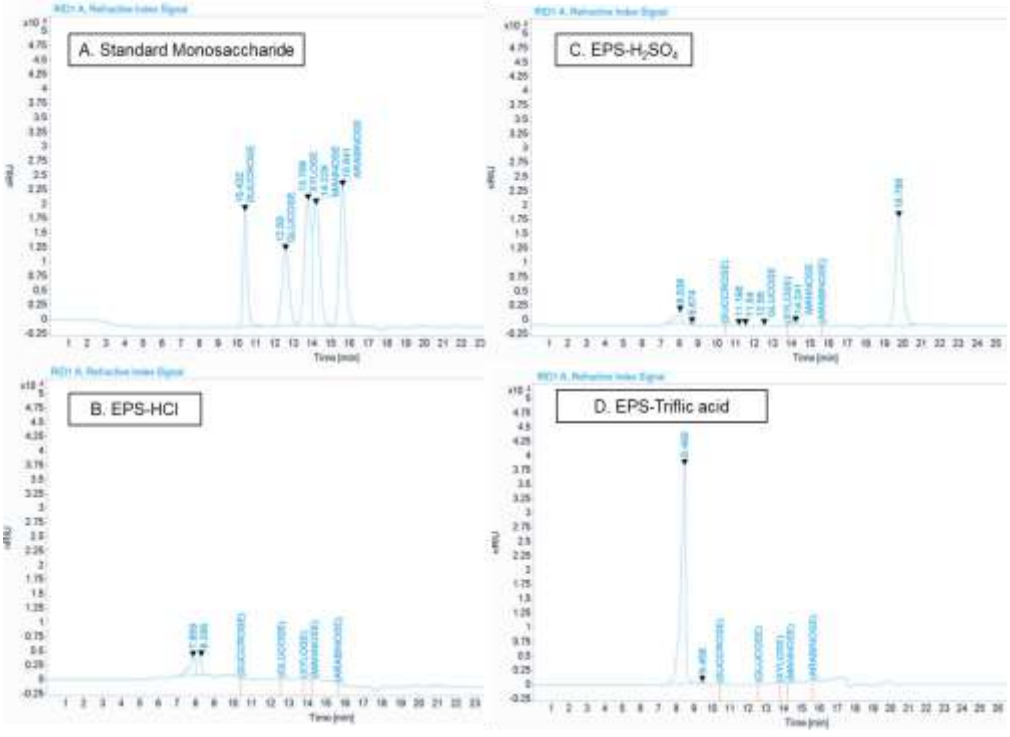


Figure 2. HPLC graphs of samples: A. Standard substance, B. EPS hydrolyzed by HCl, C. EPS hydrolyzed by H_2SO_4, D. EPS hydrolyzed by Triflic acid

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

The results of the thin-layer chromatography showed that the EPS hydrolyzed by H_2SO_4 and the EPS hydrolyzed by HCl were acetylated. The thin-layer chromatogram showed a trace on track 2 and track 4 (Figure 3), compared to the hydrolyzed EPS sample but non-acetylated (figure 3, track 1 and 3, no signal of the streak). For EPS hydrolyzed by Triflic acid, the thin-layer chromatographic (Figure 3, track 6) showed very light streaks, and the spot at the baseline was still very dark.

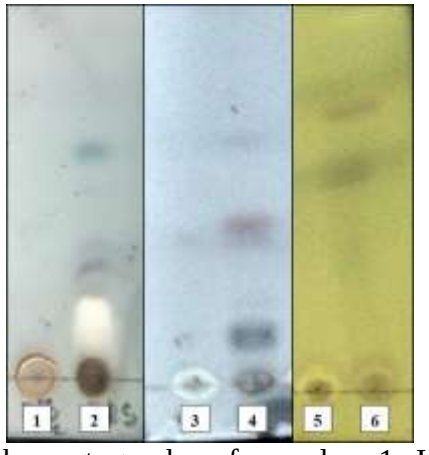
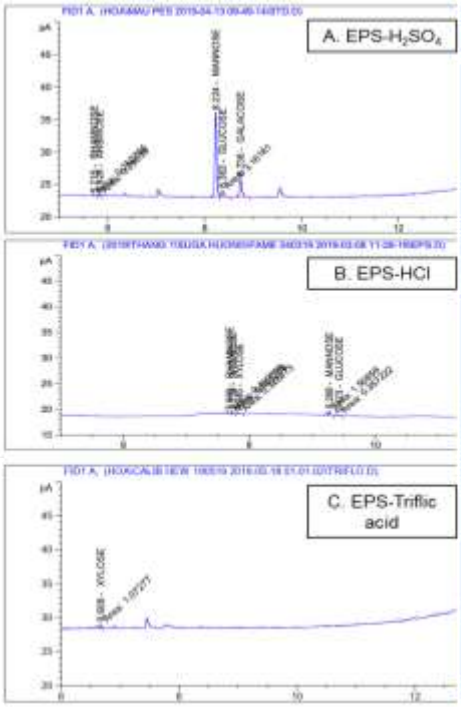


Figure 3. Thin-layer chromatography of samples: 1. H_2SO_4-hydrolyzed EPS non-acetylated, 2. H_2SO_4-hydrolyzed EPS acetylated, 3. HCl-hydrolyzed EPS non-acetylated, 4. HCl-hydrolyzed EPS acetylated, 5. Triflic acid-hydrolyzed EPS non-acetylated, 6. Triflic-hydrolyzed EPS acetylated

Results of GC-FID

GC-FID results showed that EPS hydrolyzed by H_2SO_4 had 5 kinds of monosaccharides, including rhamnose, arabinose, mannose, glucose, and galactose. The majority were mannose, galactose, and glucose; arabinose and rhamnose were a small quantity. 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; most of them are glucose, galactose, and arabinose. The EPS hydrolyzed with Triflic acid appeared many unknown signal peaks, of which only one peak corresponded to xylose (Figure 4).



EPS	Monosaccharide	Mg/g	Ratio
H2SO4-hydrolyzed	RHAMNOSE	0.17	1
	ARABINOSE	0.19	1.12
	MANOSE	22.10	130
	GLUCOSE	1.69	9.94
	GALACTOSE	7.90	46.5
HCl-hydrolyzed	RHAMNOSE	17.2	1.04
	ARABINOSE	23.7	1.45
	XYLOSE	16.3	1
	MANOSE	18.7	1.15
	GLUCOSE	65.7	4.03
	GALACTOSE	20.8	1.28
Triflic acid-hydrolyzed	XYLOSE	0.04	-

Figure 4. GC-FID graph and monosaccharide composition results of samples: A. EPS hydrolyzed with H_2SO_4, B. EPS hydrolyzed with HCl C. EPS hydrolyzed with triflic acid

Discussion

Under the effect of acid, polysaccharide hydrolyzed into smaller carbohydrate molecules by breaking down these glyosidic bonds. The results of thin-layer chromatography demonstrated that EPS samples had been hydrolyzed. However, hydrolysis process took place in varying degrees. Hydrolysis reaction with HCl and triflic acid took place incompletely, shown by the trail of substances spread along the thin plate and spots of residual material at the starting line. Hydrolysis reaction with H_2SO_4 gives better results, there was very little substance at the starting line, no stretches, spots on thin sheets were neat, concentrated. 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 monosaccharides, the product of hydrolyzing EPS samples and starch.

HPLC results showed that carbohydrates in EPS after hydrolysis with HCl, similar to hydrolysis with triflic acid, were still mostly oligosaccharides. This dedicated that hydrolysis process took place incompletely and the

hydrolysis efficiency was low. For EPS hydrolyzed with H_2SO_4, the HPLC graph showed the powerful hydrolysis of this acid, which could dissolve polysaccharide to monosaccharide.

The results of thin-layer chromatography and HPLC of the post-hydrolyzed EPS samples showed that H_2SO_4 exhibited greater hydrolysis ability than the other two acids during EPS hydrolysis.

The thin-layer chromatographic of EPSs after acetylation presented the] traces of the sample after treatment with H_2SO_4 (track 2) and HCl (track 3) might contain acetylated monosaccharides which had different poor polarities. However, the chromatographic of the HCl-hydrolyzed EPS after acetylation (track 3) showed a long, darker trail than the H_2SO_4-hydrolyzed EPS after acetylation (track 2), which dedicated that the acetylation reaction of HCl-hydrolyzed EPS had occurred incompletely. In the case of Triflic acid hydrolyzed EPS, the acetylated one also appeared a long dark streak, which indicated that the hydrolysis ability of Triflic acid on EPS was very low. The results of thin chromatography of EPSs after acetylation contributed to prove that the EPS treated with H_2SO_4 had been hydrolyzed well and facilitated successfully in acetylation reaction.

Mannose, galactose, and glucose have been the three most common monosaccharides found in most EPS extracts, and the activity of EPS is related to the ratio of these three monosaccharides (Zhang *et al.*, 2005; Soltani *et al.*, 2013). A study by Trang (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. The results of the present study were 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. The study of Zhang *et al.* (2011) analyzing simple sugars of a polysaccharide fraction from *O. sinensis* biomass also showed that this segment accounted for the majority of mannose (38.37 %), glucose (27.44 %) and galactose (24.25 %).

H_2SO_4-treated EPS detected 5 types of monosaccharides: rhamnose, arabinose, mannose, glucose, and galactose; mainly mannose, galactose, and glucose; mannose portioned the highest percentage. EPS hydrolyzed by HCl had 6 kinds of monosaccharides, including rhamnose, arabinose, xylose, mannose, glucose, and galactose; the most monosaccharide were glucose with 3.5-fold mannose and 3.1-fold galactose. The EPS hydrolyzed by Triflic acid only detected xylose.

The results from the present study showed that H_2SO_4 was a proper acid for *O. sinensis* exopolysacchride hydrolysis process, sample pretreatment before analyzing monosaccharide composition by GC-FID method.

Acknowledgement

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