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Deproteinization in purification of exopolysaccharide from *Ophiocordyceps sinensis* olive oil – stimulated culture

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Abstract Exopolysacchride (EPS) extracted from *O. sinensis culture* supplemented olive oil is analyzed not only includes polysaccharides which also have links with proteins. In this study, we evaluated the deproteinization methods from the crude EPS to increase the efficiency of exopolysaccharide extraction, and not to affect the biological activity of polysaccharide, the methods used: Sevag method, TCA (trichloroacetic acid) method, and the enzymatic method. Results showed that using protease method for efficient removal of protein on extraction, purification, and biological activity was greater than the residual methods. Deproteinized EPSs separated by Sephadex G-100 gel filtration chromatography, we obtained two segments, EPS I and EPS II. In that, polysaccharide content increased from 34.1% to 82.33% (2.4 times) for EPS I, 78.98% (2.3 times) for EPS II, protein content significantly reduced when using protease from 3.57% to 0.002%; and 0.0067%, for EPS I, EPS II respectively. Besides the antioxidant activity of the EPS segments increased significantly compared with the raw EPS.

Keywords: deproteinization, purification, exopolysacchride, *Ophiocordyceps sinensis*

Introduction

Exopolysaccharide (EPS) main role is to protect the cells in the environment. EPS are synthesized and secreted into the environment when organism's obstacle biological problems including competition with other organisms and non-biological problems such as temperature, light intensity or pH. Besides, the EPS was created to meet directly with selective environmental pressures, such as temperature, stress and light intensity. However, they do not act as energy storage and micro-organisms are not capable of metabolic EPS created (Wang *et al.,* 2002).

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Studies have demonstrated that EPS from natural or artificial cultured *O. sinensis* presents active immunomodulatory, anti-tumor, anti-oxidation and reduce blood sugar. In addition, they also have other important effects as the anti-inflammatory, reduce fatigue, protect the kidneys and protection from radiation (Hu *et al.,* 2009; Yan *et al.,* 2014). Notably, *O. sinensis* EPS at a concentration of 100 μg/ml effected to enhance the development of mice splenic lymphocytes after 48 hours of treatment, significantly increased TNF-α and IFN γ and stimulated expression of other cytokines by the immune system. In addition, Yang *et al.,* showed that EPS presented capable of inhibiting metastasis of cells B16 melanoma to the liver and lungs, and reduced the concentration of c-Myc, c-Fos, VEGF (genes coding transcription factors, regulation and growth factors) in the lungs and liver when compared to mice not used EPS ($p < 0.05$). The studies then showed EPS not only had the effect of inhibiting tumor H22 but also stimulated the immune system through increased expression of TNF -α, IFN-γ and m-RNA of splenic lymphocyte; anti-metastatic and conditioning reduces Bcl-2 protein in the liver and lungs. Considerly, EPS is potentially active group should be fully exploited to optimize the manufacturing process of this medicinal mushroom, thereby improving economic efficiency.

The composition of many EPSs from mushrooms was analyzed not only included pure saccharide, but also non-carbohydrate components such as proteins, phosphate, sulfate or amine with different proportions (Siu *et al.,* 2014). Oh *et al.* (2007) analyzed components of polysaccharide – protein complex from *C. sphecocephala* EPS, using Sepharose CL-6B chromatography obtained 3 segments was FR-I, II, III and showed component monosaccharide had mannose, galactose, glucose, and 18 amino acids.

Leung *et al.* (2009) compared the levels of proteins in polysaccharide complex and corresponding incubation duration and found significantly increased levels of polysaccharides from day 4 (46%) to 6 (70%), the protein content was reduced from 29.3% (day 4), and 27.9% (day 5). The results showed that when the molecular weight of EPS was increased, the protein was decreased during the development of mycelium. This property was maintained to phase balance, this process involves the transfer or adds 1 glucose-1-phosphate group from UDP-glucose donating substance to protein molecules and release one UMP. Base on the results, the author thought that there was a combination of polysaccharide with proteins forming a polysaccharide-protein complex from 5- 200 kDa.

To demonstrate the activity of EPS is due to the certain composition, the researchers have conducted removing protein components in the polysaccharide – protein complex. Many methods of removing protein have calculated to

increase the efficiency of polysaccharide extraction, deproteinization, the pigment composition, without affecting the biological activity of the polysaccharide, simultaneously.

Deproteinization is an essential step to analyze natural polysaccharide. However, natural polysaccharide contains many pigments and proteins with the similar molecular weight. Besides, the presence of free proteins and oligosaccharide in the sample also influence to analysis polysaccharide because spectrophotometric methods are interfered with by these components.

Many methods of deproteinization in both chemistry and biology depending on various purposes have been conducted such as Sevag method, TCA (trichloroacetic acid) method, NaCl, $CaCl_2$ method or using enzymes and combinations of these methods. Sevag method and the TCA method are most widely used by the popularity, efficiency and economic performance. However, these method causes losses of large amounts of polysaccharides (Chen *et al.*, 2012). Deproteinization by the enzymatic method is also used due to its specificity and low impact on the polysaccharide composition needed for purification.

Base on those studies and practices, and the results of our previous study demonstrated supplementing 5% of the olive oil to the culture which stimulated synthesis of fungal EPS *O. sinensis*, we used the EPS extraction process from the culture supplemented with olive oil (unpublished study) and evaluated which method was effective on removing protein and unaffected bioactive of EPS.

Materials and methods

Microorganism and cultivation

Ophiocordyceps sinensis was provided by Dr. Truong Binh Nguyen, Dalat University, Vietnam. The liquid medium was composed of 200 g/L potato, 50 g/L sucrose, 6 g/L peptone, 4 g/L yeast extract, 0.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L $CaCl_2$, 0.2 g/L $MgSO_4$, 50 mL/L olive oil, and 15 mL/L Tween-80. The strain of *O. sinensis* was inoculated in the liquid medium for 40 days (Hang *et al.*, 2017).

Isolation of crude exopolysaccharide

The fermentation broth was first treated with Petroleum ether 60 – 90 to remove excess oil, then mixed with ethanol 96% at the ratios of 1: 4 (v/v) and kept cold at 4^{o}C for 24 hours. The crude EPS was obtained by centrifuging at 5,000 rpm for 10 min, washed 3-4 times with ethanol 96% and dried at 50^{o}C. EPS was dissolved into distilled water for deproteinization.

Deproteinization by Sevag method

The mixture (0.2g EPS in 20mL distilled water) was treated with Sevag solution (v/v 1: 1), shaken vigorously for 20 min, then centrifugated at 5000 rpm for 10 min. The upper layer was carefully collected and treated as above; the solvent layer and the denatured protein were removed. The process was repeated until no denatured protein appeared at the junction of the water and organic solvent layer. Sevag solution with $CHCl_3$-n-BuOH ratio 3: 1, 4: 1, 5: 1 (v/v) were investigated for their efficacy.

Deproteinization by TCA

TCA was carried out according to Huang *et al.* (2010). TCA 10%, 20%, 30%, and 40% were added to adjust the mixture to pH 3.0, kept at 4^oC for 24 hours, then centrifuged at 10,000 rpm for 10 min, the supernatant fraction was recovered. This process was repeated twice.

Deproteinization by Enzyme Alcalase

Alcalase powder, from Brenntag Vietnam, an endoproteinase was used at 10 UI/mL, 20 UI/mL, 30 UI/mL, 40 UI/mL and 50 UI/mL. The liquid enzyme was applied to the mixture at 1:3 (v/v), incubated at 60^oC in 2 hours.

After deproteinization, the deproteinized EPS was yielded by precipitation with ethanol 96% at 1:4 (v/v) as above, then EPSs were determined the percentage of polysaccharide, percentage of protein and the scavenging effect on ABTS radicals.

Gel filtration chromatography performance

10 mL of the EPS discovered in NaCl 0.2 M (at concentrate 10 g/L) was loaded into the Sephadex G-100 gel column, solubilized the column with 0.2 M NaCl at a rate of 0.8 mL/min, then obtained 2 mL/tube. Determination of polysaccharide and protein for each tube at 490 nm and 280 nm, respectively. EPS segments were dialyzed with distilled water through 1 kDa dialysate and dried at 50^oC to be obtained.

Determination of total polysaccharide and protein

The total sugar and protein in the supernatant were measured by the phenol-sulphuric acid method using D-glucose as a standard and by the

Bradford method with bovine serum albumin as standard, respectively (Nehad *et al.,* 2010; Zhang *et al.,* 2017).

Assay for the scavenging effect on ABTS radicals

The assay was carried out according to the method of (Re *et al.,* 1999) The ABTS radical cation scavenging activity was assessed by mixing 3 mL ABTS solution (absorbance of 0.7 ± 0.02 at 743 nm) with 0.1 mL deproteinized EPS (5000 μg/mL), incubated in 30 minutes. The final absorbance was measured at 743 nm with a spectrophotometer. The percentage scavenging of was calculated by the following formula:

$$S\% = \frac{A_0 - A_1}{A_0} x100$$

Where S% - Percentage of scavenging; A◦ - Absorbance of control; A1 - Absorbance of the sample.

Statistical analysis

The data was analyzed by STATGRAPHICS Centurion XV software. A *p*-value of less than 0.05 was considered significant statistically.

Results

Survey methodology protein

Deproteinization by TCA method

The results presented that deproteinization EPS samples treated with TCA at concentrations from10% to 40% had protein content decreased from 3.57% to 1.11% (fig. 1). After removing proteins from crude EPS, polysaccharide content in the sample was increased or decreased depending on the concentration of TCA, compared to the control sample. In specificly, EPS treated TCA 10% had polysaccharide be greatest (41.23%), compared with control EPS, 34.10% only.

Deproteinization by Sevag method

Polysaccharide content in the deproteinized EPS by Sevag 3:1, Sevag 4:1, Sevag 5:1 were 45.64%, 62.72%, and 49.39%, respectively, compeared to control was 34.10%.

Protein content percentage of Sevag 4:1-treated EPS was lowest (1.65 %), while the control EPS's was 3.57%. The results presented that Sevag 4:1 was

the most effective to remove protein out of crude EPS (fig. 2). The results of polysaccharide content in deproteinized samples by Sevag 4:1 was higher and the protein was lower that make the ABTS radical – scavenging activity better than control sample (fig. 2).

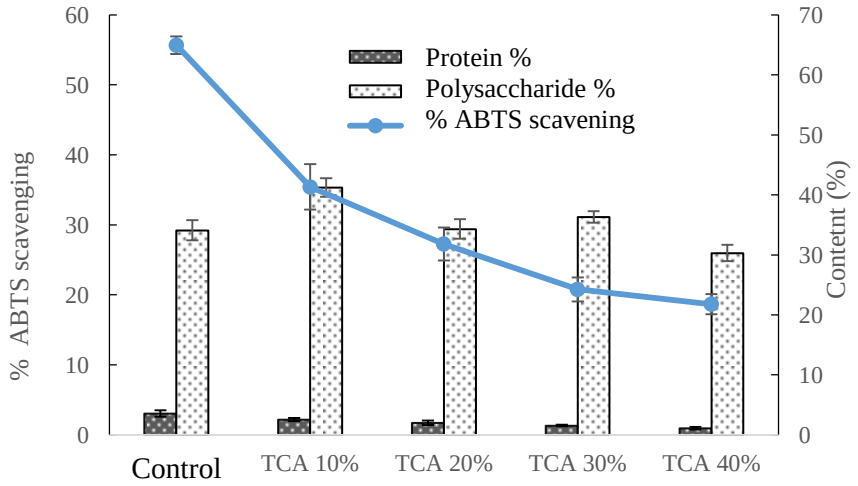

Figure 1. The total polysaccharide, protein and the ABTS radical – scavenging activity of EPSs treated with TCA method

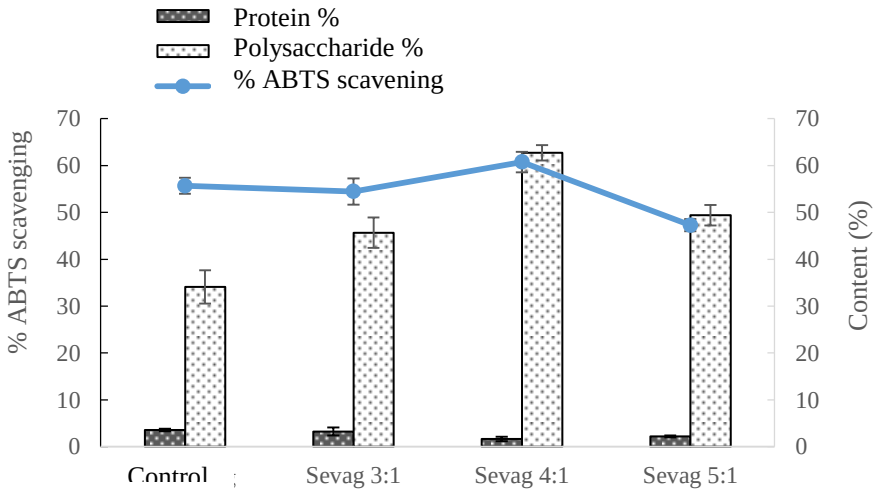

Figure 2. The total polysaccharide, protein and the ABTS radical – scavenging activity of EPSs treated with Sevag method

Deproteinization by enzymatic method

Protease enzyme agent was used at 10 UI/mL to 50 UI/mL that showed the reduced protein content than the crude EPS. Enzyme at 20 UI/mL presented the greatest concentrate. The treatment had high efficiency with protein content 1.57% compared to control 3.57%; polysaccharide content increased up to 69.2% (2 times compared to controls) and EPSs had the ABTS radical – scavenging activity greater than 50%, treated with enzyme 20UI/mL was highest, 68.98% (fig. 3).

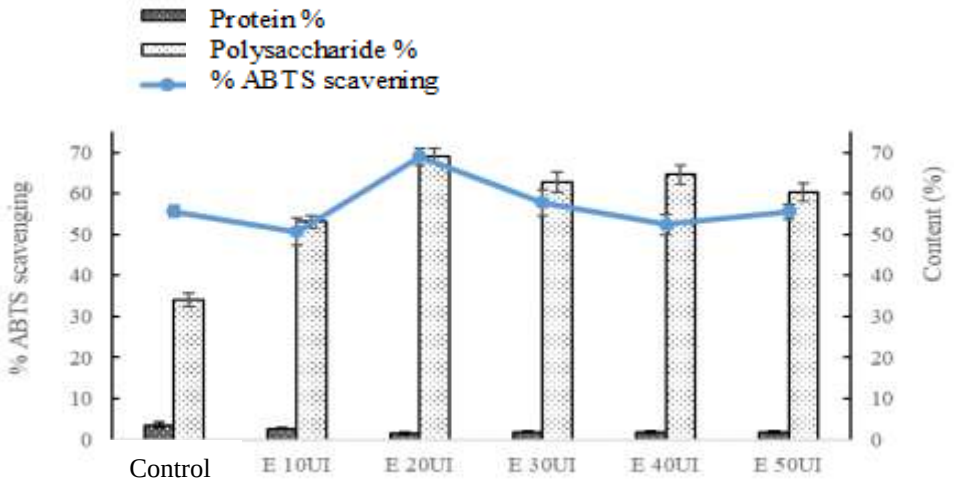

Figure 3. The total polysaccharide, protein and the ABTS radical – scavenging activity of EPSs treated with Alcalase

Examination of protein complexes polysaccharide (PSP)

The segments of the crude EPS and the EPS after deproteinization by protease at 20 UI/mL

Performing Sephadex G-100 gel filtration chromatography to separate EPS of crude and the sample after deproteinization by protease at 20 UI/ml. The results are shown in Figure 4 a, b with 2 segments EPS I (a, b) and II (a, b); value OD490nm, OD280nm used to measure the absorption of polysaccharide and the protein.

EPS was separated to obtain two segments EPS with a volume of 2 ml/tube:

EPS I from tube No. 25-35 (crude EPS) and tube No. 25-31 (EPS after deproteinization)

EPS II from tube No. 55-76 (crude EPS) and tube No. 61-76 (EPS after deproteinization)

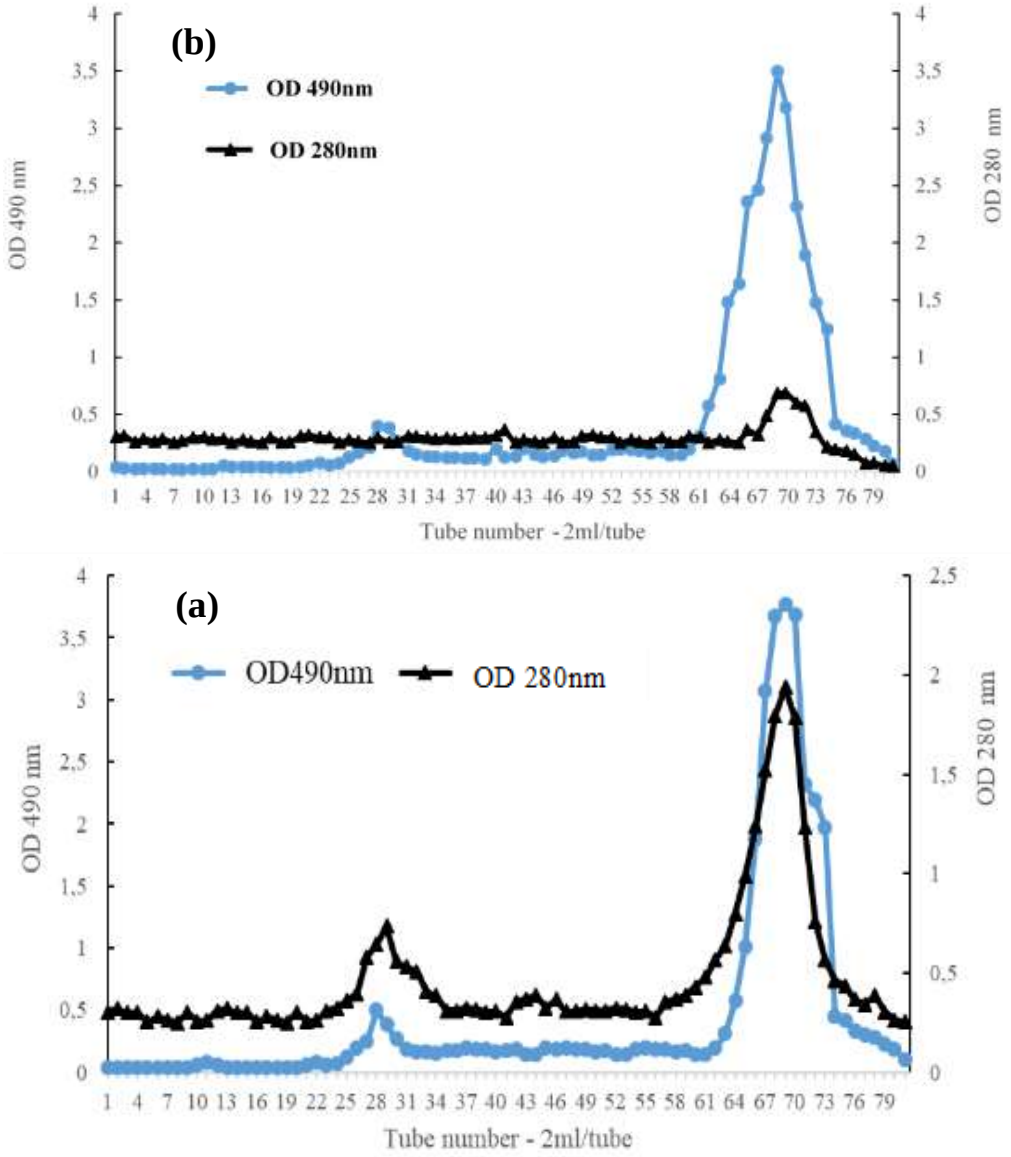

Figure 4. OD_{490nm} and OD_{280nm} values of EPS I and EPS II obtained from the crude EPS (a) and the deproteinized EPS treated by Alcalase (b) separated by Sephadex G-100

The EPS II segment had overlap polysaccharide and protein peak, the height of protein peak was lower peak lower than the peak of EPS that not using protease. This demonstrated that the polysaccharide and protein-bound together in the form of protein-polysaccharide complex, there has no space for

the protease to work, therefore could not break down all the protein still binding to the polysaccharide.

Many previous studies have shown that EPS from Cordyceps culture might contain proteins or not. Kim *et al.,* (2005) performed fractionate EPS by gel filtration chromatography Sepharose CL-6B and obtained 5 segments for EPS from *C. militaris* NG1 and 3 segments for EPS from *C. sinensis*. This difference may be due to strains origin, composition and environmental conditions or techniques and gel used in the process of separation.

The ABTS radical – scavenging activity, the total polysaccharide and protein content of EPS I and EPS II

The result showed that increasing purity of crude EPS samples by Sephadex G-100 gel filtration chromatography obtained two segments: polysaccharide content increased from 34.1% to 82.33% (2.4 times) for EPS I, 78.98% for EPS II (2.3 times), protein significantly reduced when using protease method, from 3.57% down to 0.002% and 0.0067%; EPS I and EPS II, respectively. Besides the antioxidant activity of the EPS segments increased significantly compared with the raw EPS (Tab 1.).

Table 1. Percentage of the ABTS radical – scavenging activity, the total polysaccharide and protein content of EPS I and EPS II

EPS	% ABTS scavenging	Polysaccharide content (%)	Protein content (%)
Crude EPS	55.67 ± 1.26	34.1 ± 2.69	3.57 ± 0.25
EPS I	60.23 ± 2.01	62.83 ± 0.42	2.31 ± 0.17
EPS II	62.46 ± 1.87	65.09 ± 0.53	3.57 ± 0.25
EPS-E	68.98 ± 2.05	69.2 ± 1.83	1.57 ± 0.22
EPS-E I	78.62 ± 2.94	82.33 ± 3.76	0.002 ± 0.0
EPS-E II	79.83 ± 2.66	78.98 ± 4.01	0.067 ± 0.0

Discussion

ABTS radical – scavenging activity of deproteinized EPS by TCA 10% method was much lower than the control sample (35.42% and 55.62%, respectively). The principle of the TCA method is that the protein cation can bind the TCA to form an insoluble salt for precipitation at pH < pI (isoelectric point), might break complex PSP, and destroyed part of the structure of the polysaccharide. Thereby losing the biological activity of EPS presented that

this method was not effective for deproteinization on this EPS (Huang *et al.,* 2010; Liu *et al.,* 2010; Zhang *et al.,* 2017).

Based on the ratio between the ability of removing protein, polysaccharide content, and the ABTS radical scavenging activity of the EPS samples after deproteinization by protease treatments at 10-50 UI /mL, respectively were 2.74; 1.57; 1.86; 1.75 and 1.78, we chose the protease at 20 UI/mL to compare the ability of removing protein from crude EPS samples with TCA 10% and Sevag 4:1 method.

Table 2. The total polysaccharide, protein and the ABTS radical – scavenging activity of EPSs treated with TCA 10%, Sevag 4:1 and Alcalase 20UI/mL

Methods	Polysaccharide content (%)	Protein content (%)	% the ABTS scavenging
TCA 10%	41.23 ± 1.54	2.56 ± 0.26	35.42 ± 3.24
Sevag 4:1	62.72 ± 0.18	1.65 ± 0.46	60.76 ± 2.18
Enzyme 20UI/mL	**69.20 ± 1.83**	**1.57 ± 0.22**	**68.98 ± 2.05**

The result of protein removal from the crude EPS by three methods including TCA, Sevag, and protease are present in table 2. The enzymatic method was the best method to type proteins from the crude EPS. The percentage of protein content had many differences between the 3 methods of survey, enzymatic method was the highest possible deproteinization method (only 1.57% of protein) and the highest polysaccharide content (69.20%) ($p < 0.05$) without compromising the biological activity of EPS, the ABTS scavenging was (68.98%) higher than the other methods. The TCA method impacts strongly the EPS, which caused of the greater polysaccharide destroyed, and an active decreased in comparison to the control. Remarkably, Huang *et al.,* (2010) also proved Sevag method was the good method to remove proteins for Mannan oligosaccharide, with a protein rate of 89.8% and only 12.2% Mannan Oligosaccharide lost. However, compared to the enzyme method, the probability of removing protein, polysaccharide content and bioactivity of this EPS was still higher but still not optimal. Zhu *et al.,* (2011) demonstrated the combination of the enzyme and Sevag methods was the best optimal for this kind of removing protein from polysaccharide by *Armillariella Tabescen*. Therefore, to improve the effectiveness of this process it is necessary to conduct a further survey the use of the enzyme - Sevag method in the works of later research.

Conclusion

In conclusion, we extracted the crude EPS from olive oil – supplemented *O. Sinensis* culture and treated with the enzyme method to deproteinize the crude EPS. The results of this study might be the premise, contribute to further studies of the monosaccharide composition and EPS structure for practical applications in the future.

References

Chen, Y., Li, W., Zhang, H., Nie, S., Wang, Y. and Li, C. (2012). An effective method for deproteinization of bioactive polysaccharides extracted from Lingzhi (*Ganoderma atrum*). Food Science and Biotechnology. 21:191-198.

Hang, L. T. T., Tuyet, N. T. T., Phuong, B. T. B., Trang, T. M., Thu, H., Hiep, D. M. and Thang, N. T. (2017). Effect of plant oils on mycelial biomass production, biosynthesis and antioxidants of exopolysacchride by *Ophiocordyceps sinensis*. Journal of science technology and food. 13:3-10.

Huang, G., Yang, Q. and Wang, Z. B. (2010). Extraction and deproteinization of mannan oligosaccharides. Zeitschrift für Naturforschungn C. 65:387-390.

Hu, C., Zou, Y. and Zhao, W. (2009). Effect of soybean oil on the production of mycelial biomass and pleuromutilin in the shake-flask culture of Pleurotus mutilis. World Journal of Microbiology and Biotechnology. 25:1705-1711.

Kim, H. and Yun, J. (2005). A comparative study on the production of exopolysaccharides between two entomopathogenic fungi *Cordyceps militaris* and *Cordyceps sinensis* in submerged mycelial cultures. Journal of Applied Microbiology. 99:728-738.

Leung, P. H., Zhao, S., Ho, K. P. and Wu, J. Y. (2009). Chemical properties and antioxidant activity of exopolysaccharides from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1. Food Chemistry. 114:1251-1256.

Liu, J., Luo, J., Sun, Y., Ye, H., Lu, Z. and Zeng, X. (2010). A simple method for the simultaneous decoloration and deproteinization of crude levan extract from *Paenibacillus polymyxa* EJS-3 by macroporous resin. Bioresource technology. 101:6077-6083.

Nehad, E. A. and El-Shamy, A. R. (2010). Physiological studies on the production of exopolysaccharide by fungi. Agriculture and Biology Journal of North America. 1:1303-1308.

Oh, J. Y., Cho, E. J., Nam, S. H., Choi, J. W. and Yun, J. W. (2007). Production of polysaccharide–peptide complexes by submerged mycelial culture of an entomopathogenic fungus *Cordyceps sphecocephala*. Process Biochemistry. 42:352-362.

Siu, K. C., Chen, X. and Wu, J. Y. (2014). Constituents actually responsible for the antioxidant activities of crude polysaccharides isolated from mushrooms. Journal of fuctional foods. 11:548-556.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free radical biology and medicine. 26:1231-1237.

Wang, R., Xie, J., Ji, P., Li, S., Zhan, H., Xia, J., Sun, H., Lei, L., Yu, J., Wang, Y. and Holliday, J. (2002). Clinical trial report on chronic hepatitis treatment using immune-assist brand mushroom extract mixture in conjunction with the drug Lamivudine.

Retrived from: http://www.omni-health.net/Home_Page/Products/Kemelyd/Docs/Kemelyd_Hepatitis.pdf.
Yan, J. K., Wang, W. Q. and Wu, J. Y. (2014). Recent advances in Cordyceps sinensis polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: A review. Journal of Functional Foods. 6:33-47.
Zhang, H., Wang, X., Li, R., Sun, X., Sun, S., Li, Q. and Xu, C. (2017). Preparation and bioactivity of exopolysaccharide from an endophytic fungus *Chaetomium sp*. of the medicinal plant gynostemma pentaphylla. Pharmacognosy magazine. pp.477.

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