EXTRACTION AND BIOLOGICAL ACTIVITY OF POLYSACCHARIDE AND PROTEIN FROM FRUIT BODY CORDYCEPS MILITALIS

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Abstract: *In this study, we presented the methods for polysaccharide and protein extraction from Cordyceps militaris fruit bodies. Anti-bacterial properties of protein and antioxidant activity of polysaccharides were also evaluated. The results showed that the two positive gram bacteria strains, Bacillus subtilis, Salmonella aureus, and one negative gram strain, Escherichia coli, were inhibited after being treated by extracted protein. Besides, the IC_{50} value of the polysaccharide was found to be 97.72 ppm.*

Keywords: Protein; Cordyceps militaris; Anti-bacterial; Polysaccharide; Antioxidant.

1. INTRODUCTION

Cordyceps militaris is one of more than 350 species of fungi in the genus *Cordyceps*, which belongs tothe Ascomycetes division andinfecting on insects. Since ancient times, "Cordyceps" has been used widely by Chinese people as a folk tonic to enhance health and endurance. Cordyceps contains several biologically active compounds such as cordycepin, adenosine, polysaccharide, and protein, presenting variousmedicinal properties and economic value. In the previous investigation, we successfully isolated adenosine, cordycepin in the pure form, and evaluated their anti-cancer and anti-inflammatory activities [1]. Recent studies also showed that polysaccharides extracted from *C. militaris* presented the anti-cancer ability [2]. Two main proteins,estimated at 12 kDa and 18 kDa, extracted from *C. Militaris*showed anti-bacterial properties [3, 4]. Therefore, in this work, we continue to study, extract, and assess proteins' biological activity from artificial cultured *C. militaris*.

2. MATERIALS AND METHODS

2.1. Materials

Chemicals as SDS, Albumin BSA, Acrylamide, Temed, Glycerine were purchased from Sigma-Aldrich, Protein ladder was purchased from Thermo scientific.

Cordyceps militaris(L.exFr.) Link was cultured by the Institute of Chemistry - Materials, Academy of Military Science and Technology.

2.2. Methods

2.2.1. Protein contentmeasurement [5, 6]

Protein content was measured by following the Biuret method, based on the fact that proteins (as a rule, all substances containing two or more peptidic bonds) react with copper to form a colored complex whose absorption (λ_{max}=540 nm), in the presence of excess copper, is proportional to the amount of protein present. The maximum OD value was determined by using UV-Vis UH 4150, Hitachi. Albumin BSA was used as reference standards; The concentration was calculated following calibration curvesy = 3.10^{-5} x + 0,0112, in which y was protein concentration, x

was measured OD value. The protein was extracted by electrophoresis by using Amerham.

2.2.2. Polysaccharide content measurement [7]

Polysaccharide content was measured by following the Phenol-Sulfuric method, in which polysaccharide was split into monosaccharide molecules and form a colored complex with phenol with whose absorption at λ_{max}=490 nm. The calibration curves of the reference standard, glucose, was y = 0,0064x - 0,0172, in which y was glucose concentration, x was absorbance value.

2.2.3. Polysaccharide extraction method

Polysaccharide was extracted by the following method in the previous study [8], fruit body powder was extracted 2 times under hot water at 80^{o}C, then centrifuge at 300 rpm for 10 min to remove the residue and collect the supernatant. After that, the supernatant was lyophilized to collect polysaccharide.

2.2.4. Protein extraction method

Fruit body powder of *C. militaris* was mixed with NaOH solution with the ratio at 1:10 in 4 hours. Then, the mixture was filtrated to collect the supernatant. In the following stage, acid acetic was used to adjust pH of the supernatant to the isoelectric point of protein in *C.militaris.* Settle in the fridge for 30 min then cold centrifuge at 8^{o}C and 9000 rpm in 20 min. After that, the residue was collected, rinsing under DI water, and stored in PBS or lyophilized or dried slightly.

2.2.5. Biological activities evaluation

Anti-bacterial properties evaluation method [9-11]

The anti-bacterial properties of the protein were investigated by diskdiffusion test.The test strain was activated on LB agar medium, and then one colony was inoculated to 5 ml liquid LB medium and shaken overnight at 37^{o}C. The active test dish is prepared by inoculating 200 µL of a bacterial suspension at a concentration of 4-5 × 10^8 CFU/mL on the surface of a Petri dish containing LB agar medium, allowed to dry and carve out 5 – 6 wells of 6 mm diameter andthe distance between each well is about 2-3 cm. Add 100 µL of the extract to the agar wells on the Petri dish and keep the test dishes at 40°C for 2 hours, until the extract from the wells diffuses into the bacterial culture medium; then, place the plates in an incubator at 37°C for 24 hours. A positive control is an antibiotic solution (Ampicillin for gram (-) bacteria; Kanamycin for gram (+) bacteria, Amphotericin B for yeast); The negative control was a sterile distilled aqueous solution. The anti-bacterial activity was assessed by measuring the radius (BK) of the microbial inhibition circle by the formula: BK (mm) = D-d; in which D = diameter of the sterile ring and d = diameter of the agar borehole. The experiments were performed in triplicate throughout the study.

The bacterial strains were used for testing, includingnegative gram bacteria group: *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 25923); positive gram bacteria group: *Bacillus subtillis* (ATCC 11774) and *Staphylococcus aureus subsp. aureus* (ATCC 11632), Mycelium: *Aspergillus niger* (439), Yeast: *Candida albicans* (ATCC 7754), and *Saccharomyces cerevisiae* (SH 20).

Evaluate antioxidant activity by DPPHassay [12]

Assessment of antioxidant biological activity using the DPPH assay: 1,1-Diphenyl-2-picrylhydrazyl (DPPH •) is a durable, purple-colored free radical with maximum absorption at 517 nm. In the presence of an antioxidant, DPPH is reduced to 2,2-Diphenyl-1-picrylhydrazine, yellow. The absorbance reduction measurement at 517 nmcould determine the DPPH • radical reduction capacity of the antioxidant.

A positive control was ascorbic acid (100 ppm) diluted in a solvent to dissolve the sample.

Negative control was solvent to dissolve the sample.

The percentage of antioxidant activity was determined by the following formula:

$SC\% = 100 \times (OD_c - OD_m) / OD_c$

In which:

OD_c: Absorbance value of the negative control.

OD_m: Absorbance value of sample.

Based on the percentage of DPPH • free radical, the standard linear curves were built, then determining the value of IC50 (the concentration at which 50% free radical DPPH • was captured). Purified substances showed antioxidant activity at IC_{50}<50 µg / ml. Extracts and raw compounds showed strong antioxidant activity at the IC_{50}<100 µg / ml; The lower the IC50 value, the higher the antioxidant activity.

3. RESULTS AND DISCUSSION

3.1. Protein and polysaccharide extraction

3.1.1. Protein extraction

Effect of NaOH concentration on the yield of protein

10g of *C.Militaris* powder (after using ethanol separation to collect cordycepin, adenosine; dried) was soaked in NaOH solution withconcentrations of 2%, 3%, 4%, respectively, for 4 hours. Centrifuge to remove residue, collect supernatant, adjust the solution to pH 5.1 in the fridge for 30 minutes, then centrifuge to obtain crude protein, lyophilize to obtain the following results:

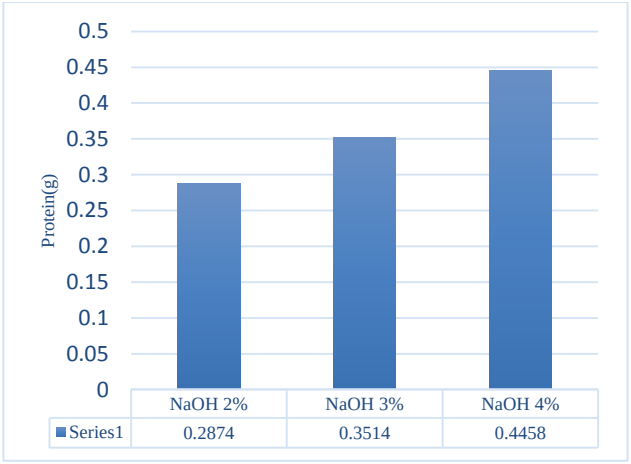

Figure 1. *Effect of NaOH concentration on the protein extraction efficiency.*

As a result above, it is found that using 4% NaOH solution obtained the highest protein content, so this concentration was used in the following experiments. In this study, we just investigated NaOH concentrations up to 4% because at higher NaOH concentration, the hydrolysis reaction between protein and NaOH occurred.

Protein content measurement

Collect 50 mg of the sample obtained in the above experiment (after freeze), dissolved in 10 ml of 0.1M NaOH, then determining the total protein content by Biuret method:

Table 1. *Protein content in the samples.*

Sample	**OD_{540nm}**	**ΔOD_{540nm}**	**Protein content in the sample (mg/ml)**	**Protein content per 1 g of fruit body (%)**
1	0,251	0,153	4,7266	15,199
Blank	0,098	0	0	0

After extraction by using NaOH 4%, the total protein accounted for 15,199% of the total weight of dried fruit body biomass.

Protein size determination

Raw protein obtained after extraction by using 4% NaOH was preserved in phosphate buffer pH 7, then be analyzed by electrophoresis on SDS page 12%, current intensity 15 mA and 25 mA, respectively; the results are as follows:

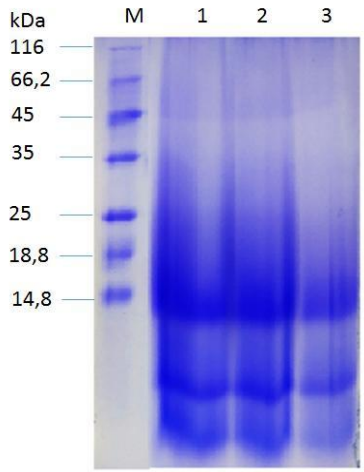

Figure 2. *Protein electrophoresis product (M: standard marker; 1,2,3: Protein isolated at 3 NaOH concentrations).*

As a result above, in comparison with the standard marker, it is found that the extracted protein presented a main dark band between 10 kDa to 18.8 kDa, the other two bands were located at lower than 10 kDa. On the other hand, Park et al. [3] had extracted protein from *C. militaris* fruiting body, purifying a protease enzyme of 12 kDa size. Similarly, Fuu Sheu et al. [4] had purified a protein with a molecular weight of 18 kDa. Both proteins of size 12 kDa and 18 kDa above have biological activity such as bacterial activity inhibition.

3.1.2. Polysaccharide extraction

Effect of temperature on the yield of polysaccharide

10g of *C.Militaris* powder (after using ethanol separation to collect cordycepin, adenosine; dried) was extracted by using hot DI water at 70°C, 80°C, 90°C in 1 hour. The results showed below:

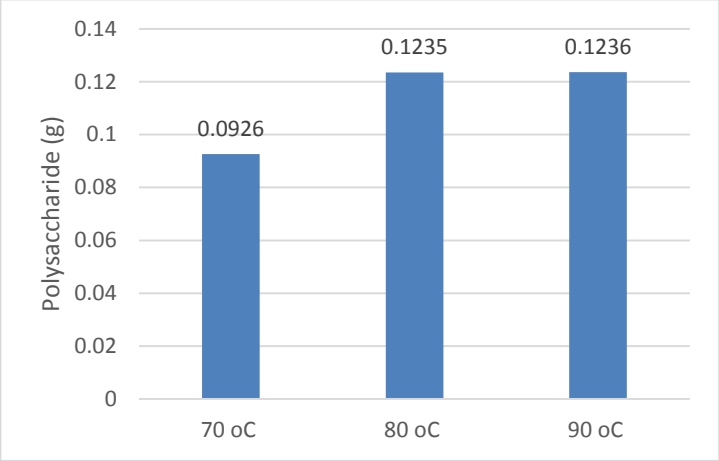

Figure 3. *Effect of temperature on polysaccharide extraction.*

The results showed that extracting at 70°C, the amount of raw polysaccharide obtained was the lowest (0.0926g), extracting at 90°C to obtain the highest content of polysaccharide (0.1266); however, at 80°C, the content of polysaccharide almost did not present any difference in comparison with 90°C. These results were consistent with the study of Du L et al. [8]. Therefore, 80°C was chosen as the optimal temperature for polysaccharide extraction to reduce production cost but still remaining efficient.

Effect ofextraction time on the yield of polysaccharide

10g of *C.Militaris* powder (after using ethanol separation to collect cordycepin, adenosine; dried) was extracted by using hot DI water at 80°C for 2 hours, 2.5 hours, 3 hours, 3.5 hours, and 4 hours. The results showed below:

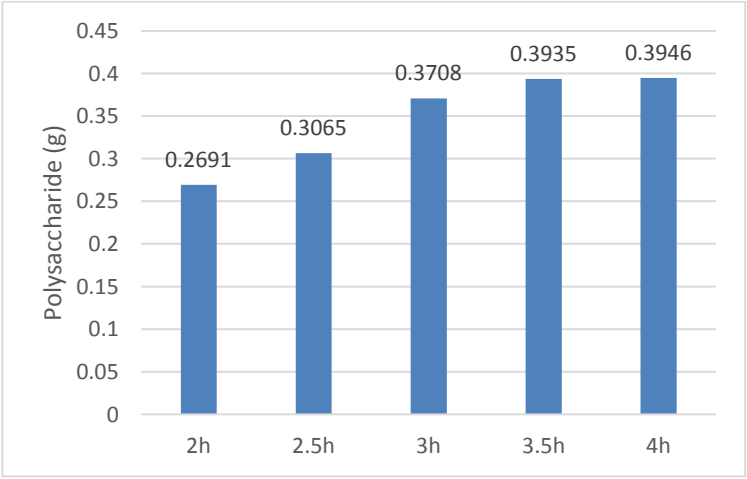

Figure 4. *Effect of extraction time on the yield of polysaccharides.*

The results showed that the highest content of polysaccharide was obtained at 4 hours (0.3946g). However, there was no significant diference between 4 hours (0.3946g) and 3.5 hours extraction (0.3935g). Therefore, it is possible to use the optimal extraction time of 3.5 hours and the extraction temperature of 80^{o}C for polysaccharide purification.

Polysaccharides content measurement in the sample

Collect 10 mg of the sample obtained in the above experiment (after lyophilization) dissolved in 100 ml of DI water; the total polysaccharide content was determined by the Phenol-sulfuric acid method. The result showed that the extracted polysaccharide content in 1 gram dried fruit of C. militaris was 9,262%. According to the report of Teng Li-rong [2], mutant *C. Militaris* (H40199) strain provides polysaccharide content up to 0.78 g/L. In addition, a polysaccharide obtained from *C. Militaris* also presented anti-inflammatory, anti-free radical, and cytotoxic effects [13]. In the next experiment, we evaluated the activity against free radicals from the collected polysaccharide samples.

Table 2. *Polysaccharides content in the sample.*

Sample	**OD_{490nm}**	**ΔOD_{490nm}**	**Polysaccharide content in the sample (mg/ml)**	**Polysaccharides content per 1 g of fruit body (%)**
1	0,537	0,462	0,0749	9,262
Blank	0,075	0	0	0

3.2. Biological activity test

3.2.1. Assessment of antimicrobial activity

We used 7 tested strains of microorganisms to test protein inoculant activity. As a result, the protein preparation was resistant to 3 in 8 strains of microorganisms, namely *B. subtillis*, *E.coli*,and *S. aureus*. Park [3] et al. reported that protein with molecular size 12 kDa was predicted as a strong protease that could inhibit *Fusarium oxysporum*. Therefore, it was possible that the total protein content in our work could contain a certain amount of protease, which could show similarly inhibitory activity of the 3 tested strains above.

Table 3. *Anti-bacterial activity test for 3 bacterial strains.*

Sample	**Concentration** (μg/mL)	**Anti-bacterial circle diameter** (cm)		
		B. subtillis	E.coli	*S. aureus*
Positive Control	5	2,5	2,5	2,4
CP Protein	100	1,5	1,3	1,2

Figure 5. *Anti-bacterial activity test for 3 bacterial strains.*

3.2.2. Evaluate antioxidant activity for obtained polysaccharide

Evaluate antioxidant activity for obtained polysaccharide was performed by using DPPH assay. The results showed in the table below.

Table 4. *Antioxidant activity for obtained polysaccharide.*

Sample	**Positive control**	**Negative control**	**1**	**2**	**3**	**4**	**5**
Concentration (ppm)	100	0	20	40	60	80	100
OD_{517nm}	0,147	0,339	0,301	0,267	0,238	0,201	0,163
SC (%)	56,63	0	11,21	21,23	29,79	40,70	51,91

As the result above, a standard curve was build as y = 0.5044x + 0.708 with correlation coefficient R2 = 0.9976. IC_{50} = 97.72 ppm. The IC_{50}<100 µg/ml showed that the obtained polysaccharide presented strong antioxidant activity. Our research results were similar to previous studies of Chen Y. J. et al. and Ohta Y. et al. [14, 15].

4. CONCLUSION

We have successfully extracted the protein under the conditions of NaOH 4% then precipitation at pH 5.1, the protein content in dried fruit bodies cultured by the Institute of Chemistry - Materialswas 15,199%. The extracted protein has inhibitory activity against three tested strains of microorganisms, namely *B. subtillis, E. coli,* and *S. aureus.*

Polysaccharide content had been extracted from dried fruit bodies, which was 9,262%. Anti-oxidation activity, IC_{50}, reached 97.72 ppm.

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REFERENCES

[1]. Trịnh Đắc Hoành, Vũ Duy Nhàn, Vũ Văn Dũng, Lê Đức Anh, *"Nghiên cứu tách chiết và hoạt tính sinh học của adenosine, cordycepin từ nấmCordyceps militaris***"**, Tạp chí Hóa học, tập 57, số 6E, (2019), tr. 316-321.

[2]. Zhang Ai-li, Lu Jia-hui, Zhang Na, Zheng Dan, Zhang Gui-rong and Teng Li-rong, *"Extraction, Purification and Anti-tumor Activity of Polysaccharide from Mycelium of MutantCordyceps Militaris",* Chem. Res., 26(5), (2010), tr. 798-802.

[3]. Byung Tae Park, Kwang Heum Na, Eui Cha Jung, Jae Wan Park, and Ha Hyung Kim, *"Antifungal and Anticancer Activities of a Protein from the MushroomCordyceps militaris",* Korean J Physiol Pharmacol, Vol 13, (2009), tr. 49 - 54.

[4]. Bai K-C, Sheu F, *"A novel protein from edible fungi Cordyceps militaris that induces apoptosis"*,Journal of Food and Drug Analysis (2016), http://dx.doi.org/10.1016/j.jfda.2016.10.013

[5]. Biuret Protein Assay, Teachers Handbook/ technical@GBiosciences.com

[6]. H. M. Rawel, Electrophoresis in Practice,*"A Guide to Methods and Applications of DNA and Protein Separations 2nd Edition"*, (1997).

[7]. Michel Dubois K. A., Gilles J. K., Hamilton P. A., Rebers and Fred Smith,*"Colorimetric Method for Determination of Sugars and Related Substances"*, Analytical Chemistry, 28(3), (1956), tr. 350-356.

[8]. Du L, Song J, Wang H, *et al*, *"Optimization of the fermentation medium forPaecilomyces tenuipes N45 using statistical approach"*, Afr J Microbiol Res 6, (2012), tr. 6130-6141.

[9]. Vlietinck A. J., *"Screening methods for detection and evaluation of biological activities of plant preparation, Bioassay Methods in Natural Product reseach and Drug development",* Kluwer acadamic publishers, USA, 1998.

[10]. Vanden Bergher D. A., and Vlietinck, *"Screening methods for Antibacterial and Ativiral Agent from Higher Plants, Methods in Plant biochemistry"*. Academic Press., USA, V. 6, 1991.

[11]. Hadacek F., Greger H., "*Testing of antifungal natural products: methodologies, comparability of results and assay choice*". Phytochem Anal., 11, (2000), tr 137-147.

[12]. Sagar B. Kedare & R. P. Singh, *"Genesis and development of DPPH method of antioxidant assay"*, J Food Sci Technol, (2011) 48(4):412–422

[13]. Wol Soon Jo, Yoo Jin Choi, Hyoun Ji Kim, Jae Yun Lee, Byung Hyouk Nam, Jae Dong Lee, Sang Wha Lee, Su Yeong Seo and Min Ho Jeong, *"The Anti-inflammatory Effects of Water Extract from Cordyceps militaris in Murine Macrophage"*, Mycobiology 38(1),(2010), tr. 46-51.

[14]. Chen Y. J., Shiao M. S., Lee S. S., Wang S.Y,"*Effect of Cordyceps sinensis on the proliferation and differentiation of human leukemic U937 cells"*. Life Sciences, 60 (25), (1997), tr.2349-2359.

[15]. Ohta Y., Lee J. B., Hayashi K., Fujita A., Dong KP, Hayashi T. *"In vivo anti-influenza virus activity of an immunomodulatory acidic polysaccharide isolated from Cordyceps militaris grown on germinated soybeans"*, Journal of Agricultural and Food Chemistry, 55 (25), (2007), tr. 10194–10199.

TÓM TẮT

NGHIÊN CỨU TÁCH CHIẾT VÀ ĐÁNH GIÁ HOẠT TÍNH SINH HỌC CỦA POLYSACCHARIDE VÀ PROTEIN TỪ NẤM CORDYCEPS MILITALIS

Trong nghiên cứu này, chúng tôi trình bầy phương pháp tách chiết polysaccharide và protein từ quả nấm Cordyceps militaris. Thử nghiệm hoạt tính kháng vi sinh vật của protein thu được kết quả, protein có khả năng ức chế hai chủng vi khuẩn gram (+) là B. subtillis, S. Aureus và một chủng gram (-) là E. coli. Thử nghiệm khả năng chống oxi hóa của polysaccharide thu được giá trị IC_{50}= 97,72 ppm.

Từ khóa: Protein; Cordyceps militaris; Kháng khuẩn; Polysaccharide; Chống oxi hóa.

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