P-ORT-08

Development of Enzymatic Assay for Adenosine Quantification in *Cordyceps militaris* Extract

Yanisa Suebsuk, Chananya Tansathaporn and Panwajee Payongsri*

Department of Biotechnology, Faculty of Science, Mahidol University 10400, Thailand

**Corresponding author. E-mail: panwajee.pay@mahidol.edu*
DOI:

ABSTRACT

Cordyceps militaris is a substituent of high price Chinese traditional medicine, *Ophiocordyceps sinensis*. This fungus is widely cultivated and consumed for health benefits in Thailand. Currently, the quality can be evaluated by the concentration of adenosine and cordycapin which relies on HPLC. This study proposed a simple enzymatic method for quantifying adenosine in *C. militaris* extract. In this research, *C. militaris* samples were extracted by hot water extraction (80ºC, 3h). Recombinant eADA (*Escherichia coli* adenosine deaminase) adenosine converted to inosine and this was continuously observed by spectrophotometer at 265 nm. The total change in OD_{265} can be used to quantify adenosine in the extract in a good agreement with quantification by HPLC. During this research, it was observed that high temperature extraction led to adenosine degradation and up to 30% reduction could be observed after 3-hour extraction. However, this phenomenon was not observed with cordycepin.

Keywords: Adenosine, Adenosine deaminase, Cordycepin, *Cordyceps militaris*, Extraction

INTRODUCTION

Cordyceps militaris is a common substituent of precious Chinese traditional medicine, *Ophiocordyceps sinensis*. This species is widely cultivated in Thailand and sold as dried fungus, capsule or an ingredient in dietary supplement as *C. militaris* exhibits various pharmaceutical effects such as anti-cancer, anti-oxidant and anti-inflammation. Such properties arise from high abundance of bioactive compounds including polysaccharides, polypeptides, erosterol and nucleosides especially adenosine and cordycepin (Lan et al., 2016).

Currently, the quality of *C. militaris* is normally evaluated by adenosine or cordycepin concentrations (Li et al., 2006). Both compounds could be extracted by water, organic solvent (e.g. methanol) together with sonication before further analysis (Yang & Li, 2008). However, the temperature was reported to reduce the amount of extracted adenosine when the temperature was higher than 40ºC (Li et al., 2015). The most widely used method for adenosine and cordycepin detection is HPLC (Chutvirasakul et al, 2017; Yang et al., 2010; Yang & Li, 2008). The levels of adenosine and cordycepin were reported to be varied depending on the sources and cultivation conditions. For example, when yeast extract was used as a nitrogen source, the level of cordycepin was reported to reach 154.8 mg/l while peptone and corn steep powder gave lower yields (Shih et al., 2007). Ikeda et al. extracted adenosine and cordycepin by sonication and examined by HPLC with UV detector. They found 1.58 – 0.70 mg/g and 3.33 – 6.36 mg/g of adenosine and cordycepin respectively in cultured *C. militaris* (Ikeda et al, 2008). Such variation in the nucleoside contents can greatly influence the price of the fungus. In addition, optimization of cultivation conditions by small and medium-sized enterprises can be limited due to the reliance on complicated analytical method.

Adenosine deaminase (ADA) (EC 3.4.1.1) catalyzes an irreversible deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine. This enzyme is commonly found in all organisms and it plays crucial role in purine salvage pathway. The ADA activity can be detected by decreasing of absorbance at 265 nm (Kalckar, 1947).

Due to high specificity of enzymatic reaction, ADA could be used for developing a new alternative method for determining adenosine in *C. militaris* extract. This enzymatic method can be done in-house and easier than HPLC.

MATERIAL AND METHODS

Materials

Dried *C. militaris* was purchased from Cordylis (CDL) and Cordy Farmacy (CFD), Thailand. Fruiting body (CFFB) and Fruiting body plus mycelium (CFFM) were purchased from Cordy Farmacy. Standard adenosine and cordycepin were purchase from Sigma Aldrich. Acetonitrile (HPLC grade) and Methanol (AR grade) were from RCI Labscan. Yeast extract and Tryptone was from Lab M.

Hot water extraction

Dried *C. militaris* was ground by pestle and mortar into fine powder. *C. militaris* samples (0.25 g) were mixed with 15-ml double-distilled water in 50 ml centrifuge tube. The mixture was boiled in hot water (80 - 85ºC) for 3 h with constant stirring. This was placed on ice for 10 minutes before centrifugation at 8000 rpm for 20 min. The supernatant was transferred into new centrifuge tube. The pellet was reconstituted in 15 ml of DDW for second extraction using the same condition as the first extraction. The supernatant was filtered through 0.45 µM membrane filter before injected into HPLC.

HPLC analysis

HPLC system consisted of WATERS 2690 separation module connected with WATERS 2487 dual wavelength detector. Thermo Acclaim™ 120 C-18 column (4.6 × 150 mm) was maintained at 30ºC. The UV detector was set at 260 nm. The injection volume was 10 µL. Mobile phase consisted of water: methanol (90:10 v/v) and flow rate was 1 ml/min. The running time was 18 min for each sample. Isocratic elution was used throughout this experiment. For *C. militaris* extract analysis, an additional 4-minute washing step was performed after cordycepin was eluted. This step used 100% acetonitrile with 1 ml/min flow rate. This was followed by equilibration step (24 – 30 min) where the mobile phase consisted of water: methanol (90:10 v/v). Flow rate was 1 ml/min.

Standard adenosine and cordycepin

The standard adenosine and cordycepin were prepared at 0.005, 0.01, 0.05, 0.1, 0.2, 0.5 and 1 mM with 0.1 M KPB pH 7.5. The solutions were passed through 0.45-µM membrane filter before being injected into HPLC. The standard curve was constructed by plotting peak areas against the standard concentrations.

***C. militaris* sample**

The HPLC conditions of *C. militaris* extract were the same as analyzing standard adenosine and cordycepin. The amount of adenosine and cordycepin were calculated from standard curve of standard adenosine and cordycepin.

LB broth

One liter of media consisted of yeast extract 5 g, tryptone 10 g, NaCl 10 g. Ampicillin 150 µg/ml was added when culturing the recombinant eADA.

eADA production

Recombinant eADA was cloned into pET-17b plasmid and expressed in *E. coli* BL21(DE3). Single colony on LB + amplicilin agar plate was selected and culture in 200 µl of LB + ampicilin at 37 ºC, 6h. Then 50 µl of culture was transferred to LB + ampicillin 10 ml, and cultured at 37 ºC, 200 rpm for 18 h. This culture was seeded into 20 ml of LB + ampicillin and grew at 200 rpm, 37 ºC. When the OD_{600} reached 0.6 to 0.8, 20 µl of 0.1 mM IPTG was added and the culture was continued at 200 rpm, 25ºC for 18 h. Cells were collected from 2 ml of culture by centrifugation at 13000 rpm. Cell pellets were mixed with 750 µl of 0.1 M KPB, pH 7.5. Then, cells were disrupted by ultrasonication at 40 Hz, 10 cycles of turn on for 10 s then turn off for 10 s. The sample was clarified by centrifugation at 13000 rpm. The supernatant was collected and kept at -80ºC until used.

Continuous spectroscopic technique for adenosine quantification

eADA assay was modified from Sigma Aldrich (Sigma, 1995). The total changes in OD265 at various adenosine concentrations were used to construct standard curve in order to quantify adenosine in extract. Each reaction consisted of 10 µl of crude lysate of recombinant eADA, 240 µl of 0.1 M KPB pH 7.5, 150 µl of DDW, 100 µl of adenosine at various concentrations. For quantification of adenosine in extract, 100 µl of extract was used instead of standard solutions (dilution might be needed) and the

reaction continuously observed at 265 nm, every 10 s, for 10 min in order to ensure that the reaction went to completion. The total change in OD265 was compared with standard curve.

$$\text{Adenosine}\left(\frac{\text{mg}}{100\text{g}}\right) = \frac{\Delta\text{OD265} \times \text{df} \times \text{extraction volumn} \times 267.24 \times 100}{\text{slope (from standard curve)} \times 1000 \times \text{sample weight}}$$

$$\Delta\text{OD265} = \text{OD265}_{t_{10}} - \text{OD265}_{t_0}$$

t_{10} means at 10 minutes and t_0 means at time 0.

RESULTS AND DISCUSSION

Hot water extraction of *C. militaris*

Four samples of *C. militaris* consisted of CDL and CFD were dried fruiting body, CFFB was grounded fruiting body and CFFM was a mix of grounded fruiting body and mycelium. The concentration of adenosine and cordycepin in hot extracts were showed in **Table 1** and **Table 2**.

Table 1. Adenosine in *C. militaris* extracted by hot water extraction (mg/100g) (n=3)

ample	0 h	1st extraction	2nd extraction	Total[a]
CDL	172.6 ± 3.02	119.7 ± 1.99	89.79 ± 0.00[b]	209.5 ± 1.99
CFD	166.8 ± 2.27	117.6 ± 9.47	90.33 ± 0.756	207.9 ± 10.2
CFFB	206.31 ± 1.51	136.3 ± 0.00[b]	91.4 ± 1.31	227.7 ± 1.31
CFFM	130.9 ± 0.756	104.8 ± 0.756	88.19 ± 0.00[b]	193.0 ± 0.756

[a] 1st extraction + 2nd extraction, [b] SD was lower than zero.

Table 2. Cordycepin in *C. militaris* extracted by hot water extraction (mg/100g) (n=3)

Sample	0 h	1st extraction	2nd extraction	Total[a]
CDL	182.4 ± 5.37	222.6 ± 20.8	37.68 ± 2.46	260.3 ± 23.3
CFD	589.4 ± 23.8	640.2 ± 198.4	96.98 ± 10.8	737.2 ± 209.2
CFFB	816.53 ± 14.3	954.2 ± 0.00[b]	122.1 ± 15.71	1076 ±15.71
CFFM	435.7 ± 2.13	492.4 ± 5.12	81.9 ± 0.00[b]	574.3 ± 5.12

[a] 1st extraction + 2nd extraction, [b] SD was lower than zero.

Before incubating the mixture at 80-85°C, 1 ml of sample was also collected to see the level of adenosine and cordycepin that were solubilized without heat treatment (0 h). It can be seen that the concentration of adenosine in all 0-h samples were higher than the 1st extraction. This suggested that some adenosine was degraded upon incubation at 80-85°C. The 2nd extraction still recovered relatively high concentration of adenosine which suggested that more than two-repeat extraction is required to quantify the total adenosine in the extract. In contrast, cordycepin could be extracted in hot water at a higher degree than adenosine. From the total cordycepin extracted, greater than 80% of cordycepin was recovered in the first extraction. It should be noted that the level of adenosine extracted was sufficient for subsequent quantification by enzymatic assay.

Although most of results were not the same as product's claim, the extraction methods would greatly affect the recovery. For example, in CDL, adenosine and cordycepin were found to be 209 ± 1.99 mg/100g and 260.28 ± 23.3 mg/100g, respectively. However, the product's claim suggested that adenosine and cordycepin contents were 145.50 mg/100g and 492.80 mg/100g, respectively. CFFB was claimed to contain adenosine and cordycepin at 256 mg/100g and 1,227 mg/100g. Our extraction showed that adenosine and cordycepin were 227.7 ± 1.31 mg/100g and 1076.3 ±15.71, respectively.

The different extraction methods were reported to greatly affect the recovery of adenosine and cordycepin. Extraction with high temperature led to

degradation of adenosine (Li et al., 2015). Compared with 0-h sample, at least 30% of adenosine was degraded after 3-h extraction. Whereas reduction of cordycepin was not observed. The results were corresponded with the finding of Yang and Li which hot water extraction can reduce adenosine content in *C. militaris* extract (Yang & Li, 2008). This phenomenon might be due to activity of some enzymes that were activated by heat. Such degradation was not observed when heating standard adenosine in the buffer under the same conditions.

Extraction by organic solvent was not included in this study because the presence of organic solvent in reaction mixture can inactivate enzyme. The extracts were further evaluated adenosine concentration by measured the total change in OD_{265} with eADA assay.

Determining adenosine by using eADA

The conversion of adenosine to inosine by eADA could be used to investigate adenosine content in the sample. The linear trend between ΔOD_{265} and concentration of standard adenosine was obtained and presented in **Error! Reference source not found.**.

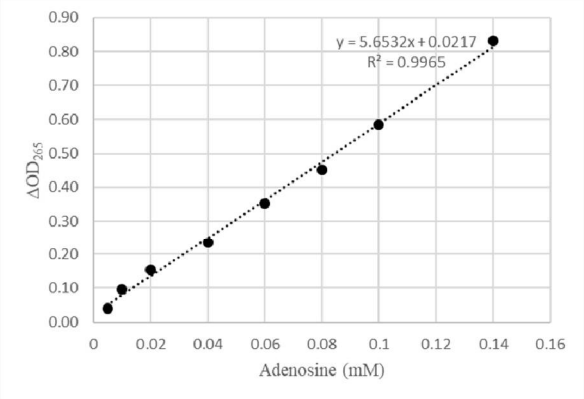

Figure 1. Standard curve of eADA activity with adenosine

CDL and CFFB extract were tested in this experiment. The hot water extracts were added in eADA reaction as a substrate. Each sample was extract once, and adenosine contents were 197.18 ± 21.16 mg/100g in CDL, and

298.12 ± 38.46 mg/100g for CFFB. Such concentrations were higher than the results from HPLC but they were in the same range as the product's claims. It could occur due to high concentration of cordycepin in extracts. Cordycepin was known as an inhibitor of adenosine deaminase (Daddona et al., 1984). However, eADA could slightly catalyze cordycepin but the rate was 10% of adenosine. The reaction might start with deamination of mainly adenosine and minimal cordycepin until the it reached equilibriums. Therefore, the total change in OD_{265} were slightly higher than individual nucleoside content.

CONCLUSION

Various extraction methods have been reported to influence the recovery of nucleosides in cordyceps. In order to develop enzymatic assay for nucleoside quantification, hot water extraction could extract adenosine and cordycepin from *C. militaris* at sufficient concentration but this could lead to lower adenosine concentration. The development of simple enzymatic assay for adenosine quantification was shown to be promising but further optimization would be required. Higher enzyme specificity may be used for either adenosine or cordycepin quantification alone.

ACKNOWLEDGEMENTS

This research work was supported by CIF grant, the Thailand Research Fund (IRG5980001) and Department of Biotechnology, Faculty of science, Mahidol university.

REFERENCES

Chutvirasakul, B., Jongmeesuk, W., Tirasomboonsiri, P., Sansandee, N., & Tadtong, S. (2017). Stability indicating method to determine bioactive nucleosides in crude drugs, extracts, and products from Cordyceps sinensis and Cordyceps militaris. *Thai Journal of Pharmaceutical Sciences*, *41*(2), 52–60.

Daddona, P. E., Wiesmanne, W. P., Lambrose, C., Kelleys, W. N., & Websterll, H. K. (1984). Human Malaria Parasite Adenosine Deaminase. *The*

Journal of Biological Chemistry, *259*(3), 1472–1475.

Ikeda, R., Nishimura, M., Sun, Y., Nakashima, K., & Wada, M. (2008). Simple HPLC-UV determination of nucleosides and its application to the authentication of Cordyceps and its allies. *Biomedical Chromatography*, *22*, 630–636.

Kalckar, H. M. (1947). Differential spectrophotometry of purine compounds by means of specific enzymes: III. studies of the enzyme of purine metabolism. *J. Biol. Chem*, (167), 461–475.

Lan, D. H., Thu, N. T. K., Lan, P. T., Nha, P. Van, & Tung, B. T. (2016). Cordyceps militaris (L.) link: Chemical bioactive compounds and pharmacological activities. *Journal of Pharmacy and Nutrition Sciences*, *6*(4), 153–159.

Li, J., Guan, M., & Li, Y. (2015). Effects of cooking on the contents of adenosine and cordycepin in Cordyceps militaris. In *Procedia Engineering* (Vol. 102, pp. 485–491).

Li, S. P., Yang, F. Q., & Tsim, K. W. K. (2006). Quality control of Cordyceps sinensis, a valued traditional Chinese medicine. *Journal of Pharmaceutical and Biomedical Analysis*, *41*(5), 1571–1584.

Shih, I. L., Tsai, K. L., & Hsieh, C. (2007). Effects of culture conditions on the mycelial growth and bioactive metabolite production in submerged culture of Cordyceps militaris. *Biochemical Engineering Journal*, *33*(3), 193–201.

Sigma. (1995). Enzymatic assay of adenosine deaminase (EC 3.5.4.4) Retrieved from https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/adenosine_deaminase.pdf

Yang, F. Q., Li, D. Q., Feng, K., Hu, D. J., & Li, S. P. (2010). Determination of nucleotides, nucleosides and their transformation products in Cordyceps by ion-pairing reversed-phase liquid chromatography-mass spectrometry. *Journal of Chromatography A*, *1217*(34), 5501–5510.

Yang, F. Q., & Li, S. P. (2008). Effects of sample preparation methods on the quantification of nucleosides in natural and cultured Cordyceps. *Journal of Pharmaceutical and Biomedical Analysis*, *48*(1), 231–235.