韓藥作誌(Korean J. Medicinal Crop Sci.) 17(6) : 434 – 438 (2009)

율무로부터 항치매성 Acetylcholinesterase 저해물질의 최적추출 조건 및 특성

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Optimal Extraction Condition and Characterization of Antidementia Acetylcholinesterase Inhibitor from Job's Tears (*Coix lachrymajobi* L.)

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ABSTRACT: For the development of a new antidementia functional food or alternative drug using agricultural products, Job's tears (*Coix lachrymajobi* L.), which shows high acetylcholinesterase (AChE) inhibitory activity (55.1%) was selected and the extraction conditions of AChE inhibitor were optimized. AChE inhibitor of Job's tears was maximally extracted when it was treated with 60% methanol at 40℃ for 6 h. The AChE inhibitor of the methanol extracts was partially purified by systematic solvent extraction, thin layer chromatography, silica gel chromatography and reverse-phase HPLC and the partial purified AChE inhibitor with inhibitory activity (IC_{50}) of 0.608 ㎍ was obtained. The partial purified AChE inhibitor was soluble in methanol and hexane, and insoluble in water. Its maximum absorption spectra was 230 ㎚ and also it was stable in the range of 30℃ and 70℃ and pH 4.0-8.0 for 1 h.

Key Words **:** Antidementia, Acetylcholinesterase Inhibitor, Job's Tears

INTRODUCTION

Alzheimer's disease (AD) is a type of dementia and it is acquired disorder and neuro degenerative disease. AD is characterized by acetylcholine depletion, amyloid β protein aggregation, and neurofibrillary tangles (Duyckaets *et al*., 1999). Acetylcholine, a neurotransmitter, has an important recognition function such as memory (Richer *et al*., 1980; Kim *et al*., 2008). Acetylcholine is synthesized incertain neurons by cholineacetyl transferase from choline and acetyl-CoA as substrates. It also hydrolyzes acetylcholine in to choline and acetate by acetylcholinesterase (AChE)[EC3.1.1.7] (Dugue *et al*., 2003). Therefore, it causes dementia by loss of AChE in brain.

Many AChE inhibitors have been developed from natural products including green tea extracts (Kwak *et al*., 2009; Jeong *et al*., 2009) and commercialized. However, commercial AChE inhibitors have some limitation for use in treatment of dementia owing to crucial problems including toxicity, inducing of vomiting, and liver toxicity. (Dubios and Albert, 2004; Muramoto *et al*., 1979). Recently, new donepezil, which shows high receptor specificity and long biological half-life as an AChE inhibitor, has been developed. Tacrine, rivastigmine, metrifonate, eptastigmine and physostigmine have also been commercialized. However, they have some side effects such as stomach disorder and liver toxicity (Vincent and Delagarza, 2003). Therefore, development of a new AChE inhibitor, made from natural products, with reduced side effect sand toxicity is necessary.

We have been working on the development of a new antidementia agent from agricultural products. In a previous paper (Song and Lee, 2008), we selected Job's tears (*Coix lachrymajobi* L.) as a potential AChE inhibitor-containing cereal. Some physiological functionalities such as anticancer by coixenolides, immunity, phytosterol derivatives, antihyperglycemia by coixans A, B, C and antihyperlipemia, antioxidant and antibacterial activity were known from extracts of Job's tear (Ryu, 2008; Lim, 2006; Ryu and

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Received 2009 October 30 / 1st Revised 2009 November 30 / Accepted 2009 December 11

Kim, 2005; Han *et al*., 2006). However, little study on AChE inhibitor from Job's tear was done. Therefore, the present study describes optimal extraction conditions of AChE inhibitor from Job's tears and some properties of the partial purified AChE inhibitor.

MATERIALS AND METHODS

1. Materials and chemicals

Job's tears (*Coix lachrymajobi L.*) which was showed very high AChE inhibitory activity (55.1%) in a previous paper (Song and Lee, 2008) was used in this study. It was purchase data local market, having been cultivated in Korea in 2007.

Unless otherwise specified, all chemicals and solvents were of analytical grade. Acetylcholinesterase (recombinant human acetylcholinesterase), acetylthiocholine chloride and 5,5'-dithiobis (2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (U.S.A.). A VERSA max microplate reader (Molecular Devices, U.S.A.) was used in the assay of AChE inhibitory activity.

2. Preparation of extracts and optimal extraction condition

Powders of Job's tears were added to water, methanol and ethanol as 1 : 15 w/v ratios and then shaken for 24 h at 40℃. Each extract were filtered using a Whatman 0.45 ㎛ membrane filter (No 7404-004) and lyophilized.

The effects of extraction temperature and time on the extraction of the AChE inhibitor were investigated in the range of 40℃ and 60℃, 3 h to 48 h.

3. Assay of the acetylcholinesterase (AChE) inhibitory activity

AChE inhibitory activity was measured spectrophotometrically applying the technique of Ellman *et al* (1961). A mixture of 110 ㎕ of assay buffer (0.1 M sodium phosphate, pH 7.3), 30 ㎕ of AChE (0.8 U/㎖), 30 ㎕ of substrate (acetylthiocholine chloride), 20 ㎕ of 5,5'-dithiobis (2-nitrobenzoic acid), and 10 ㎕ of lyophilized methanol extracts dissolved in the assay buffer (1 ㎎/1 ㎖) was incubated for 60 min at 37℃. 5-thio-2-nitrobenzoate produced from the reaction was measured at 415 ㎚ and the inhibition ratio was obtained by the following equation: inhibition (%) = [1 – {(S-S_0) / (C – C_0)}] × 100, where C is the radiation of a control (enzyme, assay buffer, DTNB and substrate) after 60 min of incubation, C_0 is the radiation of control at zero time, S is the radiation of the tested samples (enzyme, sample solution, DTNB, and substrate) after 60 min of incubation, and S_0 is the radiation of the tested samples at zero time. All data are the mean of duplicated experiments.

To evaluate the quenching effect of the samples, the sample solution was added to reaction mixture C, and any reduction in radiation by the sample was thereupon investigated. The IC_{50} value was define das the concentration of the AChE inhibitor required to inhibit 50% of the inhibitory activity.

4. Partial purification of acetylcholinesterase inhibitor

AChE inhibitor of Job's tears was partially purified by systematic solvent extraction, TLC and HPLC (Young Lin Instrument Co., Anyang, Korea), as follows.

Methanol extract of Job's tears was fractionated stepwise with n-hexane, chloroform, ethyl acetate, butanol, and water. The active fractions were applied to thin layer chromatography (TLC, $60F_{254}$ silica gel plate, Merck Co., Darmstadt, Germany) under different solvent conditions. The active fractions were then separated by chromatography on an open column pack with silica gel. (Lee *et al*., 2008). Each fraction was grouped and groups were fractionated according to their R_f value on TLC. The obtained active fractions were then subjected twice to analytical reverse-phase HPLC (3.9 ㎜ × 300 ㎜, μ-Bondapak^{TM} C_{18} column, Waters Co., MA, USA; 100% methanol as mobile phase) at a flow rate of 1 ㎖/min. The active fraction was collected and lyophilized. (Fig. 1).

RESULTS AND DISCUSSION

1. Optimal conditions for extraction of the acetylcholinesterase inhibitor

The effects of temperature, methanol concentration and time on extraction of the AChE inhibitor from Job's tears were investigated. Generally, it is known that AChE inhibitor of plants is extracted by hot water (Kwak *et al*., 2009; Jeong *et al*., 2009). However, as shown in Fig. 2 and 3. AChE inhibitory activities of Job's tear were higher in the 40℃ extracts than those of the 60℃ extracts and also were similar between 60% methanol extracts and 80% methanol extracts. Furthermore, its inhibitory activities were

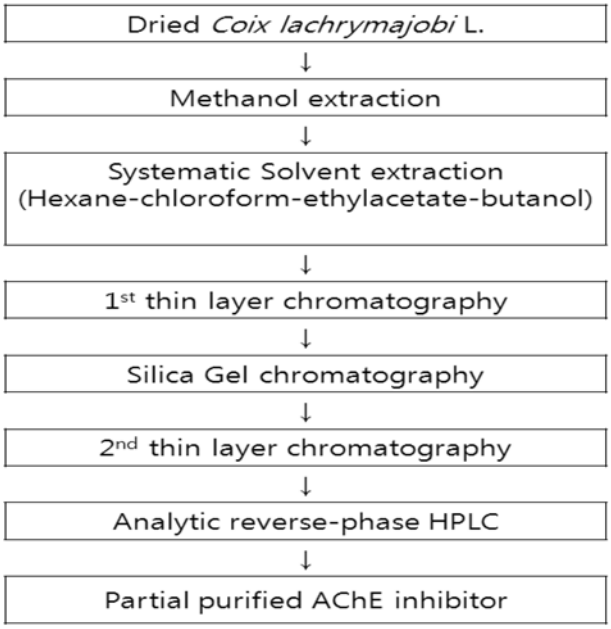

Fig. 1. Schematic diagram for the partial purification of the acetylcholinesterase inhibitor.

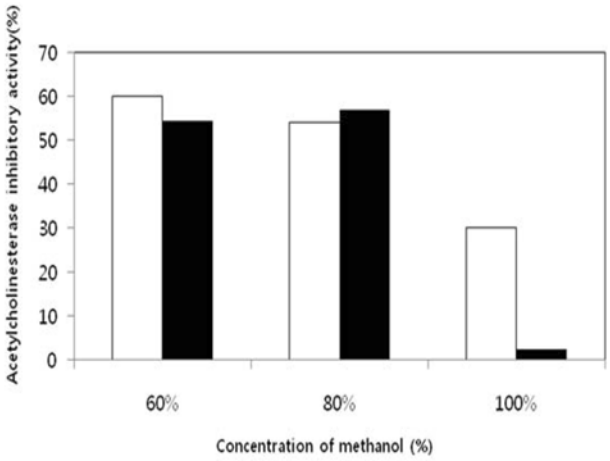

Fig. 2. Effect of extraction temperature and methanol concentration on the acetylcholinesterase inhibitory activity of methanol extract from *Coix lachrymajobi* L. (□: 40℃, ■: 60℃).

significantly decreased to below 30% in the 100% methanol extracts. It was presumed that catalytic site or allosteric site of AChE was protected by some polar compounds from extraction of Job's tear. The AChE inhibitor was maximally extracted when Job's tears was treated with 60% methanol at 40℃ for 6h and its inhibitory activity (IC_{50}) was 0.78 ㎎.

2. Partial purification of the acetylcholinesterase inhibitor

Systematic solvent extraction was performed using the methanol extract of AChE inhibitor-containing Job's tears. n-Hexane extract showed the highest AChE inhibitory activity of 75.5% (IC_{50}; 176 ㎍) and Chloroform layer and ethylacetate layer showed 12.7% and 12.1% of inhibitory activity, respectively. However, AChE inhibitory activity of butanol layer and aqueous layer were not detected (data not shown).

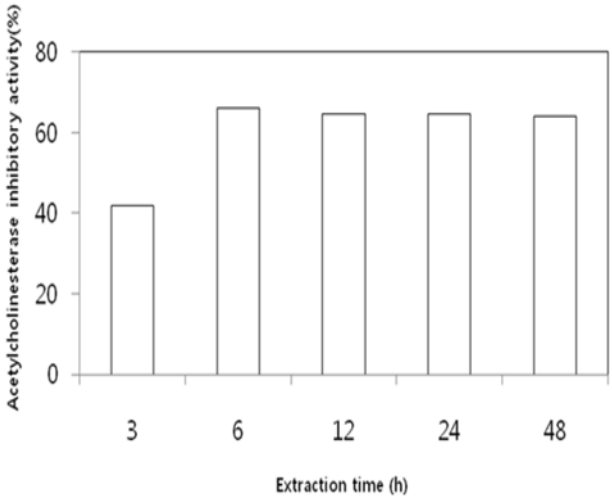

Fig. 3. Effect of extraction time on the acetylcholinesterase inhibitory activity of methanol extract from *Coix lachrymajobi* L. (60% MeOH, 40℃).

The n-hexane layer of the methanol extract was applied to a TLC plate. When it was developed with n-hexane / butanol / ethyl ether (10 : 0.5 : 0.05), six bands were obtained. Among them, the 6th band (0.48 R_f band) showed 69.5% of AChE inhibitory activity.

The n-hexane layer was fractionated using silica gel chromatography with a mixture of n-hexane / butanol / ethyl ether (10 : 0.5 : 0.05) with a flow rate of 0.5 ㎖/min for 48h, and fractions of 5 ㎖ volume were collected. Each fraction was grouped according to different R_f value on a TLC plate and the inhibitory activity of each group was investigated. The fraction S-2 showed 82.6% (IC_{50}; 1.0 ㎍) AChE inhibitory activity and 81.9% yield.

The active fraction from the above silica gel chromatography was collected and subjected to analytical HPLC and the partial purified AChE inhibitor was finally obtained with 0.608 ㎍ of AChE inhibitory activity, (IC_{50}), (Fig. 4).

3. Properties of the acetylcholinesterase inhibitor

The physicochemical properties of the partial purified

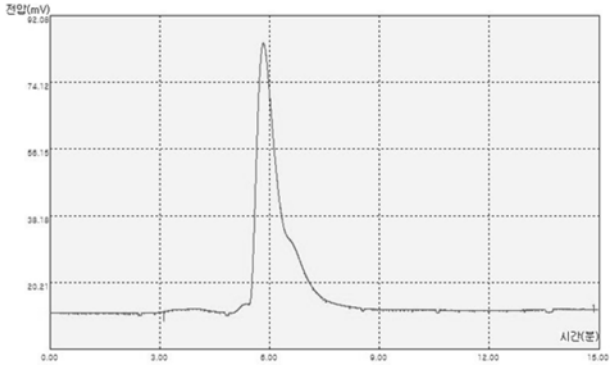

Fig. 4. Analytical reverse phase HPLC profile of the active fraction from silica gel chromatography. (Mobile phase : 100% Methanol, Flow rate : 1 ㎖/min, UV absorbance : 210 ㎚).

Table 1. Physicochemical properties of the purified acetylcholinesterase inhibitor.

Appearance	Clear oil
Soluble	Methanol, Hexane, DMSO
Insoluble	Water
UV (λ max) (MeOH)	230 ㎚

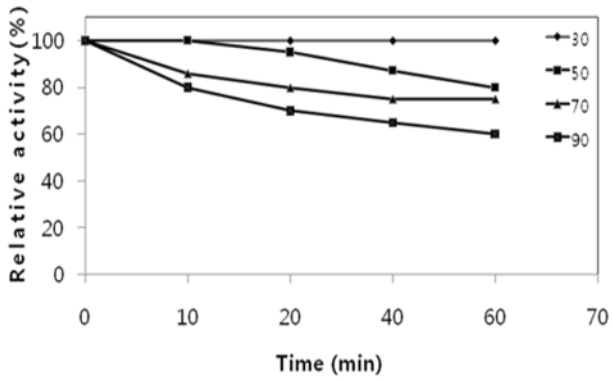

Fig. 5. Thermal stability of partial purified acetylcholinesterase inhibitor from Job's tear.

AChE inhibitor were investigated (Table 1). The purified AChE inhibitor was soluble in methanol, hexane and DMSO, whereas it was insoluble in water. The maximum absorption spectra of the purified AChE inhibitor dissolved in methanol was deduced to be 230 ㎚.

For application of the partial purified AChE inhibitor into the industry, its thermal and pH stability were investigated after treated in the range of 30℃ and 90℃, and pH 2.0 to 10.0 for 1 h. AChE inhibitory activity retained 75% of the activity at 70℃ and 60% at 90℃ for 1 h. (Fig. 5). Furthermore, the partial purified AChE inhibitor retained

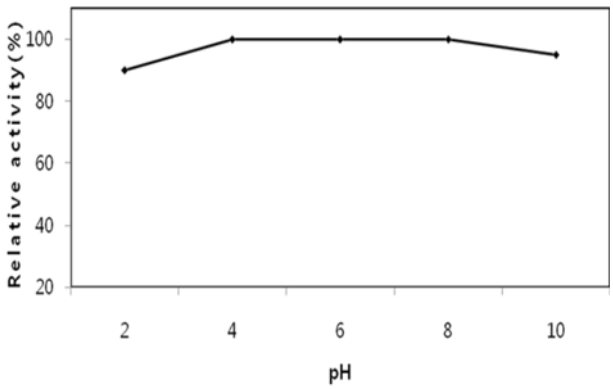

Fig. 6. pH stability of partial purified acetylcholinesterase inhibitor from Job's tear.

more than 90% of its inhibitory activity in the range of pH 4.0 to 8.0. (Fig. 6). From theses results, we concluded the AChE inhibitor is very stable against heat and pH.

In conclusion, we obtained a highly valuable antidementia AChE inhibitor that was extracted at 40℃ for 6h by 60% methanol and partially purified by TLC and HPLC, etc. It is the first report on AChE inhibitor from Job's tear and we guessed that the AChE inhibitor will be very useful development of new natural antidementia foods or nutraceuticals.

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