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Physicochemical Characteristics of Antidementia Acetylcholinesterase Inhibitor-containing Methanol Extract from *Sorghum bicolor* and Industrial Application

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항치매성 Acetylcholinesterase저해물질을 함유하고 있는 수수 메탄올 추출물의 특성 및 산업적 응용

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Abstract

Alzheimer's disease is charaterized by the acetylcholine depletion, amyloid b-protein aggregation and neurofibrillary tangles. The prevention of the breakdown of acetylcholine by acetylcholinesterase (AChE) inhibitor has the best clinically therapeutic efficacy for Alzheimer's disease patients. To develop new antidementia alternative drugs or nutraceuticals, methanol extracts of *Sorghum bicolor* was screened from various extracts of cereals and legumes as a potent AChE inhibitor-containing extract in previous paper. In this paper, physicochemical properties of the methanol extracts was investigated. The methanol extracts was soluble by water, methanol and DMSO and had 215 nm and 282nm of maximum absorption spectra. It was also stable at 20-100℃ and pH 2.0-10.0 for 1 hr. Test product was prepared by using methanol extracts from *Sorghum bicolor* and changes of its quality during storge at 20℃ and 40℃ were investigated. It was very stable for 8 weeks at 40℃.

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요 약

아세틸콜린에스터라아제(AChE)저해제에 의한 아세틸콜린 분해의 억제는 알츠하이머 질병의 환자들을 위한 치료 방법 중의 하나이다. 새로운 AChE 저해제를 개발하여 항 치매성 대체 의약품생산에 응용하기 위해, 전보에서는 다양한 곡류와 두류로부터 AChE 저해활성이 우수한 시료로 수수를 선발하여 추출 최적조건을 조사하였고 영양성 및 생리기능성 등을 조사하였다. 본 연구에서는 AChE 저해제를 함유한 수수 메탄올 추출물의 물리화학적 성질과 안정성을 조사하였다. 수수 메탄올추출물은 물, 메탄올과 DMSO등에 잘 녹았고, 215nm과 282nm의 최대흡수파장을 갖고 있었다. 또한, 추출물은 20-100℃와 pH 2.0-10.0에서 1시간동안 안정 하였다. 수수 메탄올추출물을 이용하여 시제품을 제조한 후 20℃와 40℃에서 8주간 저장하였을 때 모두 생균수와 pH에 변화가 없이 안정하였고 기호도도 우수하게 유지되었다.

Keywords: *Anti-dementia, acetylcholinesterase (AChE), Sorghum bicolor, methanol extracts*

1) Introduction

Alzheimer's disease (AD) is charaterized by the development of sensile palques and neurofibrillary tangles, which are associated with neuronal destruction, particularly in cholinergic neurons (Vincent and Delagarza, 2003). It is known that several neurotransmitters (acetylcholine, norepinephrine and dopamine) and neuropeptides (somatostatin, corticotrophin-releasing factor) are involved in AD (Brenner and Stevens, 2006, ; Gershon, 1998, ; Rossor, 1982). Especially, involvement of cholinergic neurons causes levels of acetylcholine within synapse to decline. Levels of acetylcholinesterase (AChE) also drop, perhaps to compensate for the loss of acetylcholine (Vincent and Delagarza, 2003).

Acetylcholinesterase (E.C. 3.1.1.7) hydrolyzes the neurotransmitter acetylcholine at neuromuscular junctions and brain cholinergic synapses, and thus terminates signal transmission. The major form of AChE found in brain, muscle, and other tissues is the hydrophilic species, which forms disulfide-linked oligomers with collagenous, or lipid-containing structural subunits.

AChE inhibitor is very important because it reduce the rate at which acetylcholine is

broken down and hence increase the concentration of acetylcholine in the brain. AChE inhibitor seemed to moderate symptoms but do not alter the course of the underlying dementing process.

Some AChE inhibitor have been isolated and characterized from various natural sources including Amaryllidaceae (Rhee *et al*., 2001), several plant (Pulok *et al*., 2007), *Securinega suffruticosa* (Jang *et al*., 2003), *Onosma hispida* (Ijaz *et al*., 2003) and chinese herb, *Huperzia serrata* (Xi and Yi, 1999), etc. However, they have low yield and some side effects. Therefore, the only FDA-approved drug therapy is the use of acetylcholinesterase inhibitors (e.g. Tacrine, Cognex, Aricept, Donepezil, Rivastigmine, and Galantamine) (Lahiri *et al*., 2002). But they have also cholinergic side effects such as nausea, anorexia, vomiting, and diarrhea. Therefore, clearly better are required drugs which has no harmful side effects and high efficiency (Vincent and Delagarza, 2003).

For development of new antidementia agent from cereals and legumes, we already reported on screening of antidementia acetylcholinesterase inhibitor-containing cereals and legumes and optimization of extraction condition of acetylcholinesterase inhibitor in previous paper (Song and Lee, 2008). The present study was performed to investigate physicochemical properties and stabiliy of the AChE inhibitor-containing methanol extracts from *Sorghum bicolor*. Furthermore, partial purification of the acetylcholinesterase inhibitor and its industrial application were also investigated.

2) Materials and Methods

2.1. Materials and chemicals

Sorghum bicolor was perchased at local market, that was cultivated in Korea at 2007.

Unless otherwise specified, all chemicals and solvents were of analytical grade. Acetylcholinesterase (recombinant human Acetylcholinesterase) (AChE, E.C. 3.1.1.7), butyrylcholinesterase (horse serum butyrylcholinesterase) (BuChE, E.C. 3.1.1.8), acetylthiocholine chloride (ATCh), and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from the Sigma Chemical Co (St, Louise, Mo, U.S.A.). VERSAmax microplate reader (Molecular Devices, U.S.A.) was used in acetylcholinesterase activity.

2.2. Preparation of methanol extracts

Sorghum bicolor was added in methanol as 1:10 w/v ratio and then shaked for 12 h at 40℃. The extracts was filtered by Whatman 0.45 um membrane filter (No 7404-004) and lyophilized.

2.3. Assay of the acetylcholinesterase inhibitory activity

AChE inhibitory activity was measured spectrophotometrically applying the technique of Ellman et al. (Ellman *et al*., 1961). The mixture of 110 ul of assay buffer (0.1 M sodium phosphate, pH 7.3), 30 ul of acetylcholinesterase (AChE) (0.8 U/ml), 30 μl of substrate (Acetylthiocholine chloride), 20 ul of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), and 10 ul of sample dissolved in the assay buffer was incubated for 60 min at 37℃. The reaction product 5-thio-2-nitrobenzoate produced enzymatically was measured at 415 nm.

The inhibition ratio was obtained by the following equation: inhibition (%)=[1-{(S-S_0)/(C-C_0)}] ×100, where C was the radiation of a control (enzyme, assay buffer, DTNB, and substrate) after 60 min of incubation, C_0 was the radiation of control at zero time, S was the radiation of tested samples (enzyme, sample solution, DTNB, and substrate) after 60 min of incubation, and S_0 was the radiation of the tested samples at zero time. All data are the mean of duplicated experiments.

To check the quenching effect of the samples, the sample solution was added to the reaction mixture C, and any reduction in radiation by the sample was then investigated. The IC_{50} value was defined as a concentration of the AChE inhibitor that is required to inhibit 50% of the AChE inhibitory activity.

2.4. Partial purification of acetylcholinesterase inhibitor

AChE inhibitor of *Sorghum bicolor* was partially purified by systematic solvent extraction as follows.

Methanol extracts of *Sorghum bicolor* was fractionated stepwise with n-hexane, chloroform, ethyl acetate, butanol, and water as Fig. 1.

2.5. Preparation of test product

The antidementia test product was prepared using the AChE inhibitor-containing methanol

extracts form *Sorghum bicolor* (Fig. 2, Table 1). We used vitamines, citric acid and falvors as additives for enhancing acceptability and functionality.

3) Results and Discussion

3.1. Physicochemical properties of the acetylcholinesterase inhibitor-containing methanol extracts from *Sorghum bicolor*

Physicochemical properties of the AChE inhibitor-containing methanol extracts from *Sorghum bicolor* were showed in Table 2. The AChE inhibitor-containing from methanol extracts of *Sorghum bicolor* were soluble in methanol, water and DMSO, however, it was insoluble by n-hexane, chloroform and ethyl acetate.

Meanwhile, thermal and pH stability of the methanol extracts were investigated (Fig. 3, 4). The methanol extracts were stable at 20-100℃ and pH 2.0-10.0 for 1 hr.

3.2. Partial purification of the acetylcholinesterase inhibitor from methanol extracts of *Sorghum bicolor*

The lyophilized methanol extracts of *Sorghum bicolor* was performed systematic solvent extraction. Among them, butanol fractions showed the highest AChE inhibitory activity of 81.2% (Table 3). Therefore, it suggest that the AChE inhibitor was probably hydrophilic compounds.

Quantitative examination of butanol fraction from the methanol extracts were performed to know whether partial purified AChE inhibitor in the butanol fraction is protein or sugars (Table 4). Test of protein, peptide and amino acid were positive, however sugars test was negative. Therefore, we guess partial purified AChE inhibitor may be protein or peptide compounds and it is necessary further study to identify.

Meanwhile, the stilbene oligomer viniferin from *Caragana chamlahue* (Pulok *et al*., 2007), four isoquinoline alkaloids, corynoxidine, protopine, palmatine and berberine from methanol extracts of Corydalis speciosa (Kim *et al*., 2004), dihydrosecurinine from methanol extracts of *Securinega suffruticosa* (Jang *et al*., 2003), hispidone from *Onosma hispida* (Ijaz *et al*., 2003) and herperzine A from chinese herb, *Huperzia serrata* (Xi and Yi, 1999) were recently reported as AChE inhibitor.

3.3. Stability of the antidementia test product

Stability of the antidementia test product was determined during the storage at room temperature and 40℃ for 8 weeks (Table 5).

pH, residual sugar and AChE inhibitory activity were not significantly changed during 8 weeks. Viable cell counts was also not increased significantly, though about 1.7 CFU/ml increased in the storage at 40℃ for 8 weeks. It suggest that the AChE inhibitor-containing test product would be stable during shelf-life.

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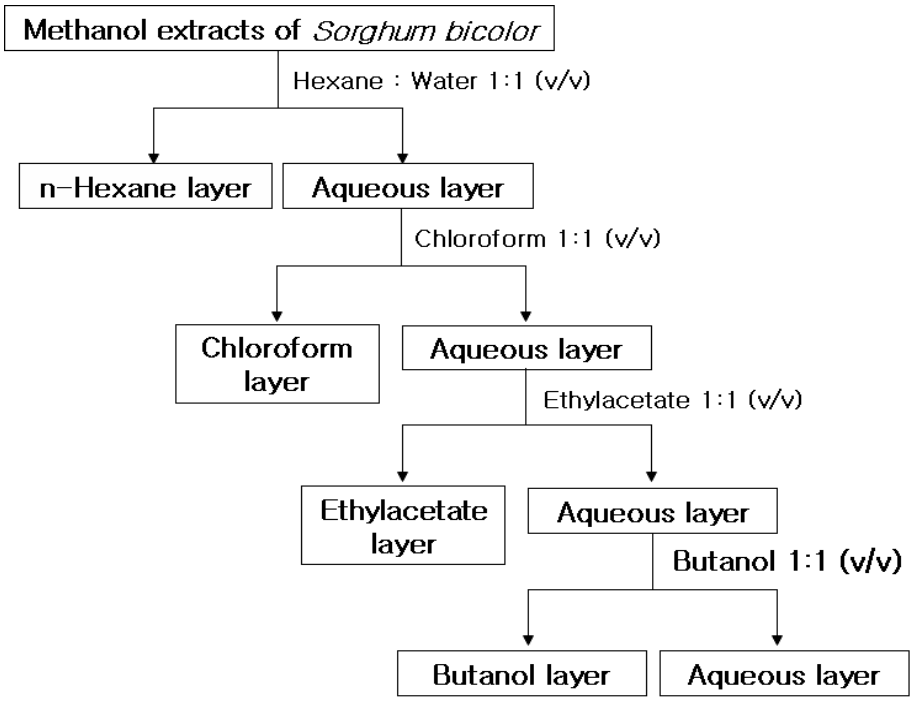

Fig. 1. Systematic solvent extraction of acetylcholinesterase inhibitor from methanol extracts of *Sorghum bicolor*

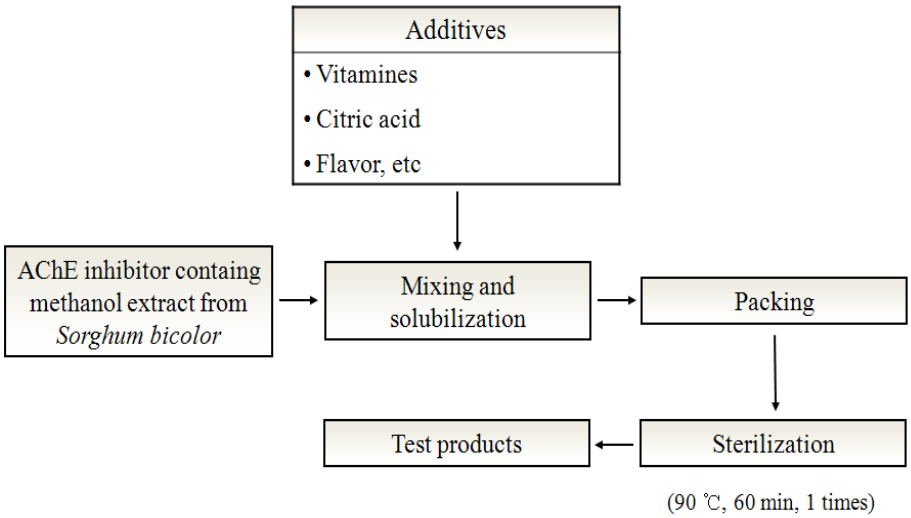

Fig. 2. Procedure for the antidementia test product preparation

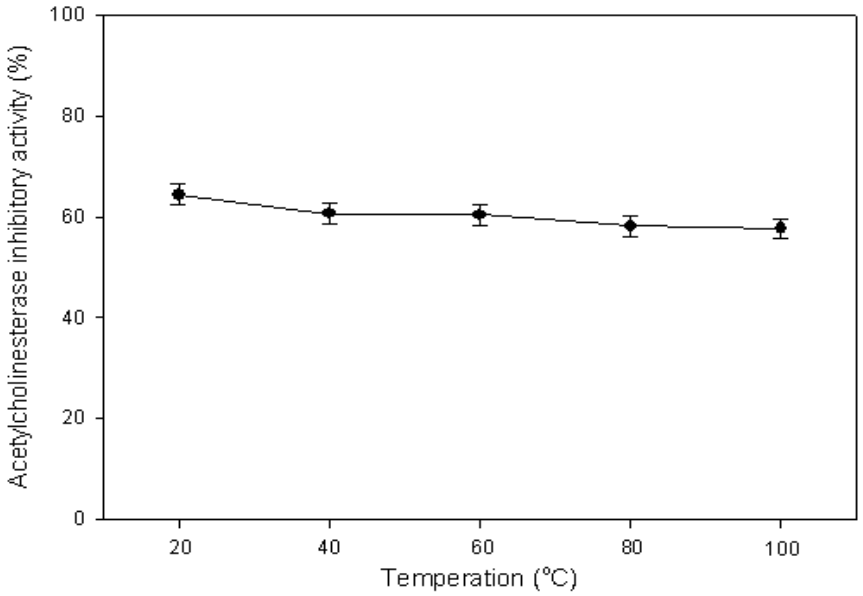

Fig. 3. Thermal stability of the methanol extracts from *Sorghum bicolor*

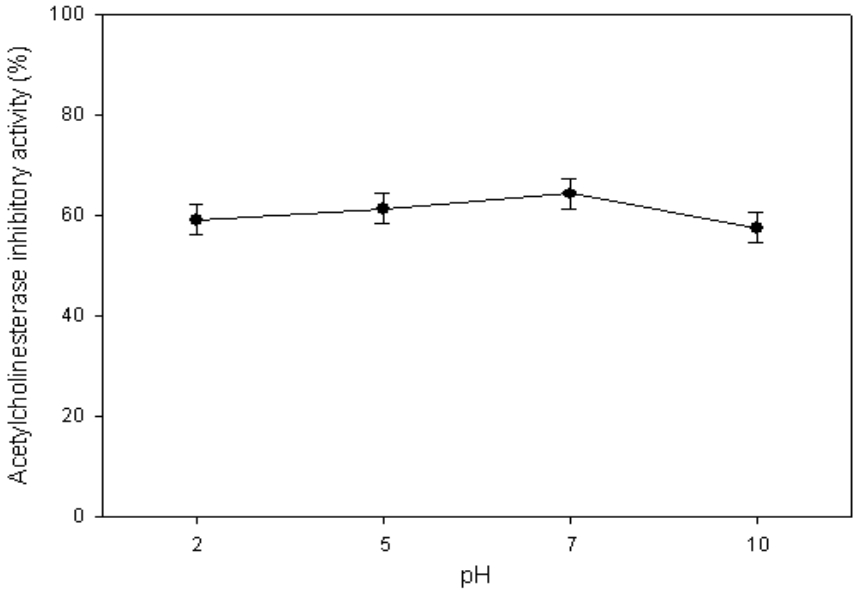

Fig. 4. pH stability of the methanol extracts from *Sorghum bicolor*

Table 1. Formula of the antidementia *Sorghum bicolor* test products.

Components		Contents
Sorghum bicolor extracts		200mg
Vitamin	B1	0.1mg
	B2	1mg
	B6	5mg
Citric acid		50mg
Liquid fructose		5g
Masking flavor		5ul
Herbal flavor		50ul
pH		4.0
Brix(°)		22.0
D.W		50ml

Table 2. Physicochemical properties of the acetylcholinesterase inhibitor-containing methanol extracts from *Sorghum bicolor.*

Appearance		brown sticky powder
Solubility -	soluble	water methanol DMSO
	insoluble	n-hexane chloroform ethylacetate
UV (λmax, nm)		215 282

Table 3. Acetylcholinesterase inhibitory activity of each solvent fractions from the methanol extracts of *Sorghum bicolor.*

(unit=%)

n-Hexane layer	Chloroform layer	Ethyl acetate layer	Butanol layer	Aqueous layer
50.7	24.8	53.3	81.2 (IC50=1.5μg)	46.6

*concentration = 1mg/ml

Table 4. Quantitative test for butanol fraction from the methanol extracts.

Tests	Results
Phenol-sulfuric acid test	positive
Folin-ciocaleu's phenol test	positive
Ninhydrin test	positive
Biorad test	positive
3,5-Dinitrosalicylic acid test	negative
Iodine test	negative

Table 5. Changes of quality characteristics during storage of room temperature and 40℃.

	0	Room temp. (20℃)			40℃		
		2Wk	4Wk	8Wk	2Wk	4Wk	8Wk
pH	4.0	3.9	3.9	3.8	4.3	4.2	4.2
Sugar(brix°)	22.0	22.0	21.2	21.1	22.5	22.0	22.1
Viable cell (counts-bacteria CFU/ml)	1.0	1.1	1.3	1.4	1.5	1.7	1.7
AChE inhibitory activity (%)	63.4	64.0	64.2	64.1	62.4	62.1	62.3
Total acceptability	4.3/5.0 (score)	4.3	4.3	4.3	4.3	4.3	4.3