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Effects of Water Extract of Smoke-dried Skipjack Tuna on Memory in a Scopolamine-induced Amnesia Animal Model

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Abstract Natural products have been used to treat many neurological illnesses such as Alzheimer's disease. In the present study, the effects of the water extract of smoke-dried skipjack tuna (WSST), which is used as a traditional seasoning in Japan, as well as its fractions on acetylcholinesterase (AChE) inhibition *in vitro* and on memory in scopolamine-induced amnesia mice *in vivo* were evaluated. Bio-Rad P-2 gel permeation chromatography revealed the presence of 7 peaks and AChE significantly inhibited peak 3 and 5. When *in vivo* behavioral studies were conducted, a passive avoidance test revealed that treatment with 50 and 100 mg/kg WSST as well as with fraction 3 and 5 improved the loss in memory retention induced by scopolamine. These results suggest that skipjack tuna extract and its fractions improve memory deficits and that these substances are suitable for use in healthy foods designed to improve memory deficits induced by aging and Alzheimer's disease.

Keywords: smoke-dried skipjack tuna, acetylcholinesterase inhibitor, memory, cognitive function, passive avoidance

Introduction

Many age-related senile central nervous system (CNS) dysfunction studies have identified disruption of the cholinergic system in the CNS as a major factor of memory loss, and as a feature of the early stages of Alzheimer's disease (1). The primary agents used to prevent or ameliorate Alzheimer's disease are acetylcholinesterase (AChE) inhibitors, which increase the availability of acetylcholine (ACh) at the cholinergic synapses (2). To date, the Food and Drug Administration has approved donepezil (3,4), galantamine (5), and rivastigmine (6) for the treatment of Alzheimer's disease, however, these drugs have only mild benefits.

Many natural products have been used by practitioners of traditional medicine in Asia to treat neurological illnesses such as Alzheimer's disease (7-9), hypertension (10,11), and stroke (12). In addition, tuna is of a great commercial importance in Asia, where skipjack tuna *(Euthymus pelamis)* is widely consumed (13). Moreover, the boiling water extract of skipjack tuna muscle is used as a traditional seasoning in Japan (14). During its production, the muscle is cleared of fat, boiled, and dried. This dried muscle is typically used to make noodle dishes, and it has been reported that dried skipjack tuna has various physiologic effects, such as the reduction of hypertension in spontaneously hypertensive rats and prophylactic effects on the development of cerebrovascular lesions in stroke-prone spontaneously hypertensive rats (15).

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Scopolamine, a muscarinic cholinergic receptor antagonist, impairs learning and memory in rodents and humans by blocking the processes of learning acquisition and short-term memory. Moreover, cholinergic neurons in the CNS are believed to be involved in learning and memory in both humans and animals (1).

In the present study, the inhibitory effects of the water extract of smoke-dried skipjack tuna (WSST) on AChE *in vitro* and in scopolamine-induced amnesia mice *in vivo* were investigated.

Materials and Methods

Preparation of smoke-dried skipjack tuna and extract
Skipjack tuna (*E. pelamis*) that were 1-2 kg in body weight and 20-25 cm in length were caught in the Pacific and then frozen at –40°C for 3 months. The fish were then thawed in tap water at room temperature, eviscerated, and filleted. The fillets were then immersed for 20 min in a seasoning solution prepared by boiling sea tangle (20 g/L) and the heads of skipjack tuna (3-4 pieces/L) for 1 hr, and then cooled to room temperature. Next, the seasoned fillets were varnished with oak vinegar (Wonmi Food Co., Wonju, Korea) and dried at room temperature for 30 min. The dried fillets were then dried in a smoker (Alto-Shaam Co., Milwaukee, WI, USA) at 80°C for 8 hr, after which they were allowed to stand at room temperature for 15 hr. This seasoning and smoking process was repeated 3 times to produce smoke-dried skipjack tuna. WSST was then prepared by adding 20 g of smoke-dried skipjack tuna (SST) to 200 mL of distilled water (DW, w/v) and boiling for 20 min. Next, extract was collected by filtration through a Whatman No. 1 filter paper and lyophilized.

Fractionation of WSST Crude WSST was further fractionated by gel filtration on a Bio-Rad P-2 gel column (2.6×70.0 cm, Bio-Rad, Hercules, CA, USA). Briefly, 1 g of sulfosalicylic acid was added to 50 mL of WSST to precipitate the proteins. The solution obtained was then ultrafiltered at 4°C using a 3,000 Da cutoff membrane (Amicon, Bedford, MA, USA). Next, the filtrate was concentrated 50 times in an evaporator (Buchi, Flawil, Switzerland) at 40°C and then loaded onto a Bio-Rad P-2 gel column and eluted with DW at a flow rate of 0.5 mL/min, with 5 mL fractions being collected. The eluted material was monitored at 214 nm, and the pooled fractions were then lyophilized to yield a powder product.

Peptide-nitrogen determinations The peptide-nitrogen contents were determined using a modified biuret method as originally described by Umemoto (16). Briefly, during column chromatography, the concentrations of low molecular weight species in fractions were determined by measuring the absorbance at 214 nm. Fractions with an absorbance higher than the upper limit of the spectrophotometer were diluted, after which the content of nitrogen in the fractions was calculated by multiplying the molar absorption coefficient by the measured absorbance.

AChE assay *in vitro* An AChE assay was performed as described by Ellman *et al.* (17) with minor modifications and using acetylthiocholine iodide as a substrate. The Ellman's reaction mixture used for the assay was comprised of 0.5 mM acetylthiocholine iodide and 1 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) in a 50 mM sodium phosphate buffer (pH 8.0). The rates at which the acetylthiocholine iodide was hydrolyzed by the AChE were monitored spectrophotometrically using a 96-well microtiter plate reader. Briefly, WSST or its fractions and 50 mM sodium phosphate buffer (30 μL) were mixed with AChE solution (10 μL). The Ellman's reaction mixture (50 μL) was then added to the mixture to give a final volume of 100 μL. Next, this mixture was incubated at 37°C for 30 min. The absorbance at 450 nm was measured immediately after Ellman's reaction mixture, and absorption measurements were repeated for 10 min at 2 min intervals to verify the linear nature of the reaction. Blanks were prepared by substituting saline solution for the enzyme.

Experimental animals Male ICR mice (8 weeks old) were obtained from the Experimental Animal Center at Hallym University, Chuncheon, Korea. The animals were housed in a conventional state under adequate temperature (23°C) and humidity (60%) with a 12-hr light/12-hr dark cycle, and provided with free access to food and water. The procedures for handling and caring for the animals were in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and were approved by the Institutional Animal Care and Use Committee at Hallym's Medical Center. All of the experiments conducted for this study were designed to minimize the number of animals used and the suffering caused by the procedures.

Treatment with WSST, its fractions, donepezil and scopolamine The animals were divided into 5 groups: a normal group (n=7), a DW-treated group (n=7), a donepezil-treated group (n=7), a WSST-treated group (n=42), and a WSST fractions-treated group (n= 49). Ninety min before being subjected to a passive avoidance trial, the animals were injected with various concentrations of WSST (1, 5, 10, 50, 100, and 500 mg/kg,), its 7 fractions (50 mg/kg) or donepezil (0.1 mg/kg) orally using a feeding needle. Sixty min after being treated, the scopolamine (1.0 mg/kg, intraperitoneal injection, i.p.) was administered to the animals to induce amnesia.

Passive avoidance test The passive avoidance test was conducted using a PACS-30 passive avoidance system (Columbus Co., New York, NY, USA). This system is comprised of 2 equal compartments (15×15×22 cm) separated by a wall with a guillotine door (4×3.5 cm). The test was conducted on 2 cocecutive days at the same time of the day (18:00). On the first day, an individual mouse was placed in the illuminated compartment. After 30 sec, the guillotine door was raised, allowing access to the dark compartment. Upon entering the dark compartment, the guillotine door was lowered and an electric shock (0.3-0.4 mA for 5 sec) was delivered. On the second day, the same procedure was followed and the time taken for the mouse to enter the dark compartment (latencies) after the guillotine door was opened was recorded. In addition, an upper cut off time of remaining in the light compartment for 300 sec was used. The latency and numbers of mice that did not enter the compartment was then evaluated.

Quantification of data and statistical analysis The data shown here represent the means±SEM of experiments performed for each experimental area. Differences among the means were statistically analyzed using a Student's t-test to elucidate the differences among the experimental groups.

Results and Discussion

Profound losses in the brain cholinergic system are associated with the cognitive deficits observed in Alzheimer's disease (1,18,19). However, increasing ACh neurotransmission by blocking AChE enzymes with drugs, such as physostigmine, tacrine, donepezil, and rivastigmine, is associated with modest improvements in memory function in both healthy volunteers (20) and patients with Alzheimer's disease (4,6).

Although Food and Drug Administration (FDA) approved AChE inhibitors are currently available for the symptomatic treatment of patients with mild to moderate Alzheimer's disease, safety and efficacy concerns limit their usefulness. Moreover, their pharmacodynamics and kinetics are not fully understood. Conversely, many studies have evaluated the ability of new active natural extracts from plants to prevent or treat neurodegenerative diseases or memory deficits due to their perceived inherent safety (7,8,21).

In this study, we fractionated the low molecular components of WSST into 7 fractions by Bio-Rad P-2 gel

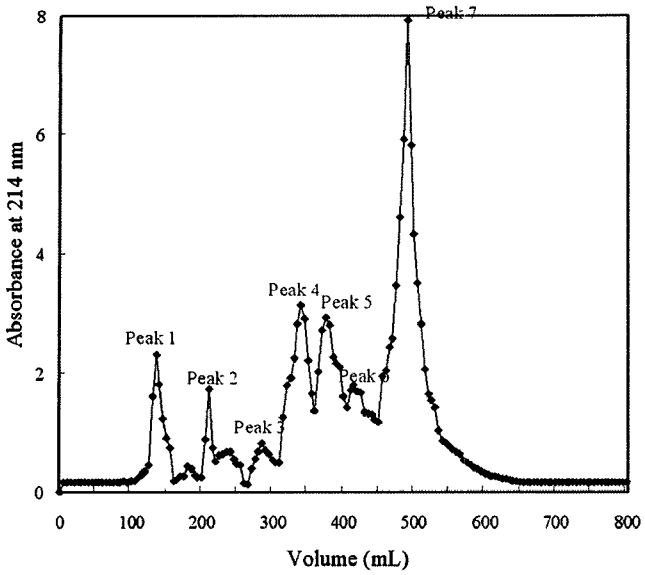

Fig. 1. Elution profile of low molecular weight substances purified from WSST on a Bio-Rad P-2 gel column.

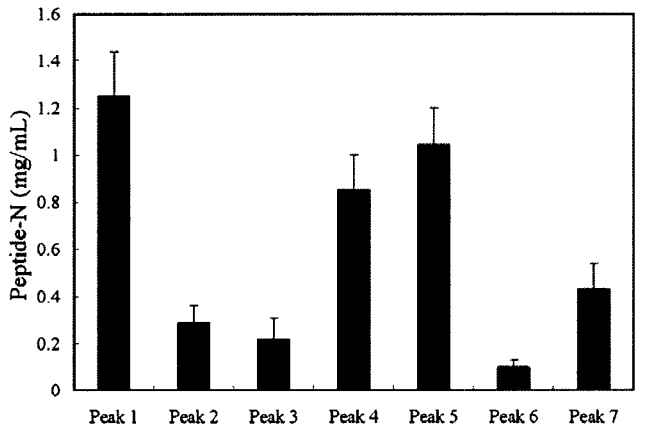

Fig. 2. Peptide-nitrogen content of low molecular substances in each peak eluted from WSST. Peak 1, 4, and 5 of WSST have more abundant peptide-N contents than the others. The bars indicate the mean±SEM.

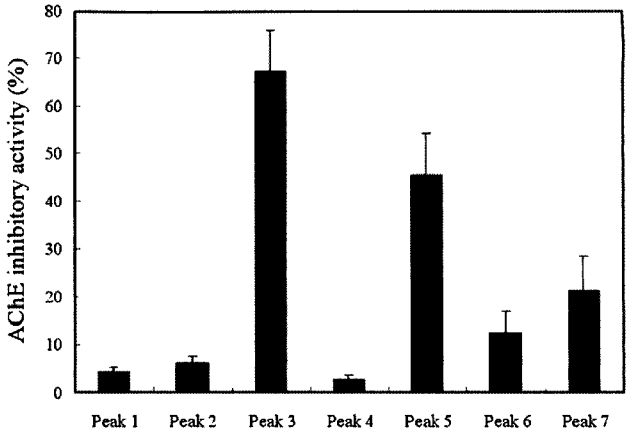

Fig. 3. AChE inhibitory activity (%) of low molecular substances in peaks eluted from WSST. Peak 3 and 5 of WSST have strong AChE inhibitory activities. The bars indicate the mean±SEM.

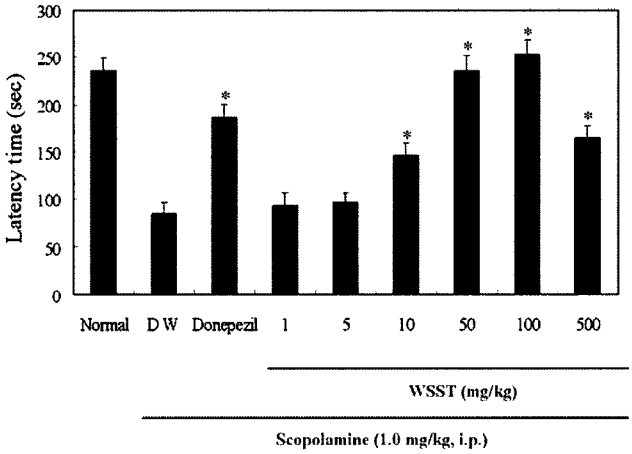

Fig. 4. Effects of WSST on scopolamine-induced memory impairment in the passive avoidance test. Note that memory impairment is reduced in the 50 and 100 mg/kg WSST-treated groups (n=7 per group; *p<0.05, significantly different from the DW-treated group). The bars indicate the mean±SEM.

permeation chromatography (Fig. 1). It is known that Bio-Rad P-2 gel can separate small molecular weight species in the range 100-1,800 Da; therefore, the peaks shown in Fig. 1 should represent low molecular weight fractions of WSST. The peptide-nitrogen contents of the 7 peaks fell in the range 0.10-1.25 mg/mL, with the highest concentration being observed in peak 1 and the lowest concentration being observed in peak 6 (Fig. 2). In addition, fraction 3 and 5 were found to have a strong inhibitory effect on AChE activity *in vitro* (Fig. 3).

When animals were treated with WSST, the mean latency time of the DW/scopolamine-treated group was significantly shorter than that of the normal group. In addition, no significant improvements in memory were observed in the mice that were treated with 1 and 5 mg/kg WSST and scopolamine (Fig. 4). However, the groups that were treated with between 10 and 500 mg/kg WSST and scopolamine were found to have a significantly greater latency time than mice that were treated with distilled water and scopolamine (Fig. 4). Furthermore, the latency time of mice treated with 50 and 100 mg/kg WSST and scopolamine was greater than that of mice treated with donepezil and scopolamine (Fig. 4). Additionally, the latency time of mice treated with fraction 3 and 5 of the eluted WSST and scopolamine was significantly greater than that of mice treated with distilled water and scopolamine (Fig. 5). We also investigated the toxicities of WSST and its fractions. No mice died (0/7 in each group) and no macroscopic pathologic findings were detected in the gastrointestinal tracts of mice that received WSST or its fractions at doses of 1 and 500 mg/kg (data not shown).

Evaluation of the effects of treatment of scopolamine-induced amnesia mice with WSST and its fractions using a mouse passive avoidance test revealed that treatment improved memory retention, which indicates that treatment with these compounds may alleviate cholinergic deficits associated with certain dementias (22). The selective inhibition of AChE in the brain may increase ACh neurotransmission in the synaptic cleft of the brain,

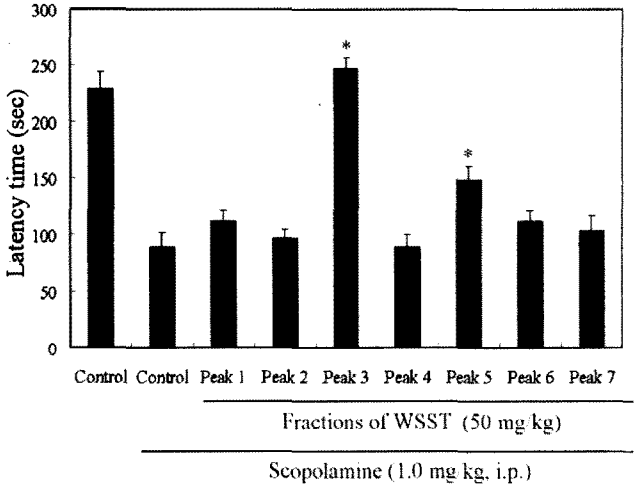

Fig. 5. Effects of WSST fractions on scopolamine-induced memory impairment in the passive avoidance test. Note that memory impairment is reduced in the peak 3 and peak 5-treated groups (n=7 per groups; *p<0.05, significantly different from the DW-treated group). The bars indicate the mean±SEM.

resulting in improvements in cognitive function (23,24). In conclusion, skipjack tuna extract contains low molecular fractions that may be effective for the treatment of memory deficits.

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