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Short communication

Inhibition of acetylcholinesterase by gallic acid-*grafted*-chitosans

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ABSTRACT

This paper discusses acetylcholinesterase inhibitory properties of gallic acid-*grafted*-chitosans (GA-*g*-chitosans) with different grafting ratios. The GA-*g*-chitosans exhibited potent acetylcholinesterase inhibitory effects in a dose-dependent manner, and their IC_{50} values ranged from 138.5 ± 0.25 to 397.6 ± 5.2 μg/mL. The acetylcholinesterase inhibition kinetics of the GA-*g*-chitosan (I) by Lineweaver–Burk plots showed a decrease in V_{max}, whereas K_m was not altered, thus suggesting a non-competitive mode of inhibition. The inhibition constant, K_i, was found to be 64.85 μg/mL by the secondary plot of the Lineweaver–Burk plots. In addition, the GA-*g*-chitosan (I) did not exert any significant cytotoxicity against PC12 cells, and the cellular acetylcholinesterase activity in PC12 cells was significantly inhibited by the GA-*g*-chitosan (I). This is the first report on acetylcholinesterase inhibition by the grafting of GA onto chitosan, and this compound might be helpful in preventing Alzheimer's disease by elevating cholinergic transmission.

1. Introduction

Alzheimer's disease is a progressive neurodegenerative disease of the brain that is characterized by memory impairment, cognitive dysfunction, and personality changes (Bartolucci, Perola, Pilger, Fels & Lambal, 2001). Based on the cholinergic hypothesis, a deficiency of cholinergic neurotransmitters in the basal forebrains is predominantly involved in Alzheimer's disease. Therefore, augmenting brain cholinergic neurotransmission is a promising strategy in the treatment of Alzheimer's disease, and it is an important approach for the inhibition of acetylcholinesterase based on the cholinergic hypothesis. Acetylcholinesterase catalyses the hydrolysis of the neurotransmitter, acetylcholine to choline and acetate, in both the peripheral nervous system and central nervous system. Therefore, using acetylcholinesterase inhibitors is among the best accepted approach toward the treatment of Alzheimer's disease by increasing cholinergic neurotransmitters by inhibiting the degradation of acetylcholine. Currently, acetylcholinesterase inhibitors are widely used for patients to inhibit the hydrolysis of acetylcholine in order to augment cholinergic neurotransmitters, as a result, the symptoms of patients with Alzheimer's disease are alleviated. Over the past few years, synthetic acetylcholinesterase inhibitors including tacrine, donepezil, and the natural product-based rivastigmine have been employed in clinical treatments. However, the insufficient activity and side effects of these drugs, including hepatotoxicity and gastrointestinal disturbances, are leading to the development of new acetylcholinesterase inhibitors from natural bioresources that are non-toxic and have outstanding activity (Schulz, 2003).

Chitosans, naturally occurring biopolymers, are linear mucopolysaccharides composed of D-glucosamine and N-acetyl-D-glucosamine units. Over the past decade, numerous biological properties of chitosans and their derivatives, including antioxidant, antihypertensive, antimicrobial, anticancer and immune-stimulating effects, have been documented (Je, Park & Kim, 2006; Jeon & Kim, 2001; Jeon, Park & Kim, 2001; Muzzarelli & Muzzarelli, 2005; Sugano, Yoshida, Hashimoto, Enomoto & Hirano, 1992). Therefore, the development of chitosan derivatives is of great importance to obtain new materials that can be applied in the industry. As part of our ongoing investigation on the development of chitosan derivatives, we grafted gallic acid onto chitosan, and herein is the first report on the anti-acetylcholinesterase activity of gallic acid-*grafted*-chitosans.

2. Materials and methods

2.1. Materials

Chitosan from crab chitin was obtained from Kitto Life Co. (Seoul, Korea), and its average molecular weight and degree of deacetylation were 310 kDa and 90%, respectively. Gallic acid, acetylcholinesterase (electric eel), and acetylthiocholine were pur-

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chased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade and commercially available.

2.2. Preparation and characterization of GA-g-chitosans

The preparation of gallic acid-*grafted*-chitosans (GA-*g*-chtiosans) was performed according to our previous report (Cho, Kim, Ahn & Je, 2010). Briefly, the chitosan (0.5 g) was dissolved in 50 mL of 2% acetic acid (v/v), and then 1 mL of 1.0 M H_2O_2 containing 0.054 g of ascorbic acid was added. After 30 min, different amounts of gallic acid were added to the mixture at the following molar ratios of residue of chitosan to gallic acid: 1:1, 1:0.5, 1:0.25 and 1:0.1. These GA-*g*-chitosans were designated as GA-*g*-chitosan (I), GA-*g*-chitosan (II), GA-*g*-chitosan (III), and GA-*g*-chitosan (IV), respectively. Finally, the mixture was allowed to sit at 25 °C for 24 h under atmospheric air, and then dialyzed with distilled water for 48 h in order to remove the unreacted gallic acid. ^{1}H NMR spectra were obtained with a JEOL JNM ECP-400 NMR spectrometer under a static magnetic field of 400 MHz.

Plain chitosan: ^{1}H NMR (400 MHz, D_2O) δ: 5.30 (1H, H-1), 3.63–4.35 (1H, H-2/6), 2.51 (H-Ac), 4.8 (D_2O). GA-*g*-chitosan: ^{1}H NMR (400 MHz, D_2O) δ: 7.63 (phenyl protons of gallic acid), 5.33 (1H, H-1), 3.65–4.36 (1H, H-2/6), 2.51–2.54 (H-Ac), 4.8 (D_2O).

2.3. Assay for inhibition of acetylcholinesterase

The acetylcholinesterase inhibition assay was conducted via the spectrophotometric method developed by Ellman, Courtney, Andres, and Featherstone (1961) with slight modification. Acetylthiocholine chloride was employed as the substrate to assay the inhibition of acetylcholinesterase. The reaction mixture contained: 140 μL of 100 mM sodium phosphate buffer (pH 8.0), 20 μL of test sample solution, and 20 μL of acetylcholinesterase (0.36 U/mL), which were mixed and incubated for 15 min at room temperature. The reactions were then initiated via the addition of 10 μL of 5-5′-thiobis-2-nitrobenzoic acid (0.5 mM) and 10 μL of acetylthiocholine chloride (0.6 mM). The hydrolysis of acetylthiocholine chloride was monitored by the following formation of yellow 5-thio-2-nitrobenzoate anion at 412 nm for 15 min, which resulted from the reaction of 5-5′-thiobis-2-nitrobenzoic acid with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine chloride.

2.4. Determination of kinetic parameters and mode of inhibition

Kinetic parameters, Michaelis constant (K_m) and maximal velocity (V_{max}) were determined via the Lineweaver–Burk plot and inhibition constant (K_i) derived from slope of primary plot vs concentration of inhibitor.

2.5. Cell culture

PC12 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cells were incubated at 37 °C in a humidified atmosphere (5% CO_2).

2.6. Cytotoxicity assay

The cytotoxicity of the GA-*g*-chitosan (I) was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were grown in 96-well plates at a density of 1×10^4 cells/well. After 24 h, the cells were treated with control medium or medium supplemented with different concentrations of GA-*g*-chitosan (I). After incubation for 24 h, MTT solution (1 mg/mL) was added and incubated for another 4 h. Finally, 100 μL of DMSO was added to solubilize the formed formazan crystals. The amount of formazan crystals was determined by measuring the absorbance at 540 nm using an ELISA reader (SpectraMax® M2/M2^e, CA).

2.7. Acetylcholinesterase inhibitory activity on PC12 cell line

PC12 cells were grown in 10 cm culture dish, and at 70–80% confluence, the cells were treated with GA-*g*-chitosan (I) with the desired concentrations and incubated for 24 h. After incubation, the cells were collected and washed three times with PBS. Then, the cells were lysed in 1 mL lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 1 M NaCl and 50 mM $MgCl_2$) for 2 h. The supernatants were obtained by centrifugation at 10,000 rpm at 4 °C for 30 min and the protein content was determined via BCA assay kit (bicinchoninic acid, Sigma Co., St. Louis, MO), using bovine serum albumin as a standard.

Acetylcholinesterase assay of the cell lysates was performed as the above mention method, and acetylcholinesterase activity was expressed as U/mg protein using standard curve.

2.8. Statistics

All assays were carried out in triplicate, and the results are reported as means ± standard deviations. The statistical significance of difference was analyzed by Student's *t*-test using SPSS (Chicago, IL, USA).

3. Results and discussion

3.1. Acetylcholinesterase inhibitory capacities of GA-g-chitosans

Acetylcholinesterase inhibitors have been well-characterized in clinical studies as the most promising Alzheimer's disease therapeutic agents by increasing endogenous levels of acetylcholine and cholinergic neurotransmission in the brains of Alzheimer's type dementia patients (Yu, Utsuki, Brossi & Greig, 1999). Therefore, we evaluated the anti-acetylcholinesterase activities of the GA-*g*-chitosans in order to help address the paucity of information on GA-*g*-chitosans as acetylcholinesterase inhibitors.

The acetylcholinesterase inhibitory capacities of the GA-*g*-chitosans are depicted in Fig. 1. First, plain chitosan, which was produced by the same preparation process as the GA-*g*-chitosan without the addition of gallic acid, showed weak acetylcholinesterase inhibitory activity as 40% inhibition at 400 μg/mL. However, the acetylcholinesterase inhibitory capacities of the GA-*g*-chitosans were higher than that of the plain chitosan. All GA-*g*-chitosans had inhibitory potency toward acetylcholinesterase in a dose-dependent manner, and their inhibition activity were recorded as 87.31% [GA-*g*-chitosan (I)], 75.77% [GA-*g*-chitosan (II)], 57.57% [GA-*g*-chitosan (III)], and 50.36% [GA-*g*-chitosan (IV)] at the concentration of 400 μg/mL. These activities were augmented with increasing gallic acid content in the grafted chitosans. Table 1 summarizes the IC_{50} values of the GA-*g*-chitosans, which are the concentrations necessary to inhibit 50% of acetylcholinesterase activity. The IC_{50} values were calculated by the non-linear regression method. As depicted in Fig. 1, GA-*g*-chitosan (I) showed the most potent acetylcholinesterase inhibitory capacity with an IC_{50} value of 138.5 ± 2.5 μg/mL, followed by GA-*g*-chitosan (II) > GA-*g*-chitosan (III) > GA-*g*-chitosan (IV), respectively.

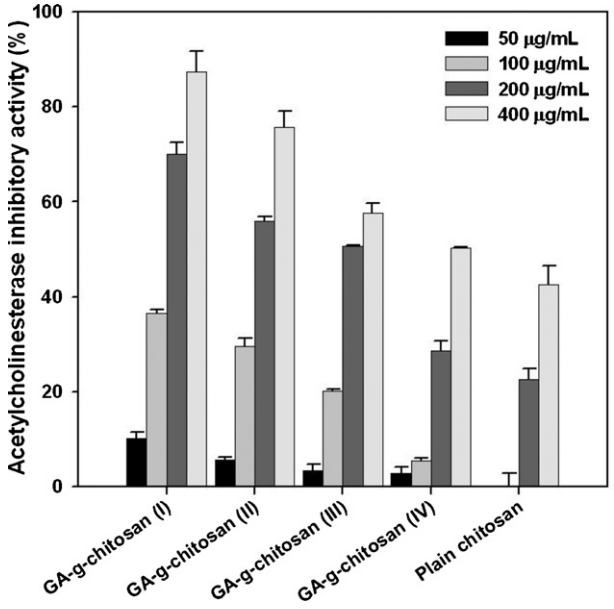

Fig. 1. Acetylcholinesterase inhibitory activities of gallic acid-*grafted*-chitosans. Results are expressed as means ± SD of three determinations.

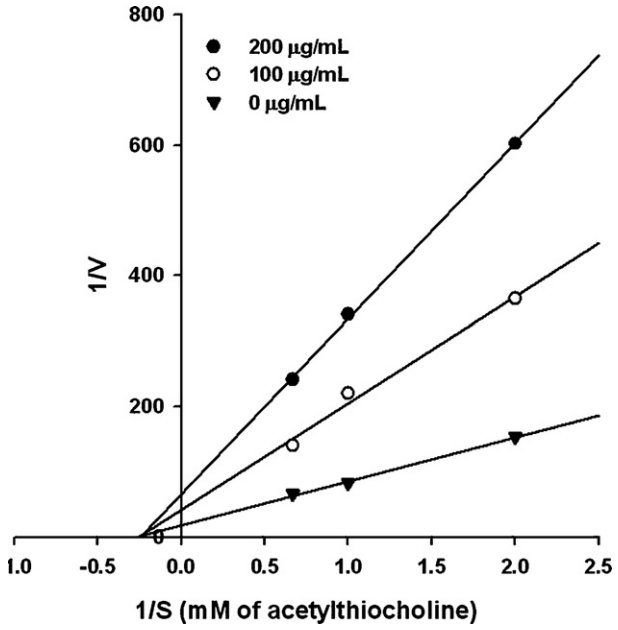

Fig. 2. Lineweaver–Burk plots of acetylcholinesterase with and without gallic acid-*grafted*-chitosan (I).

Table 1
IC_{50} values of GA-*g*-chitosans.

Samples	IC_{50} (μg/mL)
GA-*g*-chitosan (I)	138.5 ± 2.5
GA-*g*-chitosan (II)	177.9 ± 3.3
GA-*g*-chitosan (III)	196.4 ± 1.7
GA-*g*-chitosan (IV)	397.6 ± 5.2

Table 2
Kinetic parameters of acetylcholinesterase in the presence of GA-*g*-chitosan (I).

μg/mL	K_m (mM)	V_{max} (ΔOD_{412}/min)
0	4.079	0.053
100	4.079	0.023
200	4.079	0.015

Scanty information regarding polysaccharide-based acetylcholinesterase inhibitors is available, and herein we are the first to report on the acetylcholinesterase inhibitory activities of naturally occurring polysaccharide based derivatives, namely GA-*g*-chitosans, and demonstrated that GA-*g*-chitosans possess high acetylcholinesterase inhibitory capacities compared to plain chitosan. This result suggests that gallic acid improves the acetylcholinesterase inhibitory activity of plain chitosan. In our previous report, we found that chitooligosaccharides (COSs), derivatives of chitosan, possess acetylcholinesterase inhibitory activity, and their activity is dependent on their molecular weight and degree of deacetylation (Lee, Park, Kim, Ahn & Je, 2009). The IC_{50} values of the COSs ranged from 1.67 to 3.52 mg/mL. In this study, the GA-*g*-chitosans showed enhanced acetylcholinesterase inhibitory activities compared to the COSs. Yoon, Ngo, and Kim (2009) also reported that aminoalkylated COSs inhibited acetylcholinesterase, and inhibitory activity was dependent on the type of aminoalkyl groups introduced onto the COSs. The activities of these aminoalkylated COSs appear to be more potent than those of GA-*g*-chitosans. It is also developed chitosan and its oligomers as neuroprotective agents, particularly with regard to oxidative stress because increased oxidative stress is a widely accepted factor in the development and progression of Alzheimer's disease. Khodagholi, Eftekharzadeh, Maghsoudi, and Rezaei (2010) reported that chitosan prevents oxidative stress-induced amyloid β formation and cytotoxicity in NT2 neurons, and Xu, Huang, Lin, and Jiang (2010) also reported that chitosan oligomer protected rat cortical neurons by copper-induced oxidative stress. In addition, chitins and chitosans could be used for the repair of wounded nerve tissue, and reduced the oxidative stress by radical scavenging activity (Muzzarelli, 2009; Castagnino et al., 2008).

3.2. Determination of kinetic parameters and mode of inhibition

The inhibition mode of the GA-*g*-chitosans on acetylcholinesterase was analyzed using Lineweaver–Burk plots. Determination of the inhibition type is important to understand the mechanism of enzyme action and the inhibitor binding site. Considering acetylcholinesterase inhibitory activity, we used GA-*g*-chitosan (I) as a representative in kinetic analysis. The Lineweaver–Burk plots of acetylcholinesterase with and without GA-*g*-chitosan (I) (at two concentrations, 100 and 200 μg/mL) are presented in Fig. 2, showing that the lines intersect on the *x*-axis. Therefore, the inhibition mode of GA-*g*-chitosan (I) toward acetylcholinesterase is non-competitive inhibition where GA-*g*-chitosan (I) reversibly binds to an allosteric site on acetylcholinesterase, preventing the substrate from binding to the active site. This means GA-*g*-chitosan (I) acts as an acetylcholinesterase inhibitor by forming enzyme–substrate–inhibitor and enzyme–inhibitor complexes during the reaction to reduce the efficiency of catalysis. Kinetic

Table 3
Cellular acetylcholinesterase inhibitory activity of GA-*g*-chitosan (I) on PC12 cells.

	GA-*g*-chitosan (I) (μg/mL)				
	0	25	50	100	200
Acetylcholineterase activity (U/mg protein)	10.16 ± 0.27	9.18 ± 0.39	8.12 ± 0.32	7.49 ± 0.35	6.58 ± 0.27

parameters such as K_m, V_{max} and K_i were analyzed using primary and secondary Lineweaver–Burk plots, and the values are summarized in Table 2. In the presence of GA-*g*-chitosan (I), V_{max} decreased with increasing concentrations of GA-*g*-chitosan (I), but K_m was not altered. The K_i value was calculated by the secondary plot of Lineweaver–Burk, which the slopes of each line in the Lineweaver–Burk plot were plotted against different concentrations of GA-*g*-chitosan (I), and the K_i value is the intercept on the *x*-axis. The obtained K_i value was found to be 64.85 μg/mL.

3.3. Cytotoxicity and acetylcholinesterase inhibitory activity in PC12 cell line

Pheochromocytoma (PC12) cells, rich in acetylcholinesterase activity, were employed for further investigation on the effect of GA-*g*-chitosan (I) against acetylcholinesterase activity. Prior to investigate the acetylcholinesterase inhibitory activity of GA-*g*-chitosan (I) in PC12 cells, cytotoxic level of GA-*g*-chitosan (I) was determined using MTT assay. The GA-*g*-chitosan (I) did not exert any significant ($p < 0.01$) toxic effect on the PC12 cells after 24 h of treatment (0–200 μg/mL). Therefore, non-toxic concentrations of GA-*g*-chitosan (I) were applied for the effect on acetylcholinesterase activity in PC12 cells.

For determination of cellular acetylcholinesterase inhibitory activity, the GA-*g*-chitosan (I) with various concentrations was treated in PC12 cells and further incubated for 24 h. As shown in Table 3, acetylcholinesterase activities of the cell lysates including treatment with GA-*g*-chitosan (I) were observed that acetylcholinesterase activity was decreased with increased concentrations of GA-*g*-chitosan (I). In the absence of GA-*g*-chtiosan (I), acetylcholinesterase activity was observed 10.16 U/mg protein, but in the presence of GA-*g*-chtiosan (I) at the concentration of 200 μg/mL, acetylcholinesterase activity was observed 6.58 U/mg protein.

4. Conclusion

In this study, we firstly reported acetylcholinesterase inhibitory activity of GA-*g*-chitosans. The GA-*g*-chitosans exhibited potent acetylcholinesterase inhibitory activity in a dose-dependent manner compared to plain chitosan, and acetylcholinesterase inhibitory activity was dependent on GA content in the GA-*g*-chitosans. The inhibition mode of the GA-*g*-chitosan (I) was found to be non-competitive, and K_i value was found to be 64.85 μg/mL. In addition, the GA-*g*-chitosan (I) did not exert any significant cytotoxicity against PC12 cells, and the cellular acetylcholinesterase activity in PC12 cells was significantly inhibited by the GA-*g*-chitosan (I). Our present findings suggest that GA-*g*-chitosans might be beneficial materials in the prevention and treatment of Alzheimer's disease, and provide a chemical framework for the development of new acetylcholinesterase inhibitors based on chitosan.

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